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Mechanism underlying the reduced positive inotropic effects of the phosphodiesterase III inhibitors pimobendan, adibendan and saterinone in failing as compared to nonfailing human cardiac muscle preparations

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Summary. The present study was performed to compare the effects of the new positive inotropic phosphodiesterase III inhibitors pimobendan, adibendan, and saterinone on the isometric force of contraction in electrically driven ventricular trabeculae carneae isolated from explanted failing (end-stage myocardial failure) with those from nonfailing (prospective organ donors) human hearts. In preparations from nonfailing hearts the phosphodiesterase inhibitors, as well as the β-adrenoceptor agonist isoprenaline, the cardiac glycoside dihydroouabain, and calcium, which were studied for comparison, revealed pronounced positive inotropic effects. The maximal effects of pimobendan, adibendan, and saterinone amounted to 56%, 36% and 45%, respectively, of the maximal effect of calcium. In contrast, in preparations from failing hearts the phosphodiesterase III inhibitors failed to significantly increase the force of contraction and the effect of isoprenaline was markedly reduced. The effects of dihydroouabain and calcium were almost unaltered. The diminished effects of isoprenaline were restored by the concomitant application of phosphodiesterase inhibitors.

To elucidate the underlying mechanism of the lack of effect of the phosphodiesterase III inhibitors in the failing heart we also investigated the inhibitory effects of these compounds on the activities of the phosphodiesterase isoenzymes I–III separated by DEAE-cellulose chromatography from both kinds of myocardial tissue. Furthermore, the effects of pimobendan and isoprenaline on the content of cyclic adenosine monophosphate (determined by radioimmunoassays) of intact contracting trabeculae were studied. The lack of effect of the phosphodiesterase inhibitors in failing human hearts could not be explained by an altered phosphodiesterase inhibition, since the properties of the phosphodiesterase isoenzymes I–III and also the inhibitory effects of the phosphodiesterase inhibitors on these isoenzymes did not differ between failing and nonfailing human myocardial tissue. Instead, it may be due to a diminished formation of cyclic adenosine monophosphate in failing hearts, presumably caused mainly by a defect in receptor-adenylate cyclase coupling at least in idiopathic dilated cardiomyopathy. Both the basal and the pimobendan-stimulated or isoprenaline-stimulated contents of cyclic adenosine monophosphate of intact contracting trabeculae from failing hearts were decreased compared with the levels in nonfailing hearts. However, under the combined action of isoprenaline and pimobendan the cyclic adenosine monophosphate level reached values as high as with each compound alone in nonfailing preparations, and in addition the positive inotropic effect of isoprenaline was restored.

These findings may have important clinical implications. Along with the elevated levels of circulating catecholamines the positive inotropic effects of the phosphodiesterase inhibitors may be maintained in patients with heart failure. Furthermore, the concomitant application of a β-adrenoceptor agonist and a phosphodiesterase inhibitor might be beneficial in terminal heart failure refractory to conventional therapeutic regimens.

Key words: Phosphodiesterase inhibition – Failing and nonfailing human heart – Positive inotropic effect –

* Some of the results reported in this paper have already been presented in abstract form at the 61st Session of the American Heart Association, Washington, DC, Nov. 1988 (von der Leyen et al., Circulation 78 (Suppl II): II-360, 1988), at the Fall Meeting of the German Society of Pharmacology and Toxicology, Sept. 1988 (Schmitz et al., Naunyn-Schmiedeberg's Arch Pharmacol 338 (Suppl): R 16, 1988), at the 30th Spring Meeting of the German Society of Pharmacology and Toxicology, March 1989 (Meyer et al., Naunyn-Schmiedeberg's Arch Pharmacol 339 (Suppl): R 53, 1989), and at the XIII Congress of the International Society For Heart Research, Ann Arbor, MI, May 1989 (Meyer et al., J Mol Cell Cardiol 21 (Suppl II): S. 50, 1989)

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Cyclic adenosine monophosphate content – Combination of isoprenaline and phosphodiesterase inhibitors

Introduction

Current therapy for congestive heart failure includes diuretics, cardiac glycosides, and vasodilators. However, for long-term therapy cardiac glycosides still remain the only approved positive inotropic agents (Lee et al. 1982; Scholz 1984), despite their disadvantages, e.g. low therapeutic index, propensity to produce arrhythmias, and low therapeutic efficacy (Colucci et al. 1986; Robertson and Hayes 1988). In search of a replacement for digitalis, new positive inotropic agents have been introduced for clinical investigation during the last 10 years (Colucci et al. 1986). It has been suggested that the positive inotropic action of most of these agents, which also have direct vasodilatory effects, is due mainly to inhibition of type III phosphodiesterase (Colucci et al. 1986; Weishaar et al. 1987; Brunkhorst et al. 1989). These phosphodiesterase (PDE) inhibitors do not inhibit sodium-potassium ATPase; nor do reserpine-induced depletion of endogenous catecholemine stores, pretreatment with α- or β-adrenoceptor antagonists, H_2 antagonists, agents that block prostaglandin synthesis, or agents that block the fast sodium inward current diminish the positive inotropic effects of these drugs (Honerjäger et al. 1984; Colucci et al. 1986; Brunkhorst et al. 1989). At least on short-term administration, these agents produce consistent haemodynamic benefits in patients with severe heart failure whereas their long-term effects are more controversial (Colucci et al. 1986; Packer 1988b). It has been also shown recently that agents acting by elevating cyclic adenosine monophosphate (cAMP), such as β-adrenoceptor agonists and PDE inhibitors revealed reduced positive inotropic effects in preparations from failing human hearts (Wilmshurst et al. 1984; Feldman et al. 1987; Schmitz et al. 1987; Böhm et al. 1988b; Erdmann 1988), even though these compounds exerted marked positive inotropic effects in animal tissue experiments, e.g. in guinea-pig papillary muscles (Erdmann 1988; Brunkhorst et al. 1989). Therefore, it is not possible to easily extrapolate results obtained in normal animal myocardium to diseased human myocardium. In view of these facts the present study was designed to compare the effects of the new positive inotropic PDE III inhibitors pimobendan (van Meel 1985; Brunkhorst et al. 1989), adibendan (Bethke et al. 1988; Müller-Beckmann et al. 1988), and saterinone (Armah et al. 1988; Brunkhorst et al. 1988) on force of contraction in ventricular preparations isolated from failing (end-stage myocardial failure, NYHA IV) with those from nonfailing human hearts. The effects of calcium, the β-adrenoceptor agonist isoprenaline, and the cardiac glycoside dihydroouabain were studied for comparison. We also investigated the characteristics of the PDE isoenzymes I–III and the inhibitory effects on them of pimobendan, adibendan, and saterinone in both kinds of myocardial tissue. For comparison, the effects of the nonselective PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX) and the selective PDE III inhibitor milrinone were also determined (Brunkhorst et al. 1989). Additionally, we measured the influence of pimobendan and isoprenaline on the cyclic adenosine monophosphate (cAMP) content in contracting preparations.

Materials and methods

Five nonfailing human hearts were obtained from prospective multiorgan donors without heart failure who died of noncardiac causes. The patients suffered brain death, four after a head injury and one after suicide by a shot in the head. Ages of the patients (3 male, 2 female) ranged from 27 to 56 years. Their hearts could not be used for transplantation for surgical reasons and/or because of blood group incompatibility. In these donor hearts cardiac catheterization was not performed. On inspection they showed normally structured ventricles. Aortic and pulmonary valves were excised from these hearts and used later for valve replacement operations.

Six failing human hearts were obtained from patients undergoing orthotopic heart transplantation due to end-stage (NYHA IV) heart failure. In five cases the diagnosis was idiopathic dilated cardiomyopathy, and in one case, "ischaemic cardiomyopathy", i.e. heart failure resulting from coronary artery disease. Gross examination of the hearts revealed hypertrophy and dilatation of both ventricles. The heart of the patient with coronary artery disease showed large areas of fibrotic and scarred tissue resulting from myocardial infarcts. Ages of the patients ranged from 35 to 66 years. Medications being administered at the time of transplantation included diuretics ($n = 6$), cardiac glycosides ($n = 6$), angiotensin converting enzyme (ACE) inhibitors ($n = 6$), nitrates ($n = 6$), dopamine/dobutamine ($n = 1$), and amiodarone ($n = 1$). None of the patients was receiving β-adrenoceptor blockers. All patients suffered from myocardial failure clinical stage NYHA IV, had a cardiac index of less than 2.2 l m^{-2} min^{-1} (1.8 $\pm$ 0.1 l m^{-2} min^{-1}, mean $\pm$ SEM) and an ejection fraction of less than 30% (20.4 $\pm$ 2.5%, mean $\pm$ SEM). Written informed consent was obtained from the families of all organ donors and from all recipients before excision or transplantation. After explantation the hearts were placed into aerated, ice-cold bathing solution (composition see below) and transferred to our laboratory. Contraction experiments were started immediately, while PDE experiments were carried out on ventricular tissue samples taken from the same hearts but first frozen in liquid nitrogen and stored at −80°C until use.

Contraction experiments. The experiments were performed on electrically driven trabeculae carneae isolated from the right ventricles (diameter less than 1 mm, length 5–8 mm) following dissection in aerated bathing solution at room temperature. The bathing solution was a modified Tyrode's solution containing (mmol/l) NaCl 119.8, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.05. NaH_2PO_4 0.42, $NaHCO_3$ 22.6, Na_2EDTA 0.05, ascorbic acid 0.28, glucose 5.0, continuously gassed with 95% O_2 + 5% CO_2 and maintained at 35°C; the pH was 7.4. Due to poor water solubility of pimobendan, adibendan, and saterinone, these compounds were dissolved in dimethylsulfoxide (DMSO) and the bathing solution also contained 2% (v/v) DMSO and 0.04 mg/ml dimeticone (sab simplex; Parke Davis, Freiburg, FRG) as antifoaming agent. Dimeticone had no effect and DMSO had only slight effects on force of contraction (less than 10% decrease within 30 min). Appropriate aliquots of DMSO were also added in all experiments with the water soluble compounds calcium, dihydroouabain, and isoprenaline.

The preparations were attached to a bipolar platinum stimulating electrode and suspended individually in 10-ml glass tissue chambers for recording isometric contractions. Force of contraction was measured with an inductive force transducer (W. Fleck, Mainz, FRG) connected to a Hellige Helco Scriptor recorder (Hellige,

Freiburg, FRG). Each muscle was stretched to the length at which force of contraction was maximal. The resting force (approximately 5 mN) was kept constant throughout the experiment. The trabeculae were electrically paced at 0.5 Hz with rectangular pulses of 5 ms duration (Grass stimulator SD9; Grass, Quincy, Mass., USA); the voltage was about 10–20% greater than threshold. All preparations were allowed to equilibrate in drug-free 2% (v/v) DMSO containing bathing solution until complete mechanical stabilization. During this period (about 2 h) the bathing solution was changed every 15 min.

Concentration-response curves were obtained cumulatively and were expressed as increase in force of contraction in millinewtons. From each heart, several trabeculae were prepared and split up into two collectives. In the first group concentration-response curves for the PDE inhibitors pimobendan, adibendan, or saterinone were performed. After a washout the trabeculae were again exposed to a submaximally effective concentration of the PDE inhibitor (100 μmol/l pimobendan, 100 μmol/l adibendan, 10 μmol/l saterinone) and keeping this concentration constant a concentration-response curve for calcium was established. After a further washout a concentration-response curve for isoprenaline again in the presence of a PDE inhibitor was obtained. In the second group the effects of calcium, isoprenaline, and dihydroouabain in the absence of a PDE inhibitor were studied. The respective concentration-response curves were performed in the order mentioned above interrupted by washout periods. The time of exposure to each concentration of the drugs was 15 min for pimobendan, adibendan, and saterinone, 5 min for calcium or isoprenaline, and 30–45 min for dihydroouabain. The time of exposure to the various drugs was chosen as that necessary to reach steady-state conditions.

Isolation of cyclic nucleotide PDE I–III. Cyclic nucleotide PDE activities from failing and nonfailing human hearts were separated into three isoenzymes by DEAE-cellulose anion exchange chromatography using the method of Thompson et al. (1979) as adapted by Weishaar et al. (1986) and previously described with minor modifications (Brunkhorst et al. 1988). Samples of ventricular tissue which had been stored at −80° C, taken from the same hearts, as the trabeculae for the contraction experiments, were thawed on ice in 30 ml "PDE isolation buffer" (mmol/l: $MgCl_2$ 2, dithioerythritol 1, Tris-HCl 10; pH 7.5). All subsequent procedures were carried out at 4° C. After mincing with fine scissors the tissue was homogenized in a Polytron instrument (type PT 10-35; Kinematica, Littau-Luzern, Switzerland) for 10 s at setting 3 and 10 s at setting 6, and then in a Teflon glass homogenizer (Colora, Lorch, FRG) for 2 min at setting 5. The homogenate was centrifuged at 30000 *g* for 20 min. The resulting supernatant was filtered through four layers of gauze and applied to a DEAE-cellulose column (40 × 1.6 cm; bed volume about 30 ml) equilibrated with freshly prepared buffer (70 mmol/l sodium acetate and 5 mmol/l 2-mercaptoethanol; pH 6.5). After washing the column with 2–3 bed volumes of this buffer, the PDEs were eluted from the column using a continuous 70–1000 mmol/l sodium acetate gradient (containing 5 mmol/l 2-mercaptoethanol; pH 6.5; total volume 400 ml; flow rate approximately 25 ml/h). Fractions of 8 ml were collected and immediately assayed for cAMP and cGMP PDE activity, respectively (substrate concentration 1.0 μmol/l each) in the presence or absence of 2 units of calmodulin and 10 μmol/l $CaCl_2$. The cAMP PDE was additionally assayed in the presence of 0.5 μmol/l cGMP and with a substrate concentration of 25 μmol/l cAMP. Appropriate peak fractions (normally two; referred to as PDE I, II, III in order of elution) were pooled and concentrated to 14% of the original volume using an Amicon ultrafiltration cell fitted with a PM 10 membrane (Amicon, Witten, FRG). The protein was then diluted to 65% with ethylene glycol monoethyl ether and stored at −20° C. Under these conditions hydrolytic activity was stable for nearly 2–3 weeks.

Determination of PDE activity. PDE activity was determined in a two-step procedure according to Thompson and Appleman (1971) and Bauer and Schwabe (1980). The reaction mixture consisted of 5 mmol/l $MgCl_2$, 40 mmol/l Tris-HCl (pH 8.0), 1 μmol/l cAMP or cGMP, [^{3}H]cAMP or [^{3}H]cGMP (20000–30000 cpm) and enzyme preparation (diluted with 0.1% bovine serum albumin) in a final volume of 200 μl. By suitable enzyme dilution the substrate conversion was kept below 20% in order to be in the linear range of the enzyme reaction. The incubation time was 10 min at 30° C. Except for the solvent DMSO, none of the substances investigated interfered with the assay procedure. Owing to poor water solubility, pimobendan, adibendan, and saterinone had to be dissolved in 5% (v/v) DMSO. This concentration of DMSO inhibited PDE activity by about 17%. In these cases the calculation of the amount of PDE inhibition by the drugs was based on the basal activity of the enzyme in the presence of DMSO.

Protein concentrations were determined by the Bio-Rad Protein Assay according to Bradford (1976).

Determination of cAMP content. Contracting trabeculae were exposed to pimobendan (15 min), isoprenaline (5 min), pimobendan (10 min) and followed by isoprenaline for a further 5 min, or drug-free bathing solution. These times of exposure correspond to those used in the experiments where only force of contraction was measured, and were chosen to achieve steady-state. After these periods the trabeculae were quickly removed from the organ bath, freeze-clamped in liquid nitrogen, and handled for measuring the cAMP content by radioimmunoassays as described previously (Böhm et al. 1984). The average recovery of cAMP was 94.7 ± 3.5% (n = 35). Force of contraction and cAMP content were measured in the same preparations.

Materials. Drugs and reagents used were pimobendan (UD-CG 115 BS; Thomae, Biberach, FRG), adibendan (BM 14.478; Boehringer Mannheim, FRG), saterinone (BDF Beiersdorf, Hamburg, FRG), milrinone (Sterling-Winthrop, Rensselaer, NY, USA), 3-isobutyl-1-methylxanthine (IBMX; EGA Chemie, Steinheim, FRG), (±)-isoprenaline-HCl (Boehringer, Ingelheim, FRG), dihydroouabain (DHO; Hommel, Adliswil, Switzerland), calmodulin from bovine heart (Sigma, Deisenhofen, FRG), ethylene glycol monoethyl ether (Fluka, Neu-Ulm, FRG), [^{3}H]cAMP and [^{3}H]cGMP (NEN, Dreieich, FRG), DEAE-cellulose (Sephacel; Pharmacia LKB, Freiburg, FRG), DMSO (Serva, Heidelberg, FRG) and 2′-*O*-succinyl adenosine 3′,5′-monophosphate tyrosine methyl ester (Sigma, Deisenhofen, FRG) which was iodinated using Na^{125}I (Amersham-Buchler, Braunschweig, FRG) as previously described (Böhm et al. 1984). All other chemicals were of analytical or best commercial grade available. Deionized and twice-distilled water was used throughout.

Statistics. Values presented are arithmetic means ± SEM. Statistical significance was estimated using Student's t-test for paired or unpaired observations. A P-value of less than 0.05 was considered significant. Concentrations of drugs producing 50% of the maximal effect (EC_{50}) or reducing the basal activity of PDE to 50% (IC_{50}) were determined graphically. EC_{50} and IC_{50} values are given as geometric means with 95% confidence limits. SF (= selectivity factor) is the mean of the IC_{50} values of PDE I and II divided by the IC_{50} value of PDE III. The greater this ratio, the more selective is the PDE III inhibition.

Results

Effects on force of contraction

In right ventricular trabeculae isolated from *nonfailing human hearts* all three PDE inhibitors exerted a concentration-dependent positive inotropic effect. Pimobendan,

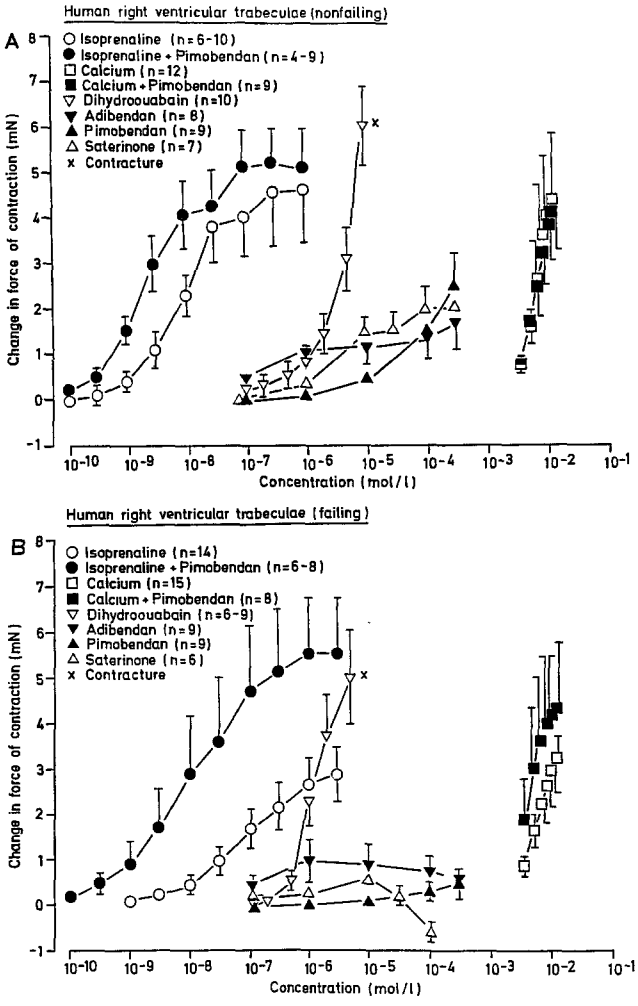

Fig. 1 A, B. Cumulative concentration-response curves for the effects of pimobendan, adibendan, saterinone, isoprenaline, dihydroouabain, and calcium on force of contraction in electrically driven (0.5 Hz) right ventricular trabeculae carneae isolated from **A** five nonfailing and **B** six faling (NYHA IV) human hearts (n = number of preparations in each case). *Ordinates,* change in force of contraction (mN). *Abscissae,* drug concentration (mol/l). In all experiments the bathing solution contained 2% (v/v) DSMO. *x,* Concentration of dihydroouabain at which contractures occurred in some preparations. Pre-drug values were: **A** 0.97 ± 0.11 mN (n = 56; ▲, ▼, △, ○, ▽, □); in the presence of 10^{-4} mol/l pimobendan 1.04 ± 0.22 mN (n = 9, ■) and 0.94 ± 0.27 mN (n = 9, ●); **B** 2.03 ± 0.30 mN (n = 62; ▲, ▼, △, ○, ▽, □); in the presence of 10^{-4} mol/l pimobendan 3.37 ± 1.14 mN (n = 8, ■) and 1.97 ± 0.47 mN (n = 8, ●)

adibendan, and saterinone enhanced the force of contraction maximally by 2.41 mN, 1.54 mN, and 1.94 mN, respectively (Fig. 1 A). The EC_{50} values were 65.9 (38.7–112.5) µmol/l (pimobendan, n = 9), 2.8 (0.2–41.8) µmol/l (adibendan, n = 8), and 4.5 (2.4–8.6) µmol/l (saterinone, n = 7). The positive inotropic effects of calcium, isoprenaline, and dihydroouabain (Fig. 1 A), which were about equieffective, were greater than the effects of the PDE inhibitors. Compared to the maximal effect of calcium, taken as the maximal inotropic response of which the muscles were capable, pimobendan, adibendan, and saterinone maximally produced only about 56%, 36%, and 45%, respectively, of this increase in force of contraction. The effects of calcium and isoprenaline were also investigated in the presence of the PDE inhibitors (Fig. 1). The calcium response was not affected by the PDE inhibitors, which is shown for pimobendan in Fig. 1 as an example. This was similar for adibendan and saterinone (data not shown). In contrast, the concentration-response curve for isoprenaline was significantly shifted to the left by pimobendan (100 µmol/l; Fig. 1, Table 1), adibendan (100 µmol/l; Table 1), or saterinone (10 µmol/l; Table 1).

In contrast to the nonfailing heart, the three PDE inhibitors were not able to significantly increase force of contraction in *failing hearts* (Fig. 1 B). In line with this, the effect of isoprenaline was also markedly reduced, whereas the responses to calcium and dihydroouabain were similar to those observed in preparations from nonfailing hearts (Fig. 1 B). In the presence of the PDE inhibitors the concentration-response curve for isoprenaline was shifted to the left and the reduced efficacy of isoprenaline in the failing heart was restored by the PDE inhibitors to the values obtained in the nonfailing heart. This is shown for pimobendan as an example in Fig. 1 B, and holds also true for adibendan and saterinone (Table 1). The maximal effect of isoprenaline alone in the failing heart amounted to 2.89 mN but to 5.53 mN in the presence of pimobendan (Table 1). The respective values in the nonfailing hearts were 4.50 mN and 5.20 mN. The PDE inhibitors also enhanced the potency of isoprenaline in the failing heart, i.e. the EC_{50} value of isoprenaline was reduced by a factor of 5.25 in the presence of pimobendan. The EC_{50} values were 65.84 and 12.65 nmol/l in the absence and presence of pimobendan, respectively. But this increase in the potency in the presence of the PDE inhibitors was not markedly different from that seen in the nonfailing heart (factor 3.78; EC_{50} values were 7.34 and 1.94 nmol/l in the absence and presence of pimobendan, respectively; Table 1). The effects of isoprenaline in the presence of adibendan or saterinone are listed in Table 1.

Effects on PDE activity

In order to elucidate whether an altered PDE activity or an altered PDE inhibition might be responsible for the lack of positive inotropic effect of the PDE inhibitors in the failing human heart we investigated the characteristics of the PDE isoenzymes I–III and the effects of the PDE inhibitors in both kinds of myocardial tissue. The samples of ventricular tissue were derived from the same hearts from which the preparations for the contraction experiments had been isolated.

Properties of the PDE isoenzymes in human myocardial preparations

PDE activities in nonfailing and failing human hearts were separated into three isoenzymes referred to as PDE I–III (Fig. 2). PDE I was a low K_m enzyme (K_m values

Table 1. Effects of isoprenaline (Iso) in the absence and presence of adibendan (Adi; 100 μmol/l), pimobendan (Pimo; 100 μmol/l), and saterinone (Sat; 10 μmol/l) on force of contraction in right ventricular trabeculae carneae isolated from nonfailing and failing human hearts. The maximal increase in force of contraction (efficacy of isoprenaline) and the EC_{50} values were obtained from cumulative concentration-response curves and are given with SEM and with 95% confidence limits, respectively. The experiments with isoprenaline alone and in the presence of pimobendan are the same as in Fig. 1. Factors = EC_{50} in the absence divided by EC_{50} in the presence of the phosphodiesterase (PDE) inhibitor; *n*, number of trabeculae

Agents	Nonfailing heart				Failing heart			
	Efficacy (mN)	*n*	EC_{50} (nmol/l)	Factor	Efficacy (mN)	*n*	EC_{50} (nmol/l)	Factor
Iso	4.50 ± 1.18	10	7.34 (2.98–18.09)	–	2.89 ± 0.60	14	65.84 (32.50–133.4)	–
Iso + Adi	6.01 ± 2.03	8	1.13 (0.74–1.71)	6.50	6.94 ± 1.03	9	10.15 (4.12–25.02)	6.49
Iso + Pimo	5.20 ± 0.71	9	1.94 (1.31–2.89)	3.78	5.53 ± 1.24	8	12.65 (3.74–42.84)	5.25
Iso + Sat	4.10 ± 0.54	6	2.68 (1.36–5.27)	2.74	7.78 ± 3.19	6	22.20 (3.54–139.3)	2.97

about 0.5 μmol/l) and was stimulated by Ca^{2+}/calmodulin (not shown). PDE II revealed high K_m values for cAMP and cGMP, about 85 and 22 μmol/l, respectively, and cAMP hydrolysis could only weakly be stimulated by 0.5 μmol/l cGMP. As reported by Reeves et al. (1987), a greater peak for PDE II was obtained with 25 μmol/l cAMP as substrate (Fig. 2). PDE III had low K_m values (about 0.2 μmol/l) for cAMP and cGMP, and its cAMP PDE activity was inhibited by 0.5 μmol/l cGMP (Fig. 2). These characteristics of the PDE isoenzymes were found to be similar in both nonfailing and failing human hearts. There were virtually no essential differences in the elution profiles (Fig. 2) and the basal activities of the PDE isoenzymes (cf. legends to Figs. 3, 4).

Effects of the PDE inhibitors on the activities of PDE I–III

The same applies to the inhibitory effects of pimobendan, adibendan, saterinone, and, for comparison, milrinone and IBMX. All compounds inhibited all isoenzymes in a concentration-dependent manner. With regard to PDE III inhibition, IBMX (Fig. 3) proved to be a nonselective PDE inhibitor, whereas all other agents were selective PDE III inhibitors (Tables 2, 3; Fig. 4); that is to say they diminished PDE III activity at concentrations 1.5 or more orders of magnitude lower than those required for the inhibition of PDE I or II. For these agents an SF of 92 (e.g. milrinone, nonfailing heart; Table 2) or greater was calculated. In contrast, IBMX had an SF of only about 3 (2.9 and 3.5 in nonfailing and failing heart, respectively; Tables 2, 3). Among the compounds tested saterinone was more than one order of magnitude more potent in inhibiting PDE III than adibendan, pimobendan, and milrinone (Tables 2, 3).

Concentration-response curves for the PDE-inhibitory effects of IBMX and pimobendan in failing and nonfailing preparations are shown as illustrations in Figs. 3 and 4, respectively. No differences between the effects of IBMX and pimobendan on the activities of the PDE isoenzymes from both kinds of human myocardial tissue could be detected. The IC_{50} values and selctivity factors of all agents tested are summarized in Tables 2 and 3. All compounds exhibited similar selectivities for PDE III inhibition and similar potencies for the inhibition of the PDE isoenzymes I–III separated from both kinds of human hearts. Thus, the PDE-inhibitory effects of all compounds were the same in failing and nonfailing human heart.

Effects on cAMP content

In these series of experiments the basal and the pimobendan- or isoprenaline-stimulated contents of cAMP in intact contracting trabeculae isolated from nonfailing and failing human hearts were compared. Force of contraction and cAMP content were measured in the same preparations. In trabeculae from *nonfailing hearts* submaximally effective concentrations of pimobendan (100 μmol/l) and isoprenaline (0.2 μmol/l) increased the force of contraction by 185% and 355% (Fig. 5B) and cAMP content by 69% and 63%, respectively (Fig. 5A). The basal values of cAMP in nonfailing human preparations corresponded to those reported in preparations from guinea-pigs (Böhm et al. 1984) and human atrial nonfailing tissues (Ikezono et al. 1987). In contrasts, in trabeculae isolated from *failing human hearts* the basal content of cAMP was only 62% of that in nonfailing preparations (Fig. 5A, d vs a). Pimobendan and isoprenaline increased cAMP content in these preparations, but the effects were only small. Even the level of the elevated cAMP content was not higher than the basal content in nonfailing ventricular preparations (Fig. 5A; e, f vs d; e, f vs a). Isoprenaline increased force of contraction by about 115% only, and pimobendan had almost no positive inotropic effect in these preparations

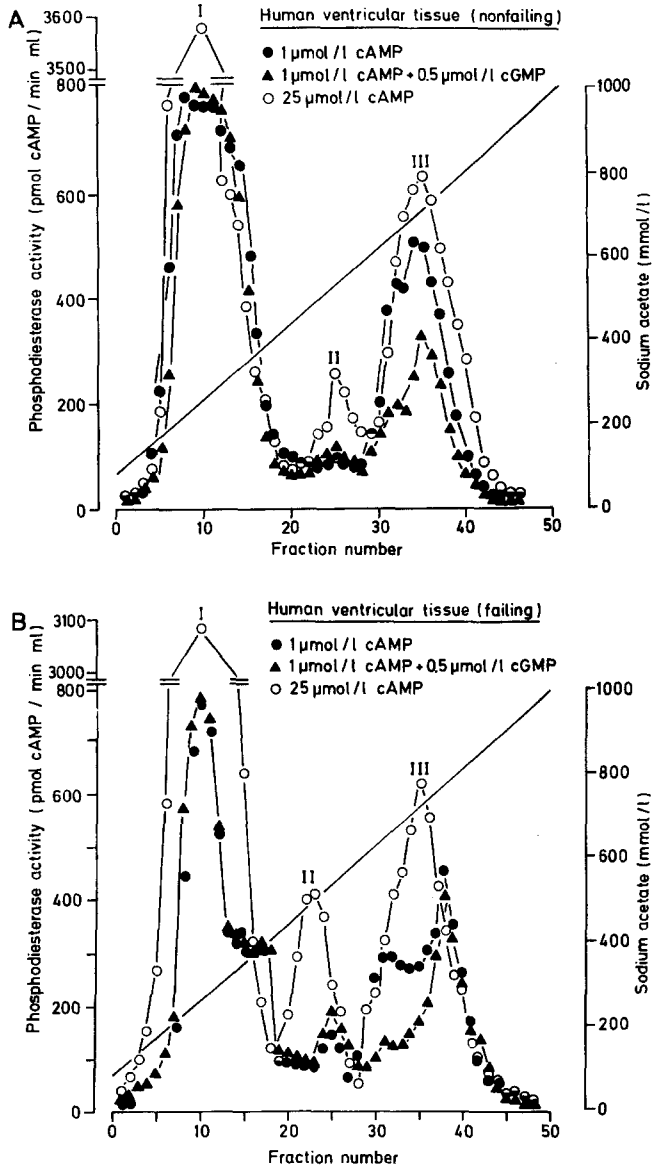

Fig. 2A, B. DEAE-cellulose anion exchange chromatographic profiles of PDE isolated from **A** nonfailing and **B** failing human hearts. Column fractions (*abscissae*; 8 ml) eluted by a 70–1000 mmol/l sodium acetate gradient (*right ordinates*) were directly assayed for PDE (phosphodiesterase) activity. Substrate concentration: 1 µmol/l cAMP (●), 1 µmol/l cAMP in the presence of 0.5 µmol/l cGMP (▲), 25 µmol/l cAMP (○). The major peaks were labelled PDE I, II and III according to the order of elution. Each *curve* represents three isolation procedures from three different hearts. *Left ordinates*, PDE activity (pmol hydrolzyed cAMP/min · ml)

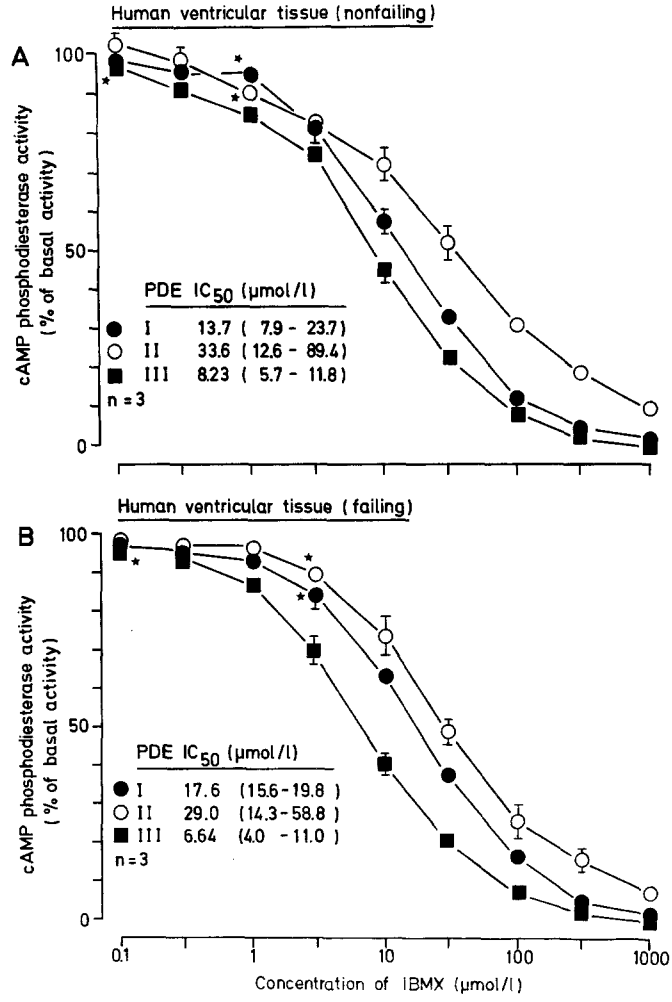

Fig. 3A, B. Effects of IBMX (3-isobutyl-1-methylxanthine) on the activity of cAMP PDE I–III isolated from ventricular tissue of **A** three nonfailing and **B** three failing human hearts. *Ordinates*, cAMP PDE activity as percentage of basal activity; *abscissae*, concentration of IBMX (µmol/l). Substrate concentration: 1 µmol/l cAMP. Incubation time 10 min at 30° C. Basal activities (pmol cAMP/mg protein · min): PDE I: 1472 ± 677.0 (n = 3; **A**), 1696 ± 755.1 (n = 3; **B**); PDE II: 246.0 ± 38.6 (n = 3; **A**), 329.3 ± 26.2 (n = 3; **B**); PDE III: 2736 ± 1239 (n = 3; **A**), 5624 ± 395.3 (n = 3; **B**). The IC_{50} values are given with 95% confidence limits in parentheses. * denotes the first concentration of IBMX producing significant ($P < 0.05$) inhibition of basal activity

(Fig. 5B). However, under the combined action of isoprenaline and pimobendan force of contraction in failing preparations was enhanced by about 260% (Fig. 5B) and cAMP content was elevated to similar values as with isoprenaline or pimobendan alone in nonfailing preparations (Fig. 5A; g vs c, b).

Discussion

In the present study the PDE III inhibitors pimobendan, adibendan, and saterinone and the β-adrenoceptor agonist isoprenaline revealed either markedly reduced positive inotropic effects or no effects at all in ventricular trabeculae derived from hearts of patients with end-stage heart failure. In contrast, the effects of calcium and of the cardiac steroid dihydroouabain were not diminished in these preparations. The concomitant application of isoprenaline and a PDE inhibitor restored the effectiveness of the β-adrenoceptor agonist. The lack of effect of the PDE inhibitors in failing human myocardium could not be explained by an altered PDE inhibition, as the properties and the inhibition of PDE activity of the PDE isoenzymes I–III were not changed in failing hearts compared with nonfailing preparations.

Usually, isolated cardiac preparations from laboratory animals are chosen to investigate the direct effects

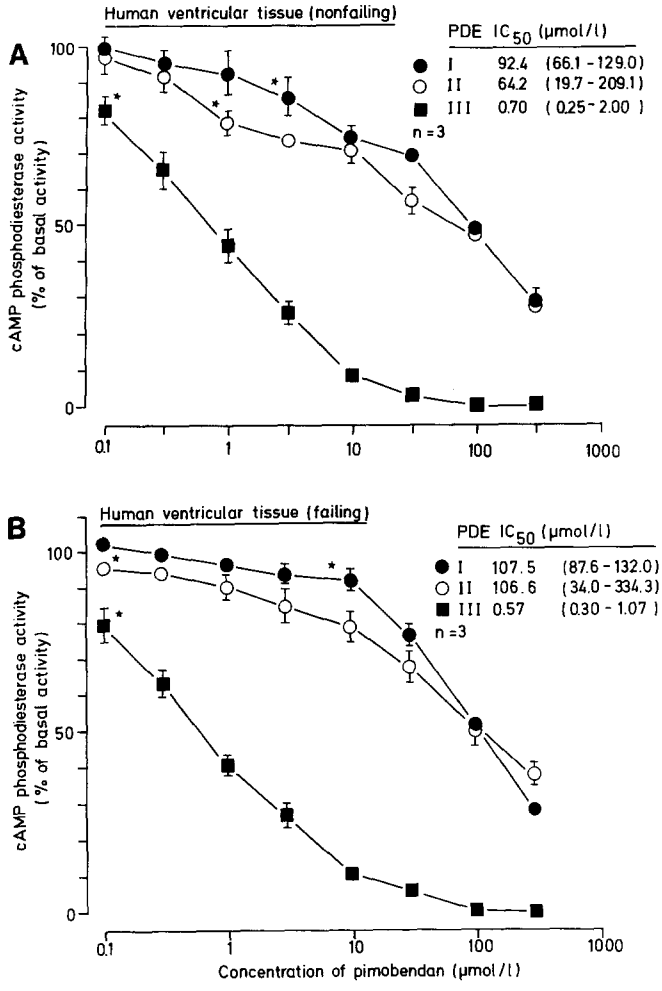

Fig. 4A, B. Effects of pimobendan on the activity of cAMP PDE I–III isolated from ventricular tissue of **A** three nonfailing and **B** three failing human hearts. *Ordinates,* cAMP PDE activity as percentage of basal activity, *abscissae,* concentration of pimobendan (µmol/l). Substrate concentration: 1 µmol/l cAMP. Incubation time 10 min at 30°C. Basal activities (pmol cAMP/mg protein · min): PDE I: 1196 ± 585.3 (n = 3; **A**), 1501 ± 677.8 (n = 3; **B**); PDE II: 207.7 ± 51.0 (n = 3; **A**), 311.0 ± 10.1 (n = 3; **B**); PDE III: 4946 ± 888.0 (n = 3; **A**), 5220 ± 224.6 (n = 3; **B**). The IC_{50} values are given with 95% confidence limits in parentheses. * denotes the first concentration of pimobendan producing significant ($P < 0.05$) inhibition of basal activity

of new compounds on force of contraction. Thus, pimobendan, adibendan, and saterinone exerted marked positive inotropic effects in guinea-pig papillary muscles (Honerjäger et al. 1984; Berger et al. 1985; Bethke et al. 1988; Brunkhorst et al. 1988, 1989). Nonetheless, several studies on preparations from failing human hearts have revealed a loss of responsiveness to positive inotropic stimulation by cAMP-increasing agents such as β-adrenoceptor agonists and PDE inhibitors, including isoprenaline, dobutamine, denopamine, zinterol, caffeine, isobutylmethylxanthine, amrinone, milrinone, and enoximone (Wilmshurst et al. 1984; Feldman et al. 1987; Schmitz et al. 1987; Böhm et al. 1988a, b; Erdmann 1988; Bristow et al. 1989). In some of these studies the reduction of the positive inotropic effect of these agents depended on the functional class of heart failure (NYHA grade); that is to say, the worse the disease, the more reduced was the effect (Wilmshurst et al. 1984; Böhm et al. 1988a, b; Erdmann 1988). Thus, it is not possible to extrapolate results obtained in normal guinea-pig myocardium to diseased human myocardium. In line with these studies the PDE inhibitors pimobendan, adibendan, and saterinone exerted hardly any positive inotropic effects in preparations from human hearts with end-stage myocardial failure (NYHA IV) in the present paper. In addition, the effect of the β-adrenoceptor agonist isoprenaline was markedly diminished. In contrast, the responses to dihydroouabain and to calcium were virtually not affected. Similarly, other groups did not find any differences in the efficacies of calcium (Ginsburg et al. 1983; Feldman et al. 1987) or cardiac glycosides (Feldman et al. 1987) between nonfailing and failing human heart preparations. Thus, isolated preparations from failing human hearts are able to generate a normal or almost normal increase in contractile force. Therefore, it is unlikely that the lack of efficacy of the PDE inhibitors was due to an impaired contractile apparatus in the failing heart. Rather, it could be due to altered basal PDE activities or impaired sensitivities of the enzymes for PDE inhibitors.

By means of DEAE-cellulose chromatography, three PDE isoenzymes have been separated from both nonfailing and failing ventricular tissue. The properties and characteristics (substrate specificity, kinetic behaviour, basal activities) of these isoenzymes did not differ between the two kinds of human myocardial tissue and were also similar to those found with comparable methods in guinea-pig hearts in our laboratory (Bethke et al. 1988; Brunkhorst et al. 1988) and by others (Weishaar et al. 1986). Recently, similar PDE isoenzymes have been observed also by DEAE-cellulose chromatography in human ventricular tissue (Ito et al. 1988). By virtue of the improved resolving properties of DEAE-Sepharose compared with DEAE-cellulose used in this and other studies, Reeves et al. (1987) were able to resolve a fourth PDE activity, PDE IV, in failing human hearts, which was eluted closely behind PDE III. The properties of PDE I, II and III activities were consistent with the properties described here and elsewhere (Weishaar et al. 1986; Ito et al. 1988). Therefore it is necessary to consider a possible contamination of the PDE III by PDE IV in the present study. However, this seems rather unlikely, since PDE IV was found to be potently inhibited by rolipram but not by the PDE III inhibitor SK & F 94120 (Reeves et al. 1987). Our results are similar to those of Reeves et al. (1987) insofar as PDE III of the present study was effectively inhibited by selective PDE III inhibitors but not by the PDE IV-selective agent rolipram, as was noted in preliminary experiments (not shown). It should be mentioned that by means of the method used, mostly soluble PDE isoenzymes have been separated. Therefore, the possibility cannot be excluded that membrane-bound PDEs, which hitherto have not been isolated in human heart muscle, may play a functional role or may undergo changes in heart failure.

As in the guinea-pig heart (Bethke et al. 1988; Brunkhorst et al. 1988, 1989), pimobendan, adibendan,

Table 2. Inhibition of cAMP PDE I–III isolated from ventricular tissue of three nonfailing human hearts by adibendan, pimobendan, saterinone, milrinone, and IBMX. IC_{50} values (concentration of drugs reducing the basal PDE activity to 50%) are given as geometrical means with 95% confidence limits in parentheses. SF (selectivity factor) is the mean of the IC_{50} values of PDE I and II divided by the IC_{50} value of PDE III. The greater this ratio, the more selective is the PDE III inhibition

Agent	cAMP PDE, IC_{50}, μmol/l, nonfailing human ventricular tissue			
	I	II	III	SF
Adibendan	275.5 (183.1–414.6)	44.4 (38.9– 50.6)	0.58 (0.40–0.86)	275.8
Pimobendan	92.4 (66.1–129.0)	64.2 (19.7–209.1)	0.70 (0.25–2.00)	111.9
Saterinone	43.8 (33.7–57.1)	6.08 (0.46–80.4)	0.05 (0.04–0.07)	498.8
Milrinone	168.6 (152.4–186.6)	41.4 (14.9–114.7)	1.14 (0.84–1.55)	92.1
IBMX	13.7 (7.9–23.7)	33.6 (12.6–89.4)	8.23 (5.7–11.8)	2.9

Table 3. Inhibition of cAMP PDE I–III isolated from ventricular tissue of three failing human hearts by adibendan, pimobendan, saterinone, milrinone, and IBMX. IC_{50} values (concentration of drugs reducing the basal PDE activity to 50%) are given as geometrical means with 95% confidence limits in parentheses.

Agent	cAMP PDE, IC_{50}, μmol/l, failing human ventricular tissue			
	I	II	III	SF
Adibendan	188.9 (92.2–386.8)	67.3 (33.2–136.3)	0.61 (0.20–1.90)	210.0
Pimobendan	107.5 (87.6–132.0)	106.6 (34.0–334.3)	0.57 (0.30–1.07)	187.8
Saterinone	35.7 (28.1–45.5)	17.2 (5.95–49.6)	0.03 (0.02–0.07)	881.7
Milrinone	204.6 (139.5–300.2)	97.9 (38.9–245.8)	1.35 (0.59–3.07)	112.0
IBMX	17.6 (15.6–19.8)	29.0 (14.3–58.8)	6.64 (4.00–11.0)	3.5

and saterinone exerted positive inotropic effects in nonfailing human hearts. Even though these compounds exerted no positive inotropic effects in preparations from failing human hearts, however, they inhibited the PDE III of these hearts in a manner similar to that of nonfailing preparations. These results are in accord with those described by Böhm et al. (1988b), who did not detect any difference in the basal activities of crude cAMP PDE from nonfailing, moderately failing (NYHA II–III), and severely failing (NYHA IV) myocardial tissues. The inhibitory effects of milrinone also did not differ between the three groups. Thus, an impairment of PDE activity and/or PDE inhibition apparently does not contribute to the pathogenesis of myocardial failure and cannot explain the lack of efficacy of the PDE inhibitors in increasing force of contraction.

The common mechanism of action of positive inotropic PDE inhibitors and of β-adrenoceptor agonists is an increase in cAMP. Therefore, it is conceivable that an inadequate rise in myocardial cAMP is involved in the decreased responsiveness of failing heart muscle preparations to these agents and in fact a reduced adenylate cyclase activity in cardiac membrane preparations after stimulation with isoprenaline was reported as long ago as 1982 (Bristow et al. 1982). However, the cAMP content in intact contracting human cardiac preparations after stimulation with PDE inhibitors has not yet been investigated. In the present study a reduced basal cAMP content was measured in failing hearts, and pimobendan enhanced the cAMP levels only as high as the basal values in nonfailing hearts. Thus, in spite of pronounced inhibition of isolated phosphodiesterases in failing myocardial tissue too, PDE inhibitors do not elevate cAMP levels to amounts sufficient to produce positive inotropism similar to that seen in nonfailing heart preparations. These data indicate that impaired cAMP formation may be a fundamental defect in hearts of patients with end-stage heart failure. It has been suggested that β-adrenoceptor down-regulation in response to chronically increased levels of circulating catecholamines in heart fail-

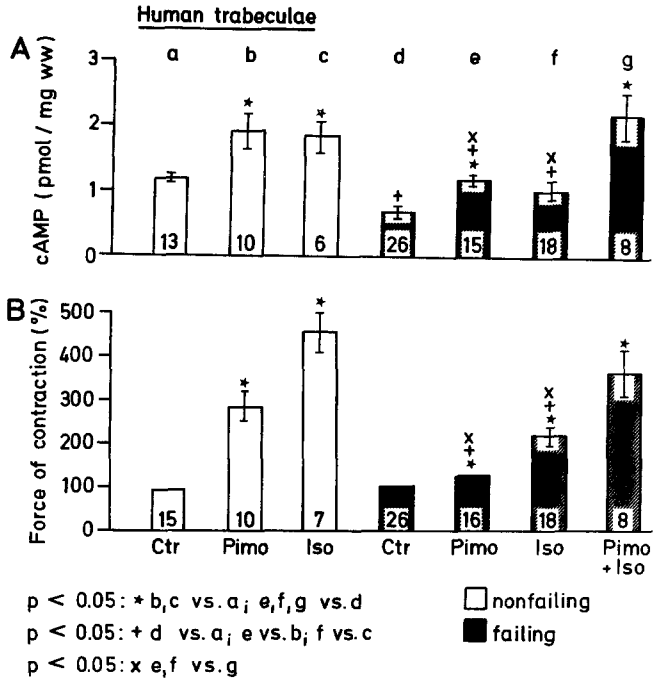

Fig. 5. **A** Cyclic AMP content in pmol/mg wet weight and **B** force of contraction as percentage of pre-drug value in electrically driven (0.5 Hz) right ventricular trabeculae carneae isolated from nonfailing and failing human hearts in drug-free bathing solution (*a* and *d*, control) and after addition of pimobendan (*b* and *e*, *Pimo*, 100 μmol/l for 15 min) or isoprenaline (*c* and *f*, *Iso*, 0.2 μmol/l for 5 min). *g*, Isoprenaline was given for a further 5 min after pimobendan had been administered for 10 min. *Numbers* in *columns* denote numbers of experiments. The pre-drug values for force of contraction were 1.26 ± 0.25 mN (32 preparations from five nonfailing hearts) and 1.58 ± 0.12 mN (68 preparations from six failing hearts)

ure may be involved (Thomas and Marks 1978; Levine et al. 1982; Bristow et al. 1982; Ginsburg et al. 1983). But only β_1-adrenoceptors and not β_2-adrenoceptors are appreciably down-regulated in failing myocardial tissue (Bristow et al. 1986), whereas the β_2-adrenergic positive inotropic response is also blunted. This has been explained by an uncoupling of the β_2-adrenoceptor from the adenylate cyclase (Bristow et al. 1989). Furthermore, the positive inotropic effects of histamine and glucagon, which activate the adenylate cyclase independently of the β-adrenoceptor, are also markedly attenuated in the failing heart (Goldstein et al. 1971; Böhm et al. 1988a; Erdmann 1988; Packer 1988a). On the other hand, forskolin, acting directly on the catalytic subunit of the adenylate cyclase (Daly 1984), retained full enzyme-stimulating activity (Bristow et al. 1984) and full inotropic response (Böhm et al. 1988a; Erdmann 1988) in preparations derived from failing hearts. All these observations suggest that besides β_1-adrenoceptor down-regulation a defect in the receptor-adenylate cyclase coupling may be responsible for the decreased activity of this enzyme (Erdmann 1988; Packer 1988a). This is supported by the recent finding that an inhibitory guanine nucleotide binding protein (G_i-protein) is increased in membranes of failing human myocardium (Feldman et al. 1988; Neumann et al. 1988). However, this increase was only found in idiopathic dilated cardiomyopathy and not in ischaemic heart disease (Neumann et al. 1988; Böhm et al. 1990). Thus, adenylate cyclase activity might be kept at a reduced level by β_1-adrenoceptor down-regulation and at least in idiopathic dilated cardiomyopathy also or mainly by the enhanced G_i-protein. As a result, the cAMP content does not adequately increase in response to β-adrenergic agents and might be insufficient to induce pronounced positive inotropic effects (Danielsen et al. 1989).

Clinical implications

In view of the present results showing no positive inotropic effects of the PDE inhibitors in preparations from failing human hearts, it might be assumed that these agents do not have any beneficial effects in the treatment of congestive heart failure. But haemodynamic improvement, which has been attributed to a vasodilatory as well as to a positive inotropic effect of the PDE III inhibitors, is obviously achieved with the use of pimobendan, adibendan, and saterinone in patients with severe heart failure (Katz et al. 1987; Rauch et al. 1988; Renard et al. 1988; Saborowski et al. 1988; Baumann et al. 1989; Hauf et al. 1989). Previous (Böhm et al. 1988b; Gilbert et al. 1989) and the present in vitro results showing that the reduced positive inotropic effect of adrenergic agonists in preparations from failing human hearts was restored in the presence of the PDE inhibitors may provide an explanation for the clinical effects. The restored positive inotropic effect can be explained by the present finding that the combination of adenylate cyclase stimulation by isoprenaline with PDE inhibition in failing hearts increased the cAMP level to similar values to those obtained with isoprenaline or pimobendan alone in nonfailing hearts. Patients with heart failure are under enhanced β-adrenergic stimulation due to elevated levels of circulating catecholamines (Thomas and Marks 1978; Levine et al. 1982). In this situation the therapy with a PDE inhibitor may increase myocardial force of contraction because of the combined action of the PDE inhibitor and the endogenous catecholamines. In addition, in patients with congestive heart failure the combined administration of a β-adrenoceptor agonist with a PDE inhibitor revealed an additive improvement in left ventricular performance, as demonstrated by the combination of dopamine with theophylline (Morgan et al. 1983), dobutamine with amrinone (Gage et al. 1986), dobutamine with milrinone (Colucci 1989), or dobutamine with enoximone (Gilbert et al. 1988, 1989). Thus, therapy with PDE inhibitors may restore β-adrenergic responsiveness in the failing myocardium overriding the G_i-protein-mediated inhibitory signal transduction. However, it must be borne in mind that the combined administration of β-adrenergic agents and PDE inhibitors may increase the arrhythmogenic risk in patients.

In conclusion, the inability of PDE inhibitors to elicit pronounced positive inotropic effects in preparations from failing human hearts is not due to an altered PDE inhibition but may rather be due to an impaired cAMP

formation resulting from a defect in receptor-adenylate cyclase coupling. The combined action of isoprenaline and a PDE inhibitor restores the positive inotropic effect of the β-adrenoceptor agonist. This may have two possible clinical implications. Firstly, the continuous β-adrenergic adenylate cyclase stimulation by elevated levels of catecholamines may be sufficient for the maintained positive inotropic effects of the PDE inhibitors in patients with heart failure. Secondly, in terminal heart failure refractory to conventional therapeutic regimens the combined application of a β-adrenoceptor agonist and a PDE inhibitor might be beneficial.

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