The identification of a new cyclic nucleotide phosphodiesterase activity in human and guinea-pig cardiac ventricle

Implications for the mechanism of action of selective phosphodiesterase inhibitors

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Four cyclic nucleotide phosphodiesterase (PDE) activities were separated from low-speed supernatants of homogenates of human cardiac ventricle by DEAE-Sephacel chromatography, and designated PDE I–PDE IV in order of elution with an increasing salt gradient. PDE I was a Ca^{2+} /calmodulin-stimulated activity, and PDE II was an activity with a high K_m for cyclic AMP which was stimulated by low concentrations of cyclic GMP. Human ventricle PDE III had K_m values of $0.14 \mu\text{M}$ (cyclic AMP) and $4 \mu\text{M}$ (cyclic GMP), and showed simple Michaelis–Menten kinetics with both substrates. PDE IV is a previously unrecognized activity in cardiac muscle, the human enzyme having K_m values of $2 \mu\text{M}$ (cyclic AMP) and $50 \mu\text{M}$ (cyclic GMP). PDE III and PDE IV were not activated by cyclic nucleotides or calmodulin. Four PDE activities were also isolated from guinea-pig ventricle, and had very similar kinetic properties. By gel filtration, the M_r of PDE III was 60000, and that of PDE IV 45000. The drug SK&F 94120 selectively and competitively inhibited PDE III with a K_i value of $0.8 \mu\text{M}$ (human), showing simple hyperbolic inhibition kinetics. Rolipram (Schering ZK 62711) and Ro 20-1724 (Roche), which have previously been reported to inhibit PDE III-like activities strongly, were shown to be weak inhibitors of human and guinea-pig PDE III enzymes (K_i values $> 25 \mu\text{M}$), but potent inhibitors of PDE IV [K_i values $2.4 \mu\text{M}$ (Rolipram) and $3.1 \mu\text{M}$ (Ro 20-1724) with human PDE IV]. The inhibition in all cases demonstrated simple hyperbolic competition. These observations suggest that the previously reported complex inhibition of PDE III-type activities from cardiac muscle was caused by incomplete separation of the PDE III from other enzymes, particularly PDE IV.

INTRODUCTION

The function of cyclic nucleotide phosphodiesterase (PDE) is to terminate the actions of cyclic 3',5'-nucleotides by catalysing their hydrolysis to nucleoside 5'-monophosphates (Butcher & Sutherland, 1962). Cyclic AMP and cyclic GMP are intracellular second messengers controlling a wide variety of processes linked to hormonal regulation (Krebs & Beavo, 1979).

The existence of multiple forms of PDE in cardiac tissues has been reviewed previously (Wells & Hardman, 1977; Beavo *et al.*, 1982). In general, cardiac-ventricle sources have been found to contain three isoforms of PDE: a Ca^{2+} /calmodulin-stimulated activity, a cyclic GMP-stimulated activity, and an activity demonstrating substrate selectivity and high affinity for cyclic AMP ('low- K_m ' form).

Much attention has been focused on cardiac-ventricle PDE isoenzymes since the recognition of a range of compounds with positive inotropic properties which potently and specifically inhibit the 'low- K_m ' PDE activity. In several reports the 'low- K_m ' PDE activity isolated from cardiac ventricle showed non-Michaelis–Menten kinetics for cyclic AMP, which were attributed to negative co-operativity of substrate binding (Russell *et al.*, 1972; Weishaar *et al.*, 1986). However, similar kinetics would also have been obtained if other PDE isoenzymes were present in the 'low- K_m ' PDE fraction,

because of incomplete resolution during the isolation procedure. Very recently, Harrison *et al.* (1986) have reported the purification of a PDE activity from bovine heart with a low K_m for cyclic AMP, which has Michaelis–Menten kinetics.

There has been one brief study of human cardiac ventricle PDEs, three activities typical of other cardiac-ventricle sources being reported (Hidaka *et al.*, 1977). In the present study we have re-investigated human cardiac-ventricle PDE in order to characterize these activities more fully. We report the identification of a new PDE activity in human cardiac ventricle, which was also found in guinea-pig cardiac ventricle. It is probable that this new PDE activity has been a contaminant of 'low- K_m ' PDE preparations previously reported, thus contributing to the observed negative co-operativity for cyclic AMP hydrolysis by this enzyme.

METHODS

Materials

[8- ^3H]Adenosine 3',5'-cyclic phosphate (15–26.5 Ci/mmol), [8- ^3H]guanosine 3',5'-cyclic phosphate (15 Ci/mmol), [U- ^{14}C]AMP (507 mCi/mmol) and [U- ^{14}C]GMP (526 mCi/mmol) were obtained from Amersham International (Amersham, Bucks., U.K.). DEAE-Sephacel CL-6B and protein M_r standards were obtained from Pharmacia (Milton Keynes, Bucks., U.K.).

Affi-Gel 601 was obtained from Bio-Rad Laboratories (Watford, Herts., U.K.), Ro 20-1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidine] was a gift from Roche Products (Welwyn Garden City, Herts., U.K.), and Rolipram [ZK 62,711; 4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone] was a gift from Schering A.G. (W. Berlin, Germany). SK&F 94120 {5-(4-acetamidophenyl)pyrazin-2[1*H*]-one} was synthesized by the Department of Medicinal Chemistry, Smith Kline and French Research Ltd., Welwyn, Herts., U.K. SK&F 94120 has been shown previously to be a potent inotropic agent in several animal species (Coates *et al.*, 1985) and man (Cameron *et al.*, 1985), and a selective inhibitor of the 'low- K_m ' PDE isoenzyme (Gristwood *et al.*, 1985). All other chemicals were obtained either from Sigma Chemical Co. or BDH Chemicals (both of Poole, Dorset, U.K.), and were normally of AnalaR or equivalent grade. Male Dunkin-Hartley guinea pigs were obtained from Porcellus Animal Breeding Ltd., Heathfield, Sussex, U.K.

Samples of left-ventricular papillary muscle were obtained from three cardiac-transplant recipients with ischaemic heart disease or cardiomyopathy undergoing surgery at Papworth Hospital, Cambs., U.K. The co-operation of Mr. T. A. H. English and Mr. J. Wallwork of Papworth Hospital in providing these samples is gratefully acknowledged.

Separation of cardiac-ventricle PDE activities

Samples of human cardiac ventricle were frozen on solid CO₂ after removal of ischaemic tissue, and stored at -70 °C until use. All subsequent procedures were carried out at 4 °C. Samples of tissue were thawed on ice, sliced and homogenized in 10 vol. of 20 mM-BisTris/5 mM-2-mercaptoethanol/2 mM-benzamidine/2 mM-EDTA/50 mM-sodium acetate, pH 6.5, in a Polytron instrument for 10 s at setting 3 and 10 s at setting 6. Phenylmethanesulphonyl fluoride was dissolved in propan-2-ol and added to the buffer immediately before homogenization to give a final concentration of 50 µM (1 µl/l). The homogenate (50 ml) was centrifuged for 20 min at 25000 *g* and the supernatant applied to a column (17 cm × 1.5 cm) of DEAE-Sepharose CL-6B pre-equilibrated with homogenization buffer. A flow rate of 80 ml/h was used throughout the ion-exchange chromatography. The column was washed with 120 ml of homogenization buffer, and the PDE activities were eluted with a linear gradient of 0.05–1.0 M-sodium acetate in homogenization buffer; 7.5 ml fractions were collected and assayed for PDE activity, and preliminary estimates of kinetic parameters obtained within 24 h by using the peak fractions. For long-term storage, ethylene glycol was added to a final concentration of 30% (v/v) and fractions were stored at -20 °C. Activity was stable for several weeks under those conditions.

Guinea-pig cardiac ventricle PDE activities were obtained in a similar manner. Guinea pigs were killed by cervical dislocation and hearts were removed and placed on ice. The ventricles were dissected and homogenized as for human ventricle. The homogenate was centrifuged at 12000 *g* for 10 min. The subsequent procedures employed were the same as for the human ventricle.

Assay of PDE activity

PDE activity was determined by a modification of the method of Davis & Daly (1979). The standard reaction

mixture contained, in a final volume of 100 µl: 50 µM-[U-¹⁴C]AMP (sp. radioactivity 6Bq/nmol), 0.5 µM-cyclic [8-³H]AMP (sp. radioactivity 40Bq/pmol), 50 mM-Tris/HCl, 5 mM-MgCl₂, pH 7.5. The reaction was initiated by the addition of 10 µl of enzyme preparation, and was incubated for 10 min at 37 °C. The reaction was terminated by heating at 100 °C for 2 min. After cooling, 500 µl of 0.1 M-Hepes/0.1 M-NaCl, pH 8.5, was added to each assay tube, and the total contents were applied to a column (1.2 ml bed volume) of Affi-Gel 601 (boronate affinity gel) previously equilibrated with Hepes/NaCl buffer. The column was freed of unchanged cyclic AMP by 15 l ml washes with the same buffer. The reaction product, AMP, was eluted in 6 ml of 0.25 M-acetic acid, and radioactivity was measured by liquid-scintillation spectrometry after the addition of 10 ml of Instagel (Packard).

The recovery of product, as estimated from the [¹⁴C]AMP recovered, was 65–85%, this being used to correct the recoveries of [³H]AMP. Assays were performed in duplicate, and corrected for blank values (approx. 0.5% of AMP produced). All assays were conducted in the linear range of the reaction, where less than 20% of the initial substrate was hydrolysed. An identical protocol was employed for assays of cyclic GMP hydrolysis.

Determination of M_r

Determination of M_r by gel-exclusion chromatography was carried out on a Superose 12/30 (Pharmacia) column in conjunction with the Pharmacia fast protein liquid chromatography system. All operations were carried out at 4 °C. With PDE III activity, two fractions of the peak containing human ventricle PDE III were combined and the activity was precipitated with 70% -satd. (NH₄)₂SO₄. The pellet was resuspended in 250 µl of 100 mM-NaCl/20 mM-BisTris/5 mM-2-mercaptoethanol/2 mM-benzamidine/2 mM-EDTA, pH 6.5, and dialysed overnight against the same buffer. With PDE IV activity, the peak fraction from the DEAE-Sepharose chromatography was used without further treatment. The samples were centrifuged at 430000 *g* in a Beckman TL-100 centrifuge for 5 min, and 300 µl of the supernatant was applied to the Superose 12/30 column at a flow rate of 0.3 ml/min. After application of the sample, the column was developed with fresh dialysis buffer at the same flow rate; 0.5 ml fractions were collected and assayed for PDE activity.

Determination of kinetic parameters

For determination of values of V_{max} , K_m and h (Hill coefficient), where the concentration of cyclic AMP (or cyclic GMP) was being varied, the amount of ³H-labelled cyclic nucleotide per assay tube was kept constant, and appropriate corrections were made for the decrease in specific radioactivity of the substrate. The data were analysed by non-linear least-squares regression analysis to obtain values for V_{max} , K_m and h .

Determination of inhibition constants was carried out by selecting a grid of substrate and inhibitor concentrations which spanned the K_m and estimated K_i values of the enzyme. The data were fitted *in toto* to various inhibitor equations by non-linear least-squares regression, and the most appropriate equation was selected by analysis of variance of the residuals (Draper & Smith, 1966).

RESULTS AND DISCUSSION

Separation of PDE activities

The profile of PDE activities obtained from chromatography of human cardiac-ventricle 25000 g supernatant is shown in Fig. 1. Four activities could be distinguished, on the basis of elution profiles measured at 0.5 μ M-cyclic AMP, 25 μ M-cyclic AMP, 1 μ M-cyclic GMP and 0.5 μ M-cyclic AMP in the presence of the selective PDE inhibitor SK&F 94120 (see below). The PDE activities present in these fractions have been named PDE I–PDE IV (see Fig. 1) for the purposes of this paper. The

Fig. 1. Profile of elution of PDE activities from human cardiac ventricle after DEAE-Sephacel chromatography

An extract of human ventricle was prepared and applied to a column (17 cm $\times$ 1.5 cm) of DEAE-Sephacel equilibrated as described in the Methods section. The column was eluted with a sodium acetate gradient ($\square$), 7.5 ml fractions being collected. Activity was assayed with 0.5 μ M-cyclic AMP ($\blacktriangle$), 0.5 μ M-cyclic AMP + 100 μ M-SK&F 94120 ($\triangle$), 25 μ M-cyclic AMP ($\bullet$) or 1 μ M-cyclic GMP ($\circ$).

total recovery of PDE activity from the column measured at 1 μ M-cyclic AMP was approx. 75% of that applied. However, the recovery of PDE III activity was only 50% (as assessed by inhibition by SK&F 94120), indicating that this isoenzyme was recovered with lower efficiency. K_m and relative V_{max} values for the four PDE activities with both cyclic AMP and cyclic GMP are shown in Table 1. Single peak fractions were assayed for determining K_m values, to lessen the possibility of cross-contamination from adjacent peaks of activity.

The kinetic properties of PDE I and II are similar to data published elsewhere for these activities [Hidaka *et al.*, 1977 (PDE I); Martins *et al.*, 1982 (PDE II); Weishaar *et al.*, 1986]. The PDE I activity demonstrated relatively high affinity for both cyclic AMP and cyclic GMP and was stimulated by Ca^{2+} /calmodulin. The effect of Ca^{2+} /calmodulin was to increase the V_{max} of PDE I for both cyclic AMP and cyclic GMP, with no effect on the K_m values. The relatively high affinity of cyclic AMP for PDE I is a property of cardiac-ventricular enzyme from many species, including guinea pig (see below) and cat (M. L. Reeves, unpublished work), and contrasts with the low affinity for cyclic AMP demonstrated by the Ca^{2+} /calmodulin-stimulated PDE from brain (Kincaid *et al.*, 1984), and previously reported for the enzyme from heart (Ho *et al.*, 1977; LaPorte *et al.*, 1979).

The presence of multiple forms of Ca^{2+} /calmodulin-dependent PDE in a single tissue has been reported previously for rat testis (Purvis *et al.*, 1981). However, we have found no evidence for the presence of multiple forms of the Ca^{2+} /calmodulin-dependent PDE in human, cat or guinea-pig cardiac ventricle, although it is possible that these activities may be lost during the chromatographic procedures. A preparation of PDE I from one patient was not stimulated by Ca^{2+} /calmodulin, although there was no change in the K_m values for cyclic AMP or cyclic GMP. This may indicate limited proteolysis of the enzyme in this one preparation. Indeed, it is well documented that proteolysis of Ca^{2+} /calmodulin-sensitive PDE generates an activity which is no longer activated by Ca^{2+} /calmodulin (Tucker *et al.*, 1981). Alternatively, this could be a consequence of the diseased state of the tissue.

The second peak of activity (PDE II) demonstrated positive co-operativity, with Hill coefficients of 1.5 for hydrolysis of cyclic GMP and 1.8 for cyclic AMP. As

Table 1. Kinetic properties of human cardiac-ventricle PDE activities

PDE activities were separated from human ventricle as shown in Fig. 1. Kinetic constants were determined as described in the Methods section. Abbreviation: n.d., not determined.

Enzyme	K_m for cyclic AMP (μ M)	K_m for cyclic GMP (μ M)	Ratio		Activator
			V_{max} with cyclic AMP	V_{max} with cyclic GMP	
PDE I	0.75	1.0	1		Ca^{2+} /calmodulin
PDE II	43*	10*	1		Cyclic GMP
PDE III	0.14	4	0.4		None
PDE IV	2.1	> 50	n.d.		None

* The kinetics for PDE II showed positive co-operativity. The numbers quoted are the $K_{0.5}$ values (concentration required to give half-maximal velocity). The Hill coefficient with cyclic AMP is 1.8, and that with cyclic GMP is 1.5.

reported previously for PDE II from bovine adrenal gland and heart (Martins *et al.*, 1982) and liver (Pyne *et al.*, 1986), cyclic AMP hydrolysis catalysed by this PDE was stimulated 5-fold by low concentrations of cyclic GMP (results not shown).

The third peak (PDE III) had a very high affinity for cyclic AMP (K_m 0.14 μM) and a relatively high affinity for cyclic GMP (K_m 4 μM). The hydrolysis of both substrates showed simple Michaelis-Menten kinetics. This is in agreement with the results of Harrison *et al.* (1986), who have reported that a highly purified PDE III-type activity from bovine ventricle exhibited Michaelis-Menten kinetics and high affinity for both cyclic AMP and cyclic GMP. This is in contrast with the properties previously reported for PDE III-like activities obtained from cardiac muscle after DEAE-cellulose ion-exchange chromatography (Kariya *et al.*, 1982; Weishaar *et al.*, 1986), where negative co-operativity for hydrolysis of cyclic AMP and poor affinity for cyclic GMP were observed. These differences are discussed in more detail below.

The fourth peak of activity (PDE IV), an enzyme that has not been described previously in cardiac ventricle, had a relatively high affinity for cyclic AMP (K_m 2.1 μM), but a low affinity for cyclic GMP ($K_m > 50 \mu\text{M}$). The kinetics for cyclic AMP hydrolysis were simple Michaelis-Menten. Although this enzyme has a K_m value that is considerably higher than that of PDE III, it is still within the physiological range of cyclic AMP values reported in the heart (England, 1976). The V_{max} of the PDE IV activity in heart homogenates, estimated with a potent inhibitor of PDE IV (Rolipram, see below), was 0.19 μmol of cyclic AMP hydrolysed/min per g of protein. This should be compared with a value of 0.14 $\mu\text{mol}/\text{min}$ per g for the V_{max} activity of PDE III in the same homogenate, estimated from inhibition by SK&F 94120. At 1 μM -cyclic AMP, therefore, the relative ratio of hydrolysis of cyclic AMP by PDE IV:PDE III would be 0.47:1 in the intact tissue, assuming that both enzymes are present in the same cell compartment. PDE IV could therefore be of considerable importance in the control of cyclic AMP concentrations (but not cyclic GMP) in the heart.

The presence of this new PDE IV activity was also shown in cardiac tissue from species other than human. Chromatography of PDE activities from guinea-pig cardiac ventricle on DEAE-Sepharose is shown in Fig. 2. Four activities, including PDE III and PDE IV, were resolved. However, the resolution of PDE III and PDE IV was only observed clearly after fractions were assayed in the presence of the selective PDE III inhibitor SK&F 94120. The properties of these activities showed considerable similarities to those of the human cardiac PDE enzymes. In particular, the K_m for cyclic AMP hydrolysis by guinea-pig PDE III was 0.33 μM , and for PDE IV, 1.3 μM (results not shown). The most important difference in the profile of guinea-pig PDE activities is that PDE IV is eluted before PDE III, in contrast with the human cardiac-ventricle PDE profile, in which PDE IV is eluted after PDE III. This difference may be caused by human PDE IV being modified during the extended surgical procedures and subsequent handling. However, tissue was frozen as quickly as possible, and proteinase inhibitors were included in the homogenization buffer. Guinea-pig ventricle preparations were applied to the DEAE-Sepharose column within 30 min of dissection

Fig. 2. Profile of elution of PDE activities from guinea-pig cardiac ventricle after DEAE-Sepharose chromatography

An extract of guinea-pig ventricle was prepared and applied to a column (17 cm $\times$ 1.5 cm) of DEAE-Sepharose equilibrated as described in the Methods section. The column was eluted with a sodium acetate gradient ($\square$), 7.5 ml fractions being collected. Activity was assayed with 0.5 μM -cyclic AMP ($\blacktriangle$), 0.5 μM -cyclic AMP + 100 μM -SK&F 94120 ($\triangle$), 25 μM -cyclic AMP ($\bullet$) or 1 μM -cyclic GMP ($\circ$).

from the animal, and thus are less likely to be modified chemically or enzymically. As with the human enzymes, both PDE III and PDE IV activities from guinea pig showed simple hyperbolic kinetics with cyclic AMP. The similarity of kinetic properties of PDE IV activities from human and guinea-pig ventricle suggest that, even if the human enzyme had undergone partial proteolysis, this would result in only slight changes in activity.

The resolution of four PDE activities from guinea-pig cardiac ventricle is in contrast with previous reports in which only three activities could be resolved, i.e. PDE IV was not identified (Weishaar *et al.*, 1986). In these reports, chromatography on DEAE-cellulose was used to separate PDE activities, in contrast with DEAE-Sepharose used by ourselves. The ability to resolve PDE activities eluted close together appears to be decreased in DEAE-cellulose chromatography. With guinea-pig ventricular preparations we have shown that, although PDE IV can be resolved from PDE III on DEAE-Sepharose (Fig. 2), on DEAE-cellulose the PDE III and PDE IV activities combine to appear as a single unresolved peak of activity (results not shown). The presence of a fourth PDE activity which chromatographs close to PDE III may explain the differences between the properties of PDE III reported by ourselves and those of other workers. In fact, the properties of the unresolved peak containing both PDE III and PDE IV activities isolated

Table 2. Kinetic constants of inhibitors of human cardiac-ventricle PDE activities

Kinetic constants were calculated either from kinetic plots similar to that shown in Fig. 3, or from IC_{50} values (concn. giving 50% inhibition) measured at $0.5 \mu\text{M}$ -cyclic AMP. Abbreviation: N.I., no inhibition up to $100 \mu\text{M}$. Values in parentheses indicate approximate values, not determined accurately because of the weak inhibition observed.

Enzyme	SK&F 94120		Rolipram		Ro 20-1724	
	$K_i (\mu\text{M})$	$IC_{50} (\mu\text{M})$	$K_i (\mu\text{M})$	$IC_{50} (\mu\text{M})$	$K_i (\mu\text{M})$	$IC_{50} (\mu\text{M})$
PDE I } PDE II }	N.I.		N.I.		N.I.	
PDE III	0.8	3.7	(25)	(100)	(25)	(100)
PDE IV	N.I.		2.4	3.0	3.1	3.8

from guinea-pig ventricle by DEAE-cellulose chromatography were similar to those reported previously for PDE III (Weishaar *et al.*, 1985b, 1986), i.e. negative co-operativity and poor affinity for cyclic GMP. This is in contrast with the simple hyperbolic kinetics and relatively high affinity for cyclic GMP reported by us for PDE III. It is our belief that the anomalous kinetics previously reported for PDE III have been caused by contamination with other PDE isoenzymes, particularly PDE IV.

M_r determinations of PDE III and PDE IV

These were determined on a Pharmacia Superose 12/30 gel-exclusion column. Chromatography of PDE III gave a single peak of activity of M_r 60000, which was totally inhibited by SK&F 94120 (results not shown). A M_r of 60000 for PDE III is in good agreement with the M_r quoted for PDE III from other sources (Yamamoto *et al.*, 1984; Grant & Colman, 1984).

Chromatography of PDE IV gave a single peak of activity of M_r 45000 (results not shown). This peak was inhibited by Rolipram, which, as discussed below, is a selective PDE IV inhibitor.

Inhibition of ventricular PDE activities by isoenzyme-selective inhibitors

Considerable evidence for the existence of PDE IV as a separate and unique activity comes from investigations using a range of inhibitors selective for specific PDE isoenzymes. SK&F 94120 is well characterized as an inotropic agent and PDE III inhibitor in the same class as compounds such as Amrinone and Milrinone (Sterling-Winthrop), CI 914 (Warner-Lambert) and MDL 17,043 (Merrell-Dow). The evidence that this class of compounds act as selective inhibitors of PDE III is well documented (Kariya *et al.*, 1982; Weishaar *et al.*, 1986). SK&F 94120 has similarly been characterized (Gristwood *et al.*, 1985; England *et al.*, 1985), and has been shown to be a potent and selective inhibitor of guinea-pig and cat ventricular PDE III. Table 2 shows the inhibition by a number of compounds of the four PDE activities isolated from human ventricle. Fig. 3(a) demonstrates the purely competitive nature of the inhibition of human PDE III by SK&F 94120, giving a K_i value of $0.8 \mu\text{M}$. Of particular interest is the lack of inhibition of PDE IV by SK&F 94120 (Table 2). Identical inhibition patterns for SK&F 94120 were obtained with PDE activities from guinea-pig ventricle (K_i $4.4 \mu\text{M}$ for PDE III; results not shown).

Rolipram and Ro 20-1724 have been implicated as selective inhibitors of the 'low- K_m ' PDE III activity (Weishaar *et al.*, 1985a). When Rolipram and Ro 20-1724 were tested as inhibitors of PDE activities from human ventricle, inhibition of PDE III was seen only

Fig. 3. Competitive nature of the inhibition of human ventricle PDE III by SK&F 94120 and of PDE IV by Rolipram

Initial rates of the PDE activities from DEAE-Sepharose chromatography were measured at various cyclic AMP concentrations in the presence of $50 \mu\text{M}$ ($\square$), $20 \mu\text{M}$ ($\blacksquare$), $5 \mu\text{M}$ ($\triangle$) or no ($\blacktriangle$) inhibitor. (a) Inhibition of PDE III by SK&F 94120; (b) inhibition of PDE IV by Rolipram. The lines drawn are those obtained from non-linear regression of the original untransformed data to the equation for competitive inhibition. The K_i values are given in Table 2.

at high concentrations (K_i approx. $25 \mu\text{M}$; Table 2), indicating that these compounds were weak inhibitors of PDE III activity. In contrast, Rolipram and Ro 20-1724 were found to be potent inhibitors of human ventricle PDE IV activity (Table 2). Inhibition of human PDE IV activity by Rolipram was purely competitive (Fig. 3b), with a K_i value of $2.4 \mu\text{M}$. The K_i value for Ro 20-1724 was $3.1 \mu\text{M}$ (competitive inhibition; results not shown). No inhibition of PDE I or PDE II was observed by either compound at concentrations up to $100 \mu\text{M}$. The observation that PDE IV is inhibited strongly, and PDE III only weakly, by Rolipram and Ro 20-1724 is further evidence that two high-affinity PDE isoenzymes exist in cardiac ventricle (i.e. PDE III and PDE IV), which can clearly be resolved by using selective PDE inhibitors. As shown in Table 2, the selectivity of Rolipram and Ro 20-1724 for PDE IV relative to PDE III is most obviously seen when the IC_{50} values are measured at physiological concentrations ($0.5 \mu\text{M}$) of cyclic AMP. There is then a 20–30-fold difference in the concentration of drug required to cause 50% inhibition.

General discussion

We have shown that human and guinea-pig cardiac ventricles contain at least four distinct PDE activities. Three of these activities have been described previously, i.e. the Ca^{2+} /calmodulin-stimulated form (PDE I), the cyclic GMP-stimulated form (PDE II) and the 'low- K_m ' PDE III form. The fourth activity, PDE IV, has been resolved from PDE III by ion-exchange chromatography by virtue of the improved resolving properties of DEAE-Sepharose compared with DEAE-cellulose, which previously has been the chromatographic medium of choice. It is therefore probable that PDE IV was a contaminant of preparations of PDE III previously described (Weishaar *et al.*, 1986), and this contamination explains the negative co-operativity for cyclic AMP hydrolysis demonstrated by these preparations. PDE IV was found to be potently inhibited by Rolipram and Ro 20-1724. SK&F 94120 is confirmed as a potent, competitive and selective inhibitor of PDE III isoenzyme from human ventricle, the same enzyme inhibited selectively by a wide range of recently identified positive inotropic agents.

The availability of human cardiac ventricle from patients with heart disease has obvious advantages when studying the mechanism of action of agents with potential clinical utility. The isolation of PDE isoenzymes from human heart permits study of drug/enzyme interactions of direct clinical relevance, and removes the potential problem of different isoenzymes being present in animal tissues. However, there are disadvantages in the use of diseased human ventricle as a source of PDE enzymes. Prior drug treatments and disease etiology may affect both the profile and properties of the PDE activities isolated. Similarly the prolonged surgical and storage conditions before homogenization and chromatography may lead to some alterations in PDE activities, perhaps by a limited proteolysis. These factors may explain the observation that in one preparation PDE I activity was not stimulated by Ca^{2+} /calmodulin. The change in the relative positions of elution of PDE III and PDE IV from human ventricle compared with those from guinea-pig ventricle may also be explained by such factors, although it is not possible to rule out genuine

species differences at present. However, we believe that our observations on the four PDE activities are of physiological significance, since the kinetic and inhibitory properties of PDE I, II and III activities reported in this study are consistent with the properties described elsewhere for these enzymes. The physiological relevance of PDE IV is supported by our observations that this activity was present in freshly isolated guinea-pig ventricle. We have also demonstrated considerable PDE IV activity in smooth-muscle preparations from pig aorta and pulmonary artery (M. L. Reeves & P. J. England, unpublished work).

It has been reported that Rolipram, which has been shown above to inhibit PDE IV potently, significantly increased cyclic AMP in guinea-pig cardiac-ventricle slices (Gristwood & Owen, 1986). This observation clearly supports the argument that PDE IV is present in cardiac ventricle. Interestingly, however, it was noted that Rolipram had no positive inotropic effect. This is consistent with our observations that Rolipram only weakly inhibited PDE III, since inhibition of PDE III is thought to be the mechanism by which compounds such as Amrinone and SK&F 94120 exert their inotropic effects.

The existence of a PDE activity with kinetic properties similar to PDE IV, demonstrating a sensitivity to inhibition by Ro 20-1724 but not to cilostamide (a selective PDE III inhibitor), has been described elsewhere (Yamamoto *et al.*, 1984; Elks & Manganiello, 1984). Two activities were described, having properties very similar to PDE III and PDE IV described in the present paper. Those authors commented on the relative sensitivity of these two forms of PDE to inhibition by cyclic GMP. The Rolipram-inhibited PDE was found to be relatively insensitive to cyclic GMP inhibition ($K_i > 100 \mu\text{M}$), whereas the PDE III isoenzyme was very sensitive to inhibition by cyclic GMP (K_i approx. $0.25 \mu\text{M}$) (Yamamoto *et al.*, 1984). We have also noted this difference, which is consistent with the K_m values of PDE III and PDE IV for cyclic GMP. This indicates that inhibition by cyclic GMP is caused by direct binding to the active sites of the enzymes.

The observation of two PDE activities in heart with relatively low K_m values for cyclic AMP prompts the question as to the functional roles of these enzymes in the intact organ. If it is assumed that both these PDEs are present in myocardial cells, why does inhibition of PDE III cause an inotropic response in the intact tissue, whereas inhibition of PDE IV does not (Gristwood *et al.*, 1985)? It is becoming clear that selective PDE inhibition can lead to a variety of discrete pharmacological responses. Rolipram and Ro 20-1724, PDE IV inhibitors, have been described as potent gastric secretagogues (Puurunen *et al.*, 1978). MB 22,948 (May & Baker), a PDE I inhibitor, is known to relax smooth muscle (Kukovetz *et al.*, 1979). MY 5445 (Mitsubishi-Yuka), a selective inhibitor of cyclic GMP-selective PDE, is a very potent inhibitor of platelet aggregation (Hidaka & Endo, 1984). The increasing amount of evidence for selective isoenzymes of PDE being involved in controlling discrete hormonal pharmacological responses raises questions as to how the cell compartmentalizes both cyclic AMP and the PDE enzymes. A thorough investigation of the numbers and properties of PDE activities in any tissue is crucial to the understanding of this complex second-messenger system.

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