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Cyclic Nucleotide Phosphodiesterases of the Renal Cortex

Characterization of Basal-Lateral Membrane Activities

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Summary. Cyclic nucleotide phosphodiesterase in the basal-lateral segment of plasma membranes from proximal tubule cells of the rabbit renal cortex was studied and compared to that in the brush border segment of the plasma membrane. Both adenosine 3′,5′-monophosphate and guanosine 3′,5′-monophosphate were hydrolyzed by the basal-lateral membrane, but activity varied differently with the two substrates in a complex concentration-dependent manner. Activity with adenosine 3′,5′-monophosphate was greater than, equal to, or less than with guanosine 3′,5′-monophosphate, at concentrations of 1000, 100, and 10 to 1 μM, respectively. Basal-lateral membrane phosphodiesterase activities at 1 and 500 μM substrate exhibited differential responses to pH, metals, heat, and a heat stable inhibitor. Stimulation by guanosine 3′,5′-monophosphate and inosine 3′,5′-monophosphate of adenosine 3′,5′-monophosphate hydrolysis was found in basal-lateral but not in brush border membranes. This stimulation was potentiated by ethyleneglycol-bis(β-aminoethyl ether)N,N′-tetraacetic acid and ethylenediaminetetraacetate, inhibited by Triton X-100, and totally blocked by Zn^{2+}. The findings indicate that multiple forms of phosphodiesterase are present in the basal-lateral segment and these differ from the activities in the brush border region of the plasma membrane. The characteristics of (i) allosteric, guanosine 3′,5′-monophosphate-sensitivity of adensoine 3′,5′-monophosphate phosphodiesterase, and (ii) relatively high guanosine 3′,5′-monophosphate phosphodiesterase activity, in basal-lateral membranes, which are also enriched in adenylate and guanylate cyclase, suggest an important physiological role for these phosphodiesterases in the regulation of net production of cyclic nucleotides in the renal cortex.

In the renal proximal tubule, cAMP[1] and hormones which stimulate renal cAMP production have been shown to affect transport of phosphate [2, 1, 10], calcium [1, 34], sodium [16, 6, 14], sugars [27, 26], and amino acids [37]. Transepithelial transport of these solutes requires passage

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1 Abbreviations used: cAMP, adenosine 3′,5′-monophosphate; cGMP, guanosine 3′,5′-monophosphate; cIMP, inosine 3′,5′-monophosphate; EGTA, ethyleneglycol-bis(β-aminoethyl ether)N,N′-tetraacetic acid; MES, 2(N-morpholino)ethanesulfonic acid.

across the luminal brush border membrane and the basal-lateral membrane of the morphologically polar proximal tubular cell. Biochemical characterizations of these membranes isolated from kidneys of the rat [24, 32, 19] and the rabbit [9, 15, 20] have also indicated polarity with respect to enzymes mediating cyclic nucleotide action. Whereas adenylate cyclase is localized predominantly in the basal-lateral membrane [24, 32, 20, 12], membrane-associated protein kinase is enriched in the brush border membrane [19, 15, 20]. Cyclic nucleotide phosphodiesterase activity in brush border membranes has been partially characterized [13]. Since in some tissues it has been reported that membrane-associated cAMP phosphodiesterase is susceptible to hormonal regulation in a manner suggestive of a close association with adenylate cyclase [22, 33, 25, 31, 38, 4, 35], it was important to examine the phosphodiesterase activity of the adenylate cyclase-enriched basal-lateral segment of the tubular cell plasma membrane. In this paper we report some properties of the enzyme in this membrane and describe differences between the activities in basal-lateral and brush border membranes.

Materials and Methods

Preparation of Membranes

Basal-lateral membranes from rabbit renal cortex were prepared by a modification of the method of Marx *et al.* [24], as described previously [20]. The final suspension of membranes, containing 40–60 μg of protein per g wet wt of renal cortex, had a Na^+, K^+-ATPase specific activity of approximately 20 μmoles/hr/mg of protein, representing an 18-fold increase relative to the specific activity in the homogenate [20]. Lactate dehydrogenase activity was not measurable [20], suggesting little, if any, contamination by the cytosol, *i.e.*, the soluble phosphodiesterases [13].

Brush border membranes were prepared as described previously from the γ-glutamyl transpeptidase-enriched region of sucrose gradients employed in the preparation of basal-lateral membranes [20], designated Method *I*, or by a standard procedure used in this laboratory [9, 13, 5], designated Method *II*. The yields were 200–300 μg and 400–800 μg per g wet wt of renal cortex with Methods *I* and *II*, respectively. The specific activity of γ-glutamyl transpeptidase was about 350 μmoles/hr/mg of protein, approximately 12-fold that in the homogenate of the cortex [20].

Assays

Cyclic nucleotide phosphodiesterase was assayed by the isotopic method of Filburn and Karn [13], with slight modification. Reaction mixtures contained 50 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, [^{3}H]cyclic nucleotide (usually 0.3–0.7 μCi) plus unlabeled cyclic nucleotide to give the indicated concentrations, and 5–20 μg of membrane protein in a total volume of 50 μl. After incubation at 30 °C for 5–40 min, the reaction was stopped by addition

of 50 μl of 0.8 N perchloric acid and the mixture then neutralized with 50 μl of 0.8 N KOH containing 0.20 M Tris-Cl previously adjusted to pH 8.2. A 20-μl aliquot of a recovery monitoring solution containing 6 mM cAMP, 1.25 mM 5′-AMP, and 175,000–250,000 cpm/ml [^{14}C]adenosine (cGMP, 5′ GMP, and [^{14}C]-guanosine were used when cyclic GMP was the substrate) in 25 mM Tris-Cl, pH 7.5 was added, followed by 0.1 unit 5′-nucleotidase and incubation at 30 °C for 15–20 min. The nucleotidase reaction was stopped by the addition of 100 μl 0.28 N acetic acid and the mixture applied to and eluted from alumina columns as previously described [13]. Eluates were counted using a detergent-containing scintillation medium and a Packard Tri-Carb scintillation spectrometer. Assays with both basal-lateral and brush border membranes were conducted under conditions of proportionality with time and amount of protein. Protein was assayed by the method of Lowry *et al.* [21].

Materials

[^{3}H]cAMP (27–30 Ci/mmole), [^{3}H]cGMP (13–21 Ci/mmole), [^{14}C]adenosine (464 mCi/mmole), and [^{14}C]guanosine (585 mCi/mmole) were purchased from Amersham-Searle. cAMP was obtained from P-L Biochemicals. cGMP, neutral alumina and 5′-nucleotidase were from Sigma.

Results

Specific and Relative Activities

Basal-lateral membrane phosphodiesterase hydrolyzed both cAMP and cGMP over a wide range of substrate concentrations (Table 1). The membrane had more activity with cAMP than with cGMP at 1 mM substrate, similar activity at 100 μM, but greater activity with cGMP than with cAMP at 10 μM and 1 μM. Both relative and specific activities of the phosphodiesterase in basal-lateral membranes differed from those in brush border membranes when compared at the various substrate concentrations. For example, at 1 mM cyclic nucleotide brush border membranes had a cAMP/cGMP activity ratio greater than basal-lateral membranes, due to a higher specific activity with cAMP. With brush border membranes the ratio decreased with decreasing substrate concentration but activity with cAMP exceeded that with cGMP even at 1 μM, in contrast to that seen with basal-lateral membranes. To be noted, however, at physiological substrate concentrations (1 to 10 μM) the two membranes had comparable activities with cAMP, but that with cGMP activity was significantly greater in basal-lateral membranes than in brush border membranes. It was found additionally (Table 1) that brush border membranes prepared by two entirely distinct methods were essentially identical in their phosphodiesterase activities, with only an apparent slight difference in activity at 1 μM cGMP.

The effect of concentration of each substrate on the activity of the

Table 1. Cyclic nucleotide phosphodiesterase activity in basal-lateral and brush border membranes of the renal cortex[a]

Substrate (μM)	Basal-lateral membrranes			Brush border membranes					
				(I)			(II)		
	cAMP	cGMP	$\frac{cAMP}{cGMP}$	cAMP	cGMP	$\frac{cAMP}{cGMP}$	cAMP	cGMP	$\frac{cAMP}{cGMP}$
	(pmole/min/mg)			(pmole/min/mg)			(pmole/min/mg)		
1000	3001 ±256[b,c]	1799 ±169	1.67	4563 ±248[b]	1857 ±88	2.46	5101 ±492[b]	1832 ±134	2.78
100	1768 ± 37	1596 ±137[c]	1.11	1620 ±142[b]	600 ±34	2.70	1496 ± 36[b]	676 ± 58	2.21
10	363 ± 48[b]	619 ± 53[c]	0.59	286 ± 25[b]	164 ± 7	1.74	275 ± 17[b]	178 ± 16	1.54
1	54 ± 3[b]	93 ± 6[c]	0.58	52 ± 6[b]	27 ± 2	1.93	55 ± 4	42 ± 5	1.31

[a] Brush border membranes were prepared by two different procedures, as described in *Materials and Methods*, and these are designated Method *I* and *II*. Values are the means ±SEM of four determinations.

[b] The activity with cAMP as substrate is significantly different from that of the same membrane with cGMP as substrate at the same concentration, $p < 0.01$.

[c] The value for basal-lateral membranes is significantly different from the corresponding activity in brush border membranes, $p < 0.01$.

basal-lateral membrane phosphodiesterase was examined in greater detail, as shown in Fig. 1. At substrate concentrations less than 0.5 μM, activity with cAMP exceeded that with cGMP. With increasing concentration, however, activity with cGMP exceeded that with cAMP up to approximately 25 μM cyclic nucleotide. Thence the relationship reversed (Fig. 1*A*) and remained so up to at least 1 mM substrate. Double reciprocal plots (Fig. 1*C*,*D*) or Eadie-Hofstee plots (not illustrated) demonstrated additionally the complexity of the membrane-associated activities and provided strong suggestive evidence for the presence of multiple phosphodiesterases in the basal-lateral membrane.

Cyclic Nucleotide Activation and Inhibition

The effects of added cyclic nucleotides upon the hydrolysis of 1 μM cAMP and cGMP by basal-lateral membranes are shown in Fig. 2. Hydrolysis of cAMP was stimulated by both cGMP and cIMP, with maximal

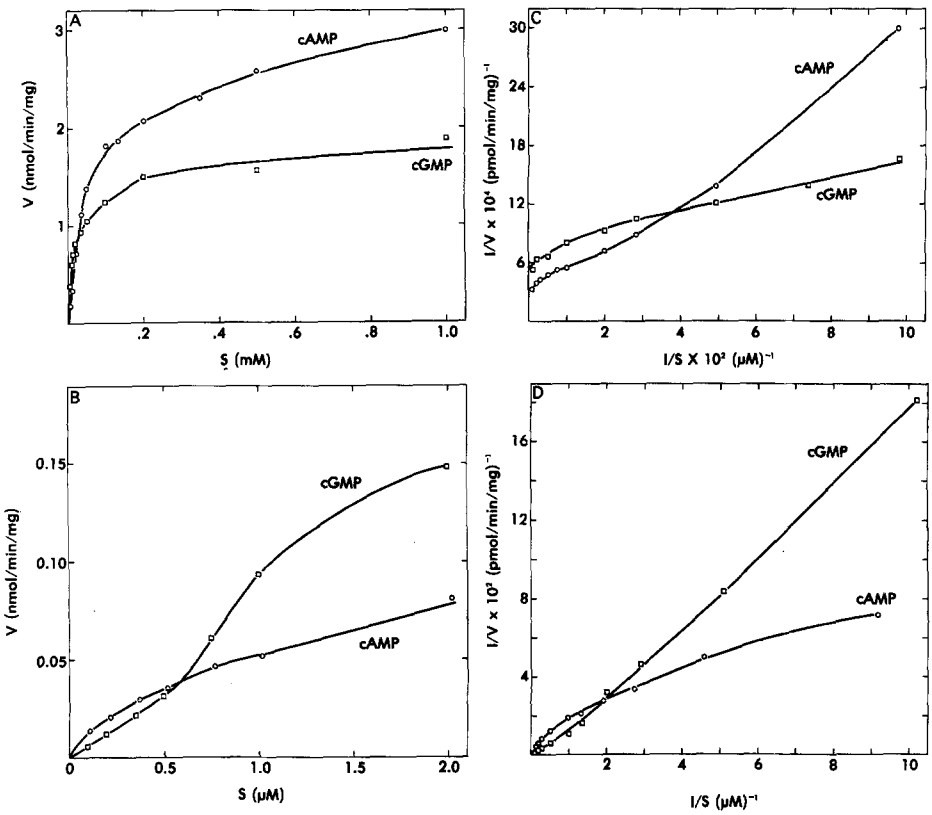

Fig. 1. Effect of substrate concentration on the rate of hydrolysis of cAMP and cGMP by basal-lateral membranes. The data in (*C*) and (*D*) are Lineweaver-Burk plots of the data in (*A*) and (*B*), respectively. ○, cAMP as substrate; □, cGMP as substrate. Values are the means of three determinations

stimulation found with 3 and 10 μM, respectively. Hydrolysis of cGMP, however, was not enhanced by cAMP or cIMP; instead phosphodiesterase activity was inhibited. The magnitude of the stimulation in rate of cAMP hydrolysis by cGMP was dependent upon the concentrations of both cyclic nucleotides. Stimulation increased with increasing cAMP up to 2 μM, and with increasing cGMP up to 3 μM (Fig. 3).

In contrast to the cGMP-dependent stimulation of hydrolysis of 1 μM cAMP by basal-lateral membranes, cGMP did not stimulate cAMP hydolysis by brush border membranes [13]. Moreover, as shown in Table 2, this insensitivity was not due to the method of preparation of the brush border membrane. Hydrolysis of 1 μM cAMP by basal-lateral membranes was inhibited 5 to 10% by 20 μM EGTA. However, when the chelator was added to the incubation medium together with cGMP, the stimula-

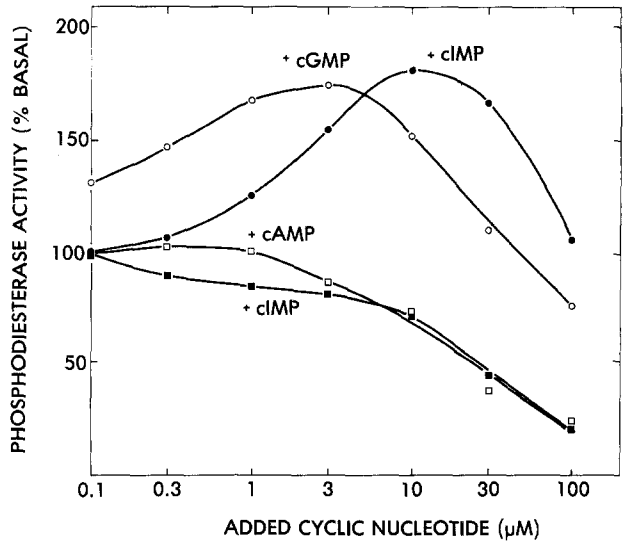

Fig. 2. Effect of added cyclic nucleotides on the hydrolysis of cAMP and cGMP by basal-lateral membrane phosphodiesterase. The concentration of ^{3}H-labeled substrate was 1 μM. With cAMP as substrate incubation times were kept short so that no more than 25% of the cAMP or added cGMP would be hydrolyzed. ○, ●, cAMP as substrate; ○, cGMP added; ●, cIMP added; □, ■, cGMP as substrate; □, cAMP added; ■, cIMP added. Values are the means of three or more determinations

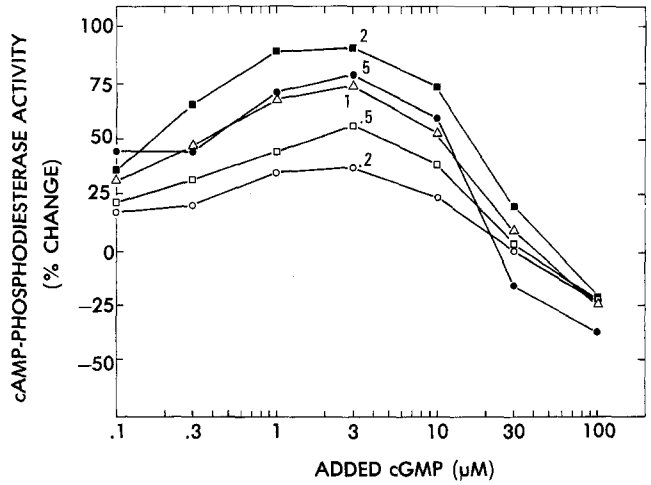

Fig. 3. Effect of varying concentrations of cGMP on the hydrolysis of different concentrations of cAMP by basal-lateral membrane phosphodiesterase. ○, □, △, ■, ●, cAMP at substrate concentrations of 0.2, 0.5, 1.0, 2.0, and 5 μM, respectively. Values are the means of three determinations

Table 2. Effect of cGMP and cGMP+EGTA on cAMP-phosphodiesterase activity of basal-lateral and brush border membranes[a]

Additions	Basal-lateral membranes		Brush border membranes			
			(I)		(II)	
	Specific activity	% Control	Specific activity	% Control	Specific activity	% Control
–	54±3	100	52±6	100	55±4	100
EGTA	48±2	88± 1	49±6	94±2	52±4	95±2
cGMP	103±7	190±12[b]	55±6	104±4	56±5	100±3
cGMP+EGTA	146±8[b,c]	269±15[b,c]	60±5	116±5	64±7	115±5

[a] Brush border membranes were prepared by two different procedures, as described in *Materials and Methods*, and these are designated Method *I* and *II*. cGMP and EGTA, when indicated, were present at 3 μM and 20 μM, respectively. Values are means ± SEM of four (brush border) or eight (basal-lateral membranes) determinations.

[b] The value is different from the control with $p < 0.001$.

[c] The value is different from that with cGMP alone with $p < 0.002$.

tory action of the cyclic nucleotide was markedly potentiated (Table 2). The combination of cGMP + EGTA affected a slight (10 to 15%), statistically insignificant, enhancement of the hydrolysis of cAMP by brush border membranes. This small enhancement could be completely accounted for by a 7 to 8% contamination of the brush border membrane preparation by basal-lateral membranes. Determination of the distribution of the basal-lateral membrane enzyme "marker" guanylate cyclase, reported elsewhere [20], indicated this level of contamination. Previously, cGMP was found to stimulate cAMP hydrolysis by particulate fractions from several different tissues [8] and EGTA was found to enhance the cGMP-stimulated hydrolysis by cytosolic phosphodiesterases [36, 17]. In other experiments with basal-lateral membranes (data not shown) it was observed that the stimulation by cGMP of cAMP hydrolysis was not significantly affected by 1 mM dithiothreitol. Also, Triton X-100 (0.06%), which did not significantly affect the hydrolysis of cAMP in the absence of cGMP, inhibited the cGMP-dependent stimulation. EGTA potentiated the cGMP-stimulated cAMP hydrolysis despite the presence of detergent, as noted earlier with a cytosolic phosphodiesterase [36].

Potentiation by EGTA of the cGMP-stimulated hydrolysis of cAMP in basal-lateral membranes was obtained with concentrations of chelator

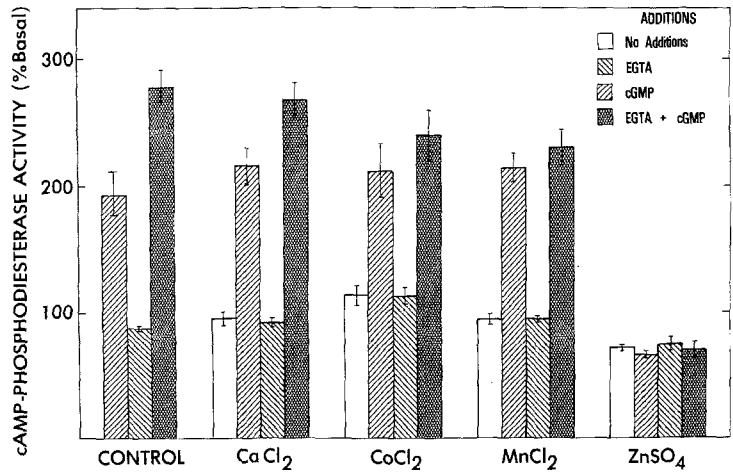

Fig. 4. Effect of divalent metal ions on EGTA potentiation of cGMP stimulation of 1 μM cAMP hydrolysis. EGTA was present at 20 μM and cGMP at 3 μM. The metals were present at a total concentration of 50 μM when included in reaction mixtures containing EGTA, or at 30 μM when EGTA was absent. $MgCl_2$ was present in all reaction mixtures at 10 mM. Values are the means ± SEM of four determinations

ranging from 5 to 1000 μM; 20 μM EDTA was equally effective (data not shown). Since EGTA binds divalent metal ions, it seemed probable that its potentiating action was related to the removal of an inhibitory metal, with Ca^{2+} being a likely candiate. Addition of 50 μM $CaCl_2$ to reaction mixtures containing 20 μM EGTA and 3 μM cGMP, however, had but negligible effect on the potentiation (Fig. 4). Addition of 50 μM $CoCl_2$ or $MnCl_2$ was similarly without effect. Inclusion of $ZnSO_4$, however, resulted in a 30% decrease in basal activity and complete elimination of the cGMP activation, irrespective of the presence of EGTA. Substitution of EDTA for EGTA did not alter the inhibitory action of Zn^{2+} (data not shown).

Distinctions between Activities at High and Low Substrate Concentration

The pH profile of basal-lateral membrane cAMP phosphodiesterase activity depended on the concentration of substrate (Fig. 5). With 1 μM cAMP, activity was maximal over a broad range, from pH 7 to 9. With 500 μM cAMP, the optimal range was narrower with a peak at pH 7.6. The sharp pH optimum for basal-lateral membrane phosphodies-

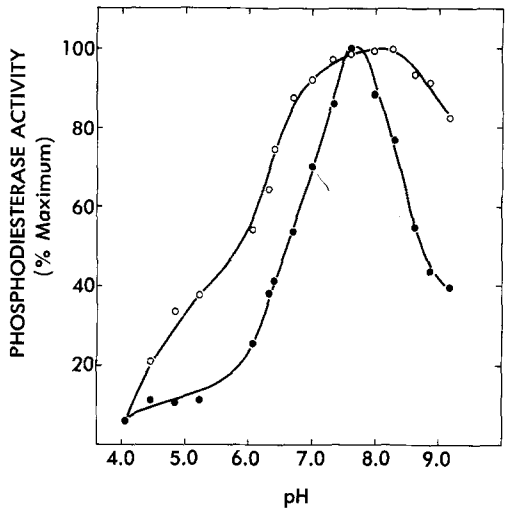

Fig. 5. Effect of pH on basal-lateral membrane phosphodiesterase activity assayed at 500 μM (●) and 1 μM (○) cAMP. All reaction mixtures contained acetate-MES-Tris-glycine, each at 40 mM, adjusted to the indicated pH at 30 °C. Specific activities at optimal pH (100%) are 2350 and 48 pmoles/min/mg for 500 and 1 μM cAMP, respectively. Values are the means of two or more determinations

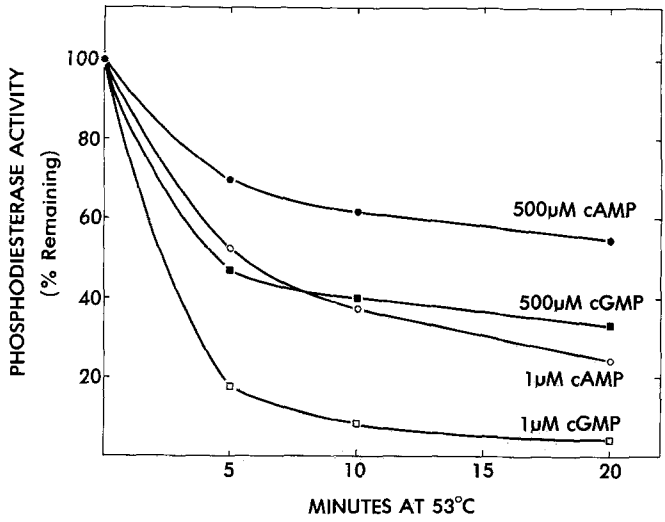

Fig. 6. Thermolability of basal-lateral membrane phosphodiesterase activity. Membranes were suspended (1.5 mg of protein/ml) in 5 mM Tris-Cl buffer (pH 7.5), preincubated for 2 min at 30 °C and then incubated at 53 °C. At the indicated times aliquots were removed to ice and subsequently assayed at 30 °C as described in Materials and Methods. ○, ●, 1 μM and 500 μM cAMP as substrate, respectively; □, ■, 1 μM and 500 μM cGMP as substrate, respectively

terase activity hydrolyzing 500 μM substrate resembled that observed for the hydrolysis of 1 mM cAMP by brush border membranes [13]. The two membranes differed, however, in that at 1 μM cAMP the brush border membrane had a more acidic and defined optimum, pH 6 to 7.

Differential thermolability of high and low substrate phosphodiesterase activities in basal-lateral membranes was also evident (Fig. 6). Upon preincubation of the membranes at 53 °C residual activity determined with 1 μM cAMP or cGMP decreased by 75 or 95%, respectively. Activity determined with 500 μM substrate, however, decreased only 45% for cAMP and 65% for cGMP. A qualitatively similar differential thermolability was found for the hydrolysis of 1 μM and 1 mM cAMP by brush border membranes [13].

A comparison of the effects of divalent metal ions on the phosphodiesterase in basal-lateral membranes with 1 and 500 μM cAMP (Table 3) illustrates additional differences between the two activities. While both activities were increased by 10 mM Mg^{2+}, Co^{2+}, and Mn^{2+}, the relative stimulations were greater with 1 μM cAMP. Also, Ca^{2+} inhibited hydrolysis of 1 μM cAMP but had no effect on the hydrolysis of 500 μM cAMP. At lower concentration of metals (0.6 mM) additional distinctions between

Table 3. Effect of divalent metal ions on cAMP-phosphodiesterase activity of basal-lateral membranes[a]

Metal	Concentration (mM)	500 μM cAMP	1 μM cAMP
		(% of control activity)	
None	–	100	100
Mg^{2+}	10	164	250
	0.6	144	169
Co^{2+}	10	138	241
	0.6	140	280
Mn^{2+}	10	157	277
	0.6	146	236
Ca^{2+}	10	98	58
	0.6	96	61

[a] The membranes were washed as described in *Materials and Methods* with 5 mM Tris-Cl (pH 7.5), prior to use in order to remove the EDTA present during preparation. Metals were used as the chloride salts. Control activities (100%) are 1463 and 21 pmoles/min/mg for 500 and 1 μM cAMP, respectively, determined in the absence of added metals. Values are the means of at least two determinations, with very close agreement between replicates.

activities at high and low substrate concentrations were seen. Thus, at 500 μM cAMP, Mg^{2+}, Co^{2+}, and Mn^{2+} were equally effective in stimulating hydrolysis. At 1 μM substrate, Mg^{2+} was less effective than the other two divalent ions. In further experiments (data not shown) a differential sensitivity to Mg^{2+} was found for the hydrolysis of 1 and 500 μM cGMP, with about a 10-fold stimulation observed at the lower concentration of substrate but only a 3-fold increase at the higher. Interestingly, in the presence of 10 mM Mg^{2+}, 1 mM EGTA stimulated hydrolysis of 1 and 500 μM cGMP 16 and 45%, respectively.

A heat stable factor, prepared from renal cortical extracts as previously described [13], markedly inhibited the hydrolysis of 100 μM and 1000 μM cAMP by basal-lateral membranes, while causing much less or no inhibition with 1 μM cAMP (data not shown). This concentration-dependent response to the factor was similar to that reported for brush border activities [13].

Discussion

The luminal brush border and the contraluminal basal-lateral segments of the renal proximal tubular cell plasma membrane are known to be morphologically, enzymically, and functionally distinct (for reviews *see* [30], [18]). The brush border membrane is enriched in disaccharidases, γ-glutamyl transpeptidase, and alkaline phosphatase, whereas the basal-lateral membrane is enriched in Na,K-ATPase. Enzymes involved in cyclic nucleotide metabolism and action also exhibit differential membrane localization, with adenylate cyclase [24, 32, 20, 12] and guanylate cyclase [20] localized predominantly in the basal-lateral membrane, but membrane-associated, intrinsic protein kinase prominantly found in the brush border membrane [19, 15]. The present study demonstrates that this differential localization of cyclic nucleotide-associated enzymes also includes phosphodiesterase. While in the two membranes the ratio of specific activities for the hydrolysis of cAMP and cGMP differs and in each activity varies in a substrate concentration-dependent manner (*see* Table 1), two distinguishing and important features emerge: (i) within a given low, micromolar range of substrate concentration cGMP-phosphodiesterase activity in the basal-lateral membrane is at least double that in brush border (Table 1); (ii) although the two membranes differ little in their activities with cAMP, at micromolar cAMP added cGMP stimulates hydrolysis of cAMP substantially in basal-lateral membranes

but is without significant affect on brush border membrane activity (Table 2). Clearly the adenylate and guanylate cyclase-enriched basal-lateral membrane has a greater potential via phosphodiesterases for regulating net production of both cAMP and cGMP than does its counterpart, the brush border membrane. This enhanced flexibility in control of cyclic nucleotide level may be of importance in the regulation of cyclic nucleotide-mediated hormonal actions in the proximal tubular cell.

The demonstration in this study that two relatively homogeneous membrane preparations, derived from the same tissue, have different substrate concentration-dependent phosphodiesterase specific activities, and that in each membrane the activity at high and low substrate responds differently to factors such as added nucleotides, heat, metals, and inhibitors (*see* [13]) is probably attributable at least in part to the presence of more than one enzyme in each of these purified membrane preparations. This tentative conclusion has relevance to the understanding of the regulation of other membrane-associated cyclic nucleotide phosphodiesterases. In rat liver and chicken embryo fibroblast, membrane-associated phosphodiesterase is relatively cAMP-specific and appears, by chromatographic criteria, to be the same as a cAMP-specific soluble phosphodiesterase [29, 28]. In these membranes and in particulate fractions from several other tissues activity with 1 μM cAMP exceeded that with 1 μM cGMP [7, 3], the only exception being the particulate activity from rat brain [7]. cGMP stimulation of cAMP hydrolysis was also reported in particulate fractions from several rat tissues [8], but cGMP was without effect in plasma membranes from isolated fat cells [31]. Thus, while membrane-associated phosphodiesterase activities in many tissues have common properties, differences seemingly exist. The present data indicate, however, that within a single cell regions of the plasma membrane may also differ. Similar distinctions may exist in other tissues containing either different cell types or in a single cell type which has a differentiated plasma membrane, e.g., intestine. It therefore seems unlikely that the particulate phosphodiesterase activities described in either crude or purified membranes from various other tissues can be accounted for by a single phosphodiesterase. Assessment of the number and type of phosphodiesterases present and of the influence of membrane association on their properties should aid in understanding their physiological function.

The ability of Zn^{2+} to prevent the cGMP-stimulation of cAMP hydrolysis by basal-lateral membranes merits some comment. Ca^{2+} has been implicated in the reversible modulation in sensitivity of a soluble cAMP-

phosphodiesterase from rat liver to cGMP [17]. The relatively high level (0.1–3 mM) required to affect this control, however, makes a physiological role for this metal questionable. With basal-lateral membranes, 30 μM Zn^{2+} completely blocked the stimulation by cGMP, even when the potentiating agents EGTA and EDTA were present. Mn^{2+} and Co^{2+}, which like Zn^{2+} complex with the chelators much more tightly than Ca^{2+}[23], had little effect on cGMP stimulation. Whether Zn^{2+} at lower levels is able to exert this inhibitory effect has not been determined. The fact that cGMP-stimulation of cAMP hydrolysis has been observed in both particulate and soluble fractions in many tissues [8] suggests that the possible role of Zn^{2+} in the overall control of cyclic nucleotide metabolism deserves further attention.

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