The catalytic subunit of protein kinase A triggers activation of the type V cyclic GMP-specific phosphodiesterase from guinea-pig lung

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The type V cyclic GMP phosphodiesterase was partially purified from the high-speed supernatant of guinea-pig lung. The isoenzyme displayed linear kinetics for cyclic GMP hydrolysis, with $K_m = 2.2 \pm 0.2 \mu\text{M}$ and $V_{\max} = 1.2 \pm 0.08 \text{ nmol/min per mg}$. The selective type V phosphodiesterase inhibitor Zaprinast inhibited cyclic GMP hydrolysis with IC_{50} (concn. giving 50% inhibition) $= 0.45 \pm 0.08 \mu\text{M}$. Isobutylmethylxanthine promoted a 3-fold increase in the binding of cyclic GMP to the isoenzyme. The addition of the catalytic subunit of protein kinase A to an activation cocktail containing the partially purified type V phosphodiesterase resulted in a marked increase in $V_{\max}$ for cyclic GMP hydrolysis (~ 10 -fold at 40 units of protein kinase A). We have suggested that protein kinase A triggers phosphorylation of the phosphodiesterase, which results in activation of phosphodiesterase activity. In addition, the sensitivity to inhibition by Zaprinast is severely decreased (the IC_{50} for inhibition is $7.5 \pm 1.1 \mu\text{M}$), suggesting that the potency of phosphodiesterase inhibitors is effected by phosphorylation of the enzyme.

INTRODUCTION

Cyclic AMP and cyclic GMP phosphodiesterases exist as a family of isoenzymes which possess distinct primary amino acid sequences and are encoded by distinct genes (Beavo & Reifsnyder, 1990). These multiple enzymes have been classified into five major groups according to kinetic, physical, immunological and regulatory properties (for review see Beavo & Reifsnyder, 1990). The phosphodiesterase plays a pivotal role in the cellular signalling process, limiting the cellular response to agents which utilize cyclic nucleotides as second messengers. However, these isoenzymes do not solely act as a brake on cyclic nucleotide synthesis, since their activity states are also regulated by a variety of hormones such as insulin, adrenaline, glucagon, prostaglandin I_2 and prostaglandin E_1 (Zinman & Hollenberg, 1974; Loten *et al.*, 1978; Makino & Kono, 1980; Heyworth *et al.*, 1983; Macphee *et al.*, 1988). The molecular events which lead to stimulation of phosphodiesterase activity result in the establishment of a new intracellular steady state of the cyclic nucleotide, alteration in the activity status of protein kinases A and G and inhibition of so-called resting-state cellular processes. The molecular mechanisms by which hormones trigger the modulation of phosphodiesterase activity is via the action of specific protein kinases.

In this regard, several phosphodiesterases have been shown to be phosphorylated by several different protein kinases. For instance, the brain type I Ca^{2+} /calmodulin-activated phosphodiesterase exists as two isoforms: one, of 63 kDa, is a substrate for a Ca^{2+} /calmodulin-dependent protein kinase, whereas the other, a 61 kDa species, is phosphorylated by protein kinase A (Sharma & Wang, 1986). The type III cyclic GMP-inhibited cyclic AMP phosphodiesterase is also a substrate for protein kinase A (Harrison *et al.*, 1986; Kilgour *et al.*, 1989), the phosphorylation of this isoenzyme leading to a profound activation of cyclic AMP phosphodiesterase activity. The type IV cyclic AMP-specific phosphodiesterase has been shown to be phosphorylated and activated by the insulin-receptor tyrosine kinase in intact

hepatocytes (Pyne *et al.*, 1989). Finally, the type V cyclic GMP-specific phosphodiesterase is a substrate for phosphorylation by both protein kinase G and protein kinase A. The magnitude of phosphorylation is dependent on the binding of cyclic GMP to a non-catalytic site in the phosphodiesterase (Thomas *et al.*, 1990a,b). However, as yet no subsequent activity changes have been observed upon phosphorylation of this isoenzyme by either kinase.

In this paper, we report that the catalytic subunit of protein kinase A triggers phosphorylation and activation of the type V phosphodiesterase from guinea-pig lung. In addition, we have characterized the effect of this phosphorylation on both kinetic and inhibitor properties of this phosphodiesterase. These results suggest that, in the lung, a level of cross-talk between cellular signalling processes utilizing cyclic AMP and cyclic GMP exists.

MATERIALS AND METHODS

Materials

The purified catalytic subunit of protein kinase A was purchased from Sigma Chemical Co. (Poole, Dorset, U.K.); its purity was $> 95\%$. Cyclic $[8\text{-}^3\text{H}]\text{GMP}$ was obtained from Amersham International (Amersham, Bucks., U.K.). Biochemicals were from Boehringer Mannheim (Germany) and all chemicals were from Sigma. Zaprinast (M & B 22948) was a gift from Dr. J. Souness (Rhone-Poulenc Rorer, Dagenham, Essex, U.K.). Purified rat brain protein kinase C was from Lipidex (New York, NY, U.S.A.) and contains a mixture of isoenzymes.

Assay of cyclic GMP phosphodiesterase activity

Assay of phosphodiesterase activity was performed as described by Thompson & Appleman (1971). This is a two-step radioactive assay using cyclic $[8\text{-}^3\text{H}]\text{GMP}$, which is incubated with the enzyme preparation for 10 min at 30°C . Routinely, the assay components are $0.5 \mu\text{M}$ -cyclic $[8\text{-}^3\text{H}]\text{GMP}$, 5 mM - MgCl_2 and 10 mM -Tris/HCl, pH 7.4. The total assay volume was $100 \mu\text{l}$,

Abbreviation used: IC_{50} , concn. giving 50% inhibition.

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and approx. 100 000 c.p.m. of cyclic [8-³H]GMP was added per assay. The incubation was terminated by boiling the assay tubes for 2 min. After cooling the samples, 25 μ l of *Hannah ophiophagus* snake venom (1 mg/ml) was added to each, and a further incubation was performed at 30 °C for 10 min. After this incubation, 400 μ l of Dowex-1 (Cl⁻ form) slurry was added per assay and the samples were left at 4 °C for 15 min, with constant vortex-mixing. The samples were then centrifuged at 15 000 *g* for 2 min in a Beckman microcentrifuge and 150 μ l of the supernatant was taken for liquid-scintillation counting of [8-³H]guanosine. In all cases, the assay of phosphodiesterase activity was performed under conditions where product formation was linear with both time and protein concentration. Less than 10% of the substrate was utilized under these assay conditions.

The effects of Ca²⁺ and calmodulin, Zaprinast and the catalytic subunit of protein kinase A on the phosphodiesterase activity were assessed. Lineweaver–Burk plots were performed over the range 0.05–100 μ M-cyclic [8-³H]GMP. Assay of cyclic AMP phosphodiesterase activity was performed by identical protocols.

Cyclic GMP-binding assay

Cyclic GMP binding was measured in a total volume of 100 μ l containing 10 mM-sodium phosphate, pH 6.8, 1 mM-EDTA, 12 mM- β -mercaptoethanol, 0.5 μ M-cyclic [³H]GMP (600 000 c.p.m.) and 10 μ M-cyclic AMP, with or without 0.2 mM-isobutylmethylxanthine. Reactions were initiated by the addition of a partially purified phosphodiesterase preparation and incubated at 4 °C for 60 min. Assay mixtures were then filtered on to Millipore HAWP filters (pore size 0.45 μ m), which were washed three times with 10 mM-potassium phosphate (pH 6.8)/1 mM-EDTA at 4 °C. The filters were dried and their radioactivity was determined.

Phosphorylation procedure

Routinely, preparations of partially purified cyclic GMP phosphodiesterase (30 μ g) were combined with an activation cocktail containing (final concns.) 25 mM-Hepes, pH 7.0, 5 mM-MgCl₂ and 25 μ M-ATP. To initiate the incubation, the catalytic subunit of protein kinase A (1–100 units; 1 unit = 16 pmol of ³²P incorporated/min) was added. The protein kinase A was stored in dithiothreitol (6 mg/ml). In experiments where no kinase was added, dithiothreitol (6 mg/ml) was added to the activation cocktail. The total assay volume was 100 μ l. Samples were incubated at 37 °C for 20 min and the assays terminated by cooling to 4 °C. Samples were then withdrawn for assay of cyclic GMP phosphodiesterase activity.

For incubations with protein kinase C, the cyclic GMP phosphodiesterase (30 μ g) was combined with an activation cocktail containing (final concns.) 25 mM-Hepes, pH 7.5, 5 mM-MgCl₂, 0.75 mM-CaCl₂ and 25 μ M-ATP. To this activation cocktail was added phosphatidylserine (50 μ g/ml), which had been previously sonicated in 10 mM-Tris/HCl, pH 7.4. To initiate the reaction, purified protein kinase C (0.3 unit; 1 unit = 1 nmol of ³²P incorporated/min) was added and the incubation performed at 37 °C for 20 min. Reactions were terminated by placing samples on ice at 4 °C. Samples were then removed for assay of cyclic GMP phosphodiesterase. The protein kinase C was stored at –20 °C in 10 mM-Tris/HCl (pH 7.4)/0.5 mM-EGTA/0.5 mM-EDTA/10 mM- β -mercaptoethanol/10% (v/v) glycerol/0.05% Triton X-100 and 400 mM-NaCl. In incubations where no kinase was added, the components of the storage buffer were added to the incubation cocktail.

Purification procedure

Step 1: preparation of high-speed supernatant. Routinely one lung (perfused in Krebs–Henseleit buffer, pH 7.4) was removed

from a guinea pig and homogenized in buffer A (containing 20 mM-sodium phosphate, pH 6.8, 2 mM-EDTA and 25 mM- β -mercaptoethanol) with a Turrex homogenizer (4–5 strokes, up and down). All procedures were performed at 4 °C, unless otherwise stated. The homogenate was filtered through cheesecloth and centrifuged at 10 000 rev./min for 10 min in a SW 55.2 rotor. The resulting high-speed supernatant was filtered through cheesecloth and taken for chromatography by a modified procedure developed by Francis & Corbin (1988).

Step 2: chromatography on DEAE-Sepharose column. DEAE-Sepharose was pre-equilibrated in buffer A, after which the filtered supernatant was loaded on to the column. The DEAE-Sepharose was washed with 6 vol. of buffer A until no protein was eluted, as determined by measurement at 280 nm. The phosphodiesterase activity was then eluted with a 400 ml salt gradient, consisting of buffer A supplemented with 0–0.5 M-NaCl. The fractions were pooled and dialysed against 4 litres of buffer A to remove NaCl.

Step 3: Affi-Gel Blue column. The pooled and dialysed fraction was applied to an Affi-Gel Blue-agarose column (2 cm $\times$ 3 cm) which had been previously equilibrated in buffer A. The column was washed sequentially with 6 vol. of buffer A, 5 vol. of buffer A + 0.8 M-NaCl, and 5 vol. of buffer A, after which the phosphodiesterase activity was eluted with buffer A + 0.35 M-KSCN. The active fractions were pooled and dialysed against buffer A overnight.

Step 4: zinc chelate absorbent. The pooled enzyme was dialysed for a further 16 h against buffer B containing 20 mM-sodium phosphate, pH 6.8, to remove EDTA and mercaptoethanol. The resulting material was loaded on to a 1 ml column of zinc chelate (Boehringer Mannheim) that had been previously equilibrated in 20 mM-sodium phosphate, pH 6.8. The flow-through fractions containing phosphodiesterase activity were assayed and pooled. Enzyme was stored at –20 °C and was stable for 4 weeks.

RESULTS

Partial purification of the type V phosphodiesterase

The cyclic GMP phosphodiesterase activity from guinea-pig lung high-speed supernatants was purified 27-fold by using three chromatographic separation techniques, which included DEAE-Sepharose, Affi-Gel Blue-agarose and zinc chelate absorbent (Table 1). The cyclic GMP phosphodiesterase activity was eluted from DEAE-Sepharose with a 0–0.5 M-NaCl gradient. A single peak of cyclic GMP phosphodiesterase activity was resolved from this chromatographic column. Phosphodiesterase activity was eluted between 0.1 and 0.2 M-NaCl, and the pooled active fractions had a specific activity of 25 pmol/min per mg at

Table 1. Purification of the cyclic GMP-specific phosphodiesterase from guinea-pig lung 'high-speed' supernatant fraction

Phosphodiesterase assays were performed at 0.5 μ M-cyclic [³H]GMP. Active fractions from each chromatographic separation were pooled and the specific activity was determined. This is a purification scheme typical of one performed at least three times.

Purification step	Total activity (pmol/min)	Specific activity (pmol/min per mg)	Purification (fold)
Supernatant	9609	8	
DEAE-Sepharose	2377	25	3
Affi-Gel Blue	207	121	14
Zinc chelate	123	236	27

Table 2. Kinetic and inhibitor properties of the partially purified cyclic GMP phosphodiesterase from guinea-pig lung 'high-speed' supernatant fraction

Phosphodiesterase activity was assessed between 0.01 and 100 μM -cyclic GMP, for determination of K_m and $V_{\max}$. IC_{50} values for Zaprinast were assessed at 0.5 μM -cyclic GMP. For cyclic GMP binding, experiments were performed at 0.5 μM -cyclic GMP in the presence and absence of isobutylmethylxanthine (IBMX; 200 μM). The $t_{1/2}$ was determined from an exponential decay constant at 50 $^{\circ}\text{C}$ and is a measure of 50% inactivation of phosphodiesterase activity. Assays for cyclic GMP phosphodiesterase were performed at 0.5 μM -cyclic GMP.

Property	Value
K_m	$2.2 \pm 0.2 \mu\text{M}$
$V_{\max}$	$1.2 \pm 0.08 \text{ nmol/min per mg}$
IC_{50} (Zaprinast)	$0.45 \pm 0.08 \mu\text{M}$
$t_{1/2}$ (50 $^{\circ}\text{C}$)	$1.2 \pm 0.1 \text{ min}$
Binding activity + IBMX	$300 \pm 40 \text{ pmol/mg}$ $1012 \pm 109 \text{ pmol/mg}$

Fig. 1. Kinetic properties of partially purified cyclic GMP phosphodiesterase

This is a double-reciprocal plot ($1/v$ versus $1/s$) of cyclic GMP phosphodiesterase activity measured between 0.01 and 100 μM cyclic GMP. Limiting values for K_m and $V_{\max}$ were determined by using linear regression analysis and best-curve fitting computer programs. The inset shows a Hill plot ($h_{\text{app}} = 1$), where $\log[\gamma/(1-\gamma)]$ is plotted against $\log[\text{cyclic GMP}]$, where $\gamma = (V_{\max} - v)/V_{\max}$ at any given [cyclic GMP]. This is a typical result of an experiment performed at least three times on three different preparations of partially purified cyclic GMP phosphodiesterase.

0.5 μM -cyclic GMP, which represents a 3-fold purification from the high-speed supernatant (Table 1). After dialysis to remove NaCl, the cyclic GMP phosphodiesterase was applied to Affi-Gel Blue-agarose, and 95% of the total phosphodiesterase activity bound to this matrix. Cyclic GMP phosphodiesterase activity was sequentially eluted with buffer A plus 0.35 M-KSCN. Less than 10% of the total phosphodiesterase activity applied to Affi-Gel Blue-agarose was eluted with 0.35 M-KSCN (Table 1), whereas only a 14-fold purification was achieved from the high-speed supernatant. Application of pooled fractions containing cyclic GMP phosphodiesterase activity to the zinc chelate adsorbant resulted in direct passage of the phosphodiesterase through

Fig. 2. Effect of the catalytic subunit of protein kinase A on cyclic GMP phosphodiesterase

Dose-response curve of the activation of cyclic GMP phosphodiesterase activity induced by adding various amounts of protein kinase A catalytic activity (0.05–100 units) to an activation cocktail containing partially purified cyclic GMP phosphodiesterase. Assays were performed at 0.5 μM cyclic GMP. The fold stimulation is plotted against the amount of catalytic subunit added. Data are expressed as means $\pm$ S.D. ($n = 6$).

the matrix. A 27-fold purification of cyclic GMP phosphodiesterase activity from the high-speed supernatant was achieved by this procedure (Table 1) and the resulting preparation of cyclic GMP phosphodiesterase activity had a specific activity of 236 pmol/min per mg at 0.5 μM cyclic GMP. The partially purified preparation of phosphodiesterase contained a single isoenzyme, as assessed from first-order-decay exponentials of thermal inactivation at 50 $^{\circ}\text{C}$; $t_{1/2}$ values for the phosphodiesterase at 50 $^{\circ}\text{C}$ were $1.2 \pm 0.1 \text{ min}$ (Table 2).

Kinetic properties of partially purified phosphodiesterase

In order to identify the class of cyclic GMP phosphodiesterase partially purified by the above procedure, a number of experiments were performed. These included determination of substrate specificity, Ca^{2+} /calmodulin-sensitivity, inhibitor sensitivities and the effect of phosphodiesterase inhibitors on cyclic GMP binding.

The partially purified phosphodiesterase hydrolysed cyclic GMP with 'high affinity' and displayed linear Michaelis-Menten kinetics. Limiting kinetic constants were determined from double-reciprocal plots by using linear-regression and best-curve-fitting computer models. The isoenzyme had $K_m = 2.2 \pm 0.2 \mu\text{M}$ and $V_{\max} = 1.2 \pm 0.08 \text{ nmol/min per mg}$ ($n = 7$) (Table 2, Fig. 1). Cyclic GMP phosphodiesterase activity was potently inhibited by a type V-specific inhibitor, Zaprinast. Inhibition of cyclic GMP phosphodiesterase activity was dose-dependent and yielded IC_{50} (concn. giving 50% inhibition) = $0.45 \pm 0.08 \mu\text{M}$ ($n = 6$) (Table 2), at 0.5 μM -cyclic GMP. The non-selective phosphodiesterase inhibitor isobutylmethylxanthine also potently inhibited the cyclic GMP phosphodiesterase activity, yielding $\text{IC}_{50} = 5 \pm 0.9 \mu\text{M}$ ($n = 6$) (results not shown), at 0.5 μM -cyclic GMP. However, isobutylmethylxanthine (200 μM) was a potent activator of cyclic GMP binding to the phosphodiesterase, resulting in a 3-fold increase in cyclic GMP binding, as measured at 0.5 μM -cyclic GMP (Table 2; binding activity without isobutylmethylxanthine = $300 \pm 40 \text{ pmol/mg}$, + 200 μM -isobutylmethylxanthine = $1012 \pm 109 \text{ pmol/mg}$). Ca^{2+} /calmodulin (1–10 units) had no effect on cyclic GP phosphodiesterase activity (results not shown), and the phosphodiesterase did not hydrolyse cyclic AMP (results not shown).

Fig. 3. Kinetic properties of phosphorylated cyclic GMP phosphodiesterase

This is a double-reciprocal plot ($1/v$ versus $1/s$) of cyclic GMP phosphodiesterase activity (●) measured between 0.01 and 100 μM cyclic GMP. K_m and $V_{\max}$ values were determined by using best-curve fitting computer models. Phosphodiesterase was incubated with either 20 units of purified catalytic subunit of protein kinase A (▼) or 40 units of catalytic subunit (▲) and double-reciprocal plots were constructed for the subsequently activated cyclic GMP phosphodiesterase activity. This is an experiment representative of one performed at least three times.

Table 3. Kinetic properties and inhibitor characteristics of the phosphorylated cyclic GMP phosphodiesterase

Phosphodiesterase was assessed as described previously with a substrate concentration range of 0.01–100 μM -cyclic GMP. K_m and $V_{\max}$ values were determined for activated phosphodiesterase by using 40 units of purified catalytic subunit of protein kinase A. For IC_{50} determination of the sensitivity to inhibition by Zaprinast, the phosphodiesterase was activated with 40 units of catalytic subunit. The IC_{50} for Zaprinast was assessed at 0.5 μM -cyclic GMP.

Property	Value
K_m	1.6 μM
$V_{\max}$	11.9 nmol/min per mg
IC_{50} (Zaprinast)	$7.5 \pm 1.1 \mu\text{M}$

Effect of protein kinase A catalytic subunit

Incubation of partially purified cyclic GMP phosphodiesterase (30 μg) with activation cocktail containing the catalytic subunit of protein kinase A (1–100 units) resulted in dose-dependent activation of cyclic GMP phosphodiesterase activity (Fig. 2). At 0.5 μM -cyclic GMP, the EC_{50} (concn. giving 50% of maximum effect) for activation by protein kinase A was 20 units of catalytic subunit. The maximal stimulation (13-fold) of cyclic GMP phosphodiesterase activity was achieved with 100 units of catalytic sub-unit. The catalytic subunit preparation contained negligible (< 0.1%) phosphodiesterase activity. Omission of ATP from the activation cocktail decreased the activation of cyclic GMP phosphodiesterase activity (results not shown).

Kinetic properties of phosphorylated phosphodiesterase

We have assessed the effect of two specific activities of the catalytic subunit of protein kinase A (40 units and 20 units) on the kinetic properties of the partially purified cyclic GMP phosphodiesterase (Fig. 3, Table 3). Addition of 40 units of the catalytic subunit of protein kinase A triggered a 10-fold stimulation of cyclic GMP phosphodiesterase activity. The phosphoryl-

Fig. 4. Inhibitor-sensitivity of phosphorylated cyclic GMP phosphodiesterase

A dose-response plot of the inhibition of cyclic GMP phosphodiesterase induced by various concentrations of Zaprinast (●). Phosphodiesterase was incubated with 40 units of catalytic subunit of protein kinase A, and samples were withdrawn for analysis of inhibitor sensitivity to Zaprinast (▲). In both cases the dose range of Zaprinast was 10^{-8} – 10^{-4} M (final concns.). This is an experiment representative of one performed at least three times.

ated phosphodiesterase displayed linear kinetics for cyclic GMP hydrolysis, with $K_m = 1.6 \mu\text{M}$ and $V_{\max} = 11.9$ nmol/min per mg. Addition of 20 units of the catalytic subunit of protein kinase A triggered a 6-fold stimulation of cyclic GMP phosphodiesterase activity, which was again due to a marked increase in the $V_{\max}$ for cyclic GMP hydrolysis. The phosphodiesterase activity has $K_m = 1.6 \mu\text{M}$ and $V_{\max} = 9.6$ nmol/min per mg.

Inhibitor-sensitivity of the phosphorylated phosphodiesterase

Addition of 40 units of the catalytic subunit of protein kinase A to the partially purified cyclic GMP phosphodiesterase triggers activation of cyclic GMP hydrolysis. We assessed whether the phosphorylated enzyme displayed altered sensitivity to inhibition by the type V-selective inhibitor Zaprinast. Zaprinast inhibited dose-dependently the cyclic GMP phosphodiesterase activity (Fig. 4, Table 3) with $\text{IC}_{50} = 7.5 \pm 1.1 \mu\text{M}$ -cyclic GMP.

Effect of protein kinase C

Incubation of the partially purified preparation of cyclic GMP phosphodiesterase with protein kinase C did not significantly alter phosphodiesterase activity (results not shown).

DISCUSSION

We have partially purified the cyclic GMP phosphodiesterase from high-speed supernatants of guinea-pig lung. The partially purified enzyme displayed kinetics and inhibitor-sensitivities which closely resembled those of the type V cyclic GMP-specific phosphodiesterase purified from both bovine and rat lung high-speed supernatants (Francis & Corbin, 1988; Thomas *et al.*, 1990a). For instance, Thomas *et al.* (1990a) determined a K_m for cyclic GMP hydrolysis by the bovine lung isoform of 5.6 μM . In comparison, the cyclic GMP phosphodiesterase partially purified from guinea-pig lung high-speed supernatants has a K_m of $2.2 \pm 0.2 \mu\text{M}$. The bovine lung isoform is potently inhibited by Zaprinast, and likewise the guinea-pig lung phosphodiesterase is also markedly inhibited by this drug. Zaprinast is also a potent inhibitor of the type I Ca^{2+} /calmodulin-stimulated cyclic GMP phosphodiesterase (Souness *et al.*, 1989). We suggest that the isoenzyme partially purified from guinea-pig lung is not a Ca^{2+} /calmodulin-activated type I phosphodiesterase, since Ca^{2+} and calmodulin did not stimulate cyclic GMP phosphodiesterase

activity. The type I isoenzyme also displays specificity for cyclic AMP, whereas the lung isoform exclusively hydrolyses cyclic GMP. The most definitive evidence that the guinea-pig lung cyclic GMP phosphodiesterase is a type V isoenzyme is that isobutylmethylxanthine stimulates cyclic GMP binding to the enzyme. This is an established characteristic of type V phosphodiesterases (Francis & Corbin, 1988). The ability of isobutylmethylxanthine to promote cyclic GMP binding while eliciting inhibition of cyclic GMP hydrolysis is due to enhanced binding at a non-catalytic site (Francis & Corbin, 1988).

The partially purified cyclic GMP phosphodiesterase from guinea-pig lung is a substrate for phosphorylation by the catalytic subunit of protein kinase A. We have based this conclusion on the dose-dependent activation of cyclic GMP phosphodiesterase activity. Thomas *et al.* (1990b) observed that the bovine lung type V phosphodiesterase was a substrate for protein kinase A, although no activity change was detected upon phosphorylation of the isoenzyme. Cyclic GMP appeared to enhance the susceptibility of the isoenzyme to act as a substrate for protein kinase A, but did not enhance stimulation of phosphodiesterase activity. Likewise, the isoenzyme is also a substrate for protein kinase G. The protein kinase G-dependent phosphorylation of the isoenzyme occurs at the same site as that for protein kinase A, albeit with a faster rate. The present study therefore supports the notion that the type V isoenzyme is a substrate for protein kinase A, although in addition we have observed a marked activation of cyclic GMP hydrolysis. That activation results from a marked increase in the $V_{\max}$ for cyclic GMP hydrolysis. Consequently, the sensitivity to inhibition by Zaprinast is apparently decreased. This mechanism of activation of cyclic GMP phosphodiesterase activity is similar to the cyclic AMP-dependent activation of the type III cyclic AMP phosphodiesterase activity (Loten *et al.*, 1978; Kilgour *et al.*, 1989). These observations may therefore reflect a generalized mechanism by which protein kinase A-mediated phosphorylation of phosphodiesterase results in a marked increase in catalytic efficiency. In contrast, protein kinase C had no effect on cyclic GMP hydrolysis by the partially purified cyclic GMP phosphodiesterase from guinea-pig lung. Again, these results are in accord with those of Thomas *et al.* (1990b), where the type V bovine lung isoenzyme was shown to be a very poor substrate for protein kinase C.

These results suggest that the guinea-pig lung type V phosphodiesterase may be a substrate for protein kinase A in cells. The potent activation of cyclic GMP hydrolysis as a consequence of phosphorylation would have a significant effect on both basal and agonist-stimulated intracellular cyclic GMP levels. The change in the $V_{\max}$ for cyclic GMP hydrolysis would have a substantial effect on cyclic GMP degradation in these cells. We have previously shown (Burns *et al.*, 1991) that the lung type V isoenzyme appears to perform an important role in suppressing cyclic GMP levels in airways, since Zaprinast is capable of uncoupling pre-contracted airway smooth muscle via inhibition of the type V isoenzyme. There is substantial evidence to support the notion that elevated intracellular levels of both cyclic AMP and cyclic GMP can provoke excitation-contraction uncoupling (Torphy *et al.*, 1988; Harris *et al.*, 1989; Nakatsu & Diamond,

1989), although it is ill-defined if these agents do so via similar or different mechanisms. Our results imply that there may be cross-talk between the cyclic GMP and cyclic AMP signalling cascades. However, the implied necessity for why suppressed cyclic GMP levels are produced upon elevation of intracellular cyclic AMP, and the physiological significance of this, require further study. One possibility is that two pools of cyclic GMP exist in lung cells. One elicits uncoupling of excitation-contraction coupling, whereas the other serves to inhibit cyclic AMP accumulation. Thus, as soon as cyclic AMP levels in these cells reach a sufficient value to promote protein kinase A activation, the type V phosphodiesterase hydrolyses the inhibitory cyclic GMP pool. This would allow greater amplification of cyclic AMP accumulation in response to β -adrenoceptor agonists. In support of this notion, contractile agonists, while promoting mobilization of intracellular Ca^{2+} , also promote cyclic GMP accumulation (Nakatsu & Diamond, 1989). Indeed, for certain agonists such as methacholine, there exists a reciprocal antagonism between muscarinic tone and relaxation by agents that increase cyclic AMP (Torphy *et al.*, 1988), and this may be related to the extent of methacholine-induced cyclic GMP accumulation.

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