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IDENTIFICATION AND SELECTIVE INHIBITION OF FOUR DISTINCT SOLUBLE FORMS OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITY FROM KIDNEY

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Abstract—Homogenization of rat kidney under isotonic conditions and in the presence of protease inhibitors showed that some 92% of the cyclic AMP phosphodiesterase activity and some 83% of the cyclic GMP phosphodiesterase activity was released into the soluble fraction. Analysis of soluble phosphodiesterase activity by FPLC on a Mono-Q column resolved four distinct fractions expressing cyclic nucleotide phosphodiesterase activity. Lineweaver–Burk plots for the hydrolysis of both cyclic GMP and cyclic AMP yielded linear results. The first two peaks (KPDE-MQ-II, KPDE-MQ-III) showed higher activities towards cyclic GMP than cyclic AMP with the ratio of their V_{max} values for the hydrolysis of cyclic AMP/cyclic GMP being 0.66 and 0.16, respectively. For the second two peaks (KPDE-MQ-IV, KPDE-MQ-V) the V_{max} ratios for the hydrolysis of cyclic AMP/cyclic GMP were 6.4 and 16.7, respectively. All enzymes exhibited similar low K_m values for both cyclic AMP and cyclic GMP but had very different V_{max} values. KPDE-MQ-II was activated by Ca^{2+}/calmodulin. The cyclic AMP phosphodiesterase activity of KPDE-MQ-III was augmented by the presence of low concentrations of cyclic GMP. Thermal denaturation studies showed that the phosphodiesterase activity of each fraction decayed as a single exponential indicating that each phosphodiesterase fraction contained but a single phosphodiesterase activity. The inhibitors IBMX, zaprinast, milrinone, amrinone, buquineran, carbazeran, ICI 118233, ICI 63197 exerted selective effects on the activities of these enzymes. We compared the action of these compounds on cyclic GMP phosphodiesterases from bovine retina. Over the concentration ranges used, the bovine retinal enzyme was only inhibited by IBMX, zaprinast and carbazeran. The cytosolic isoenzymes of cyclic AMP phosphodiesterases play a much more important role in metabolizing cyclic AMP in kidney compared with liver, where the activity of membrane-bound isoenzymes predominate.

Hormones and neurotransmitters can exert actions on target cells by altering the intracellular concentration of cyclic nucleotides. The only known route of degrading these second messengers is through the action of the ubiquitous cyclic nucleotide phosphodiesterases. With the exception of retinal rod and cone cells, where a single cyclic GMP-specific enzyme appears to be expressed, all other cells investigated appear to express multiple forms of cyclic AMP and cyclic GMP phosphodiesterase activity [1, 2]. These can be soluble or associated with distinct membrane fractions and, in some instances, their activity can be rapidly modulated by hormones [2, 3]. Appreciable evidence has been obtained to suggest that multiple forms of cyclic nucleotide phosphodiesterases are indeed isoenzymes that can be selectively expressed in various cell types [1, 3, 4]. The possibility of producing selective inhibitors for specific types of cyclic nucleotide phosphodiesterases has been explored by various investigators [5–8] in order to produce novel therapeutic agents. Indeed, much work has focussed on the isoenzymes of cardiac tissue where selective inhibitors of 'cyclic AMP specific' phosphodiesterases appear to exert actions as positive inotropic agents [8, 9]. Many investigators have resolved tissue extracts on DEAE-ion exchange chromatography to yield three fractions called PDE-I, which appears to be a Ca^{2+}/calmodulin-stimulated enzyme, PDE-II a form able to hydrolyse both cyclic AMP and cyclic GMP and where low concentrations of cyclic GMP augment the hydrolysis of cyclic AMP and PDE-III, a cyclic AMP-specific form [6–9]. Such analyses have not attempted to identify the relative contribution of membrane to cytosol forms which, at least in hepatocytes, can be approximately equally distributed [10]. Also, the low resolution of the DEAE chromatography techniques employed in such studies have not adequately resolved the various isoforms present. Thus, a more detailed analysis of cardiac tissue has shown that two rather than one cyclic AMP-specific high affinity enzyme is present [11]. We have recently [12] employed FPLC techniques to resolve the cytosol phosphodiesterases of liver and hepatocytes, whereupon we have not only confirmed the existence of two high affinity cyclic AMP-specific forms eluting at high ionic strength but have also identified a novel cyclic AMP-specific form eluting at low ionic strength.

Kidney is an important organ involved in diuresis and where cyclic nucleotides play a pivotal role. Thus hormones such as vasopressin can stimulate adenylate cyclase and thus increase the intracellular concentration of cyclic AMP whilst the substance ANF (atrial natiuretic factor) activates guanylate cyclase and increases the intracellular concentration of cyclic GMP. Here we show that the majority of cyclic nucleotide phosphodiesterase activity is in the

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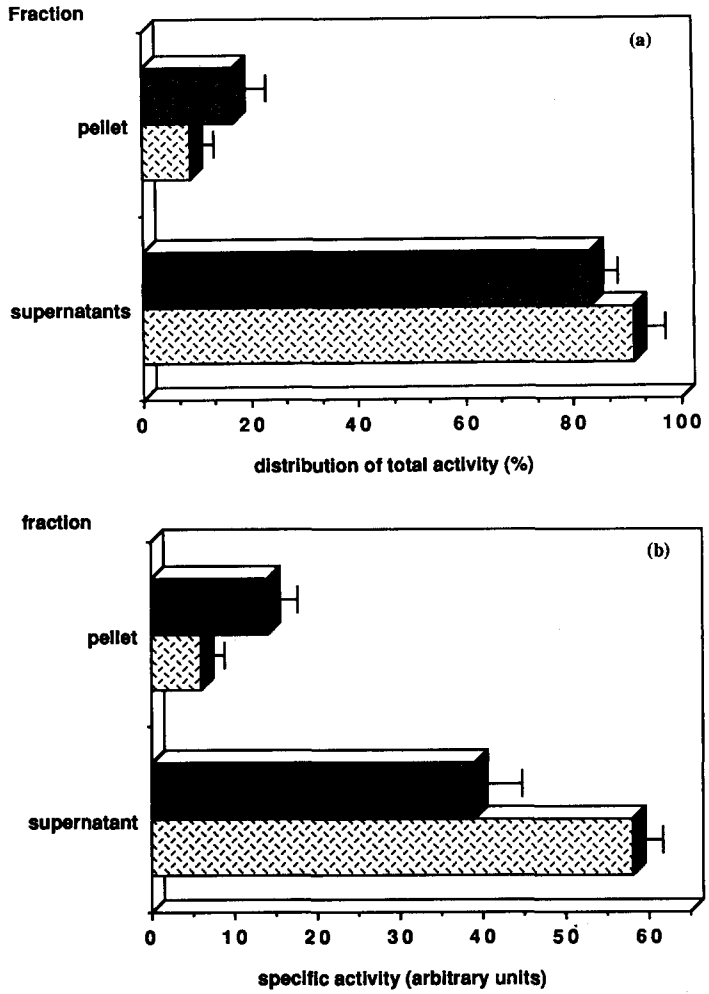

Fig. 1. Distribution of cyclic nucleotide phosphodiesterase activity between soluble and membrane fractions. Panel (a) shows the distribution of phosphodiesterase activity as a percentage of the total; panel (b) gives the specific activity (arbitrary units) using either cyclic AMP (stippled) or cyclic GMP (diagonal stripes) as substrates. The supernatant fraction is that activity found in the supernatant after centrifuging the homogenate for 1 hr at 100,000 *g* together with the activity in the supernatant after washing the resultant pellet with isotonic media. The pellet fraction is that obtained after such an isotonic wash. The specific activities shown are (activity/protein) for the original 100,000 *g* supernatant and the final washed pellet. Errors are given for three experiments.

soluble fraction of rat kidney and that such activity can be resolved upon ion-exchange chromatography into four apparently distinct forms. These show different kinetics of substrate utilization, distinct responses to stimulators and inhibitors and have different thermolabilities.

A range of so-called selective and non-selective inhibitors have been employed in this study. Thus the methylxanthine, IBMX was chosen as an example of a non-selective inhibitor, although a phosphodiesterase isoform in hepatocytes has been found to be unaffected by this species [12]. Zaprinast was chosen as an example of a selective inhibitor of cyclic GMP-specific phosphodiesterases [8]. ICI 118233 was chosen because of its extreme selectivity, being one of the novel, 'second generation' pyridazinone species developed to be an inhibitor of cyclic AMP-specific phosphodiesterases and subsequently shown by us [4] to inhibit exclusively the membrane-bound cyclic GMP-inhibited ('dense-vesicle') cyclic AMP-specific phosphodiesterase in rat hepatocytes. ICI 63197 is a triazolo pyrimidine compound developed as an antibroncho-constrictor agent and orginially thought to be non-specific phosphodiesterase inhibitor with a potency akin to that of IBMX [13]. More recently, however, it has been shown to be a highly potent inhibitor of the cyclic AMP-specific peripheral plasma membrane phosphodiesterase and not the 'dense-vesicle' enzyme in rat hepatocytes [10, 14]. Amrinone and milrinone are structurally-related bipyridines which exert positive inotropic actions and increase the cyclic AMP content of heart [6, 7, 15, 16] as do the structurally-related piperidines, buquineran and carbazeran [7]. Such cardiotonic agents were suggested to act by inhibiting cyclic AMP-specific phosphodiesterases [6, 7, 8, 17] although, more recently, a precise target of the cyclic GMP-inhibited cyclic AMP-specific phosphodiesterase has

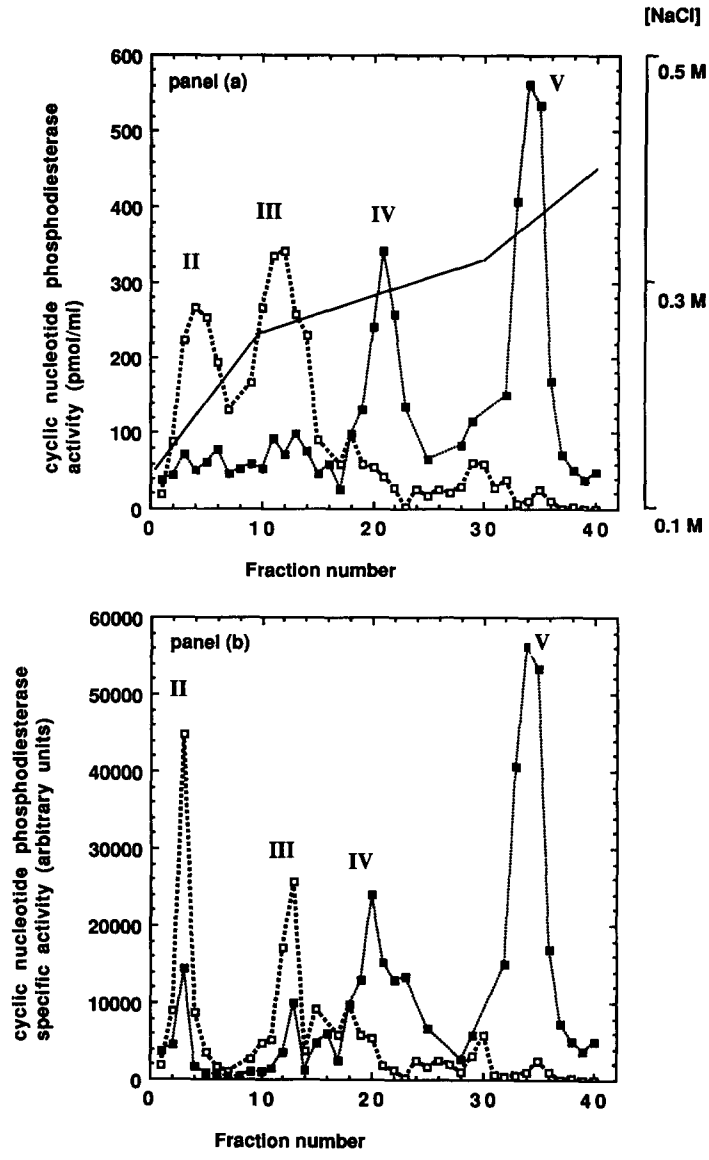

Fig. 2. Separation of phosphodiesterase isoenzymes by fast protein liquid chromatography (FPLC) on a Mono-Q column. Panel (a) shows the distribution of phosphodiesterase activity expressed as pmol produced/min/mL of assay and panel (b) shows the data corrected for protein in the samples giving specific phosphodiesterase activity in arbitrary units. Fractions were assayed using either cyclic GMP (--□--) or cyclic AMP (··■··) as substrate. Peaks are labelled as II, III, IV and V in order to correspond with those obtained from our studies done on cytosol fractionation of both rat liver and hepatocyte [12]. The NaCl elution gradient is given as a solid line in panel (a).

been mooted [4, 17, 18]. In studies on cyclic GMP-inhibited activity, milrinone was shown to serve as a much more potent inhibitor than amrinone [17, 18] as has also been noted for their relative potency as positive inotropic agents [19]. Both buquineran and carbazeran, which are effective cardiac stimulants and vasodilators in man, appear to exert selective inhibitory effects on cyclic AMP-specific phosphodiesterases [7, 20] and have an inhibitory profile akin to that of milrinone [9]. Ro-20-1724 was studied as it is a poor inhibitor of the membrane-bound cyclic GMP-inhibited cyclic AMP specific phosphodiesterase [14, 17, 21] and can discriminate between the two cyclic AMP-specific forms found in cardiac cytosol [11]. In that instance, the species eluting at the lower ionic strength was less sensitive to inhibition than the one eluting at higher ionic strength [11].

MATERIALS AND METHODS

Two kidneys from male Sprague–Dawley rats were cleaned of fat, minced finely in 15 mL of isotonic buffer consisting of 0.25 M sucrose, 1 mM EDTA, 2 mM benzamidine, 0.2 mM PMSF and 10 mM Tris–HCl final pH 7.4. This was homogenized by 8–10 passages through a Teflon/glass homogenizer. The homogenate was centrifuged at 1000 *g* for 10 min and

Table 1. Elution of kidney cytosol cyclic nucleotide phosphodiesterase from a Mono-Q FPLC column

Activity form Nomenclature 1*	Rat liver cytosol [NaCl] (M)	Rat kidney cytosol [NaCl] (M)	General properties	Nomenclature 2†
PDE-MQ-I	0.085	None eluted	Cyclic AMP-specific, IBMX-insensitive	—
PDE-MQ-II	0.16	0.18	Ca^{2+}/calmodulin activated, cyclic GMP preferring	PDE-I
PDE-MQ-III	0.24	0.24	Low [cyclic GMP] stimulate the hydrolysis of cyclic AMP	PDE-II
PDE-MQ-IV	0.34	0.31	Cyclic AMP specific	PDE-III
PDE-MQ-V	0.40	0.36	Cyclic AMP specific, Ro-20-1724 and rolipram selective	PDE-IV

Data compares the concentration of NaCl used to elute phosphodiesterase isoforms from the high speed supernatant (cytosol) fractions of rat kidney with our previous studies performed on rat liver and hepatocytes [12]. The concentrations of NaCl given are those at which the peaks of activity eluted are found.

* Nomenclature of the fractions eluting from the Mono-Q column is as defined by us before [12] and relates to their relative position of elution from the column upon increasing the ionic strength.

† Relates to the nomenclature used by Reeves *et al.* [11] in their study on cardiac isoforms eluted from a DEAE column.

the resultant 1000 *g* supernatant centrifuged for 1 hr at 100,000 *g*. The 100,000 *g* pellet was washed twice with isotonic sucrose and re-centrifugated. The supernatants were combined (CS).

Portions (10 mL) of the combined supernatants (CS) were filtered on Millipore Millex-GV single filters and applied to a Mono-Q column (10 mL bed volume) on a Pharmacia FPLC system. The column was first equilibrated in buffer A (1 mM EDTA, 0.2 mM PMSF, 2 mM benzamidine and 10 mM Tris–HCl final pH 7.4) and elution was carried out using a 0–0.4 M NaCl gradient in buffer A (final pH 7.4) collecting 0.5 mL fractions.

Assays of cyclic nucleotide phosphodiesterase activity were performed routinely by a modification [22] of the method of Thompson and Appleman [23]. The Dowex-1 resin was resuspended in EtOH:H_2O in order not to underestimate phosphodiesterase activity [24]. We added 400,000 cpm of labelled cyclic nucleotide to each assay. Initial rates from linear time courses performed at 30° were analysed.

The cyclic GMP-specific cyclic nucleotide phosphodiesterase was purified from bovine rod outer segments [25]. This resulted in a highly purified enzyme of specific activity 3.6 μmol/min/mg at 1 μM cyclic GMP as substrate. Analysis on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) showed the 86 kDa subunit of this enzyme as the major protein present.

Samples of the various peak fractions from the Mono-Q column were subjected to SDS–PAGE with subsequent immunoblotting with the specific antiserum directed against the 'dense-vesicle' phosphodiesterase from rat liver as detailed before by us [4]. Hypotonic lysis of membrane fractions was performed as described Ref. 26.

[^{3}H]Cyclic AMP and [^{3}H]cyclic GMP were from Amersham International (Amersham, U.K.). Dowex-1 resin, PMSF, benzamidine, isobutylmethylxanthine (IBMX), *Ophiophagus Hannah* venom were from the Sigma Chemical Co. (Poole, U.K.). All general chemicals were of AR grade from BDH Chemicals (Poole, U.K.). ICI 118233 6-[*p*-(3-methyl-ureido)phenyl]-3[2*H*]-pyridazione and ICI 63197 (2-amino-6-methyl-5-oxo-4-*n*-propyl-4,5-dihydro-*S*-triazolo [1,5*a*] pyrimidine were a kind gift from Dr M. Collis, ICI Pharmaceuticals, Alderley Park, U.K. Ro-20-1724 (4-(3-butoxy-4-methoxybenzyl)-2-imidazoline) was a kind gift from Roche Products Ltd (Welwyn Garden City, U.K.). All other inhibitors were synthesized by Pfizer Central Research (Sandwich, U.K.). IBMX is 3-isobutyl-1-methylxanthine; milrinone is 1,6-dihydro-2-methyl-6-oxo-[3,4′-bipyridine]-5-carbonitrile; amrinone is 5-amino-[3,4′-bipyridine]-6(1*H*)-one; buquineran is 6,7-dimethoxy-4-[4-(3-*n*-buty-ureido) piperidino] quinazolone; carbazeran is 6,7-dimethoxy-1-[4-(ethyl carbonoxy) piperidino] phthalazine.

RESULTS

Kidneys were homogenized under isotonic conditions to prevent the breakage of lysosomes. This is because the release of proteases has been shown to solubilize certain phosphodiesterase isoenzymes [26, 27] and to alter the kinetic properties as regards

Table 2. Properties of the kidney cytosol cyclic nucleotide phosphodiesterases

	KPDE-MQ-II	KPDE-MQ-III	KPDE-MQ-IV	KPDE-MQ-V
K_m (μM) cyclic AMP	5.0 ± 0.2	1.1 ± 0.2	2.8 ± 0.9	1.2 ± 0.3
V_{max} cyclic AMP*	880 ± 42	295 ± 23	2190 ± 155	2720 ± 345
K_m (μM) cyclic GMP	7.6 ± 1.9	6.8 ± 2.4	10.2 ± 3.4	2.2 ± 0.6
V_{max} cyclic GMP*	2020 ± 150	1337 ± 187	345 ± 28	163 ± 32
Ratio $V_{max}^{\text{cyclic AMP}}/V_{max}^{\text{cyclic GMP}}$	0.44	0.22	6.35	16.69
Ratio $K_m^{\text{cyclic AMP}}/K_m^{\text{cyclic GMP}}$	0.66	0.16	0.27	0.55
$T_{0.5}$† (min)	3.3 ± 0.2	31 ± 5	8.5 ± 0.8	5.9 ± 0.4

Kinetic constants were obtained from three separate enzyme preparations analysed over a substrate concentration range of 0.025 to 100 μM using determinations of initial rates at 28 different substrate concentrations in duplicate.

* V_{max} values are given in arbitrary units.

† Half-lives for the decay of enzymes followed the loss of greater than 90% of enzyme activity with determinations of activity made at 10 time-points using cyclic GMP as substrate for enzymes II and III and cyclic AMP as substrate for enzymes IV and V. Data is given for three separate experiments.

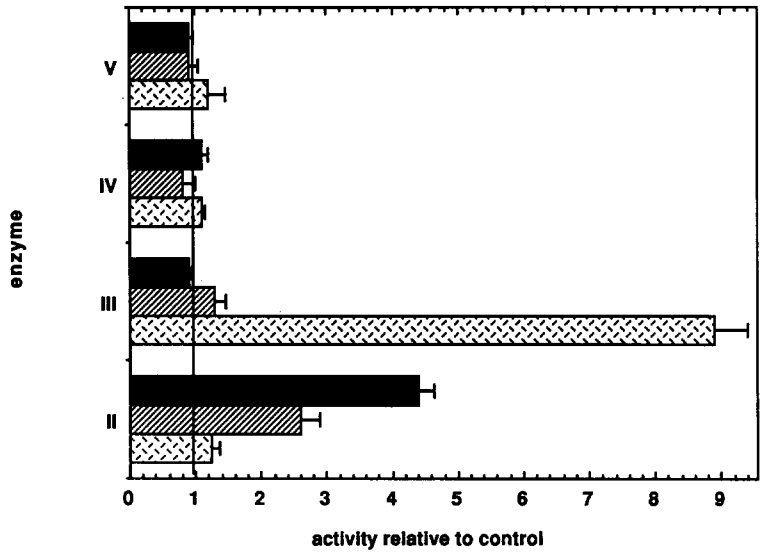

Fig. 3. Regulation of phosphodiesterase activity by effectors. Phosphodiesterase activity is shown relative to the control activity using 0.05 μM cyclic nucleotides as substrate. Shown is the effect of 2 μM cyclic GMP on the hydrolysis of cyclic AMP (stippled) and the effect of Ca^{2+}/calmodulin on the hydrolysis of both cyclic AMP (diagonal stripes) and cyclic GMP (dark block). Data is from three separate experiments and the labelling of phosphodiesterase species is as defined in the legend to Fig. 2.

both activator and inhibitor sensitivity and substrate ultization of certain species [9, 28]. Protease inhibitors were also added to ensure the inactivation of any residual protease activity. Under such conditions we observed that the majority of both the cyclic AMP and cyclic GMP phosphodiesterase activity was found in the soluble and thus, presumably, cytosolic-derived fraction (Fig. 1).

The soluble phosphodiesterase activity was resolved into four distinct fractions by FPLC ion exchange chromatography on a Mono-Q column (Fig. 2). The gradient employed appeared to elute all of the applied activity (greater than 95% of total using either cyclic AMP or cyclic GMP as substrate).

We noted that in contrast with our previous studies on rat liver [12] and hepatocytes, no phosphodiesterase activity was eluted at concentrations of NaCl lower than 0.14 M. Thus the so-called PDE-MQ-I, which eluted at *ca.* 0.085 M from rat liver (hepatocyte) was apparently absent from rat kidney cytosol. This phosphodiesterase form (PDE-MQ-I) was shown by us [12] to be a cyclic AMP-specific enzyme which was resistant to inhibition by IBMX. Consistent with the absence of this enzyme from kidney, we were able to demonstrate that greater than 95% of the phosphodiesterase activity of the kidney high-speed supernatant fraction as inhibited in the presence of 100 μM IBMX when either 1 μM-cyclic AMP or 1 μM-cyclic GMP were used as substrate (three separate preparations).

Four phosphodiesterase activities were resolved from kidney cytosol on the Mono-Q column. The concentrations of NaCl which were used to elute these corresponded to those employed to elute the cytosol forms from rat liver termed KPDE-MQ-II, III, IV and V (Table 1; [12]). We thus refer to the resolved, cytosolic kidney enzymes as KPDE-MQ-II, -III, -IV, and -V. This identifies them not only by their elution characteristics but also the general properties (Table 1) of the comparable enzymes

resolved from rat liver cytosol by Mono-Q chromatography [12].

As can be seen from Fig. 2, when assayed at 1 μM substrate concentration, the first two peaks (II and III) predominantly hydrolysed cyclic GMP and the second two peaks (IV and V) predominantly hydrolysed cyclic AMP. The ratios for the hydrolysis of cyclic GMP and cyclic AMP are given in Table 2. Lineweaver–Burk plots for the hydrolysis of cyclic nucleotides over a substrate concentration range of 0.025 to 100 μM were linear for all the enzymes analysed. The data given in Table 2 shows that all the enzymes expressed low K_m values for both cyclic nucleotides as substrates but exhibited very different V_{max} values for cyclic AMP and cyclic GMP.

We observed that the activity of KPDE-MQ-II, when assayed using either cyclic GMP or cyclic AMP as substrate, was potently augmented by the addition of Ca^{2+}/calmodulin. In contrast, the activities of the other enzymes were unaffected (Fig. 3). When a low concentration (2 μM) of unlabelled cyclic GMP was added to assay of cyclic AMP (labelled) phosphodiesterase activity then a marked increase in the cyclic AMP phosphodiesterase activity of KPDE-MQ-III was observed (Fig. 3). At concentrations of 0.2–5 μM (range) of cyclic GMP there was no indication of any inhibition (less than 6%) of either of the two cyclic AMP-preferring enzymes, namely either KPDE-MQ-IV or KPDE-MQ-V, respectively (Fig. 2). In contrast, the membrane-bound cyclic GMP inhibited phosphodiesterase from rat hepatocytes and from the kidney membrane fraction could be inhibited by *ca.* 50% in the presence of μM cyclic GMP [14]. Material from each of the four peaks was also subjected to SDS–PAGE with subsequently immunoblotting with an antisera directed against the membrane-bound, cyclic GMP-inhibited 'dense-vesicle' cyclic AMP phosphodiesterase from rat liver [4]. No immunoreactivity was detected in these soluble extracts from kidney (data not shown).

Thermal inactivation studies were performed at 50° on the enzyme activities from each of the fractions. In each instance activity decayed as a single exponential yielding linear semi-logarithmic decay plots. This indicated that the activities in each of the isolated fractions was a single enzyme protein [29]. The half-lives for decay of each of these activities were very different and are given in Table 1.

The effect of potential selective inhibitors were assessed on the cyclic GMP phosphodiesterase activity of KPDE-MQ-II and KPDE-MQ-III (Table 3) and this was compared with the effect of such inhibitors on the activity of the cyclic GMP-specific phosphodiesterase from bovine retina (Table 3). The action of such inhibitors was also evaluated on the cyclic AMP phosphodiesterase activity of KPDE-MQ-IV and KPDE-MQ-V (Table 3).

An indication of the relative importance of the various cyclic nucleotide phosphodiesterases in degrading cyclic GMP is shown in Fig. 4. The assumptions made in this analysis are: (i) that the relative amounts of each isoform are as found by determining recoveries from total peak areas of the various fractions recovered from the Mono-Q column; (ii) that the enzymes observe Michaelis kinetics *in situ* ($V = V_{max}[S]/(K_m + [S])$) with similar kinetic constants to those determined in this study (Table 2); and (iii) that cyclic AMP is not compartmentalized inside cells with each enzyme being exposed to similar concentrations. For this comparative exercise, the cyclic AMP substrate concentration was set at 1 μM, a value below that expected to activate protein kinase A and thus more representative of basal or resting intracellular concentrations. The relative activities are thus calculated from the Michaelis–Menten equation and the fractional activity of the total expressed for each isoform (Fig. 4). In the case of the kidney data, the isoform distribution analysed averages that seen in all cell types rather than, as with the hepatocyte data [10] reflecting a single cell type. This is clearly an oversimplification nevertheless, that over 80% of cyclic nucleotide phosphodiesterase activity is cytosolic and the majority of the remainder can be attributed to the membrane-bound cyclic GMP inhibited ('dense-vesicle') enzyme [4] suggests that isoform heterogeneity is rather restricted in kidney compared with, for example, hepatocytes [4, 12] where there is a much greater range and magnitude of activity of membrane-associated species. We have observed (M. D. Houslay and M. Woods, unpublished), however, that the kidney MDCK epithelial cell line shows a similar range of phosphodiesterase isoforms as we described in this study and, similarly, the major fraction of activity is found in the cytosol fraction.

Our analysis of relative activities shows, intriguingly, it is PDE-MQ-V, the Ro-20-1724 cyclic AMP-specific isoform which appears to play the overall major role in degrading cyclic AMP, closely followed by the other cyclic AMP-specified species, PDE-MQ-IV.

DISCUSSION

The majority of cyclic nucleotide phosphodiesterase activity is found in the soluble fraction of rat kidney. This situation contrasts markedly with liver (hepatocytes), where around 60% of the total cyclic nucleotide phosphodiesterase activities are membrane-bound [12, 22].

Resolution of soluble phosphodiesterase activity from kidney upon ion-exchange chromatography on a Mono-Q FPLC column yielded four distinct fractions (Fig. 2). The elution characteristics, substrate preferences and sensitivities to regulation by effectors prompted us to refer to these enzymes as KDPE-MQ-II, -III, -IV and -V in order to allow comparison with those species we have identified from rat cytosol fractions from both liver and hepatocytes (Table 1). Thus we see that KPDE-MQ-II is a cyclic GMP-preferring Ca^{2+}/calmodulin-activated enzyme; KPDE-MQ-III is an enzyme where low concentrations of cyclic GMP activate cyclic AMP hydrolysis and KPDE-MQ-IV and -V are cyclic AMP-specific enzymes (Fig. 3). All four fractions of phosphodiesterase activity appear to reflect distinct, single enzyme activities as they showed very different thermostabilities and the activity in each fraction decayed as a single exponential, indicating a single component (Table 1).

That kidney and liver high speed supernatants prepared under isotonic conditions each demonstrate

Table 3. Selective inhibition of cyclic nucleotide phosphodiesterase from kidney

	KPDE-MQ-II	KPDE-MQ-III	KPDE-MQ-IV	KPDE-MQ-V	Retinal cyclic GMP-specific PDE
IBMX	44.6 ± 0.8	5.8 ± 0.8	46.8 ± 7.5	9.4 ± 5.8	20 ± 5
Zaprinast	11 ± 2	65 ± 11	326 ± 33	89 ± 5	22 ± 3
Milrinone	658 ± 37	NI	38 ± 4	78 ± 8	NI
Amrinone	950 ± 92	NI	260 ± 25	789 ± 67	NI
Buquineran	388 ± 42	885 ± 87	23 ± 3	32 ± 4	NI
Carbazeran	150 ± 30	280 ± 84	8.0 ± 3	6 ± 2	168 ± 27
ICI 118233	986 ± 95	NI	809 ± 97	912 ± 57	NI
ICI 63197	487 ± 45	NI	9 ± 3	10 ± 3	NI
Ro-20-1724	NI	NI	32 ± 5	2.3 ± 0.6	ND

KPDE-MQ-II and KPDE-MQ-III assayed using cyclic GMP as substrate and KPDE-MQ-IV and KPDE-MQ-V were assayed using cyclic AMP as substrate. Determination of K_i values was performed on three separate preparations with values given as μM.

NI, no inhibition in the presence of 1 mM agent with 0.05 μM substrate.

ND, not determined.

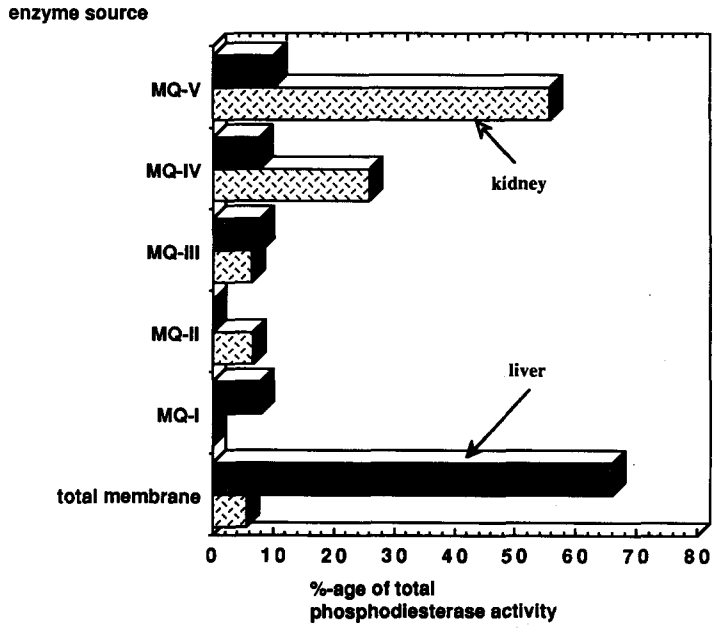

Fig. 4. Relative phosphodiesterase activities in kidney and liver. The relative rates of cyclic AMP degradation were calculated for the various cytosolic phosphodiesterase isoforms together with the total membrane activity. This was done with 1 μM cyclic AMP as substrate. Data for the relative amounts of kidney enzymes were determined experimentally in this study from Mono-Q elution profile shown in Fig. 1 and for the kinetic constants denoted in Table 2. Calculations were done assuming Michaelis–Menten kinetics were obeyed as found experimentally (Table 2). Data for liver (hepatocytes) was taken from our previous studies [9].

phosphodiesterase activities of similar general characteristics as regards substrate specificity, sensitivity to effectors and elution properties suggests that these activities truly reflect distinct cytosol isoforms. We feel it is unlikely that such multiplicity arises from any proteolysis of these enzymes as (i) fractions were prepared under isotonic conditions in the presence of protease inhibitors; (ii) the 'dense-vesicle' enzyme, whose release is exquisitely sensitive to protease action [4, 14, 26, 27] remained membrane-bound; and (iii) that Ca^{2+}/calmodulin activation, which is lost upon proteolysis [1], was clearly evident in KPDE-MQ-II.

Interestingly, in contrast to our studies on liver [12] we failed to observe a cyclic AMP-specific enzyme eluting at low ionic strength from the Mono-Q column. This species, called PDE-MQ-I, was remarkably resistant to inhibition by IBMX. Thus kidney appears not to express this isoform and consistent with this was our ability to inhibit over 95% of the phosphodiesterase activity of both kidney homogenates and cytosol with 100 μM IBMX. It would thus appear that PDE-MQ-I shows a tissue specific expression in rat.

There has been considerable interest in cardiac tissue that the target for positive inotropic agents is a cyclic AMP-specific phosphodiesterase [6–8] whose activity can be potently inhibited by cyclic GMP [4,

14, 17]. Clearly, there is no evidence for such a species occurring in kidney cytosol (Fig. 3). However, we [4, 14] have suggested that this enzyme is a membrane-bound species called the 'dense-vesicle' enzyme which can be released from its membrane environment by the action of proteases [14]. Such a species has clearly not been released into the kidney soluble fractions by our preparative procedure as (i) there is no cyclic AMP-specific species where cyclic GMP potently inhibits activity; (ii) ICI 118233, the potent and specific inhibitor of this enzyme [4] is ineffective against all the species identified here (Table 3); and (iii) Western blotting analysis of the Mono-Q fractions, using a specific antiserum directed against this enzyme [4], failed to detect any immunoreactive species. This is consistent with our previous data [4] which demonstrated the presence of such a protein in both homogenates of rat kidney and kidney membrane fractions. On the basis of inhibition of kidney homogenate cyclic AMP phosphodiesterase activity by ICI 118233 this enzyme appeared to contribute only a small fraction of the total cyclic AMP phosphodiesterase activity of kidney [4]. The present study confirms this and shows quite clearly that the soluble, cytosol phosphodiesterases are the key enzymes involved in metabolizing cyclic nucleotides in kidney. This emphasizes the fact that functional role of specific isoenzymes differs between tissues.

We noted that all of the soluble, kidney enzymes appeared to show very low and similar K_m values for both cyclic AMP and cyclic GMP as substrates. The differences in ability to metabolize these two cyclic nucleotides is thus determined by the magnitude of their V_{max} values for each cyclic nucleotide (Table 2). This is particularly noticeable for KPDE-MQ-IV and KPDE-MQ-V which exhibit V_{max} values for the hydrolysis of cyclic AMP which are some 6- and 17-fold higher, respectively, than those for cyclic GMP. In contrast KPDE-II and KPDE-III exhibit V_{max} values which are some 2- and 5-fold higher, respectively, for cyclic GMP than cyclic AMP. The K_m values for the MQ-II (cyclic GMP), MQ-IV (cyclic AMP) and MQ-V (cyclic AMP) were very similar for both the kidney (Table 3) and liver (hepatocyte) [12] enzymes and each particular species from the respective two tissues showed similar sensitivities to inhibition by selective inhibitors (Table 3; [12]).

The use of a range of selective inhibitors indicated that zaprinast showed selectivity for inhibiting the cyclic GMP-specific enzymes over those metabolizing cyclic AMP (Table 3). The reverse of this was exemplified by milrinone, carbazeran, buquineran Ro-20-1724 and ICI 63197 which potently inhibited the cyclic AMP-specific isoforms yet were ineffective against the cyclic GMP-specific species. The elution profile of soluble kidney enzymes, subjected to chromatography on Mono-Q, bears a good resemblance to that found for cardiac soluble isoforms [11] separated using a DEAE-sepharose ion-exchange system which had been modified [11] to enhance the resolving power over that used by many investigators in earlier studies [16, 21]. A characteristic pattern emerging from high resolving studies such as those described here and elsewhere [12] using FPLC and by Reeves *et al.* [11], identifies, in order of elution, a Ca^{2+}/calmodulin-stimulated isoform, a cyclic GMP-stimulated isoform and then two, high affinity cyclic AMP-specific isoforms. Reeves *et al.* [11] have suggested that the two high affinity cyclic AMP-specific isoforms can be distinguished on the basis of inhibitor sensitivity and that, for example, the species eluting last i.e. at higher ionic strength, is more sensitive to inhibition by Ro-24-1724 than the first of this pair of enzymes. This observation appears to hold true for both rat hepatocytes [12] and for kidney (Table 3) isoforms with (K)PDE-MQ-V being more sensitive to inhibition by Ro-20-1724 than (K)PDE-MQ-IV. In contrast both ICI 118233, the specific inhibitor of the cyclic GMP-inhibited 'dense-vesicle' enzyme [4] and amrinone exerted little or no inhibitory effect on any of these species. Indeed, such observations are similar to those observed in cardiac tissue [9], where amrinone was a much weaker inhibitor of cytosolic cyclic AMP-specific enzyme compared with the other so-called cyclic AMP selective inhibitors. It is interesting that both ICI 118233 and amrinone can exert positive inotropic effects on cardiac tissue [4–8], which provides support for our argument [4, 14] that the membrane-bound 'dense-vesicle' enzyme in cardiac tissue is a primary 'target' for these agents and not the cyclic AMP-specific soluble forms.

In this study we considered that it may be of interest to compare the inhibitor sensitivity of an enzyme which was as totally specific for cyclic GMP as substrate with the cyclic GMP preferring Ca^{2+}/calmodulin activated phosphodiesterase (KPDE-MQ-II). To do this we chose the retinal phosphodiesterase, which was isolated here from a bovine source [25]. The inhibitor sensitivities of these two enzymes were remarkably similar, with inhibition effected easily by IBMX and zaprinast and more weakly with carbazeran. This shows that the therapeutic use of cyclic AMP-specific phosphodiesterase inhibitors is unlikely to cause problems with visual transduction. However, the design of cyclic GMP-specific inhibitors, perhaps with the notion of potentiating the action of ANF, may lead to the possibility of interfering with the visual system in an untoward fashion. The kinetic properties of isoforms II, IV and V were very similar in both liver and kidney. In contrast, we observed that there was a significant difference in the K_m values for both cyclic AMP and cyclic GMP of the PDE-MQ-III species from kidney (5 and 8 μM, respectively) and hepatocytes (38 and 36 μM, respectively). Indeed, the properties of solely this isoform was seen to differ in our comparative analysis of cytosolic phosphodiesterases in whole liver and isolated hepatocytes [12]. This indicates that the form of PDE-MQ-III (the cyclic GMP activated species) expressed in various cell types may be subtly altered, leading to small changes in both the kinetics of substrate utilization, as indicated above, and in sensitivity to inhibitors, as KPDE-MQ-III is inhibited by zaprinast whereas liver PDE-MQ-III is not [12]. It may be that cell-specific isoforms of the cyclic GMP-activated cyclic nucleotide enzyme are produced through differential splicing of a particular phosphodiesterase gene [30].

Our evaluation of the distribution and kinetics of the soluble phosphodiesterase isoforms from two

organs, kidney and liver, from the same species together with a knowledge of membrane phosphodiesterase activities allows us to make estimations of the relative contributions of each of these enzymes to the metabolism of cyclic AMP in liver and kidney (Fig. 4). It is evident from such analyses that there are dramatic differences between the relative importance of specific phosphodiesterase isoforms to cyclic AMP metabolism in these two organs. Thus we see that the membrane-bound forms appear to play by far the major role in metabolizing cyclic AMP in liver whereas, in contrast, it is the high affinity, cyclic AMP specific, soluble (K)PDE-MQ-IV and (K)PDE-MQ-V forms which are of major importance in the kidney. Interestingly, the cyclic GMP-stimulated cytosol form (PDE-MQ-III) appears to be of similar relative importance to both organs whereas the role of PDE-MQ-I in liver appears to be taken by PDE-MQ-II in kidney. Such an analysis implies that the phosphodiesterase isoform distribution is uniquely tailored to particular organs/tissues, indicating that selective gene expression occurs. However, whilst corroborative biochemical studies have been performed on hepatocytes to support the predictions of the computer modelling studies [10], this remains to be done for the kidney. Furthermore, whilst data and analyses have been done on a single cell type, namely hepatocytes, we should note that kidney consists of a number of cell types. Whilst it is likely that they all express an established range of known phosphodiesterase isoforms, it is equally likely that the proportions of such species will differ. Nevertheless, a common feature is likely to be a preponderance of soluble over membrane-bound forms, as over 80–90% of the total activity is found in the supernatant fraction.

The importance of a particular tissue-specific profile of phosphodiesterase isoforms to cellular functioning remains to be determined. However, that it occurs indicates that so-called selective phosphodiesterase inhibitors are likely to exert tissue-selective effects on cyclic nucleotide metabolism.

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