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Effects of Phosphodiesterase Inhibitors on Cyclic Nucleotide Levels and Relaxation of Pig Coronary Arteries

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SUMMARY

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A series of xanthine derivatives and papaverine have been studied to determine their abilities to alter tissue levels of cyclic AMP and cyclic GMP, inhibit phosphodiesterase activities, and cause relaxation of pig coronary arteries. The agents selected for this study exhibited a wide range of potencies to inhibit phosphodiesterase activities in the coronary artery supernatant fraction. In addition, some of these agents were up to 10 times more potent as inhibitors of cyclic GMP hydrolysis than of cyclic AMP hydrolysis, while others were 2-4 times more potent as inhibitors of cyclic AMP than of cyclic GMP hydrolysis. The rank order of potencies of these agents to cause relaxation of coronary artery strips was similar to the rank order of potencies to inhibit cyclic nucleotide phosphodiesterase activities. There were, however, some notable exceptions to the correlation between inhibition of cyclic nucleotide phosphodiesterase activities and relaxation. 1-Isoamyl-3-isobutylxanthine was a more potent relaxing agent than might be expected from its relatively low potency to inhibit cyclic nucleotide hydrolysis in tissue extracts. On the other hand, 1-methyl-3-isobutyl-7-(3-chlorobenzyl)-xanthine was one of the more potent inhibitors of cyclic nucleotide hydrolysis but was not as potent in causing relaxation as might have been expected. Exposure of the coronary artery strips to inhibitors caused increases in tissue levels of cyclic AMP and cyclic GMP and there was a statistically significant multiple linear regression of cyclic AMP and cyclic GMP levels on percent relaxation after 5 min of exposure to the agents. Cyclic AMP and cyclic GMP levels made approximately equal contributions to the regression of changes in percent relaxation, as determined by analysis of variance methods. While 1-isoamyl-3-isobutylxanthine did not fit the correlation between phosphodiesterase inhibition and potency to relax the arterial strips as well as the other agents, this agent also was found to cause unexpectedly large increases in cyclic AMP levels. Some agents caused relaxation accompanied by significant elevation of cyclic GMP levels and no significant change in cyclic AMP levels while other agents caused relaxation accompanied by significant increases in cyclic AMP but not cyclic GMP. These data offer some support for a hypothesis that both cyclic AMP and cyclic GMP are involved in the relaxation processes of pig coronary arteries.

INTRODUCTION

The hypothesis that cyclic nucleotides play a role in the regulation of smooth muscle tone must be characterized as controversial. There is considerable evidence

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to suggest that adenosine-3',5'-monophosphate (cyclic AMP) may mediate smooth muscle relaxation (1). There are, however, a number of reports in which agents have been found to raise cyclic AMP levels without concomitant relaxation or to cause relaxation with no measurable elevation of cyclic AMP levels (2). A number of observations have shown associations between smooth muscle contraction in response to some agents and increases in guanosine-3',5'-monophosphate (cyclic GMP) levels, leading to speculation about a causal relationship between these phenomena (3). Here too, however, there are numerous reports of dissociations between contraction and elevation of cyclic GMP levels (2). Further, it has recently been suggested that cyclic GMP may be involved in processes which lead to smooth muscle relaxation (4, 5), but evidence contrary to this hypothesis has also been presented (6).

Studies of the role of cyclic AMP in smooth muscle and other tissues have often been undertaken using methylxanthines and other agents that inhibit cyclic AMP hydrolysis by phosphodiesterases. Where examined, however, such agents have also been shown to inhibit cyclic GMP hydrolysis. This complication necessarily limits the utility of these phosphodiesterase inhibitors as tools in the investigation of the roles of cyclic AMP and cyclic GMP in biological systems.

We have demonstrated in pig coronary arteries the presence of two forms of phosphodiesterase activity that can be separated by DEAE-cellulose chromatography of the supernatant fraction (7). One of these forms (peak I) hydrolyzes both cyclic AMP and cyclic GMP but has a higher affinity for cyclic GMP. The activity of this peak is increased 3- to 8-fold in the presence of an activator protein, which also can be isolated by this procedure and is presumably the same as or similar to the calcium-dependent regulatory protein isolated from other tissues (8). The other form of phosphodiesterase activity (peak II) hydrolyzes cyclic AMP but has very low activity against cyclic GMP.

JNW as an Established Investigator of the American Heart Association.

We have designed and synthesized a series of xanthine derivatives in an effort to obtain compounds that selectively inhibit the hydrolysis of cyclic AMP or cyclic GMP by the phosphodiesterase activities of pig coronary arteries (9–11). Several of these newly synthesized xanthine derivatives have shown selectivity toward inhibition of cyclic GMP hydrolysis by virtue of their greater potency to inhibit peak I than peak II activity. Some selectivity toward inhibition of cyclic AMP hydrolysis has also been demonstrated with papaverine and 1-isoamyl-3-isobutylxanthine (IIX) (9, 11). The purpose of the present study was to ascertain if there was a relationship between the potency of these agents to inhibit cyclic nucleotide phosphodiesterase activities of coronary arteries and their potency to cause relaxation of coronary artery strips. In addition, we wished to determine if the potency to inhibit phosphodiesterase activities is reflected by increased levels of the cyclic nucleotides in the artery strips.

MATERIALS AND METHODS

Materials. Cyclic AMP and cyclic GMP (Sigma Chemical Co.) were prepared as stock solutions and used without further purification. Tritiated cyclic nucleotides obtained from New England Nuclear Corp. were purified on Dowex-50 cation exchange resin columns (12). Papaverine was purchased from Eli Lilly, theophylline from Mallinckrodt, and caffeine from Merck. 1-Methyl-3-isobutyl-xanthine (MIX)[2] was prepared by the method of Kramer *et al.* (11). All other agents were synthesized according to previously published methods (10, 11).

Tissue preparation. Hearts from pigs of both sexes and varying ages were obtained from a local slaughterhouse within 20 min after slaughter of the animals and kept packed in ice until dissection, which was completed within 2 hr. A segment of the right coronary artery was dissected and the fat removed. Helical strips were cut into 3 × 25 mm segments, taking care to avoid stretching the tissues. Tissues were incubated at 37°, and the medium was repeat-

[2] The abbreviations used are: MIX, 1-methyl-3-isobutyl-xanthine; DMSO, dimethyl sulfoxide;

edly changed over a 2 hr period. The incubation medium was Krebs-Ringer bicarbonate buffer with 10 mM glucose and 1 mM pyruvate, bubbled with 95% O_2–5% CO_2 at pH 7.4. After the 2 hr incubation, tissues were stored in this medium for 18 to 24 hr at 4° (the bicarbonate concentration was adjusted to maintain pH 7.4 at this temperature) before use.

Measurement of muscle tension responses. Tissue segments were mounted in organ baths in 25 ml of the incubation medium at 37° with 1 g resting tension. Isometric tension was measured by a Statham strain gauge transducer connected to a Gould-Brush 2400 recorder. Tissues were preincubated for 2 hr. During this time the medium was changed 3 times and tension was periodically readjusted to 1 g until no more spontaneous relaxation was observed. Tissues were then further preconditioned by the addition of K^+ (50 mM) to the incubation buffer (maximal stimulation) for 1–2 min followed by equilibration in incubation buffer for 25 min, then exposure to 20 mM K^+ (about 30% maximal stimulation) for 15 min, followed by equilibration in incubation buffer for 25 min. This preconditioning procedure helped to bring the tissues more quickly to a state of constant responsiveness to the contraction stimulus. Phosphodiesterase inhibitors were tested for their ability to cause relaxation of K^+- or histamine-contracted strips in the following manner: The incubation buffer was replaced by buffer in which K^+ had been substituted for an equal concentration of Na^+ to give 20 mM total K^+ or histamine was added to give 1 μM final concentration. The tissue was allowed to reach peak contraction, at which point the phosphodiesterase inhibitor in 50 μl of DMSO was added. In untreated tissues contractions decreased less than 5% in 5 min. The tissue was exposed to agent for 5 min. The tissue was then washed twice with incubation medium and reequilibrated for 25 min with additional washings at 5 and 15 min after washing out the agent. After 15 min, tension was readjusted to 1 g if necessary. Control treatments with 50 μl of DMSO (giving 0.2% or 28 mM final concentration in the bath) were carried out during every third contraction so that each concentration of an agent was tested either immediately before or immediately after a control contraction. Some (about 5% of maximum) relaxation in response to DMSO was observed; therefore, the response due to DMSO was subtracted from the response to each agent in the presence of DMSO. MIX was used as the reference agent in these studies. Relaxation of each tissue in response to 100 μM MIX, added as described above, was taken as 100% and responses to the other agents are reported with reference to this response. Concentrations of the agents that caused 50% relaxation of the K^+-induced contraction (EC_{50}) were determined from concentration-response curves obtained by averaging the responses of 8–12 tissues.

Cyclic AMP and cyclic GMP levels. Tissues were mounted and preconditioned as described for measurement of tension responses. Tissues were then contracted by addition of 20 mM K^+-substituted buffer, and the test agents were added at peak tension. At the appropriate time, the bath was lowered from the tissue, and within 2 sec (with no observable change in tension), the tissue was clamped with aluminum blocks that had been cooled in liquid nitrogen. Two separate tissue samples treated in an identical manner were pooled and stored at −70° until assayed. Tissue samples were homogenized by placing the frozen sample in a stainless steel capsule with a stainless steel ball (all cooled in liquid nitrogen) and shaking the capsule and its contents in a Wig-L-Bug dental amalgamator (Crescent Dental Mfg. Co.) at maximum speed 3 times for 20 sec. The capsule was cooled in liquid nitrogen before and after each shaking. The resulting frozen, powdered tissue was added to 4 ml of 0.1 M perchloric acid in 70% ethanol, at −78°. Radiolabeled cyclic AMP and cyclic GMP were added to monitor recoveries which were about 50% for cyclic AMP and 70% for cyclic GMP. The mixture was suspended by agitation, and the suspension was centrifuged at 27,000 × *g* for 20 min. The supernatant fraction was placed on a 0.7 × 25 cm Dowex-50 column for purification and separation of cyclic AMP and cyclic GMP (13). Cyclic AMP was assayed by the method of Gilman (14)

and cyclic GMP by radioimmunoassay (15) and the amount of cyclic nucleotide added to monitor recoveries was corrected for in the calculation of the assay results. Control samples treated with phosphodiesterase showed no detectable amounts of cyclic AMP or cyclic GMP. The perchloric acid insoluble pellet was resuspended in 5 ml of 1 M sodium hydroxide and heated at 100° for 30 min. The mixture was centrifuged at 28,000 × *g* for 20 min and the supernatant fluid was assayed for protein by the method of Lowry *et al.* (16).

Enzyme preparation. The crude supernatant phosphodiesterase activities from pig coronary arteries were obtained as described previously (7). 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 and media, was removed and homogenized in 4 ml of buffer (20 mM Tris·HCl, pH 7.5, 2 mM MgOAc, and 1 mM dithiothreitol) per g of tissue (wet weight). After centrifugation at 40,000 × *g* for 30 min at 0° the supernatant fraction was used directly.

Phosphodiesterase assay. The agents were assayed as inhibitors of the phosphodiesterase activities in the 40,000 × *g* supernatant fraction. Characterization of this fraction and the assay procedures have been reported (7, 9). Sufficient activator protein and calcium are present in this fraction to give maximal cyclic AMP and cyclic GMP hydrolytic activity (7, 9). Assays were performed with 1 μM substrate at 30° for 30 min at enzyme dilutions, which gave 10–20% hydrolysis of substrate in the absence of inhibitor. MIX (10 μM) was included in each experiment to assure that the enzyme preparations were responding in a constant manner. The agents were dissolved in 30% DMSO, and 25 μl of this solution was added to the assay tube (final volume was 250 μl). All activities were measured in the presence of 3% DMSO; the product accumulation was linear for at least 30 min under the conditions of assay. Concentrations of the agents that inhibited by 50% the hydrolysis of 1 μM substrate (I_{50}) were determined from concentration-percent inhibition curves, utilizing concentrations of the agents from 0.1–100 μM (or 0.1–1000 μM if the agent was sufficiently soluble). None of the agents altered the efficacy of the nucleotide step or subsequent steps in the assay.

Statistical methods. All statistical treatments were carried out by methods outlined and referenced in *SPSS: Statistical package for the social sciences* (17). The data in Table 2 were treated by ln transformation followed by one-way analysis of variance. Homogeneity of variances after ln transformation was confirmed using Cochrain's C test and the Bartlett-Box F test ($p > 0.05$). Groups were compared by multiple range test using the Tukey-HSD multiple range procedure.

RESULTS

Inhibition of phosphodiesterase activities in crude supernatant fractions. Potencies of the agents to inhibit cyclic GMP hydrolysis by phosphodiesterase activities in the supernatant fraction (Table 1) were indistinguishable from those for inhibition of cyclic GMP hydrolysis by peak I activity isolated by DEAE-cellulose chromatography (9–11). Cyclic AMP hydrolytic activity, however, is found in about equal amounts in the fractions isolated by DEAE-cellulose chromatography (peak I and peak II). Therefore, potencies of the agents to inhibit cyclic AMP hydrolysis by activities in the supernatant fraction (Table 1) were intermediate between those for inhibition of cyclic AMP hydrolysis by peak I and by peak II activities.

Relaxation of pig coronary artery strips. All agents tested caused relaxation of K^+-contracted coronary artery strips in a concentration-dependent manner (data not shown). Potencies of the agents to cause relaxation is indicated by EC_{50} values and also by percent relaxation caused by single agent concentration (30 μM) are presented in Table 1 in approximate rank order. This order of potency is similar to that for inhibition of phosphodiesterase activities as may be seen from I_{50} values also presented in Table 1. The low solubility of 7-(3-Cl Bzl) MIX and 8-CF_3 MIX (see Table 1 for abbreviations) did not permit determina-

TABLE 1

Effects of agents to cause relaxation of pig coronary artery strips and to cause inhibition of cyclic nucleotide phosphodiesterase activities in crude supernatant fractions of pig coronary arteries

Agent	Relaxation		Phosphodiesterase inhibition, I_{50}[c]	
	EC_{50}[a]	Percent[b] at 30 μM agent	cyclic AMP	cyclic GMP
1-Methyl-3-isobutyl-6-thioxanthine (6-Thio MIX)	4.6	83 ± 4 (7)	4.5 ± 1.5	4.0 ± 1.0
1-Isoamyl-3-isobutylxanthine (IIX)	6.1	70 ± 6 (7)	40 ± 4[d]	97 ± 3[d]
1,8-Dimethyl-3-isobutylxanthine (8-Methyl MIX)	10	73 ± 7 (8)	21 ± 2	2.0 ± 1.0
1-Methyl-3-isobutylxanthine (MIX)	14	72 ± 2 (7)	11 ± 2[d]	5.6 ± 0.8[d]
1-Methyl-3-isobutyl-8-*tert*-butylxanthine (8-*t*-Bu MIX)	15	69 ± 4 (8)	18 ± 4[d]	1.9 ± 0.4[d]
Papaverine	15	69 ± 4 (8)	3.7 ± 0.5[e]	17 ± 1[e]
1-Methyl-3-isobutyl-7-benzylxanthine (7-Bzl MIX)	23	56 ± 5 (12)	30 ± 6[d]	3.9 ± 0.6[d]
1-Methyl-3-isobutyl-7-(3-chlorobenzyl)-xanthine [7-(3-Cl Bzl) MIX]	>100	32 ± 4 (8)	19 ± 6	2.7 ± 0.3
Theophylline	350	8 ± 2 (6)	140 ± 10[e]	140 ± 15[e]
1-Methyl-3-isobutyl-8-trifluoromethylxanthine (8-CF_3 MIX)	>100	4 ± 1 (6)	>100	>100
Caffeine	1000	3 ± 1 (7)	500 ± 70[e]	390 ± 30[e]

[a] EC_{50} is the concentration (μM) of agent required to cause 50% relaxation of 20 mM K^+-contracted coronary artery strips.

[b] Percent relaxation of 20 mM K^+-contracted coronary artery strips caused by 30 μM agents. Values are means ± standard errors of the number of determinations given in parentheses.

[c] I_{50} is the concentration (μM) of agent required to inhibit by 50% the hydrolysis of 1 μM substrate. Values are means ± standard errors of at least three determinations with different preparations.

[d] Originally reported in reference 11.

[e] Originally reported in reference 9.

tion of EC_{50} values for these agents.

Potencies of agents to relax histamine-contracted coronary artery strips were also determined and were essentially identical to those for relaxation of K^+-contracted strips (data not shown).

Cyclic AMP and cyclic GMP levels. Cyclic AMP and cyclic GMP levels in tissues contracted with 20 mM K^+ and clamp-frozen at peak tension (12–15 min of exposure to 20 mM K^+) and 1 and 5 min after peak tension were not significantly different from levels in uncontracted tissues (data not shown). In some tissues DMSO at higher concentration than used here has been shown to elevate cyclic AMP levels (18, 19). In our studies DMSO caused no significant change in cyclic AMP levels at any time point, but cyclic GMP levels were significantly higher ($p < 0.05$) after 5 min of exposure to DMSO than after 20 sec or 1 min of exposure (Table 2). In general cyclic GMP levels increased more rapidly and to a greater extent relative to control levels than did cyclic AMP.

The data in Table 2 demonstrate an overall similarity between the abilities of the agents to raise cyclic nucleotide levels and their potencies to cause relaxation. Thus, theophylline, which was the least potent in causing relaxation of the agents chosen for cyclic nucleotide levels studies, also caused less change in cyclic nucleotide levels at 100 μM than any other agent tested at this concentration, and 6-thio MIX, which was the most potent agent tested to cause relaxation, caused relatively large changes in cyclic nucleotide levels. IIX, which was a potent relaxing agent, is of particular interest in that at 10 and 30 μM it caused larger increases in cyclic AMP levels after 5 min than all other agents at these concentrations despite its relatively low potency as a phosphodiesterase inhibitor.

TABLE 2

Cyclic AMP and cyclic GMP levels in response to agents

Agent	Concentration (μM)	20 sec		1 min		5 min	
		Cyclic AMP[a]	Cyclic GMP[a]	Cyclic AMP[a]	Cyclic GMP[a]	Cyclic AMP[a]	Cyclic GMP[a]
DMSO	28000	1.58 ± 0.17 (12)	0.13 ± 0.02 (11)	1.61 ± 0.07 (20)	0.15 ± 0.02 (21)	1.61 ± 0.08 (20)	0.20 ± 0.02 (19)
6-Thio MIX	10	ND[b]	ND	ND	ND	2.18 ± 0.22 (6)	0.80 ± 0.23[c] (6)
	30	ND	ND	2.16 ± 0.15 (5)	0.61 ± 0.10[c] (6)	2.15 ± 0.23 (6)	0.86 ± 0.10[c] (5)
	100	ND	ND	2.14 ± 0.17 (6)	0.87 ± 0.20[c] (6)	2.97 ± 0.37[c] (6)	0.73 ± 0.17[c] (6)
IIX	10	ND	ND	ND	ND	2.78 ± 0.29[c] (8)	0.35 ± 0.05 (7)
	30	2.08 ± 0.27 (8)	0.20 ± 0.01 (8)	1.73 ± 0.10 (10)	0.21 ± 0.02 (11)	2.47 ± 0.14[c] (7)	0.38 ± 0.07 (6)
MIX	10	ND	ND	ND	ND	2.13 ± 0.19 (8)	0.37 ± 0.07 (8)
	30	1.63 ± 0.12 (8)	0.24 ± 0.04 (7)	1.69 ± 0.08 (10)	0.33 ± 0.04[c] (10)	1.99 ± 0.13 (8)	0.36 ± 0.04 (8)
	100	1.66 ± 0.16 (6)	0.29 ± 0.07[c] (5)	2.09 ± 0.12 (6)	0.42 ± 0.11[c] (5)	2.77 ± 0.41[c] (6)	0.34 ± 0.06 (6)
8-*t*-Bu MIX	10	ND	ND	ND	ND	1.82 ± 0.10 (8)	0.47 ± 0.06[c] (8)
	30	ND	ND	2.04 ± 0.10 (8)	0.74 ± 0.13[c] (8)	1.96 ± 0.07 (6)	0.82 ± 0.12[c] (6)
	100	ND	ND	1.75 ± 0.12 (7)	0.72 ± 0.14[c] (8)	2.38 ± 0.30 (6)	1.27 ± 0.36[c] (5)
Papaverine	10	ND	ND	ND	ND	2.22 ± 0.14 (8)	0.30 ± 0.04 (7)
	30	ND	ND	1.82 ± 0.11 (6)	0.54 ± 0.12[c] (6)	2.00 ± 0.25 (6)	0.34 ± 0.08 (6)
	100	ND	ND	2.54 ± 0.37[c] (6)	0.41 ± 0.06[c] (6)	3.28 ± 0.24[c] (6)	0.65 ± 0.07[c] (6)
7-Bzl MIX	10	ND	ND	ND	ND	1.93 ± 0.14 (8)	0.46 ± 0.07[c] (8)
	30	ND	ND	1.71 ± 0.08 (6)	0.48 ± 0.07[c] (6)	1.74 ± 0.13 (8)	0.67 ± 0.09[c] (8)
	100	ND	ND	1.63 ± 0.15 (6)	0.70 ± 0.13[c] (6)	2.58 ± 0.19[c] (6)	1.10 ± 0.27[c] (5)
Theophylline	100	ND	ND	1.70 ± 0.10 (8)	0.17 ± 0.03 (7)	2.02 ± 0.12 (6)	0.27 ± 0.05 (6)
	1000	ND	ND	1.92 ± 0.12 (6)	0.29 ± 0.07 (6)	2.33 ± 0.25 (6)	0.29 ± 0.05 (6)

[a] Values are expressed as picomoles/mg protein ± standard errors of the number of determinations given in parentheses.

[b] Not determined.

[c] Significantly different from response to DMSO ($p < 0.05$) (see STATISTICAL METHODS).

As might be predicted from their ability to inhibit cGMP hydrolysis more potently than cAMP hydrolysis (I_{50} values, Table 1), 8-*t*-Bu MIX and 7-Bzl MIX were relatively more potent to alter cyclic GMP than cyclic AMP levels in the artery strips. 6-Thio MIX, which was equipotent as an inhibitor of cyclic AMP and cyclic GMP phosphodiesterase activities, also caused relatively larger changes in cyclic GMP than in cyclic AMP levels. MIX caused surprisingly small changes in cyclic GMP in view of its potency to inhibit cyclic GMP phosphodiesterase.

DISCUSSION

As a preliminary assessment of the potential ability of the agents chosen for this study to alter cyclic nucleotide levels in intact smooth muscle of coronary arteries it was desirable to test these agents as inhibitors of total phosphodiesterase activities in the intima + media layers. Since it has been demonstrated that greater than 80% of the phosphodiesterase activity of the whole homogenate of the intima + media layers is present in the soluble fraction (7), agents were selected for cyclic nucleotide levels studies on the basis of their potencies to inhibit cyclic AMP and cyclic GMP hydrolysis by the crude soluble enzyme preparation (Table 1).

The abilities of agents to cause relaxation of coronary artery strips appeared with some exceptions to parallel their abilities to inhibit phosphodiesterase. In Figure 1, I_{50} values for inhibition of cyclic AMP (left panel) and cyclic GMP (right panel) hydrolysis by the supernatant fractions are plotted against EC_{50} values for relaxation of K^+-induced contractions (7-[3-Cl Bzl] MIX and 8-CF_3 MIX were omitted because they were too insoluble to permit determination of EC_{50} values). In both cases (Fig. 1), there is a significant positive correlation.

Although the correlation of relaxation with inhibition of cyclic AMP or cyclic GMP hydrolysis seems reasonably strong, observations with some agents deviate from this overall relationship. For instance, IIX is substantially more potent as a relaxing agent than expected considering its I_{50} values for inhibition of cyclic AMP or cyclic GMP hydrolysis. 7-(3-Cl Bzl) MIX, on the other hand, is less effective as a relaxing agent than its potency to inhibit cyclic AMP or cyclic GMP hydrolysis might suggest. Such variations could be interpreted as evidence that these agents have effects other than phosphodiesterase inhibition to alter smooth muscle tone. However, a number of assumptions must be made in attaching significance to a correlation between phosphodiesterase inhibitory potency and potency to cause relaxation and in requiring a given agent to adhere quantitatively to the overall relationship. One assumption is that all agents have equal access to the site of action inside the smooth muscle cell. Then for instance deviation by IIX from the relationship might be explained if its greater alkyl bulk in the 1-position as compared with the other xanthines allowed

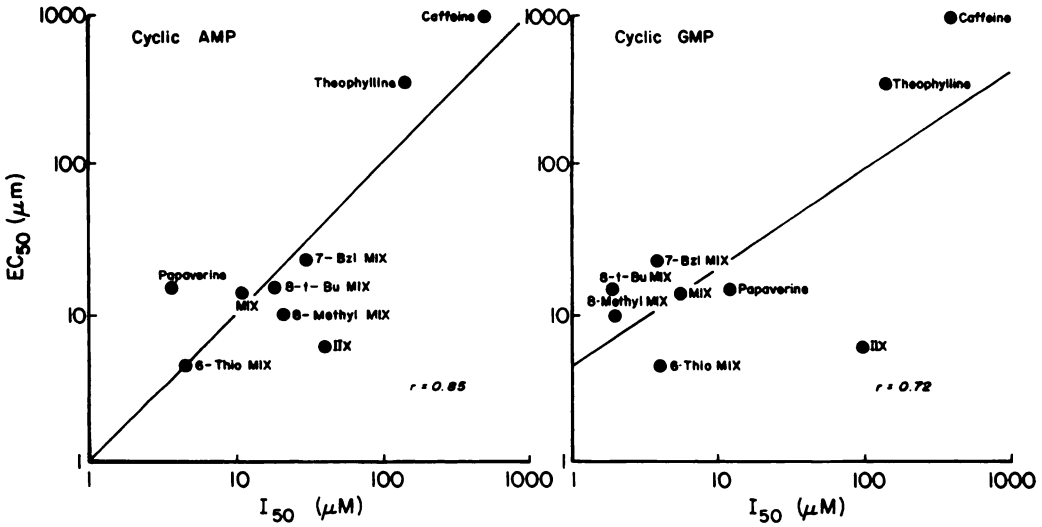

FIG. 1. *Relationships between the potency of agents to inhibit cyclic nucleotide phosphodiesterase and the potency to cause relaxation of coronary artery strips*

The I_{50} of agents to inhibit the hydrolysis of 1 μM cyclic AMP (left panel) and 1 μM cyclic GMP (right panel) by supernatant fractions from pig coronary arteries were plotted against the potency of agents to cause relaxation of pig coronary artery strips. Both were significant positive correlations ($p < 0.05$).

more facile penetration of the plasma membrane and, thus, greater access to the intracellular phosphodiesterase. It is assumed that the phosphodiesterases in the supernatant fraction are the same as, or representative of, the physiologically important enzymes in the intact cell and that assay conditions approximate those at the intracellular site of action. At present there is no strong support for these assumptions. It appears that no proteolytic modification of the supernatant enzymes has taken place[3] and these enzymes appear to be similar to those found in a wide variety of mammalian cell types (2, 8). On the other hand, the particulate phosphodiesterase has not been well characterized and the inhibitory properties of agents have not been tested on this fraction. Although the total activity of this membrane associated phosphodiesterase is low, its relative physiological importance is not known. Under the depolarizing conditions used to induce contractions presumably there is a relatively high calcium concentration in the cell. Under our assay conditions there is maximum activation of the calcium-dependent activator protein-sensitive enzyme but it is not known if this mimics the conditions in the cell or even if the calcium-dependent activator is physiologically important. It has been shown, however, that the agents used have the same inhibitory effects on the activator-sensitive enzyme in the presence or absence of the activator protein (9–11). Thus, while positive correlations between the abilities of phosphodiesterase inhibitors to inhibit cyclic nucleotide hydrolysis and to cause relaxation in this and in other smooth muscle systems (19–23) offer some support for a cause-effect hypothesis, they must not be regarded as proof of such a relationship.

In view of these difficulties a comparison of abilities to raise tissue cyclic nucleotide levels with abilities to cause relaxation would appear to be a more direct indication of the extent of involvement of cyclic AMP or cyclic GMP in the relaxation process. Few such studies have been reported using vascular tissues, and these have given inconsistent results (22, 25–27). The data in Table 2 generally reflect the potencies and selectivities of the agents as cyclic AMP and cyclic GMP phosphodiesterase inhibitors. It was not possible to obtain EC_{50} values for the ability of agents to increase cyclic AMP or cyclic GMP levels because low solubility of the agents did not allow determination of maximum effects. We have, therefore, analyzed the 152 observations of picomoles/mg protein cyclic AMP and cyclic GMP content of muscle strips and accompanying percent relaxation after 5 min of exposure (data not shown) using multiple regression (16). A significant ($p < 0.005$) regression of cyclic AMP and cyclic GMP levels on percent relaxation was found to which both cyclic nucleotides contributed about equally.[4] There is, however, considerable variance in this regression ($r^2 = 0.41$), which may indicate contributions to the regression of percent relaxation by variables other than cyclic AMP and cyclic GMP. In view of the evidence that cyclic AMP levels may be elevated above those necessary to bring about maximum effective activation of the protein kinase through which it apparently acts (28), a linear relationship between cyclic nucleotide levels and percent relaxation is not necessarily expected over the entire range of percent relaxation values. However, in view of the relatively large scatter of the cyclic AMP and cyclic GMP values in this study, attempts to fit a curvilinear regression did not appear to be justified. However, the positive relationship between cyclic nucleotide levels and percent relaxation complements the positive correlations between the ability of the agents to cause relaxation and their ability to inhibit cyclic nucleotide phosphodiesterase activities.

A further indication that both cyclic AMP and cyclic GMP might be independently involved in the relaxation process is the cyclic nucleotide levels after 5 min ex-

[3] T. Michon-Kerevis, J. G. Hardman and J. N. Wells, manuscript in preparation.

[4] The partial regression coefficients obtained were 10.2 for cyclic AMP and 13.6 for cyclic GMP. The standard partial regression coefficients were 0.43 for cyclic AMP and 0.38 for cyclic GMP. An F-test of the partial regression coefficients indicates that both cyclic AMP and cyclic GMP contribute significantly ($p < 0.005$ for both) to the regression.

posure to IIX and 8-*t*-Bu MIX (Table 2). After 5 min of exposure to 10 or 30 μM IIX significant elevation of cyclic AMP but not of cyclic GMP has taken place. On the other hand, after 5 min of exposure to 8-*t*-Bu MIX (10, 30 and 100 μM) cyclic GMP is significantly higher while cyclic AMP is unchanged. It must be noted, however, that the results in Table 2 strongly indicate that in many or perhaps most instances where tissues have been exposed to agents, increases in both cyclic AMP and cyclic GMP levels have occurred which, while not statistically significant, may be real. Presumably the relative changes in cyclic AMP and cyclic GMP observed at 5 min are an indication of the situation at earlier and more important time points where unmeasurable but perhaps important changes may have taken place. Thus, it is most probably the difficulties inherent in measuring cyclic nucleotide levels which prevents us from finding the cyclic nucleotide changes which accompany relaxation in response to theophylline at all time points and concentrations or to other agents, particularly at 1 min and 20 sec.

Interpretations of the data presented here are further complicated since xanthines are known to have effects other than phosphodiesterase inhibition, which could affect muscle contraction. For instance, caffeine has been shown to cause release of Ca^{++} from rabbit aorta segments in a manner that is apparently cyclic nucleotide independent (29), and methylxanthines have been shown to antagonize the effects of adenosine in smooth muscle and other systems (30). In addition, although these agents are all xanthine derivatives (except papaverine), they have differing physical properties resulting from various alkyl substituents that could affect relative abilities to enter the cell or to partition into various intracellular compartments. Such properties could explain, for instance, the discrepancy between the potencies of IIX to inhibit phosphodiesterase and to cause relaxation and elevate cyclic AMP levels. We are, therefore, presently involved in obtaining radiolabeled agents for the purpose of investigating their relative abilities to enter the smooth muscle cell.

Despite the difficulties in interpretation, a consistent pattern emerges from these studies suggesting the involvement of both cyclic AMP and cyclic GMP in the relaxation process of pig coronary arteries. There is a positive correlation between the ability of agents to cause relaxation of coronary artery strips and to inhibit phosphodiesterase activity of tissue extracts. There is a significant positive relationship between cyclic AMP and cyclic GMP levels and accompanying relaxation. There are instances in which relaxation takes place accompanied by elevated cyclic GMP levels but by no significant change in cyclic AMP levels or conversely by elevated cyclic AMP levels but by no significant change in cyclic GMP levels. The most straightforward interpretation of these lines of evidence is that they offer some support for the hypothesis that both cyclic AMP and cyclic GMP have a role in causing relaxation in this tissue but most certainly cannot be regarded as conclusive. Further work is necessary to identify other variables involved in the relaxation processes that are altered by these agents before they may properly be used to elucidate the roles of the cyclic nucleotides in this process. Nevertheless, the value of selective phosphodiesterase inhibitors is demonstrated by the ability of some of these inhibitors to selectively elevate only cyclic GMP or only cyclic AMP levels in pig coronary arteries.

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