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Effects of pH on Allosteric and Catalytic Properties of the Guanosine Cyclic 3′,5′-Phosphate Stimulated Cyclic Nucleotide Phosphodiesterase from Calf Liver

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ABSTRACT: We have investigated effects of pH on the catalytic and allosteric properties of the cGMP-stimulated cyclic nucleotide phosphodiesterase purified from calf liver. In the "activated" state, i.e., with 0.5 μM [^{3}H]cAMP plus 1 μM cGMP or at saturating substrate concentrations (250 μM [^{3}H]cAMP or [^{3}H]cGMP), hydrolysis was maximal at pH 7.5–8.0 in assays of different pH. Hydrolysis of concentrations of substrate not sufficient to saturate regulatory sites and below the apparent Michaelis constant (K_m^{app}), i.e., 0.5 μM [^{3}H]cAMP or 0.01 μM [^{3}H]cGMP, was maximal at pH 9.5. Although hydrolysis of 0.5 μM [^{3}H]cAMP increased with pH from 7.5 to 9.5, cGMP stimulation of cAMP hydrolysis decreased. As pH increased or decreased from 7.5, Hill coefficients (n_{app}) and V_{max} for cAMP decreased. Thus, assay pH affects both catalytic (V_{max}) and allosteric (n_{app}) properties. Enzyme was therefore incubated for 5 min at 30 °C in the presence of $MgCl_2$ at various pHs before assay at pH 7.5. Prior exposure to different pHs from pH 6.5 to 10.0 did not alter the V_{max} or cGMP-stimulated activity (assayed at pH 7.5). Incubation at high (9.0–10.0) pH did, in assays at pH 7.5, markedly increase hydrolysis of 0.5 μM [^{3}H]cAMP and reduce K_m^{app} and n_{app}. After incubation at pH 10, hydrolysis of 0.5 μM [^{3}H]cAMP was maximally increased and was similar in the presence or absence of cGMP. Thus, after incubation at high pH, the phosphodiesterase acquires characteristics of the cGMP-stimulated form. Activation at high pH occurs at 30 °C but not 5 °C, requires $MgCl_2$, and is prevented but not reversed by ethylenediaminetetraacetic acid. These results indicate that incubation at high pH in the absence of substrates and/or effectors promotes allosteric transitions (n_{app}) and a decrease in K_m^{app} in the absence of changes in V_{max} and suggest independent regulation of topographical features and domains responsible for these properties.

A cGMP-stimulated cyclic nucleotide phosphodiesterase (PDE)[1] has been purified from bovine heart, adrenal, and liver tissue (Martins et al., 1982; Yamamoto et al., 1983a). The enzyme hydrolyzes both cAMP and cGMP with positively cooperative kinetics. At substrate concentrations well below the apparent K_m (K_m^{app}), hydrolysis of one cyclic nucleotide is stimulated by the other; cGMP is the preferred substrate or effector (Martins et al., 1982; Yamamoto et al., 1983a; Moss et al., 1977). Earlier studies have suggested that substrates and effectors induce allosteric transitions that lead to enhanced catalytic activity as well as increased susceptibility to proteolytic attack (Moss et al., 1977). As might be expected from the cooperative behavior of the PDE, certain substrate analogues and competitive inhibitors can, like substrates, induce allosteric transitions that result in "activation" of the PDE and stimulation of hydrolysis of low substrate concentrations. Substrate analogues and inhibitors can also compete with substrate at catalytic sites and competitively inhibit hydrolysis of substrate by the activated form of the enzyme (Moss et al.,

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[1] Abbreviations: PDE, phosphodiesterase; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; BSA, bovine serum albumin; PMSF, phenylmethanesulfonyl fluoride; IBMX, isobutylmethylxanthine; K_m^{app}, apparent Michaelis constant; n_{app}, apparent number of cooperatively interacting sites (Hill coefficient); K_I, inhibition constant; EDTA, ethylenediaminetetraacetic acid; BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; BICINE, *N*,*N*-bis(2-hydroxyethyl)glycine; MES, 4-morpholineethanesulfonic acid.

1977; Erneux et al., 1981, 1982; Yamamoto et al., 1983b).

In the experiments reported here, effects of pH on cooperative interactions (Hill coefficient, n_{app}) and catalytic activity (V_{max}) were investigated. After incubation at high pH (9.5 or 10) in the presence of $MgCl_2$, in assays at pH 7.5 n_{app} is reduced without changes in V_{max}, and the PDE exhibits characteristics of the cGMP-stimulated state (Martins et al., 1982; Yamamoto et al., 1983a; Moss et al., 1977; Erneux et al., 1981). These findings suggest that incubation at high pH promotes allosteric transitions and activates the PDE in the absence of substrates or effectors and that the molecular interactions involved in promoting allosteric transitions can be regulated independently of those involved in regulation of V_{max}.

Experimental Procedures

Materials. [2,8-^{3}H]cAMP (~30 Ci/mmol) and [8,5-^{3}H]cGMP (35.6 Ci/mmol) purchased from New England Nuclear were purified by thin-layer chromatography on cellulose with 0.5 M ammonium acetate/ethanol, 2:5 (v/v), and on columns of DEAE-Sephadex A-25 (Pharmacia). BES (Ultrol), CAPS (Ultrol), and HEPES (Ultrol) were purchased from Calbiochem-Behring; BICINE and MES were from Boehringer Mannheim. All other materials were purchased from the sources described (Yamamoto et al., 1983a,b).

Preparation and Assay of cGMP-Stimulated Cyclic Nucleotide PDE. The cGMP-stimulated PDE was purified from calf liver to apparent homogeneity, as judged by SDS gel electrophoresis (Yamamoto et al., 1983a). PDE activity was assayed at 30 °C (usually for 10 min) in a final volume of 0.3 mL at the indicated pH containing 15 μmol of specified buffer, 2.5 μmol of $MgCl_2$, 15 nmol of EGTA, 150 μg of ovalbumin, 20 000–30 000 cpm of [^{3}H]cAMP or [^{3}H]cGMP, and the indicated concentrations of unlabeled cAMP and/or cGMP (Yamamoto et al., 1983a,b). PDE activity was constant for at least 20 min with 1 or 250 μM [^{3}H]cAMP at either pH 7.5 or pH 9.5. To verify constant rates in individual experiments where hydrolysis of a wide range of cAMP concentrations was measured, assays were carried out for 10 and 20 min at several substrate concentrations. The reaction was terminated, and the ^{3}H-labeled nucleoside was isolated for radioassay as described previously (Yamamoto et al., 1983a,b). For pH 6.0 and 6.5 buffers, MES (pK_a = 6.15 at 20 °C) was used; for pH 7.0, 7.5, and 8.0 buffers, HEPES (pK_a = 7.55); for pH 8.5, 9.0, and 9.5, BICINE (pK_a = 8.35); for pH 10.0 and 10.5, CAPS (pK_a = 10.4). pH was determined at room temperature by using the Orion Research Model 210A and the Orion Research Ross pH electrode. In some experiments, the cGMP-stimulated PDE was incubated (usually at ~2.5 μg/mL) in buffer A at the indicated pH [50 mM buffer, 3 mg/mL BSA (fatty acid free), 0.1 mM EDTA, 1 mM NaN_3, 0.4 mM PMSF, 1 μM pepstatin, and 2 μM leupeptin] in the presence or absence of $MgCl_2$ (usually 5 mM) at 30 °C. After dilution (~35–150-fold) with buffer A at pH 7.5, samples (0.05 mL) were assayed at pH 7.5. Dilutions were checked (with identical solutions which did not contain enzyme) to ascertain that the desired pH was achieved in these sequential manipulations. Unless otherwise noted, data reported are means of values of duplicate assays in representative experiments. Protein concentration was determined with Coomassie Brilliant Blue G-250 (Bio-Rad) using bovine serum albumin as the standard (Bradford, 1976).

Results

Effects of Assay pH on cAMP and cGMP Hydrolysis, cGMP-Stimulated cAMP Hydrolysis, V_{max}, and n_{app}. In assays with saturating substrate concentrations (i.e., 250 μM [^{3}H]cAMP or [^{3}H]cGMP), or at substrate or effector concentrations sufficient to saturate regulatory sites, induce allosteric transitions, and "activate" the PDE (i.e., 1 μM [^{3}H]cGMP or 0.5 μM [^{3}H]cAMP plus 1 μM cGMP) (Martins et al., 1982; Yamamoto et al., 1983a; Moss et al., 1977), PDE activity was maximal at pH 7.5. At lower substrate concentrations, below K_m^{app} for each and not sufficient to saturate regulatory sites and activate the PDE (i.e., 0.5 μM [^{3}H]cAMP or 0.01 μM [^{3}H]cGMP), activity increased as pH increased and was maximal at pH 9.5 (Figure 1A,B). Stimulation of cAMP hydrolysis (0.5 μM [^{3}H]cAMP) by cGMP (1 μM) was much less in assays at pH 9.5 than at pH 7.5 because as activity in the presence of cGMP decreased, hydrolysis of 0.5 μM [^{3}H]cAMP increased (Figure 1).

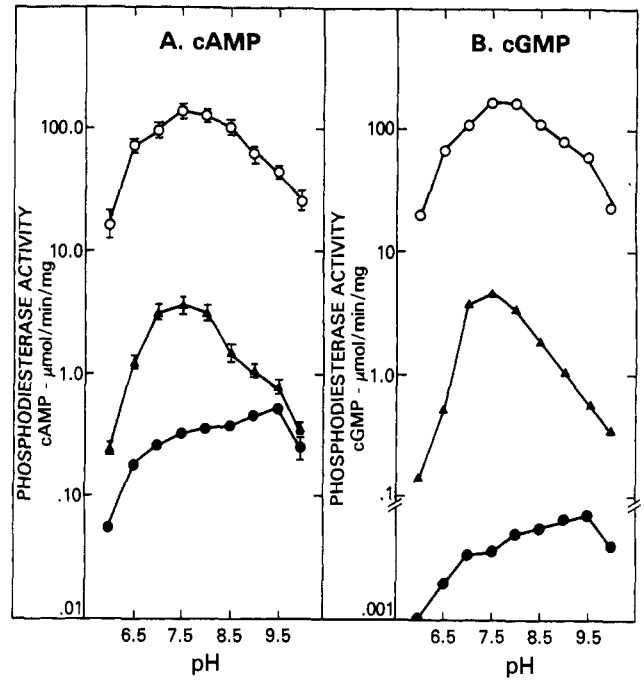

FIGURE 1: Effects of assay pH on hydrolysis of cAMP and cGMP. Enzyme was assayed (A) at the indicated pH with 0.5 μM (●) or 250 μM (○) [^{3}H]cAMP or with 0.5 μM [^{3}H]cAMP plus 1 μM cGMP (▲) [values presented represent mean ± SEM (n = 3 experiments, except at pH 6.0, 10.0, and 10.5 which are means of duplicate experiments)], or (B) at the indicated pH with 0.01 μM (●), 1 μM (▲), or 250 μM (○) [^{3}H]cGMP (values presented represent means of two experiments, except those for 1 μM [^{3}H]cGMP, an experiment performed only once).

In assays at pH 6.5, 7.5, 8.5, and 9.5 but not at pH 10, increasing concentrations of cGMP increased hydrolysis of 0.5 μM [^{3}H]cAMP (data not shown). With maximally effective concentrations of cGMP (5–10 μM), cGMP-stimulated hydrolysis of cAMP was much higher at pH 7.5 than at either pH 6.5 or pH 8.5. cGMP stimulation of cAMP hydrolysis was minimal at pH 9.5 and virtually absent at pH 10. Although at pH 9.5 both x-fold stimulation and maximal stimulation of cAMP hydrolysis by cGMP were much less than at pH 7.5, the concentration of cGMP required for half-maximal stimulation of cAMP hydrolysis was similar (0.25–0.5 μM cGMP) at both pH values (data not shown).

When hydrolysis of a wide range of cAMP concentrations (0.5–1000 μM) was measured in assays at different pHs V_{max} was highest at pH 7.5 (Figure 2). As seen in the insert to Figure 2, the Hill coefficient (n_{app}) for cAMP hydrolysis, an index of cooperative interactions, was highest (~1.8) in assays at pH 7.5 and decreased to ~1 at either pH 6.0 or pH 9.5; K_m^{app} [i.e., log ($v/V - v$) = 0] was lower at pH 7.5 than at pH 9.5 or 6.0. In view of these rather complex effects of assay

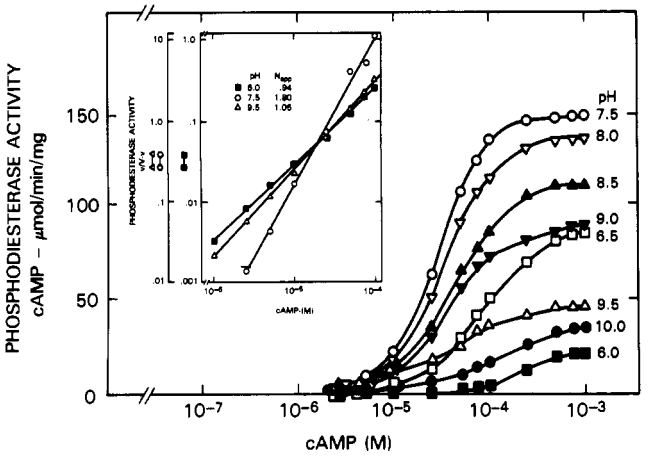

FIGURE 2: Effects of assay pH on cAMP hydrolysis. Enzyme (12.5 ng at pH 6.0 and 6.5; 6.25 ng at all others) was assayed at the indicated pH in the presence of the indicated concentrations of [^{3}H]cAMP: pH 6.0 (■), 6.5 (□), 7.5 (○), 8.0 (▽), 8.5 (▲), 9.0 (▼), 9.5 (△), 10.0 (●). Inset: Hill plots. Hill coefficients (n_{app}) at pH 6.0 (■), 0.94; pH 7.5 (○), 1.80; pH 9.5 (△), 1.05.

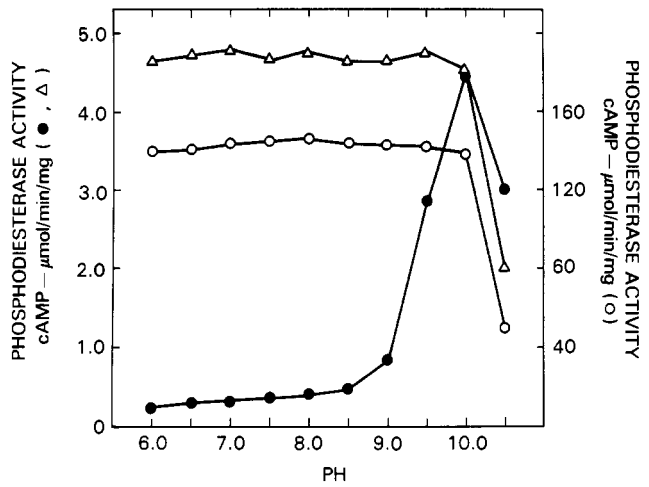

FIGURE 3: Effects of incubation at various pHs on cAMP hydrolysis in subsequent assays at pH 7.5. Enzyme (2.5 μg/mL) was incubated in buffer A at the indicated pH for 5 min at 30 °C with 5 mM $MgCl_2$ prior to dilution with buffer A at pH 7.5 and assay at pH 7.5 in the presence of 0.5 μM (●) or 500 μM (○) [^{3}H]cAMP or 0.5 μM [^{3}H]cAMP plus 1 μM cGMP (△).

pH, enzyme was therefore first incubated at a different pH and then assayed at pH 7.5, which was optimal for the activated form of the enzyme.

Effects of Incubation at Different pHs on cAMP Hydrolysis in Assays at pH 7.5. Incubation at pH 6.5–10.0 for 5 min at 30 °C in the presence of 5 mM $MgCl_2$ before assay at pH 7.5 did not affect hydrolysis of 500 μM [^{3}H]cAMP or 0.5 μM [^{3}H]cAMP plus 1 μM cGMP (Figure 3). Incubation (5 min, 30 °C, 5 mM $MgCl_2$) at pH 6.5–8.0 also had little or no effect on hydrolysis of 0.5 μM [^{3}H]cAMP in assays at pH 7.5. Thus, changes in these parameters of cAMP hydrolysis with pH (Figures 1 and 2) were apparently readily reversed.

After incubation (5 min, 30 °C, 5 mM $MgCl_2$) at pH 8.5–10, however, hydrolysis of 0.5 μM [^{3}H]cAMP at pH 7.5 was markedly increased (Figure 3); prior exposure to pH 10 produced maximal increases (~15-fold) in cAMP hydrolysis at pH 7.5. In fact, hydrolysis of 0.5 μM [^{3}H]cAMP at pH 7.5 was increased to the same extent by direct addition of 1 μM cGMP to the assays or by prior incubation at pH 10 (5 min, 30 °C, 5 mM $MgCl_2$) (Figure 3).

Incubation (5 min, 30 °C) at pH 9.5 in the presence of increasing concentrations of $MgCl_2$ resulted in increased hydrolysis of 0.5 μM [^{3}H]cAMP at pH 7.5; maximal increases were produced with 5 mM $MgCl_2$ (half-maximal increases with ~2 mM $MgCl_2$). After incubation at pH 9.5 (5 min, 30 °C) with $MgCl_2$, activation was not reversed by dilution or incubation with EDTA; EDTA present during incubation at pH 9.5 with $MgCl_2$, however, prevented the increase in hydrolysis of 0.5 μM [^{3}H]cAMP in assays at pH 7.5 (data not shown). Hydrolysis of 250 or 500 μM [^{3}H]cAMP or 0.5 μM [^{3}H]cAMP plus 1 μM cGMP at pH 7.5 was not affected by prior incubation at pH 9.5 in the presence or absence of $MgCl_2$, with or without EDTA (Figure 3, data not shown).

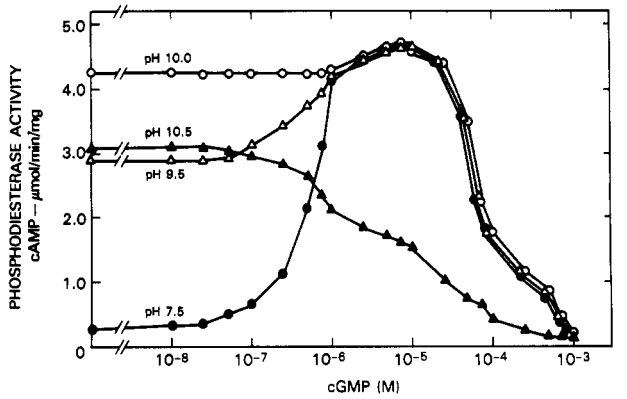

FIGURE 4: Effects of incubation at various pHs on cGMP stimulation of cAMP hydrolysis in subsequent assays at pH 7.5. Enzyme (2.5 μg/mL) was incubated in buffer A at pH 7.5 (●), 9.5 (△), 10.0 (○), or 10.5 (▲) for 5 min at 30 °C with 5 mM $MgCl_2$ prior to dilution with buffer A at pH 7.5 and assay at pH 7.5 in the presence of 0.5 μM [^{3}H]cAMP plus the indicated concentrations of cGMP.

Effects of Incubation at High pH on cGMP-Stimulated Activity Assayed at pH 7.5. With enzyme incubated at pH 7.5 for 5 min at 30 °C with 5 mM $MgCl_2$ (as with unincubated enzyme), increasing concentrations of cGMP increased cAMP hydrolysis at pH 7.5 (Figure 4). Maximal stimulation (~18-fold) was produced by 5–10 μM cGMP; higher concentrations reduced activity. Incubation at pH 9.5, 10, and 10.5 for 5 min at 30 °C with 5 mM $MgCl_2$ markedly increased hydrolysis of 0.5 μM [^{3}H]cAMP at pH 7.5, with a concomitant reduction in x-fold stimulation by cGMP (Figure 4). Stimulation of cAMP hydrolysis was much lower after incubation at pH 9.5 than at pH 7.5 and was nearly absent after exposure to pH 10.0. Regardless of the pH of the first incubation, i.e., 7.5, 9.5, or 10, maximally effective concentrations of cGMP stimulated hydrolysis of 0.5 μM [^{3}H]cAMP at pH 7.5 to the same level. Thus, maximal hydrolysis of 0.5 μM [^{3}H]cAMP at pH 7.5 was produced either by incubation at pH 10 or by addition of cGMP (up to 5–10 μM) to assays of control enzyme (or to enzyme incubated at pH 7.5). Higher concentrations of cGMP reduced [^{3}H]cAMP hydrolysis. The concentration dependency for inhibition of hydrolysis of 0.5 μM [^{3}H]cAMP for higher (>10 μM) concentrations of cGMP was similar, whether the enzyme was maximally activated by addition of cGMP to assays of control enzyme or after incubation at pH 9.5 or 10.0 (Figure 4). This would indicate that the interaction of cGMP at the catalytic site of the activated enzyme is similar whether the enzyme is activated by incubation at pH 9.5 or 10.0 or by addition of cGMP to assays of control enzyme (or enzyme incubated at pH 7.5). After exposure to pH 10.5, which reduced the V_{max} and the cGMP-stimulated cAMP hydrolysis in assays at pH 7.5 (Figure 3), cGMP did not stimulate by only reduced cAMP hydrolysis (Figure 4). Incubation at pH 10.0 also produced increased hydrolysis of 0.1 μM [^{3}H]cAMP at pH 7.5 and decreased x-fold stimulation by cGMP (data not shown).

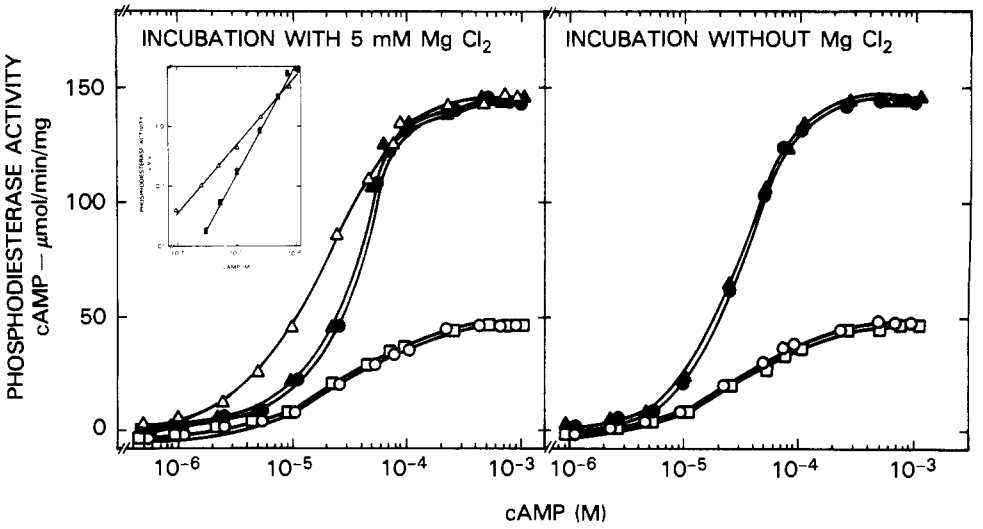

FIGURE 5: Effects of incubation at pH 7.5 or 9.5 on cAMP hydrolysis in subsequent assays at pH 7.5 or 9.5. (Left panel) Enzyme (2.5 μg/mL) was incubated in buffer A at pH 7.5 (○, ●) or at pH 9.5 (▲, △, □) for 5 min at 30 °C (△, ●, ○, □) or at 0 °C (▲) in the presence of 5 mM $MgCl_2$ prior to dilution with buffer A and assay at either pH 7.5 (△, ▲, ●) or pH 9.5 (○, □). Inset: Hill plot. Coefficients (n_{app}): (▲) 1.1; (●, ▲) 1.8. (Right panel) Enzyme was incubated in buffer A at pH 7.5 (○, ●) or pH 9.5 (▲, □) for 5 min at 30 °C in the absence of $MgCl_2$ prior to dilution with buffer A at the appropriate pH and assay at pH 7.5 (●, ▲) or pH 9.5 (○, □).

Effects of Incubation at High pH on V_{max} and n_{app}. Kinetics of cAMP hydrolysis, assayed at pH 7.5, were virtually identical with enzyme first incubated for 5 min at 30 °C at pH 7.5 with (Figure 5, left) or without (Figure 5, right) 5 mM $MgCl_2$, with 5 mM $MgCl_2$ at 0 °C at pH 9.5 (Figure 5, left), or without $MgCl_2$ at 30 °C at pH 9.5 (Figure 5, right). After incubation at pH 9.5 for 5 min at 30 °C with 5 mM $MgCl_2$, however, hydrolysis of low cAMP concentrations at pH 7.5 was increased, K_m^{app} was reduced, and n_{app} was decreased from 1.7 to 1.2, but V_{max} was not altered (Figure 5, left). Incubation at pH 10 for 5 min at 30 °C in the presence of 5 mM $MgCl_2$ prior to assay at pH 7.5 produced a greater decrease in n_{app} (from ~1.7 to ~1.1) and K_m^{app} than did incubation at pH 9.5, without a change in V_{max} (data not shown). As also seen in Figure 5, kinetics of cAMP hydrolysis in assays at pH 9.5 were not affected by incubation at either pH 7.5 or pH 9.5 for 5 min at 30 °C in either the presence or the absence of $MgCl_2$.

For 1–1000 μM [^{3}H]cGMP, incubation at pH 10 did not alter V_{max} or n_{app} (~1.4) for cGMP. Hydrolysis of low concentrations of cGMP was markedly enhanced, i.e., 8–10-fold at 0.05–0.1 μM [^{3}H]cGMP, and over the concentration range 0.01–1 μM [^{3}H]cGMP, n_{app} was reduced from ~1.6 to 1.0 (data not shown). After incubation at pH 9.5–10, hydrolysis of <1 μM [^{3}H]cAMP at pH 7.5 equalled that of [^{3}H]cGMP; at higher substrate concentrations, cGMP hydrolysis exceeded that of cAMP (data not shown). Kinetics of cGMP hydrolysis at pH 9.5 were virtually identical after incubation (5 mM $MgCl_2$, 5 min, 30 °C) at pH 9.5 or 7.5 (data not shown). Similarly, kinetics of cAMP hydrolysis at pH 9.5 were virtually identical after incubation at either pH 9.5 or pH 7.5 (Figure 5).

Effects of pH on Inhibition by IBMX and Papaverine. As previously demonstrated (Yamamoto et al., 1983b; Erneux et al., 1982; Miot et al., 1985), in assays at pH 7.5, certain competitive inhibitors, such as IBMX and papaverine, can, like substrates, induce allosteric transitions to the activated state and increase hydrolysis of low cAMP concentrations (Figure 6). After incubation of the enzyme at 30 °C at pH 10 with 5 mM $MgCl_2$, however, IBMX and papaverine did not stimulate hydrolysis of low cAMP concentrations but only inhibited (Figure 6). With control enzyme, or after incubation at pH 10, both IBMX and papaverine inhibited hydrolysis of 50 μM [^{3}H]cAMP at pH 7.5 (data not shown).

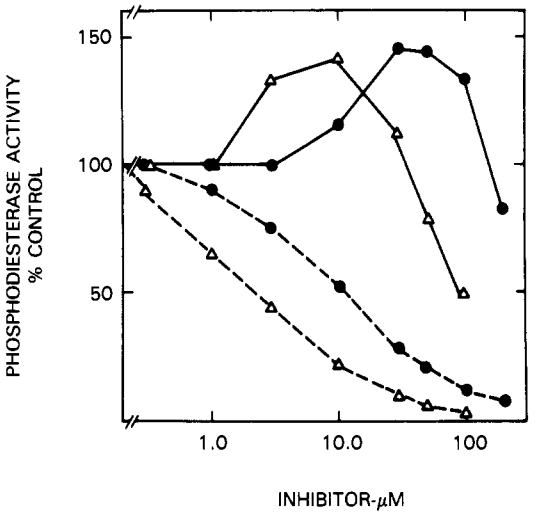

FIGURE 6: Effects of papaverine and IBMX on cAMP hydrolysis in assays at pH 7.5 after incubation at pH 7.5 or 10.0. Enzyme (5.0 μg/mL) was incubated at 30 °C for 5 min with 5 mM $MgCl_2$ in buffer A at pH 7.5 (solid line) or at pH 10.0 (dashed lines) prior to dilution and assay at pH 7.5 in the absence or presence of the indicated concentrations of IBMX (●) or papaverine (△) with 1 μM [^{3}H]cAMP. Activity in the presence of inhibitors is plotted relative to that in the absence of inhibitor (taken as 100%) which was 0.52 μmol min^{-1} mg^{-1} after incubation at pH 7.5 and 8.4 μmol min^{-1} mg^{-1} after incubation at pH 10.0.

For cAMP hydrolysis at pH 7.5 by control enzyme (not incubated at pH 9.5 or 10), n_{app} was ~1.7 (cf. Figure 3), and, as might be expected, Lineweaver–Burk plots for cAMP hydrolysis were curvilinear, making it difficult to estimate K_m^{app} by graphical analysis (data not shown). As also reported (Yamamoto et al., 1983b; Erneux et al., 1981), in the presence of high substrate concentrations (i.e., the activated enzyme) and IBMX, Lineweaver–Burk plots of cAMP hydrolysis by control enzyme were linear. From graphical analysis of these data, a K_m/K_I ratio of 1.3 was calculated (data not shown). After incubation (5 min, 30 °C, 5 mM $MgCl_2$) at pH 9.5, Lineweaver–Burk plots of cAMP hydrolysis at pH 7.5 were linear in the absence or presence of various concentrations of IBMX (observations consistent with pH-induced changes in n_{app}). Graphical analysis of these data indicated a K_m/K_I ratio of 1.4. With control enzyme not incubated at pH 9.5, Line-

weaver–Burk plots of cAMP hydrolysis in assays at pH 9.5 with or without IBMX were also linear; graphical analysis indicated a K_m/K_I ratio of 0.63 (data not shown).

Discussion

The cGMP-stimulated cyclic nucleotide PDE hydrolyzes cAMP and cGMP with positively homotropic cooperative kinetics. Previous studies are consistent with the idea that substrates, effectors, and certain substrate analogues and competitive inhibitors bind to the "low affinity" or "ligand-free" state and induce allosteric transitions that lead to "activation", i.e., increased catalytic activity at low substrate concentrations. Using a series of cyclic nucleotide derivatives, Erneux et al. (1981) reported that there was no obvious correlation between the rank order of effectiveness in the ability of various analogues to serve as substrates, effectors, or inhibitors. More recently, these workers have found that potencies in the ability of analogues to activate the PDE correlate with their displacement of cGMP from presumed regulatory cGMP binding sites (Erneux et al., 1985; Miot et al., 1985). Thus, different structural determinants of analogues and inhibitors seem to influence catalysis, allosteric activation, and inhibition (Erneux et al., 1981, 1985; Yamamoto et al., 1983b; Miot et al., 1985).

In assays at substrate or effector concentrations sufficient to saturate presumed regulatory sites, induce allosteric transitions, and activate the PDE, the pH optimum was 7.5. V_{max} values for cAMP hydrolysis and cGMP-stimulated activity were maximal at pH 7.5, and the Hill coefficient (n_{app}) for cAMP hydrolysis was ~1.7 at pH 7.5 and ~1 at pH 6.0 or 9.5. At concentrations of cAMP or cGMP not sufficient to saturate regulatory sites, the pH optimum was 9.5. Although cGMP stimulated cAMP hydrolysis (0.5 μM [^{3}H]cAMP) from pH 6.5 to pH 9.5, activity in the presence of cGMP was maximal at pH 7.5. Fold stimulation was minimal at pH 9.5, and cGMP did not stimulate cAMP hydrolysis at pH 10. Thus, assay pH has complex effects on cooperative behavior (i.e., n_{app}, cGMP-stimulated activity) and expression of catalytic activity by the "ligand free" or "low-affinity" state (i.e., hydrolysis of low cAMP concentrations) as well as the "activated" state (i.e., V_{max}, cGMP-stimulated cAMP hydrolysis).

In the interpretation of these effects of pH on cAMP hydrolysis, changes in enzyme structure as well as the protonation states of cAMP and cGMP must be considered. For nucleotide monophosphates such as cAMP and cGMP, the critical ionizing groups are the cyclic phosphate and amino groups of the purine ring of cAMP (N^1) and cGMP (N^7), as well as the enolic function of cGMP (Chargaff & Davidson, 1955). In the experiments presented in this report, (i.e., assays at pH >6), protonation of the amino groups of cAMP and cGMP is not an important consideration, since the dissociation constants for these groups range between 2 and 4 (Chargaff & Davidson, 1955). With a dissociation constant of ~6 (Chargaff & Davidson, 1955), ionization of the phosphate group, which would be virtually completed at pH 7.4, might be related to the pH dependence of V_{max} for cAMP and maximal stimulation of cAMP hydrolysis by cGMP. From pH 7.5 to 10.5, changes in the protonation state of cAMP would not be expected to contribute to the observed effects of high pH on cAMP hydrolysis (Chargaff & Davidson, 1955). On the other hand, with a dissociation constant of ~9.5 (Chargaff & Davidson, 1955), tautomerization of the enol group (N^1–C^6–OH) of cGMP, which would occur at higher assay pH values, might contribute to the pH-dependent effects of cGMP on cAMP hydrolysis.

To minimize the contribution of possible effects of protonation of cAMP and cGMP on pH-induced changes in cAMP hydrolysis, enzyme was first incubated at one pH and then assayed at pH 7.5, which was optimal for the activated form of the enzyme (V_{max}, cGMP-stimulated activity). Under these conditions, effects of pH on cooperative behavior (n_{app}) were observed in the absence of changes in V_{max}. After incubation at high pH (i.e., 9.0–10.0), in assays at pH 7.5 there was a marked increase in hydrolysis of low concentrations of cAMP (but no change in V_{max}), a decrease in n_{app} from ~1.7 to 1.1–1.2, and a concomitant decrease in x-fold stimulation, but not maximal stimulation, of cAMP hydrolysis by cGMP. After incubation at pH 10, hydrolysis of low (<5 μM) concentrations of cAMP equalled that of cGMP in assays at pH 7.5. Maximal hydrolysis of low cAMP concentrations at pH 7.5 was achieved either by prior incubation of enzyme at pH 10 or by direct addition of cGMP to assays of control enzyme at pH 7.5.

Direct addition of cGMP to assays of control enzyme at pH 7.5 has been shown not only to increase hydrolysis of low cAMP concentrations but also to cause a shift from positively cooperative to normal Michaelis–Menten kinetics and reduce n_{app} (Martins et al., 1982; Yamamoto et al., 1983a; Moss et al., 1977; Erneux et al., 1981). Thus, after incubation with $MgCl_2$ at high pH, the PDE exhibits a number of characteristics of the cGMP-stimulated or activated form, i.e., increased hydrolysis of low cAMP concentrations, reduced n_{app}, reduced x-fold stimulation but not maximal stimulation with cGMP, and reduced K_m^{app} with no change in V_{max}. Whether incubation with $MgCl_2$ at high pH induces the same allosteric transitions as those presumably produced by cGMP is not known. All these data are also consistent with the view that allosteric activation of this PDE is related more to changes in affinity for cAMP rather than to changes in V_{max}. Activation induced by high pH was dependent on temperature (30 °C) and $MgCl_2$ concentration (5 mM). EDTA could prevent, but not reverse, activation, suggesting that whereas $MgCl_2$ may be required for the transition to the "high affinity" or "activated" state, $MgCl_2$ is not required for maintenance of the "activated" state or is sequestered in a site relatively inaccessible to EDTA.

In assays at pH 7.5, certain competitive inhibitors can, like substrates and effectors, bind to regulatory sites, induce allosteric transitions to the activated state, and thus increase hydrolysis of low cAMP concentrations (Yamamoto et al., 1983b; Erneux et al., 1982; Moss et al., 1985). At higher substrate concentrations sufficient to saturate regulatory sites and activate the enzyme, competitive inhibitors compete with substrate at catalytic sites and reduce cAMP hydrolysis in a competitive fashion (Yamamoto et al., 1983b; Erneux et al., 1982; Miot et al., 1985). The hypothesis that incubation at high pH promotes transitions to the "activated" state is supported by the observation that after incubation at pH 9.5–10, the competitive inhibitors IBMX and persantine did not stimulate hydrolysis of 1 μM cAMP but inhibited hydrolysis of both 1 and 50 μM cAMP.

Analysis of Lineweaver–Burk plots of enzyme activity in the presence of various concentrations of competitive inhibitors can yield a K_m/K_I ratio; for allosteric enzymes, this K_m/K_I ratio can be interpreted as an index of the relative affinities of substrate and inhibitor for the "activated" state (Mastrantonio et al., 1983). In assays at pH 7.5 in the presence of IBMX, we find a K_m/K_I ratio of ~1.3 for either the control enzyme or the enzyme incubated at high pH. Thus, two parameters which presumably reflect the interaction of IBMX with the PDE, i.e., the K_m/K_I ratio and inhibition of hydrolysis of both

low and high concentrations of cAMP, also indicate that incubation at high pH "activates" the cGMP-stimulated PDE.

The data presented in this report suggest that for the cGMP-stimulated PDE, molecular interactions involved in transitions to the "high affinity" or "activated" state (n_{app}) can occur in the absence of substrate or effector; these transitions can also apparently be regulated independently of those important for catalytic activity (V_{max}). These findings support the notion that "high" and "low affinity" states are characterized by distinct binding and regulatory domains with discrete topographical features. It may also be inferred that under physiologic conditions transitions to the "activated" state might be promoted not only by cyclic nucleotides but also by other effectors and substrate-independent mechanisms, including covalent modification. Were such events to occur in the intact cell, substrate specificities of this PDE would be altered in the sense that submicromolar concentrations of both cAMP and cGMP would be more effectively hydrolyzed. Since hydrolysis of low (micromolar) concentrations of cAMP is greatly enhanced when the PDE is in the "activated" state, activation of this PDE could be especially significant in regulation of cAMP concentration, which has been reported to be in the micromolar range in liver (Steiner et al., 1970). Such a conversion would also limit regulation of the cGMP-stimulated enzyme by cGMP, since the activated PDE is no longer stimulated by cGMP and hydrolysis of cAMP and cGMP at micromolar concentrations is virtually identical. It is also possible that interconversions similar to that described here may occur with other forms of PDE and could perhaps be responsible for some of the multiplicity of PDE forms isolated from various tissues (Wells & Hardman, 1977; Manganiello et al., 1984).

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Registry No. PDE, 9040-59-9; cAMP, 60-92-4; cGMP, 7665-99-8; IBMX, 28822-58-4; Mg, 7639-95-4; papaverine, 58-74-2.

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