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TISSUE DISTRIBUTION AND SELECTIVE INHIBITION OF SUBTYPES OF HIGH AFFINITY cAMP PHOSPHODIESTERASE

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Abstract—High affinity cAMP phosphodiesterase (PDE), also referred to as PDE III, or low K_m PDE occurs as two subtypes. One subtype is sensitive to inhibition by cGMP while the other is relatively insensitive. To be consistent with previously recommended nomenclature, these subtypes were designated Types IV and V PDEs respectively. Tissue distribution of these subtypes of high affinity cAMP PDE was investigated using comparative potencies of specific inhibitors. Of the tissues examined, dog heart contained the highest proportion of the cGMP inhibitable form (Type IV PDE), whereas dog kidney cortex and brain were composed almost entirely of the cGMP non-inhibitable form (Type V PDE). Enoximone and other new cardiotonic drugs that inhibit high affinity cAMP PDE were shown to be specific for the cGMP inhibitable form, whereas rolipram was specific for the cGMP non-inhibitable form. The apparently partially competitive kinetics shown by one of these drugs, enoximone, was due to the presence of both subtypes of the enzyme. When the activity of the cGMP non-inhibitable form was suppressed by rolipram, competitive inhibition of the cGMP inhibitable subtype by enoximone was observed. Rat heart high affinity cAMP PDE activity contained a higher proportion of the cGMP non-inhibitable subtype than did the enzyme from dog heart. It is suggested that this may account for the relative insensitivity of rats to the cardiotonic PDE inhibitors.

Multiple molecular forms of cyclic nucleotide phosphodiesterases (PDEs†) having distinct physical and catalytic properties have been isolated from a variety of tissues [1]. These PDEs were classified several years ago according to their substrate preference and regulatory properties [2]. That classification is as follows:

Type I: Calmodulin-sensitive cyclic nucleotide PDE. This enzyme hydrolyzes both cAMP and cGMP, and its activity is enhanced by Ca^{2+} calmodulin.

Type II: cGMP-sensitive cyclic nucleotide PDE. This enzyme also hydrolyzes both cAMP and cGMP, but its cAMP hydrolytic activity is enhanced by cGMP.

Type III: Rhodospin-sensitive cGMP PDE.

Type IV: Cyclic AMP PDE. This enzyme is relatively specific for cAMP and had been referred to as the low K_m or high affinity cAMP PDE.

The high affinity cAMP PDE (Type IV enzyme, by above nomenclature) isolated from cardiac tissue has been shown to be selectively inhibited by many of the new cardiotonic drugs, e.g. enoximone (MDL 17,043), piroximone (MDL 19,205), amrinone, milrinone, imazodan (CI-914) [3–7] and others (for reviews, see Refs. 8 and 9). The type of inhibition produced by these agents is somewhat controversial since it has been reported to be partially competitive by some, and competitive by others [3, 4, 6, 10]. The high affinity cAMP PDE has been shown recently to consist of two subtypes—one that is very sensitive to inhibition by cGMP (cGMP inhibitable) and one that is rather insensitive to inhibition by cGMP (cGMP non-inhibitable) [11–13]. The high affinity cAMP PDE isolated from cardiac tissue was reported to contain predominately the cGMP inhibitable form, whereas the enzyme isolated from kidneys contained almost entirely the cGMP non-inhibitable subtype.‡ The cGMP inhibitable form is sensitive to inhibition by the cardiotonic PDE inhibitors [13], whereas the cGMP non-inhibitable form is rather insensitive to these inhibitors but is inhibited by RO-20-1724 [11, 12], rolipram [12] and SQ-65442.‡

The identification of the two subtypes of the high affinity cAMP PDE has added to the rather confusing nomenclature in this area. Initially, there was some confusion because the three enzymes (Types I, II and IV by above nomenclature) isolated from the heart and separated by anion exchange column chromatography were designated PDE I, PDE II and PDE III, respectively, by their order of elution from a chromatographic column. More recently, the nomenclature has become more confusing when the two subtypes of the high affinity cAMP PDE (Type IV by above nomenclature and PDE III by elution

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† Abbreviations: PDE, phosphodiesterase; DEAE, diethylaminoethyl; cAMP, adenosine 3′:5′-cyclic monophosphate; cGMP, guanosine 3′:5′-cyclic monophosphate; IBMX, isobutylmethylxanthine; TLCK, tosyl lysine chloromethyl ketone; MOPS, 3-[*N*-morpholino]propanesulfonic acid; and DTT, dithiothreitol.

‡ S. J. Strada, C-C. Shen and W. J. Thompson, VIth International Conference on Cyclic Nucleotides, Calcium and Protein Phosphorylation, Abstr. No. 115 (1986).

order) were isolated from cardiac tissue and described as PDEs III and IV, according to their order of elution from a chromatographic column [11, 13]. To be consistent with the recommended nomenclature, these two subtypes of cAMP PDE would best be designated Types IV (the cGMP inhibitable form) and Type V (the cGMP non-inhibitable form) and they will be referred to this way in this manuscript.

In the present study, the distribution of these two subtypes of high affinity cAMP PDE in various tissues of the dog and in cardiac tissues of dogs and rats has been investigated using the comparative potencies of specific inhibitors of these two subtypes. Also, the inhibition of Type IV PDE by enoximone has been re-investigated in the presence of a specific inhibitor of the cGMP non-inhibitable PDE (Type V enzyme).

MATERIALS AND METHODS

Materials. Tissues were obtained from mongrel dogs of either sex and from male Sprague–Dawley rats for isolation of PDEs. DEAE-cellulose was purchased from Bio-Rad Laboratories. Snake venom (*Ophiophages hannah*), TLCK, leupeptin, pepstatin, cAMP and cGMP were obtained from the Sigma Chemical Co. [2,8-^{3}H]Cyclic AMP was obtained from New England Nuclear and purified by thin-layer chromatography before use [1]; Universol liquid scintillation fluid was from ICN Biomedicals. The drugs used were obtained from the following sources: rolipram, Schering Corp.; amrinone and milrinone, Sterling Winthrop Research Institute; RO 20-1724, Hoffmann-LaRoche, Inc.; and IBMX, Aldrich Chemical Co. Enoximone was synthesized at Merrell Dow Research Institute. All other chemicals were reagent grade or of the highest purity commercially available.

Homogenization of tissues. Samples of fresh tissues were minced and homogenized in an ice-cold medium containing 10 μM TLCK and 1 μg/ml each of leupeptin and pepstatin using three bursts of 10 sec each of a Polytron homogenizer with a PT20 generator. The homogenate was centrifuged at 20,000 *g* for 20 min at 4°.

Isolation of cAMP PDE. Isolation of high affinity cAMP PDE was based on the DEAE-cellulose anion exchange chromatographic procedure of Thompson *et al.* [10]. DEAE-cellulose was cycled with 0.25 N NaOH, 0.25 N HCl and 0.25 N NaOH as recommended by the supplier, and 1.5 × 15 cm columns were poured. After equilibration of each column with 70 mM sodium acetate in a buffer containing 5 mM MOPS, 5% glucose, 50 mM NaF, 1 mM DTT, 10 μM TLCK, 1 μg/ml leupeptin and 1 μg/ml pepstatin, pH 6.5 in 30% ethylene glycol (EG buffer), the homogenate supernatant was applied. Each column was washed with 60 ml of 70 mM sodium acetate in EG buffer, and the PDEs were eluted with 220 mM sodium acetate in EG buffer (Type I PDE), 350 mM sodium acetate in EG buffer (Type II PDE) and 750 mM sodium acetate in EG buffer (high affinity cAMP PDE containing the two subtypes, Types IV and V PDEs) collecting 8, 12 and 12 fractions, respectively, of 5 ml each. All buffers were pH 6.5. Each fraction was assayed for PDE activity using a substrate concentration of 1 μM cAMP, and the fractions containing high affinity cAMP PDE activity were pooled. The pooled fractions were diluted with EG buffer without sodium acetate to reduce the sodium acetate concentration to 350 mM and applied to a second DEAE-cellulose column. Any Type II PDE activity that may have been present was washed out with 400 mM sodium acetate in EG buffer (10 × 5 ml fractions), and the high affinity cAMP PDE activity was eluted with 750 mM sodium acetate in EG buffer (12 × 5 ml fractions). The fractions containing peak high affinity cAMP PDE activity were pooled and stored at −20°.

Assay for phosphodiesterase activity. PDE activity was measured as described by Thompson *et al.* [1] in a medium containing 40 mM MOPS (pH 7.4), 5 mM $MgCl_2$ and 0.4 mg/ml of bovine serum albumin. The concentration of substrate was 0.5 μM cAMP in all experiments. All assays were done in duplicate, using three or four different enzyme preparations, except for the femoral artery, which was done on a single preparation isolated from pooled tissues from five animals. Incubation time was 10 min and enzyme concentrations were used that utilized no more than 20% of the substrate during the incubation period.

Assay for protein. Enzyme fractions were assayed for protein content by the method of Bradford [14], using a dye reagent concentrate from Bio-Rad Laboratories. Bovine serum albumin was used for standards.

RESULTS

Inhibition of activity of high affinity cAMP PDE isolated from different tissues by 1 μM cGMP varied widely (Fig. 1). Canine cardiac ventricular tissue high affinity cAMP PDE contained predominately

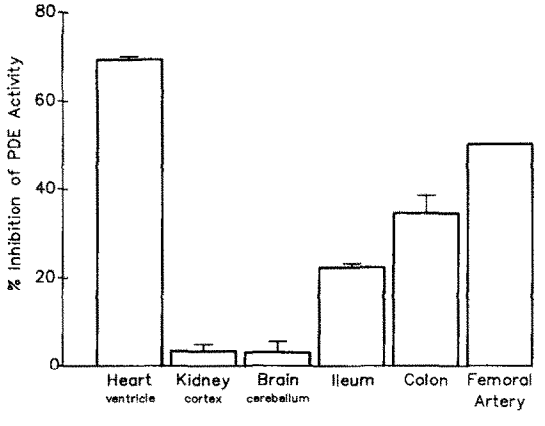

Fig. 1. Inhibition of high affinity PDE activity from dog tissues by cGMP. Enzymes from different tissue were incubated in the absence and presence of 1 μM cGMP, using 0.5 μM [^{3}H]cAMP as the substrate. Each value represents the mean ± SE of three or four preparations, except femoral artery, which is a single preparation made from pooled tissues from five animals. Average specific activities (in nmol cAMP hydrolyzed/min/mg protein) of the enzyme preparations in the absence of cGMP were: heart, 9.8; kidney, 7.8; brain, 0.84; ileum, 1.0; colon, 1.10; and femoral artery, 1.41.

Table 1. Effects of inhibitors on the high affinity cAMP PDE activity from dog tissue

Drug	IC_{50} (μM) for high affinity cAMP PDE activity from: Heart	Kidney	Brain
Enoximone	2.9 ± 0.2	>150 (34 ± 6%)	>150 (26 ± 1%)
Amrinone	41.0 ± 2.1	>150 (24 ± 5%)	>150 (14 ± 2%)
Milrinone	1.2 ± 0.02	11.3 ± 2.6	32.3 ± 4.4
IBMX	3.7 ± 0.1	13.0 ± 0.5	14.6 ± 0.7
Rolipram	411 ± 16	1.2 ± 0.2	1.8 ± 0.4
RO 20-1724	165 ± 5	5.9 ± 0.9	8.2 ± 2.6

The IC_{50} values were calculated from Dixon plots ($1/v$ vs inhibitor concn.) of assays run with various concentrations of inhibitors at a substrate concentration of 0.5 μM cAMP. Values are means ± SE from three or four preparations; numbers in parentheses indicate percent inhibition of the enzyme at the drug concentration shown.

cGMP inhibitable activity (Type IV PDE), and the enzyme isolated from kidney cortex was almost exclusively cGMP non-inhibitable (Type V PDE) in agreement with the findings of Strada *et al.** High affinity cAMP PDE isolated from the brain (cerebellum) was similar to that from kidney cortex in that the enzyme was not inhibited by cGMP. Enzymes isolated from intestinal smooth muscle (ileum and colon) were predominately the cGMP non-inhibitable subtype, while one-half of the activity of the enzyme from femoral arteries was inhibited by 1 μM cGMP.

Selective inhibition by several inhibitors of high affinity cAMP PDE from dog tissue is shown in Table 1. Enoximone and amrinone, which are cardiotonic drugs that selectively inhibit cardiac high affinity cAMP PDE, were specific inhibitors of the enzyme from the heart, which contains predominately Type IV PDE. Milrinone, another cAMP PDE inhibitor and cardiotonic drug, and IBMX, a non-specific PDE inhibitor, inhibited high affinity cAMP PDEs isolated from heart, kidney and brain, but both drugs were more potent inhibitors of the heart enzyme(s) than those from kidney and brain. In contrast, rolipram, an antidepressant drug, and RO 20-1724 were specific inhibitors of the high affinity cAMP PDE from kidney or brain, which is almost exclusively the Type V enzyme.

To test the effect of enoximone on Type IV PDE, inhibition curves with enoximone were determined using dog heart enzymes in the absence and presence of 50 μM rolipram, which selectively inhibits activity of Type V PDE with only a slight effect on Type IV PDE. The results are shown in Fig. 2. In the absence of rolipram, the Dixon plot was a convex curve. However, in the presence of 50 μM rolipram, the Dixon plot of enzyme inhibition was nearly linear.

The effects of cGMP and three inhibitors were tested on the high affinity cAMP PDE activity isolated from dog and rat hearts to see if there was a species difference in their response to inhibitors. As shown in Table 2, the dog heart enzyme preparation contained more cGMP inhibitable PDE activity, i.e. Type IV PDE, than did the rat heart preparation. This was confirmed by the effects of enoximone and rolipram, which are selective inhibitors of Types IV and V PDEs, respectively, and IBMX, a relatively non-selective PDE inhibitor. Enoximone was a much more potent inhibitor of the dog enzyme, whereas

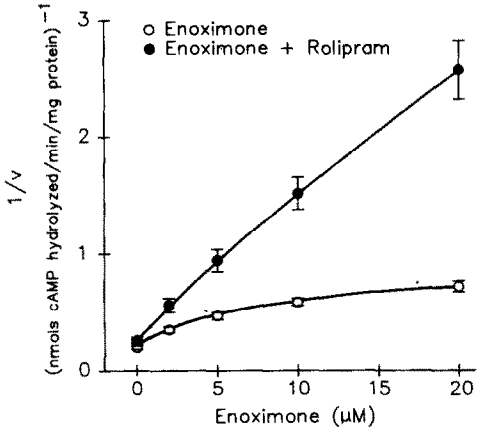

Fig. 2. Dixon plots of dog cardiac high affinity cAMP PDE inhibition by enoximone and by enoximone + rolipram. Assays were done in the presence of the indicated concentrations of enoximone in the absence and presence of 50 μM rolipram. Each point represents the mean ± SE of four different enzyme preparations.

Table 2. Comparative responses of dog heart and rat heart high affinity cAMP PDE activity to inhibitors

	% Inhibition		IC_{50} (μM)	
	Dog	Rat	Dog	Rat
cGMP (1 μM)	67 ± 1	40 ± 2.5		
Enoximone			3 ± 0.2	30 ± 11
Rolipram			411 ± 15	66 ± 25
IBMX			4 ± 0.1	6 ± 0.1

The values for dogs are the means ± SE from four different enzyme preparations; the values for rats are from three enzyme preparations made from pools of five to nine rat hearts. The IC_{50} values were determined at a substrate concentration of 0.5 μM cAMP as described for Table 1.

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rolipram was a more potent inhibitor of the rat enzyme and a weak inhibitor of the dog enzyme. IBMX was a potent inhibitor of the high affinity cAMP PDE activity from both species.

DISCUSSION

The data presented are in agreement with the finding by Strada *et al.** that there are two subtypes of high affinity cAMP PDE and that these can be distinguished by cGMP inhibition of cAMP hydrolysis and by their response to selective inhibitors. Dog heart high affinity cAMP PDE activity contained predominately the cGMP inhibitable subtype (Type IV PDE), whereas this activity isolated from kidney cortex and from the cerebellum contained almost exclusively the cGMP non-inhibitable form (Type V PDE). Enzymes isolated from intestinal tissues and from femoral arteries of the same species contained intermediate amounts of cGMP inhibitable PDE activity. These findings indicate that the proportion of these subtypes of high affinity cAMP PDE can vary between tissues and these differences may be important for pharmacological specificity of Types IV and V PDE inhibitors. It appears, for example, that inhibition of Type IV PDE is important for cardiotonic activity [13] and that inhibition of the Type V PDE may be important for antidepressant [15] and gastric secretogogue activity [16].

Earlier experiments suggested that IBMX is a competitive inhibitor of dog heart high affinity cAMP PDE, and that enoximone is a partially competitive inhibitor of the enzyme [3]. The latter conclusion was based on the finding that enoximone yielded double-reciprocal plots typical of competitive inhibition, but convex rather than linear curves were obtained when the data were plotted according to Dixon ($1/v$ vs inhibitor concn.) [17, 18]. Our present findings indicate that enoximone was a competitive inhibitor of Type IV PDE, the cGMP inhibitable form of the high affinity cAMP PDE, in agreement with the findings of Harrison *et al.* [13] and that the convex curvature of the Dixon plot is caused by the presence of Type V PDE, the cGMP non-inhibitable form of the enzyme. The latter was very insensitive to the inhibitory effect of enoximone. However, when the activity of the cGMP non-inhibitable form was suppressed by rolipram, the Dixon plot was almost linear, suggesting competitive inhibition. Any slight remaining curvature may have been due to incomplete inhibition of the cGMP non-inhibitable subtype by rolipram.

The lower proportion of the Type IV PDE to Type V PDE in the rat heart preparation, when compared with dog heart, may be the basis for the relative insensitivity of rats to the cardiotonic effects of the selective Type IV PDE inhibitors [19, 20]. Alternatively, differences in the intracellular localization of Type IV PDE may be the determining factor for the differences in species sensitivity to these agents. Weishaar *et al.* [21] recently reported that imazodan-sensitive PDE is membrane-bound in the dog ventricular muscle, whereas the enzyme is in the soluble fraction in rat and guinea pig hearts. Imazodan-sensitive PDE appears to be identical to Type IV PDE. Positive inotropic effects were produced by imazodan in dogs but not in rats and guinea pigs. Thus, the intracellular location of the enzyme may also be important for the cardiotonic effect of the drug.

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