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Resistance of a separated form of canine ureteral phosphodiesterase activity to inhibition by xanthines and papaverine *†

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Adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and guanosine 3′:5′-cyclic monophosphate (cyclic GMP) appear to play regulatory roles in smooth muscle. In smooth muscle, as in other tissues, the only known pathway for the degradation of the cyclic nucleotides is via one or more phosphodiesterases. Multiple forms of these enzymes occur in many if not all tissues, including smooth muscle [1, 2]. A characteristic of cyclic nucleotide phosphodiesterases of mammalian origin is sensitivity to inhibition by xanthine derivatives and by papaverine. The abilities of these drugs to relax smooth muscle are thought to derive, at least in part, from inhibition of cyclic nucleotide phosphodiesterases. The various, and multiple forms of these enzymes in vascular smooth muscle differ in their relative sensitivities to xanthines and papaverine [3, 4]. We were interested in comparing the effects of these agents on separated multiple forms of phosphodiesterase activity in the canine ureter. During the course of these studies, we detected a peak of cyclic AMP phosphodiesterase activity from DEAE-cellulose columns that was highly resistant to inhibition by xanthines and papaverine. To our knowledge, such resistance to inhibition by these agents is novel for phosphodiesterases of mammalian origin. Phosphodiesterases from some unicellular organisms [5, 6] and perhaps from epithelial cells of the toad bladder [7], however, have been show to be insensitive to xanthines.

Materials and methods

DEAE-cellulose (0.95 m-equiv/l mg, coarse grade) was obtained from the Sigma Chemical Co. (St. Louis, MO); tritiated cyclic nucleotides were obtained from the New England Nuclear Corp. (Boston, MA) and were purified on Dowex-50 cation exchange resin columns [8]. Cyclic AMP and cyclic GMP were purchased from Sigma and used without further purification. Theophylline, dithiothreitol, *Crotalus atrox* venom, and bovine serum albumin (fraction V) were from Sigma. 1-Methyl-3-isobutylxanthine (MIX) was obtained from the Aldrich Chemical Co. (Milwaukee, WI), papaverine from Eli Lilly & Co. (Indianapolis, IN), and leupeptin from the U.S.–Japan Cooperative Cancer Research Program. 8-Methoxymethyl MIX was synthesized by methods described previously [4].

Adult dogs (10–15 kg) were anesthetized with phenobarbital and their ureters were rapidly excised and placed in cold saline. Following removal of surrounding connective tissue, fat and blood vessels, the ureters were quickly frozen in liquid nitrogen and stored for 2–6 weeks at −70°. At the time of homogenization, the ureters were weighed and homogenized (1 g/4 ml) in a solution containing 20 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$ and 1 mM dithiothreitol with an Ultra-Turrax homogenizer (Jahnke & Kunkel, Staufen, Germany) at 4°. Supernatant and particulate fractions were obtained by centrifugation at 48,000 *g* for 30 min at 0°. The particulate fraction was washed twice in the homogenization solution and resuspended in the solution.

DEAE-cellulose was washed as described by Cheung [9] and then was equilibriated with the homogenization buffer. The supernatant fraction of the tissue homogenate (8 ml) was applied to a 20 × 0.9 cm column of DEAE-cellulose, and the column was then washed with 100 ml of homogenization solution. The wash contained no phosphodiesterase activity. The column was developed with the homogenization solution containing an exponential gradient of $(NH_4)_2 SO_4$ [9]. Initial separations were obtained with the mixing tank containing 400 ml of homogenization buffer and the addition tank containing 400 ml of a 0.5 M $(NH_4)_2 SO_4$ solution made with the same buffer. The flow rate was 0.6 ml/min, and fractions of 7 ml each were collected. Each collection tube contained 8 mg of bovine serum albumin in 0.2 ml of water. When a shallower gradient was employed, the mixing tank contained 500 ml of homogenization buffer and the addition tank 500 ml of 0.1 M $(NH_4)_2 SO_4$ made with the same buffer. Ninety fractions of 8 ml each were collected, and then 192 ml of the buffer solution containing 0.3 M $(NH_4)_2 SO_4$ was added directly to the column. Fractions containing peak phosphodiesterase activity were pooled and dialyzed for 18 hr against 1000 ml of homogenization medium (three changes) to reduce and equalize the salt concentration prior to studying the inhibitory effects of the xanthines and papaverine on phosphodiesterase activity. In some instances, fractions from separated peaks of phosphodiesterase activity were pooled, concentrated to approximately 14% of the original volume in an Amicon ultrafiltration cell with an UM-10 membrane, and dialyzed for 24 hr against 1000 ml (three changes) of the homogenization solution.

Phosphodiesterase activity was determined as described previously [10]. Enzyme preparations were assayed after dilution in 40 mM Tris–HCl (pH 7.5) containing 2 mg/ml bovine serum albumin to give a 5–30 per cent conversion of substrate to product in 30 min at 30°. Cyclic nucleotide (cyclic AMP or cyclic GMP) substrate concentration was 1 μM. In addition to tritiated cyclic nucleotides, the assay mixture contained 40 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$ and, when indicated, phosphodiesterase inhibitors and/or calmodulin. Calmodulin was purified from pig brain [11]. Product accumulation was linear as a function of incubation time and protein concentration. All determinations were made in duplicate.

Results and discussion

Approximately 85 per cent of the cyclic AMP and cyclic GMP hydrolytic activity was found in the 48,000 *g* supernatant fraction of homogenates of canine ureter. The ratio of cyclic GMP hydrolysis to cyclic AMP hydrolysis (at 1 μM initial concentrations) in the supernatant fraction was approximately 4.5:1.

DEAE-cellulose chromatography of the 48,000 *g* supernatant fraction using 0.5 M $(NH_4)_2 SO_4$ in the addition tank] resolved two peaks of cyclic nucleotide phosphodiesterase activity (Fig. 1A), and (not shown on the figure) a heat-stable activator fraction, presumably calmodulin. Peak I (fractions 4–10) catalyzed hydrolysis of cyclic AMP

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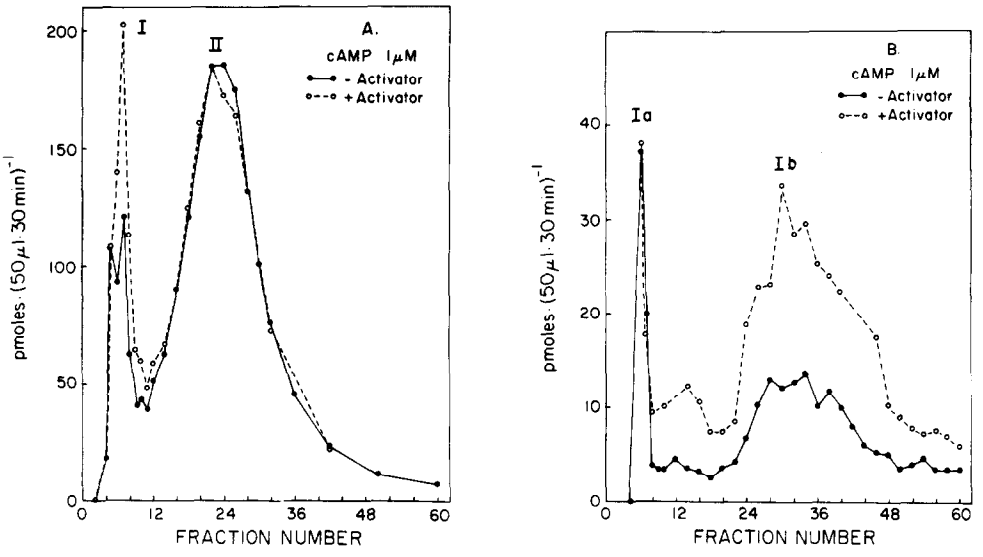

Fig. 1. DEAE-cellulose chromatography of cyclic AMP phosphodiesterase activity from canine ureter. In panel A, gradient elution was carried out as described in Materials and Methods with 0.5 M $(NH_4)_2SO_4$ in the mixing tank, and in panel B with 0.1 M $(NH_4)_2SO_4$. Fractions were assayed with 1 μM cyclic AMP as substrate with and without pig brain calmodulin (activator) as indicated.

and (not shown on the figure) cyclic GMP; the ratio of cyclic GMP hydrolysis to cyclic AMP hydrolysis was approximately 15:1. Peak II (fractions 14–36) was relatively specific for cyclic AMP. The hydrolysis of cyclic AMP and cyclic GMP by peak I was moderately stimulated by calmodulin, but that by peak II was not. Assay of individual tubes for cyclic AMP hydrolytic activity in the absence of calmodulin suggested the presence of two subpeaks under peak I. The two subpeaks could be further separated using a shallower gradient of $(NH_4)_2SO_4$. With 500 ml of 0.1 M $(NH_4)_2SO_4$ in the addition tank (Fig. 1B), peak I cyclic AMP phosphodiesterase activity was resolved into a small calmodulin-insensitive peak Ia (fractions 4–8) and a larger, calmodulin-sensitive peak Ib (fractions 22–48). In addition, a small peak of cyclic AMP hydrolytic activity was observed between peaks Ia and Ib. After collection of ninety 8-ml fractions, addition of 0.3 M $(NH_4)_2SO_4$ to the column eluted the calmodulin-insensitive peak II cyclic AMP phosphodiesterase activity (not shown). Although the shallower gradient expanded the number of fractions in which peak I cyclic GMP hydrolytic activity could be assayed, separate subpeaks were not observed; all of the detectable cyclic GMP hydrolytic activity eluted with peak Ib. Addition of 5 μM leupeptin, an inhibitor of proteolysis, to the buffer used for homogenization and DEAE-cellulose chromatography had no effect on the profile of phosphodiesterase activities or on the sensitivity of the separated forms to inhibition by xanthines and papaverine.

Figure 2 shows inhibition of the separated forms of cyclic AMP phosphodiesterase activities by papaverine, theophylline, MIX and 8-methoxymethyl MIX. Papaverine and theophylline were slightly more effective as inhibitors of cyclic AMP hydrolysis by peak II (I_{50} = 1.5 and 200 μM respectively) than as inhibitors of either cyclic AMP hydrolysis by peak Ib (I_{50} = 3 and 550 μM respectively) or cyclic GMP hydrolysis by Ib (I_{50} = 13 and 650 μM respectively; data not shown). Conversely, MIX was slightly less effective as an inhibitor of cyclic AMP hydrolysis by peak II (I_{50} = 15 μM) than as an inhibitor of either cyclic AMP hydrolysis by peak Ib (I_{50} = 1.2 μM) or of cyclic GMP hydrolysis by peak Ib (I_{50} = 2.5 μM, data not shown). 8-Methoxymethyl MIX was a much more potent inhibitor of cyclic AMP hydrolysis by peak Ib (I_{50} = 1.0 μM) and of cyclic GMP hydrolysis by peak Ib (I_{50} = 3.2 μM) than of cyclic AMP hydrolysis by peak II (I_{50} = 60 μM).

Hydrolysis of cyclic AMP by peak Ia was resistant to all inhibitors studied. Papaverine and MIX produced no inhibition at concentrations up to 1 mM. 8-Methoxymethyl MIX did not inhibit at concentrations up to 0.5 mM, and theophylline was ineffective at concentrations up to 10 mM. Papaverine and 8-methoxymethyl MIX actually caused a small stimulation of activity at lower concentrations.

Except for the resistance of cyclic AMP hydrolysis by peak Ia to inhibition by papaverine and xanthines, the cyclic nucleotide phosphodiesterases of canine uterer were inhibited by papaverine and the three xanthines in a manner qualitatively similar to that seen with the porcine coronary artery phosphodiesterases [3, 4].

Neither the importance of the inhibitor-resistant phosphodiesterase activity in the ureter nor its occurrence in other tissues is known. In one other smooth muscle-containing tissue, the porcine coronary artery, such an activity was sought but not found. In extracts of canine ureter, the inhibitor-resistant enzyme accounts for only a small fraction of the total cyclic AMP hydrolytic activity as measured at an apparent physiological concentration. If this form of phosphodiesterase activity actually exists as such within intact cells, it might be more important in controlling cyclic AMP concentrations within a subcellular pool or compartment than within the entire cell. Alternatively, this phosphodiesterase activity might be abundant in a cell type that makes up only a small fraction of the total mass of the ureter. The possibility that a xanthine- and papaverine-resistant form of phosphodiesterase activity could be involved in regulating cAMP concentrations, even if in very limited situations, should be kept in mind in using these drugs as tools to evaluate potential roles of cyclic AMP in mammalian cells.

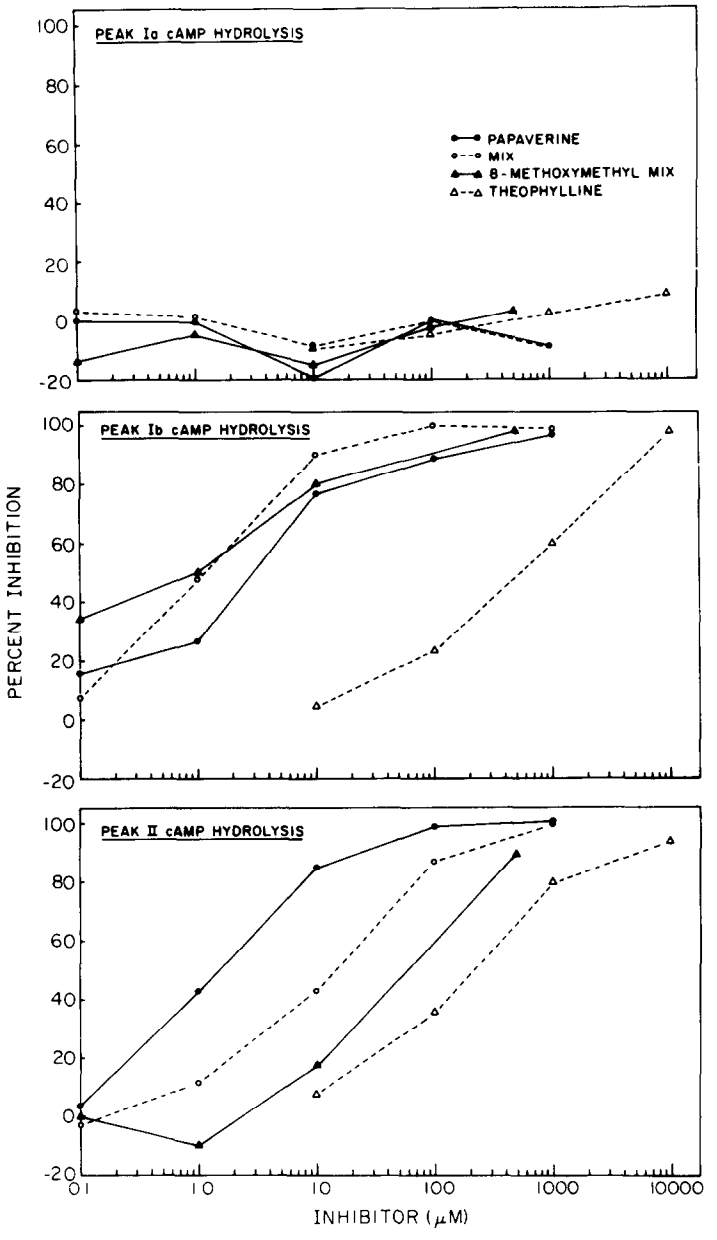

Fig. 2. Inhibition by xanthines and papaverine of separated forms of cyclic AMP phosphodiesterase from canine ureter. Peaks Ia (top panel), Ib (middle panel), and II (lower panel) were obtained by DEAE-cellulose chromatography as shown in Fig. 1. Fractions containing peak activity were pooled, dialyzed, concentrated, and assayed for phosphodiesterase activity as described in Materials and Methods with and without the indicated concentration of the inhibitors.

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Actions of 3-deazaguanine and 3-deazaguanosine on variant lines of Chinese hamster ovary cells

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3-Deazaguanine and its 9-ribosyl derivative, 3-deazaguanosine, were synthesized recently by Cook, *et al.* [1] as potential antitumor and antiviral agents. 3-Deazaguanine has exhibited significant activity against a variety of rodent neoplasms including several mammary adenocarcinoma lines [2–4]. The several cell types that are inhibited *in vitro* by 3-deazaguanine include L1210 [2], HeLa [2], and Ehrlich ascites tumor cells [5]. These compounds also exhibit activity against bacteria and a variety of DNA and RNA viruses [6–8].

In a study of the effects of 3-deazaguanine and its derivatives on purine nucleotide biosynthesis in Ehrlich ascites cells, Streeter and Koyama [5] describe the utilization of hypoxanthine-[^{14}C] in the presence of these agents at a concentration of 1 mM. Of the enzymes considered, they found IMP dehydrogenase (EC 1.2.1.14) to be the most sensitive to inhibition by all compounds tested—3-deazaguanine, 3-deazaguanosine and 3-deazaguanylic acid. HGPRT* was partially inhibited by 3-deazaguanosine and 3-deazaguanylic acid but only slightly by the free base, 3-deazaguanine. Thus, at 1 mM, 3-deazaguanine partially interfered with the biosynthesis of guanine nucleotides in these cells. These investigators also reported that 3-deazaguanosine was cleaved to 3-deazaguanine by extracts of Ehrlich ascites cells during an 18-hr incubation. Schwartz *et al.* [4] have reported the metabolism of 3-deazaguanine to 3-deazaguanosine-5′-triphosphate as well as its incorporation into nucleic acids in L1210 cells.

More recently Cook *et al.* [1] have compared the antiviral and antibacterial activities of 3-deazaguanine, 3-deazaguanosine, 3-deazaguanylic acid, and their imidazolecarboxamide precursors. Although the latter were more active, the antiviral spectra were similar for all. These investigators also reported partial inhibition of IMP dehydrogenase in Ehrlich ascites cells by 3-deazaguanine and its derivatives.

We have developed procedures to study the biochemical transformations of 3-deazaguanine and its derivatives using CHO cell mutants that are deficient in specific enzymes involved in purine nucleotide synthesis and interconversion. This type of approach, which has been employed effectively by Bennett *et al.* [9] and others, can be very useful in clarifying biochemical routes, particularly in comparing agents of similar structure.

CHO cells were carried in monolayer culture using McCoy's 5A growth medium supplemented with 10% fetal calf serum (Grand Island Biological Co., Grand Island, NY) as described previously [10]. Dialyzed fetal calf serum was used in all experiments relating to drug inhibition and utilization to avoid competition by natural serum purines.

Conventional methods were employed for mutagenesis and subsequent isolation of drug resistant cell lines [11]. CHO cells were dispensed into 250-ml plastic flasks (5×10^6 cells/flask) and treated with ethyl methanesulfonate (0.1 to 0.5 mg/ml) for 18 hr. The medium was removed, the cells were washed, and fresh medium was added to the flask. After 5 days, single-cell suspensions of the cells from those lines exhibiting 10–20 per cent survival were obtained by trypsinization and were dispensed into 250-ml flasks (2×10^5 cells/flask) in medium containing dialyzed fetal calf serum and an inhibitory concentration of the desired selective agent. After incubation for 10 days those flasks containing resistant clones were treated with trypsin, and single-cell suspensions were obtained by repeated pipetting (mechanical pipet aids were used throughout these procedures). A portion of each cell suspension was diluted and dispensed into 25-cm^2 flasks such that there was approximately 1 cell per flask. After incubation for 10 days those flasks containing single clones were kept, and the cells were allowed to propagate as pure cell lines.

For the determination of a minimum inhibitory drug concentration, logarithmically growing CHO cells were trypsinized and appropriately diluted with medium containing dialyzed fetal calf serum. They were dispensed into

* Abbreviations: HGPRT, hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8); APRT, adenine phosphoribosyltransferase (EC 2.4.2.7); AK, adenosine kinase (EC 2.7.1.20); dCK, deoxycytidine kinase (EC 2.7.1.74); CHO cells, Chinese hamster ovary cells; 8-azaGua, 8-azaguanine; 8-azaAde, 8-azaadenine; TCN, tricyclic nucleoside (3-amino-1,5-dihydro-5-methyl-1-β-D-ribofuranosyl-1,4,5,6,8-pentazaacenaphthylene); ara-C, arabinosylcytosine; EHNA, *erthro*-9-(2-hydroxy-3-nonyl)adenine; DTT, dithiothreitol; and PRPP, phosphoribosyl pyrophosphate.