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Phosphodiesterases from Porcine Coronary Arteries: Inhibition of Separated Forms by Xanthines, Papaverine, and Cyclic Nucleotides

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SUMMARY

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This study was conducted to evaluate the possibility of selective inhibition of either guanosine cyclic 3′,5′-monophosphate or adenosine cyclic 3′,5′-monophosphate phosphodiesterase activities of pig coronary arteries. Two peaks (I and II) of cyclic nucleotide phosphodiesterase activity and a heat-stable, nondialyzable activator of peak I can be resolved from the intima plus media layer of pig coronary arteries by DEAE-cellulose chromatography. Peak I catalyzes the hydrolysis of both cyclic GMP and cyclic AMP, but it has a much lower apparent K_m (1–4 μM) for cyclic GMP than for cyclic AMP (40–100 μM). Peak II activity is relatively specific for cyclic AMP and exhibits apparent negative cooperative behavior. Papaverine, 1-methyl-3-isobutylxanthine (MIX), 3′,5′-cyclic nucleotides, and some 3′,5′-cyclic nucleotide derivatives inhibit these phosphodiesterases to different degrees. For example, papaverine is 3–4 times more potent as an inhibitor of peak II than of peak I while MIX is 5–6 times more potent as an inhibitor of peak I than of peak II activity. Theophylline shows little selectivity for either form. The degree of inhibition of peak I by each agent is virtually independent of the cyclic nucleotide used as substrate. Hydrolysis of cyclic AMP by peak I activity is decreased by cyclic GMP more effectively than cyclic GMP hydrolysis is decreased by cyclic AMP. K_i values for inhibition of peak I activity by cyclic GMP and cyclic AMP correspond to their respective apparent K_m values. Cyclic GMP also inhibits the hydrolysis of cyclic AMP by peak II, and it is as effective as MIX in this regard. The effects of none of the above agents on peak I were altered by the presence or absence of heat-stable, nondialyzable activator. N^2,2′-*O*-Dibutyryl cyclic GMP, 8-bromo cyclic GMP, and N^6,2′-*O*-dibutyryl cyclic AMP are, in general, more potent than theophylline as inhibitors of both forms of phosphodiesterase. 8-Bromo cyclic GMP and dibutyryl cyclic GMP are about 10 times more potent as inhibitors of peak I activity with either substrate than of peak II activity, whereas dibutyryl cyclic AMP is about 10 times more potent as an inhibitor of peak II activity than of peak I activity. 8-Bromo cyclic GMP is 4–6 times more potent as an

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inhibitor of fully activated peak I with either substrate than of activator-deficient peak I activity, but effects of dibutyryl cyclic AMP or dibutyryl cyclic GMP are changed very little by the presence of the activator. These data suggest the possibility of selective pharmacological regulation of cyclic GMP or cyclic AMP levels through selective inhibition of coronary artery phosphodiesterase activities.

INTRODUCTION

An increasing amount of evidence suggests a regulatory role for adenosine cyclic 3',5'-monophosphate in vascular and other smooth muscle (1–4); its levels are increased by *beta* adrenergic agents and some other substances that cause relaxation of smooth muscle. Although the physiological role of guanosine cyclic 3',5'-monophosphate in smooth muscle and other tissues is not clear, its levels are increased by cholinergic and other agents that produce contraction of smooth muscle (5–9). However, levels of both cyclic nucleotides are increased in smooth muscular tissues by 1-methyl-3-isobutylxanthine, a cyclic nucleotide phosphodiesterase inhibitor that produces relaxation of smooth muscle (7, 10). Phosphodiesterase inhibitors that are selective for either cyclic AMP or cyclic GMP hydrolysis could be useful tools in defining the roles of these nucleotides and in understanding the relationship between their roles in smooth muscle and other tissues.

We have separated and partially characterized cyclic nucleotide phosphodiesterase activities from the intima plus media layer of pig coronary arteries (11). Two peaks of phosphodiesterase activity and a heat-stable, nondialyzable activator can be separated by DEAE-cellulose chromatography. Peak I phosphodiesterase activity exhibits classical kinetic behavior and catalyzes the hydrolysis of both cyclic GMP and cyclic AMP, but it has a much lower apparent K_m for cyclic GMP. Peak II phosphodiesterase activity exhibits apparent negative cooperativity and is relatively specific for cyclic AMP; its activity with cyclic GMP as a substrate has been too low to permit practical kinetic studies. Peak I activity is stimulated 3–8-fold by a heat-stable, nondialyzable activator, but peak II activity is not altered by this material.

The aim of the present study was to investigate the inhibition of cyclic AMP and cyclic GMP phosphodiesterase activities of pig coronary arteries by 1,3-disubstituted xanthines, cyclic nucleotides and their derivatives, and other agents in order to assess the potential for selective inhibition of the hydrolysis of either cyclic nucleotide. Phosphodiesterase activities separated by DEAE-cellulose as well as those in crude supernatant and particulate fractions were studied. The use of separated forms of phosphodiesterase allows a more valid and straightforward interpretation of the selectivity and nature of inhibition than does the use of whole homogenates. However, in examining only the separated enzymes, it might be possible to overlook inhibition of significant activities that are not present among the separated forms. Moreover, the patterns of inhibition seen in crude systems may be more representative of the composite patterns that occur in intact cells.

METHODS

Materials. These were as previously described (11). 1-Methyl-3-isobutylxanthine was purchased from Aldrich; papaverine, from Eli Lilly and Company; and N^6,2'-*O*-dibutyryl cyclic AMP, N^2,2'-*O*-dibutyryl cyclic GMP, 8-bromo cyclic GMP, and bovine serum albumin fraction V, from Sigma.

Assay procedures. The assay of cyclic nucleotide phosphodiesterase activity was in principle that described by Butcher and Sutherland (12) with the tritiated substrate modification of Beavo *et al.* (13). Details have been presented elsewhere (11). The assay mixture (250 μl) consisted of 10 μmoles of Tris-HCl (pH 7.5), 0.5 μmole of $MgCl_2$, 0.26 nmole, except where otherwise indicated, of tritiated cyclic nucleotide, and the indicated amounts of inhibitors. Phosphodiesterase preparations were diluted in 40 mM Tris-HCl (pH 7.5)

TABLE 1

Inhibition of cyclic nucleotide phosphodiesterase activities in supernatant and washed particulate fractions of homogenates of pig coronary arteries

Inhibitor	I_{50}[a]			
	Supernatant fraction		Particulate fraction	
	Cyclic GMP	Cyclic AMP	Cyclic GMP	Cyclic AMP
	μM	μM	μM	μM
Papaverine	17 ± 1	3.7 ± 0.5	10 ± 1	3.6 ± 1.5
MIX	4.4 ± 0.9	3.7 ± 0.6	2.8 ± 0.3	11 ± 4
Theophylline	140 ± 10	140 ± 15	—[b]	—[b]
Caffeine	390 ± 30	500 ± 70	480 ± 16	790 ± 200
Adenosine	>1000	>1000	>1000	>1000

[a] I_{50} value is that concentration of the agent required to produce 50% inhibition of the hydrolysis of 1 μM substrate. Values are means ± standard errors of six determinations with three different preparations for MIX and papaverine and of four determinations with two different preparations for caffeine, theophylline, and adenosine. Adenosine at 1 mM inhibited both fractions with either substrate by approximately 30%; higher concentrations were not used.

[b] Not determined.

containing 2 mg/ml of BSA[1] to concentrations that gave 10–30% breakdown of substrate in 30 min at 30°. Peak I activity was measured in the presence and absence of an optimal level of activator (11) by adding either 10 μl (10–15 μg of protein) of boiled, dialyzed 40,000 × *g* supernatant fluid from an intima-media homogenate (consisting of 1 g of tissue per 4 ml of 20 mM Tris-HCl, pH 7.5, containing 2 mM $MgSO_4$) (11) plus 40 μl of 40 mM Tris-HCl (pH 7.5) containing 2 mg/ml of BSA or 50 μl of the Tris-BSA solution without activator. Product accumulation was linear for at least 30 min under the above conditions.

Enzyme preparation. Washed particulate and supernatant fractions were obtained as described previously (11). Briefly, the right coronary, anterior descending, and circumflex arteries from fresh cold pig hearts were everted, and an inner layer which consisted predominantly of microscopically identifiable intima plus media tissue was removed by dissection using small, pointed forceps. The remaining layer was predominantly adventitia. The inner layer was homogenized in 9 ml of buffer (40 mM Tris-HCl, pH 7.5, 0.25 M sucrose, and 0.1 mM EDTA) per gram of tissue (wet weight) and centrifuged at 40,000 × *g* for 30 min at 0°. Particulate fractions were washed twice in the homogenizing medium. The inner layer contained 85% and 90%, respectively, of the cyclic AMP and cyclic GMP phosphodiesterase activities of the total coronary artery (11).

[1] The abbreviations used are: BSA, bovine serum albumin; MIX, 1-methyl-3-isobutylxanthine.

RESULTS

Inhibition of phosphodiesterase activities in crude supernatant and particulate fractions. MIX and papaverine were about 2 orders of magnitude more potent than caffeine as inhibitors of phosphodiesterase activities in both supernatant and particulate fractions at μM substrate (Table 1). Theophylline was 3–4 times more potent than caffeine as an inhibitor of supernatant phosphodiesterase activities, while adenosine, a potent coronary vasodilator (14), displayed weaker potency than caffeine with both fractions (Table 1).

The relative potencies of MIX and papaverine varied with the fraction and with the substrate (Table 1). With the supernatant fraction, the two agents were essentially equipotent as inhibitors of cyclic AMP hydrolysis, but MIX was about 4 times more potent than papaverine as an inhibitor of cyclic GMP hydrolysis. With the particulate fraction, papaverine was about 3 times more potent than MIX as an inhibitor of cyclic AMP hydrolysis, while, as with the supernatant fraction, the reverse was true for cyclic GMP hydrolysis. Both

MIX and papaverine raised the apparent K_m but did not change the V_{max} for cyclic GMP hydrolysis by supernatant phosphodiesterase activity (data not shown). Because of the complex kinetic plots and the multiple forms of phosphodiesterase associated with cyclic AMP hydrolysis in the crude fractions (11), the nature of the inhibition with this nucleotide as substrate could not be meaningfully studied.

Inhibition of chromatographically separated phosphodiesterase activities by theophylline, MIX, and papaverine. In order to assess more clearly the nature and potential selectivity of inhibition, MIX, papaverine, and theophylline were further studied with the phosphodiesterase activities that had been separated by DEAE-cellulose chromatography (11).

Peak I phosphodiesterase activity has an apparent K_m value of 1–4 μM for cyclic GMP and 40–100 μM for cyclic AMP and is dependent on a heat-stable, nondialyzable material for maximum activity (11). Although the substrate concentration routinely used for cyclic AMP (1 μM) was below the apparent K_m for this nucleotide, it was selected because it approximates physiological concentrations. With either cyclic nucleotide as substrate, peak I activity was more sensitive to inhibition by MIX than by papaverine or theophylline (Table 2). The absolute and relative potencies of the three inhibitors were the same in both the absence and presence of a saturating amount of the heat-stable, nondialyzable activator. As shown in Fig. 1, the type of inhibition produced by the xanthines and papaverine was competitive with both sub-

TABLE 2
Inhibition of peak I and II phosphodiesterase activities by MIX, papaverine, and theophylline

Inhibitor	I_{50}[a]		
	Peak I[b]		Peak II: cyclic AMP
	Cyclic GMP	Cyclic AMP	
	μM	μM	μM
MIX	4.2 ± 0.7	2.6 ± 0.3	11 ± 1
Papaverine	13 ± 1	13 ± 2	2.8 ± 0.3
Theophylline	100 ± 9	58 ± 4	190 ± 6

[a] I_{50} value is the concentration of agent required to produce 50% inhibition of the hydrolysis of 1 μM substrate. Values are means ± standard errors of four determinations with four different preparations.

[b] Peak I activity was measured in the presence of an optimal amount of activator (15 μg of protein).

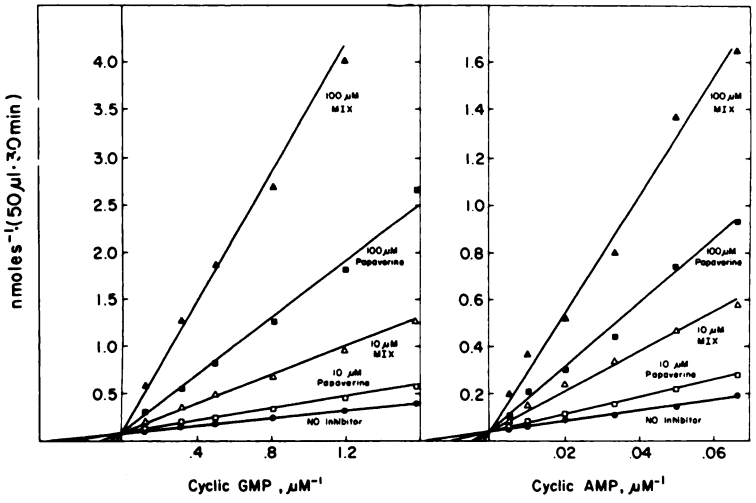

FIG. 1. *Reciprocal plots of cyclic AMP (right) and cyclic GMP (left) hydrolyses by peak I in the presence of MIX or papaverine*

Activity was measured as described in METHODS, in the presence of a saturating amount of the activator (15 μg of protein). Substrate ranges shown are 0.6–10 μM and 15–200 μM for cyclic GMP and cyclic AMP, respectively, but the linearity of the kinetic plot was maintained with concentrations as low as 0.1 μM cyclic AMP. Results are means of duplicate determinations and are representative of experiments with two different preparations.

strates (the theophylline data are not shown). K_i values, which were obtained from Dixon plots and are therefore numerically different from the I_{50} values determined at 1 μM substrate, were essentially the same with either cyclic nucleotide as substrate and ranged from 5 to 10 μM for MIX, 12 to 20 μM for papaverine, and 100 to 200 μM for theophylline in three different experiments.

Peak II phosphodiesterase activity displays apparent negatively cooperative behavior with cyclic AMP as substrate; this peak has low activity with cyclic GMP, but it has not been practical to study this nucleotide in detail as a substrate (11). The activity of peak II phosphodiesterase is not affected by the heat-stable, nondialyzable activator of peak I. In contrast to the activity of peak I, that of peak II was more sensitive to inhibition by papaverine than by either MIX or theophylline at 1 μM cyclic AMP (Table 2). Both Lineweaver-Burk and Dixon plots of peak II activity were curved; therefore it is not possible to establish precisely either the type of inhibition or K_i values by simple graphical analyses. Peak II activity was several times more sensitive to inhibition by papaverine and less sensitive to inhibition by MIX than was peak I activity at 1 μM substrate or within the range of 0.1 to 100 μM substrate (data not shown).

Inhibition of chromatographically separated phosphodiesterase activities by cyclic nucleotides and cyclic nucleotide derivatives. If peak I phosphodiesterase activity consists of one enzyme with a common catalytic site for both cyclic AMP and cyclic GMP, the two nucleotides should competitively inhibit the hydrolysis of each other with relative inhibitory potencies predictable from their apparent K_m values as substrates. As shown in Fig. 2, lower panel, both nucleotides were indeed inhibitors of the hydrolysis of each other by peak I activity, and cyclic GMP was much more potent in inhibiting cyclic AMP hydrolysis than the converse. The type of inhibition by both nucleotides was competitive (Fig. 3), and the apparent K_i values, 2 μM for cyclic GMP and 60 μM for cyclic AMP, were indistinguishable from the apparent K_m values for each nucleotide as substrate (11).

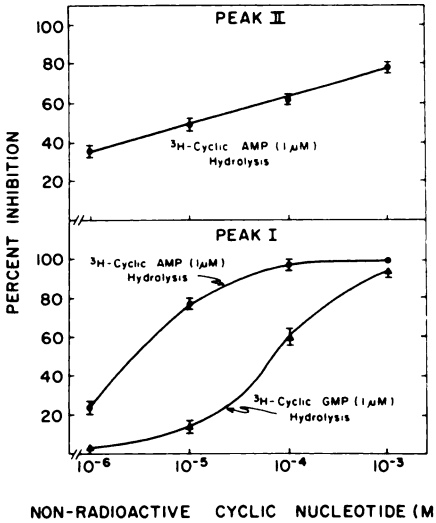

FIG. 2. *Inhibition of separated coronary artery phosphodiesterases by cyclic nucleotides*

Tritiated substrate hydrolysis was measured as described in METHODS, and the alternate nonradioactive cyclic nucleotide was added as inhibitor in the concentrations indicated. Peak I activities were measured in the presence of an optimal amount of activator (15 μg of protein). Values with vertical bars are means ± standard errors of eight determinations; the other values are means of four determinations.

Although cyclic GMP is a poor substrate for peak II activity (11), it was found to be an inhibitor of this enzyme (Fig. 2, upper panel). The hydrolysis of 1 μM cyclic AMP was inhibited by 50% by about 10 μM cyclic GMP. The data in Fig. 4 are plotted according to Hofstee (15) in order to accommodate the wide substrate range (0.14–500 μM) used in kinetic studies of peak II. These data are presented to demonstrate that graphical analysis cannot be used to determine the type of inhibition of peak II. Lines tangential to the low substrate portions of the curves appear to intersect at a common point on the ordinate, indicating competitive inhibition, while lines tangential to other portions of the curves do not converge at a common V_{max} value. Thus, because of the anomalous kinetic behavior of peak II activity, the type of inhibition by cyclic GMP cannot be precisely determined by simple graphical analyses.

A number of derivatives of cyclic AMP and cyclic GMP have been applied to intact cell systems in attempts to mimic ef-

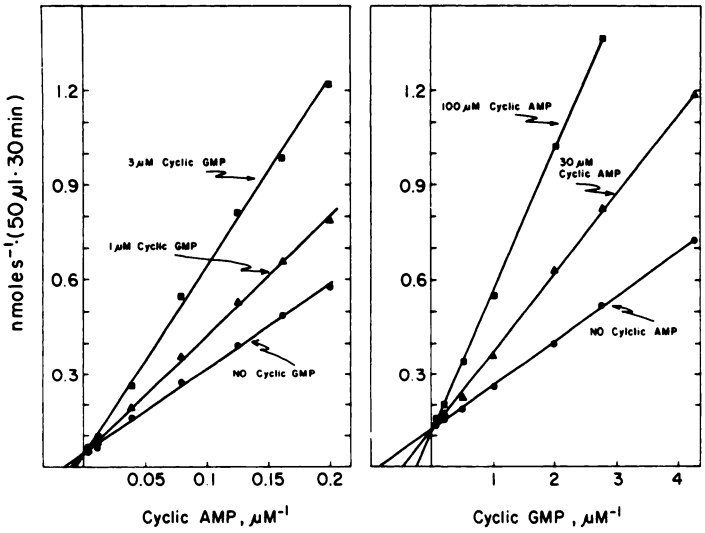

FIG. 3. *Reciprocal plots of ^{3}H-cyclic AMP (left) and ^{3}H-cyclic GMP (right) hydrolyses by peak I in the presence and absence of nonradioactive cyclic GMP and cyclic AMP, respectively*

Activity was measured as described in METHODS, in the presence of a saturating amount of the activator (15 µg of protein). Results are means of duplicate determinations and are representative of experiments with two different preparations.

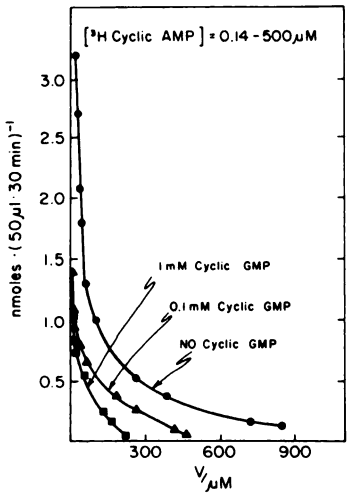

FIG. 4. *Hofstee plots of ^{3}H-cyclic AMP hydrolysis in the presence and absence of nonradioactive cyclic GMP*

Activity was measured as described in METHODS. Results are means of duplicate determinations and are representative of experiments with two different preparations.

fects of the endogenous cyclic nucleotides. Some of these derivatives are known to inhibit cyclic AMP hydrolysis by crude phosphodiesterase preparations (16, 17). Three of the more commonly used derivatives were studied as inhibitors of peak I and peak II activities. N^2,2′-*O*-Dibutyryl cyclic GMP and 8-bromo cyclic GMP were potent inhibitors of the hydrolysis of both cyclic GMP and cyclic AMP by peak I phosphodiesterase, and the two derivatives of cyclic GMP were nearly 2 orders of magnitude more potent as inhibitors of peak I activity than was N^6,2′-*O*-dibutyryl cyclic AMP (Fig. 5). 8-Bromo cyclic GMP was some 4–6 times more potent as an inhibitor of fully activated peak I phosphodiesterase than as an inhibitor of activator-deficient enzyme. Concentrations of the derivatives required to produce 50% inhibition of the hydrolysis of 1 µM cyclic AMP or cyclic GMP by fully activated peak I enzyme were 30–40 µM for N^2,2′-*O*-dibutyryl cyclic GMP, 20–30 µM for 8-bromo cyclic GMP, and 1 mM for N^6,2′-*O*-dibutyryl cyclic AMP. Thus, at 1 µM substrate, the cyclic GMP derivatives are only slightly less potent than papaverine as inhibitors of peak I activity, while the cyclic AMP derivative is about one-tenth as potent as theophylline.

In contrast to the results with peak I, dibutyryl cyclic AMP was about 3 times as potent as either of the cyclic GMP deriva-

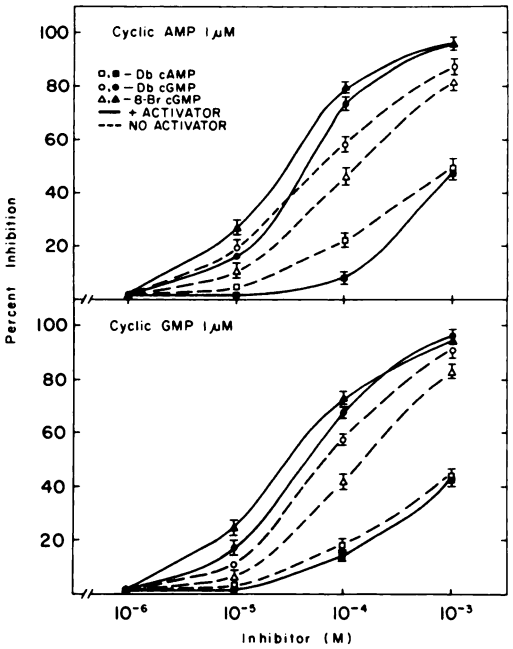

FIG. 5. *Inhibition of peak I cyclic nucleotide phosphodiesterase activities by cyclic nucleotide derivatives*

Hydrolysis of cyclic AMP or cyclic GMP in the absence of added cyclic nucleotide derivative was compared with hydrolysis in the presence of the indicated cyclic nucleotide derivative with and without added protein activator as described in METHODS. Values are means of four determinations (eight at 10^{-4} M inhibitor). Vertical lines represent standard errors. In a typical experiment control rates of hydrolysis were 94.8 and 14.1 for cyclic AMP and 850 and 187 pmoles·(50 μl 30 min)$^{-1}$ for cyclic GMP in the presence and absence of added activator, respectively. Abbreviations: Db cAMP, N^6,2′-*O*-dibutyryl cyclic AMP; Db cGMP, N^2,2′-*O*-dibutyryl cyclic GMP; 8-Br cGMP, 8-bromo cyclic GMP.

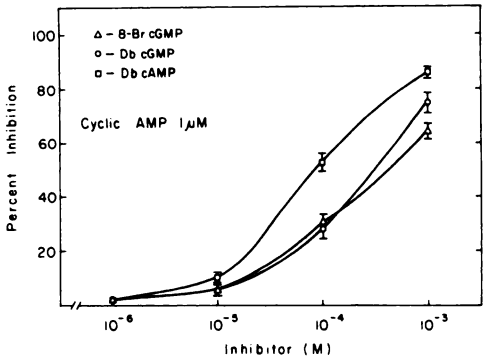

FIG. 6. *Inhibition of peak II phosphodiesterase by cyclic nucleotide derivatives*

Values are means of four determinations (eight at 10^{-4} M inhibitor). Vertical lines represent standard errors. In a typical experiment the control rate of substrate hydrolysis was 46.3 pmoles·(50 μl 30 min)$^{-1}$. Abbreviations are defined in the legend to Fig. 5.

tives as an inhibitor of peak II cyclic AMP phosphodiesterase activity (Fig. 6). The cyclic GMP derivatives were about one-tenth as potent, and the cyclic AMP derivative about 10 times more potent, as inhibitors of this peak of activity than as inhibitors of peak I. The cyclic GMP derivatives displayed about the same inhibitory potency as theophylline with peak II, and dibutyryl cyclic AMP was intermediate in potency between MIX and theophylline.

DISCUSSION

Of the compounds studied, MIX was the most potent inhibitor of cyclic GMP hydrolysis in the crude supernatant fraction and of peak I phosphodiesterase activity with either cyclic AMP or cyclic GMP as substrate. This agent was 30–60 times more potent than theophylline as an inhibitor of supernatant and of peak I and peak II phosphodiesterase activities when either 1 μM cyclic AMP or 1 μM cyclic GMP was used as substrate. Similar differences in potency have been reported for MIX and theophylline as inhibitors of cyclic AMP phosphodiesterase activities from other tissues (18–20). Papaverine, on the other hand, was a more potent inhibitor of peak II phosphodiesterase than was MIX.

Although the specificities of MIX and papaverine for peaks I and II, respectively, were not absolute, the effects of these two compounds do demonstrate that some degree of selectivity in inhibition of the hydrolysis of the two cyclic nucleotides by vascular phosphodiesterases is possible. Other workers also have seen relatively selective inhibition of the hydrolysis of cyclic GMP and cyclic AMP in crude systems from other tissues by papaverine (21) and other agents (17, 22, 23). By structural modifications of the xanthines (24) and/or papaverine, more highly selective pharmacological control of the degradation of the two cyclic nucleotides may be feasible. However, highly selective inhibition of phosphodiesterase will probably be neces-

sary in order to alter cyclic nucleotide levels selectively in intact cells, since in pig coronary arteries, at least, the total cyclic AMP phosphodiesterase activity of fully activated peak I (at 1 μM substrate) is approximately equal to the total activity of peak II (11); therefore regulation of either activity could potentially affect cyclic AMP levels in this tissue. Studies with pig coronary artery strips[2] and rat ductus deferens (6) showed that while MIX does raise cyclic GMP levels to a greater relative degree than it elevates cyclic AMP levels, it nonetheless raises levels of both nucleotides in these intact tissues.

The 8-bromo and dibutyryl derivatives of cyclic AMP and cyclic GMP have been used exogenously to simulate presumed effects of endogenous cyclic nucleotides in various intact cell preparations. There are severe limitations in interpreting the data obtained from such studies. Good correlation has been reported for some cyclic AMP derivatives between protein kinase activation and the biological effect expected of cyclic AMP (25, 26). However, the data reported here and elsewhere emphasize that inhibition of phosphodiesterases also should be considered as a possible mechanism of action of cyclic nucleotide derivatives. N^6,2′-*O*-Dibutyryl cyclic AMP and 8-bromo cyclic GMP have been shown previously to inhibit cyclic nucleotide phosphodiesterases from several sources (16, 17). In this study these agents, along with dibutyryl cyclic GMP, were found to be inhibitors of both peak I and II phosphodiesterases. An added uncertainty about the effects of these agents in intact cells is raised by the finding that 8-bromo cyclic GMP is more potent on fully activated than on activator-deficient peak I. Cyclic GMP, which can accumulate to high levels when added exogenously to the perfused liver (27), was a potent inhibitor of both peak I and peak II cyclic AMP phosphodiesterase activities. Therefore any studies involving the use of cyclic nucleotides or their derivatives must be interpreted with caution because of the possibility that levels of both cyclic AMP and cyclic GMP could be raised by inhibition of phosphodiesterases.

[2] C. A. Sutherland and J. G. Hardman, unpublished observations.

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