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Xanthines and Skeletal Muscle: Lack of Relationship between Phosphodiesterase Inhibition and Increased Twitch Tension in Rat Diaphragms

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SUMMARY

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No relationship was found between the abilities of a series of xanthine derivatives to increase twitch tension of directly stimulated rat hemidiaphragms and their abilities to inhibit hydrolysis *in vitro* of cyclic AMP or cyclic GMP or to change tissue levels of these cyclic nucleotides. The order of potency of the agents to increase twitch tension by 20% did not resemble the order of potency to inhibit either cyclic AMP or cyclic GMP hydrolysis. Further, a wide range of twitch tension responses was observed when agents were applied at concentrations that were five times those required for 50% inhibition of cyclic AMP and cyclic GMP hydrolysis. At concentrations that caused 20% increases in twitch tension, two of the agents were found to cause no significant changes in the tissue levels of cyclic AMP or cyclic GMP while two other agents caused significant increases in levels of both cyclic nucleotides. On the other hand, two agents that caused similar increases in cyclic AMP levels and no significant changes in cyclic GMP levels caused very different tension responses. Thus, under the conditions of this study, cyclic nucleotide metabolism does not appear to be involved in the mechanism of action of xanthines to increase twitch tension of rat diaphragms.

INTRODUCTION

The methylxanthines, caffeine and theophylline, enhance contractions of skeletal muscle. These agents are known to have both presynaptic and postsynaptic effects that may participate in this enhancement. These effects, in turn, have been linked to the ability of methylxanthines to inhibit cyclic nucleotide phosphodiesterases. Breckenridge *et al.* (1) first suggested that facilitation of neuromuscular transmission by theophylline might be related to alteration of cyclic AMP metabolism and this relationship has since been the subject of numerous studies (2-9). On the other hand, the direct action of caffeine and theophylline on muscle fibers as first suggested by Huidobro and Amenbar (10) has most often been explained in terms of an interaction of the xanthine with the sarcoplasmic reticulum or the plasma membrane to mobilize calcium ions [see Bianchi (11) for review]. Recently, however, it has been suggested that changes in cyclic AMP levels in the muscle cell, brought about through phosphodiesterase inhibition by caffeine or theophylline, may be involved in the regulation of metabolic processes that affect the contractile elements (12-14).

The purpose of the present study was to determine if there is a relationship between the abilities of a series of xanthine derivatives to increase twitch tension of rat hemidiaphragm preparations and their abilities to inhibit phosphodiesterase activities from this tissue. Studies were carried out: (1) to identify a series of xanthine derivatives with a wide range of potencies and selectivities for inhibition of rat diaphragm cyclic AMP and cyclic GMP phosphodiesterase activities; (2) to determine the abilities of these compounds to increase twitch tension of directly stimulated rat hemidiaphragm preparations; and (3) to measure changes in cyclic AMP and cyclic GMP tissue levels in response to these agents.

MATERIALS AND METHODS

Materials. Cyclic AMP and cyclic GMP (Sigma) were prepared as stock solutions and used without further purification. Tritiated cyclic nucleotides obtained from New England Nuclear Corporation were purified on Dowex 50 cation exchange resin columns (15). Theophylline was purchased from Mallinckrodt; caffeine from Merck; and *d*-tubocurarine chloride from Boebringer-

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Mannheim. 1-Methyl-3-isobutylxanthine [MIX (1)][1] and 1-methyl-3-isobutyl-8-methoxymethylxanthine [8-methoxymethyl MIX (7)] were prepared by Dr. John E. Garst by previously published methods (16). All other agents were also synthesized by published methods (16, 17).

Tissue preparation. Male Wistar rats (200–300 g) were decapitated, and phrenic nerve–hemidiaphragm preparations were made according to the method of Bülbring (18). The hemidiaphragms were suspended in a 25-ml bath at 37° in Krebs–Ringer bicarbonate buffer with 10 mM glucose and 1 mM pyruvate, bubbled with 95% O_2–5% CO_2 at pH 7.4. The muscles were attached to isometric force transducers (Statham) connected to a Gould–Brush 2400 recorder. The preparations were adjusted to a tension of 1 g and directly stimulated through two platinum wire loops placed in contact with the muscles. The muscles were stimulated with 12 single rectangular pulses per minute of 5-msec duration. In some experiments, the phrenic nerve of the left hemidiaphragm was stimulated through platinum loops with 12 single rectangular pulses per minute of 0.5-msec duration.

For denervation studies, rats were anesthetized with chloral hydrate (0.4 g/kg). The rib cage on the left side was opened and 2–5 mm of the left phrenic nerve was removed. Studies were carried out 24 hr after denervation and responses were compared with those of the innervated right hemidiaphragm of the same rat.

Measurement of changes in twitch tension. The preparations described above were allowed to equilibrate until they attained constant twitch tension in response to electrical stimulation. The xanthine derivative in 50 μl of DMSO was added directly to the bath. The tissue was exposed to the agent for 3 min by which time about 95% of full twitch tension response had been reached. The tissue was then washed twice in succession with the incubation medium and then washed again 5 min later. A total of 15 min after the initial washing was allowed before the next addition of agent. Percentage increase in twitch tension after 3-min exposure is expressed relative to twitch tension just prior to exposure to the agent. The effect on twitch tension of DMSO alone was negligible. Limited water solubility of the agents did not allow determination of maximum responses.

Cyclic AMP and cyclic GMP levels. Both the right and left hemidiaphragms of a rat were prepared and directly stimulated. The preparations were allowed to equilibrate until constant twitch tension in response to electrical stimulation was obtained. One of the hemidiaphragms was exposed to agent in dimethyl sulfoxide (DMSO) as described for measurement of change in twitch tension. After 3 min of exposure, electrical stimulation was stopped, the bath was lowered from the tissue and the tissue was clamped with aluminum blocks that had been cooled in liquid nitrogen. The other hemidiaphragm was exposed to DMSO alone and treated in the same manner. No differences between responses in cyclic nucleotide levels or twitch tension of the right and left hemidiaphragms were found. Right and left hemidiaphragms were used alternately for exposure to a given agent.

Tissue samples were homogenized by placing the frozen sample and a stainless steel ball in a stainless steel capsule (all cooled in liquid nitrogen) and shaking the capsule and its contents in a Wig-L-Bug dental amalgamator (Crescent Dental Mgf. Co.) at maximum speed three 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 −79°. Radiolabeled cyclic AMP and cyclic GMP were added to monitor recoveries. 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 (19). Cyclic AMP and cyclic GMP were assayed by radioimmunoassay (20) 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 27,000*g* for 20 min and the supernatant fluid was assayed for protein by the method of Lowry *et al.* (21).

Enzyme preparation. Male Wistar rats (200–300 g) were decapitated and the diaphragms removed. Nerve and connective tissues were trimmed from the right and left hemidiaphragms. The hemidiaphragms were homogenized in 4 ml/g of a solution containing 20 mM Tris·HCl (pH 7.5), 2 mM $MgCl_2$ and 1 mM dithiothreitol at 4° with an Ultra-Turrax homogenizer (Jahnke and Kunkel, Stanfen, Germany). Supernatant and particulate fractions were obtained by centrifugation of this homogenate for 30 min at 48,000*g* at 0°.

Phosphodiesterase assay. The agents were assayed as inhibitors of the phosphodiesterase activities in the 48,000*g* supernatant fraction. This fraction was found to contain about 80% of the cyclic AMP hydrolytic activity and about 90% of the cyclic GMP hydrolytic activity of the whole homogenate. The assay procedures have been reported (22, 23). Assays were performed with 1 μM substrate at 30° for 30 min at enzyme dilutions that gave 10–20% hydrolysis of substrate in the absence of inhibitor. 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 the assay. Concentrations of the agents that inhibited by 50% the hydrolysis of 1 μM substrate (I_{50}) were determined from concentration–percentage 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 nucleotidase step or subsequent steps in the assay.

RESULTS

Inhibition of phosphodiesterase activities in supernatant fractions. Potencies of the xanthine derivatives

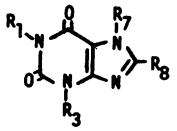
[1] The abbreviations used are: MIX, 1-methyl-3-isobutylxanthine (1); 8-methoxymethyl MIX, 1-methyl-3-isobutyl-8-methoxymethylxanthine (7); DMSO, dimethyl sulfoxide; IIX, 1-isoamyl-3-isobutylxanthine (2).

to inhibit the hydrolysis of cyclic AMP and cyclic GMP by phosphodiesterase activities in the 48,000*g* supernatant fraction from rat diaphragm homogenates are presented in Table 1. A wide range of inhibitory potencies was found. MIX (1) was the most potent inhibitor, and caffeine (9) the least potent inhibitor of cyclic AMP and cyclic GMP hydrolysis. MIX was 175 times as potent as caffeine as an inhibitor of cyclic AMP hydrolysis and 244 times as potent as caffeine as an inhibitor of cyclic GMP hydrolysis. A range of selectivities was also found. 8-Methoxymethyl MIX (7) was 6.5 times more potent as an inhibitor of cyclic GMP than of cyclic AMP hydrolysis. On the other hand, 1-isoamyl-3-isobutylxanthine [IIX (2)], while too insoluble to assay above 50 μM, was greater than 5 times more potent as an inhibitor of cyclic AMP than of cyclic GMP hydrolysis. The agents are listed in Table 1 in order of potency to inhibit cyclic AMP hydrolysis. As a result of the variety of selectivities, this order is different from the order of potency to inhibit cyclic GMP hydrolysis.

Effects on twitch tension. The data reported in the top panel of Table 2 show the effects of xanthines on indirectly (nerve) stimulated hemidiaphragms and on directly stimulated tissues. These experiments were carried out using left hemidiaphragms that were stimulated through the phrenic nerve and right diaphragms from the same animals that were directly stimulated in the presence of a concentration of *d*-tubocurarine chloride (5 μM) that completely blocked twitch responses to nerve stimulation. The percent increases in tension of the nerve-stimulated preparations were not signficantly different from those of the directly stimulated hemidiaphragms for each agent examined. In the lower panel of Table 2, responses of normally innervated right hemidiaphragms are compared with those of 24-hr denervated left hemidiaphragms from the same animals. Both preparations were directly stimulated. Agents were added to both types of preparations in the absence of *d*-tubocurarine chloride, and responses of innervated and denervated tissues to a given agent were essentially the same. The data in the remainder of the studies were obtained using directly stimulated, normally innervated hemidiaphragms in the absence of *d*-tubocurarine chloride.

TABLE 1

Phosphodiesterase inhibition by xanthine derivatives



No.	Agent	I_{50}[a] Cyclic AMP	I_{50}[a] Cyclic GMP
1	1-Methyl-3-isobutylxanthine (MIX)	3.9 ± 0.6	2.1 ± 0.5
2	1-Isoamyl-3-isobutylxanthine (IIX)	10 ± 4	>50 (7.7%)
3	7-(3-Chlorobenzyl) MIX	17 ± 1	10 ± 2
4	7-Cyclopropylmethyl MIX	19 ± 7	42 ± 8
5	7-Benzyl MIX	27 ± 2	20 ± 4
6	7-Phenethyl MIX	74 ± 10	101 ± 9
7	8-Methoxymethyl MIX	124 ± 12	19 ± 1
8	Theophylline	385 ± 41	360 ± 48
9	Caffeine	683 ± 63	513 ± 39

[a] I_{50} value is defined as the concentration (μM) of the agent required to produce 50% inhibition of the hydrolysis of 1 μM substrate by the 48,000*g* supernatant fractions from rat hemidiaphragms. Values are means ± SEM of duplicate determinations with four different preparations. Agent 2 (1-isoamyl-3-isobutylxanthine) was insoluble above 50 μM, therefore percentage inhibition at 50 μM is given in parentheses.

TABLE 2

Effects of d-tubocurarine or denervation on response to xanthines

No.	Agent	Percentage increase in twitch tension: Nerve stimulated[a]	Directly stimulated + *d*-tubocurarine[a]
1	MIX (500 μM)	15.7 ± 2.2	17.3 ± 1.5
6	7-Phenethyl MIX (100 μM)	11.3 ± 4.1	12.3 ± 1.2
7	8-Methoxymethyl MIX (1050 μM)	16.7 ± 3.4	16.3 ± 1.5
9	Caffeine (360 μM)	13.7 ± 2.2	11.7 ± 2.0

No.	Agent	Innervated[b]	Denervated[b]
1	MIX (500 μM)	28, 25	32, 23
5	7-Benzyl MIX (100 μM)	25, 18	19, 15
7	8-Methoxymethyl MIX (1050 μM)	24, 28	24, 24
9	Caffeine (360 μM)	17, 14	14, 10
9	Caffeine (2570 μM)	132, 148	123, 123

[a] Values are means ± SEM of determinations with three different pairs of preparations. Each pair consisted of a left hemidiaphragm that was stimulated electrically through the phrenic nerve (nerve stimulated) and the right hemidiaphragm from the same rat that was directly electrically stimulated and was constantly exposed to 5 μM *d*-tubocurarine. Agents were added to the bath of both hemidiaphragms to give the final concentration shown in parentheses. The percentage increase in twitch tension was recorded after 3 min of exposure to the agent.

[b] Values are determinations with two different pairs of preparations. Each pair consisted of a left hemidiaphragm that had been denervated 24 hr prior to removal (see MATERIALS AND METHODS) and the right hemidiaphragm from the same rat which remained innervated. Agents were added and twitch tension recorded after 3 min as above.

Concentrations (μM) of agents required to cause tension to increase by 20% are given in Table 3. IIX (2) is not included because no change in twitch tension in response to this agent was observed at any concentration up to its solubility limit (50 μM). Agents are listed in the order of potency to increase tension. The 7-substituted MIX derivatives (3, 4, 5, and 6) were the most potent agents and were about equipotent with each other. 8-Methoxymethyl MIX was the least potent agent to increase tension and was about one tenth as potent as the 7-substituted MIX derivatives. Comparison of the data in Table 3 with that in Table 1 reveals no similarity between the orders of potency to increase tension and to inhibit cyclic AMP hydrolysis or cyclic GMP hydrolysis.

As a second approach to relate the abilities of xanthine derivatives to increase tension with inhibition of cyclic nucleotide hydrolysis, the effects on tension of drugs at concentrations 5 times their I_{50} value for inhibition of cyclic AMP and cyclic GMP hydrolysis *in vitro* were measured (Fig. 1). 7-Phenethyl MIX (6) was not included

nor were 5 × I_{50} concentrations for inhibition of cyclic GMP hydrolysis for IIX (2) and 7-cyclopropylmethyl MIX (4) because these agents are not sufficiently water-soluble to give the desired concentrations. No changes in tension could be observed in response to 5 × I_{50} concentrations for inhibition of cyclic AMP hydrolysis with MIX (1) (20 μM) or IIX (2) (50 μM). Similarly, no changes in tension were observed at 5 × I_{50} concentrations for inhibition of cyclic GMP hydrolysis with MIX (1) (10 μM) or 8-methoxymethyl MIX (7) (95 μM). On the other hand, caffeine at both the 5 × I_{50} concentration for cyclic AMP hydrolysis (3415 μM) and for cyclic GMP hydrolysis (2625 μM) caused over 150% increases in twitch tension.

TABLE 3

Concentration of xanthines required to cause 20% increase in twitch tension

No.	Agent	Concentration[a]
		(μM)
6	7-Phenethyl MIX	103 ± 2
3	7-(3-Chlorobenzyl) MIX	107 ± 18
5	7-Benzyl MIX	109 ± 10
4	7-Cyclopropylmethyl MIX	116 ± 8
9	Caffeine	360 ± 51
1	MIX	498 ± 18
8	Theophylline	933 ± 25
7	8-Methoxymethyl MIX	1050 ± 90

[a] Concentration of the agent required to cause a 20% increase in the twitch tension of directly stimulated hemidiaphragm preparations after 3 min of exposure. Values are means ± SEM of determinations made on four different preparations.

Cyclic AMP and cyclic GMP levels. No significant differences in basal cyclic AMP or cyclic GMP levels were found between right and left hemidiaphragm preparations (data not shown). Variations between cyclic nucleotide levels in preparations from different animals were greater than those between right and left hemidiaphragms from the same animals. For this reason, paired studies were conducted. The data in Table 4 are cyclic AMP and cyclic GMP levels in hemidiaphragms exposed to agents (added in DMSO) and hemidiaphragms from the same animals exposed to DMSO only. DMSO caused no change in levels of either cyclic AMP or cyclic GMP.

Cyclic nucleotide levels were measured in response to four agents at concentrations that caused 20% increases in tension in order to determine if similar tension changes in response to different agents were accompanied by similar changes in cyclic nucleotide levels. As shown in Table 4, two of the agents (500 μM MIX and 1050 μM 8-methoxymethyl MIX) caused significant increases in both cyclic AMP and cyclic GMP levels while the other two (360 μM caffeine and 100 μM 7-phenethyl MIX) did not cause changes in levels of either cyclic nucleotide. In addition, cyclic nucleotide levels were measured in response to 20 μM MIX and 3415 μM caffeine which are 5 × I_{50} concentrations of these agents for inhibition of

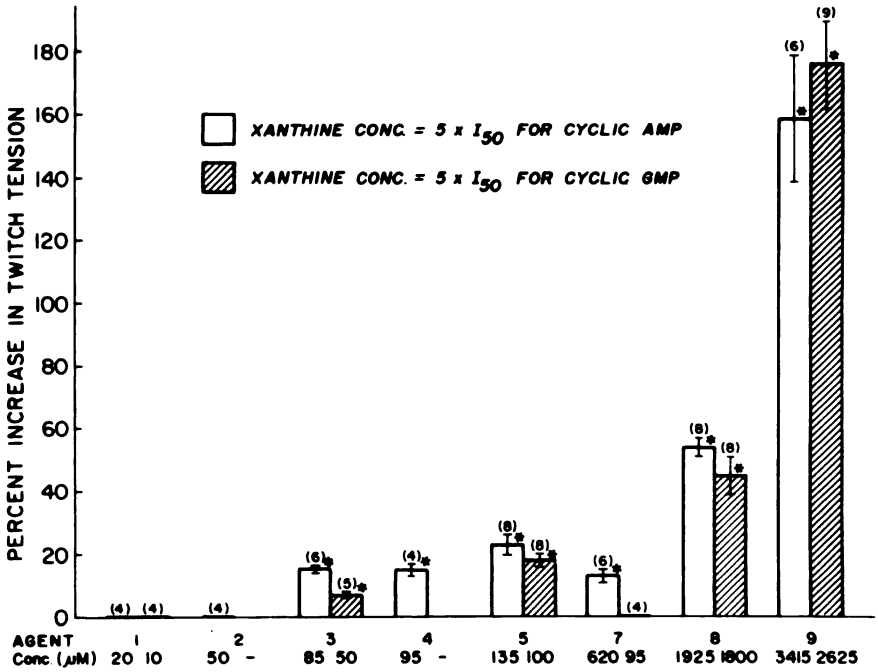


FIG. 1. *Percentage increases in twitch tension in response to agents at 5 × I_{50} concentrations*

Rat hemidiaphragms were directly stimulated and the agent in DMSO was added to the bath to give the final concentration shown. The effect on twitch tension of DMSO alone was negligible. Twitch tension after 3 min of exposure to the agent was measured and expressed as the percentage increase relative to the twitch tension before addition of the agent. Each agent was tested at 5 × I_{50} concentration for inhibition of cyclic AMP hydrolysis (open bars) and at 5 × I_{50} concentration for inhibition of cyclic GMP hydrolysis (shaded bars). I_{50} is defined as the concentration (μM) of the agent required to produce 50% inhibition of the hydrolysis of 1 μM substrate by the 48,000*g* supernatant fraction from rat hemidiaphragms. I_{50} values and agent numbers are given in Table 1. Compound 6 was not tested at either concentration and compounds 2 and 4 were not tested at 5 × I_{50} concentration for inhibition of cyclic GMP hydrolysis because all were too insoluble to give the required concentrations. Bar represents ±SEM of the number of determinations shown in parentheses. Asterisks (*) indicate statistically significant increases in twitch tension ($p < 0.05$).

TABLE 4

Cyclic AMP and cyclic GMP levels after three minutes of exposure to agents

Addition	Cyclic AMP[a]	Cyclic GMP[a]	n
DMSO	2.01 ± 0.20	0.175 ± 0.061	5
None	2.06 ± 0.24	0.212 ± 0.063	
DMSO	2.06 ± 0.08	0.326 ± 0.058	5
MIX (1), 20 μM	4.17 ± 0.66*	0.278 ± 0.038	
DMSO	2.29 ± 0.16	0.160 ± 0.049	5
MIX (1), 500 μM	11.9 ± 2.1*	0.314 ± 0.063*	
DMSO	2.22 ± 0.20	0.199 ± 0.062	5
Caffeine (9), 360 μM	2.14 ± 0.23	0.268 ± 0.164	
DMSO	1.98 ± 0.16	0.195 ± 0.032	5
Caffeine (9), 3415 μM	2.96 ± 0.17*	0.257 ± 0.052	
DMSO	2.03 ± 0.30	0.129 ± 0.042	5
7-Phenethyl MIX (6), 100 μM	2.61 ± 0.21	0.157 ± 0.047	
DMSO	2.31 ± 0.23	0.248 ± 0.090	9
8-Methoxymethyl MIX (7), 1050 μM	5.81 ± 0.95*	0.438 ± 0.108*	

[a] Values are expressed as pmol/mg protein ± SEM of determinations from the number of pairs of hemidiaphragm preparations shown in the column headed by *n*. Each pair consisted of the right and left hemidiaphragms of a rat. These were directly stimulated and one hemidiaphragm was treated with DMSO and the other hemidiaphragm was treated with the agent in DMSO.

* Significantly different from response to DMSO ($p < 0.05$).

cyclic AMP hydrolysis. This was done in order to test the assumption that different agents at the same multiple of their I_{50} concentrations will cause similar changes in tissue cyclic nucleotide levels. The two agents caused significant increases in cyclic AMP levels but no significant changes in cyclic GMP levels. It should be noted that as shown in Fig. 1, these agents at $5 \times I_{50}$ concentrations had very different effects on tension.

In general, increases in cyclic nucleotide levels are dependent upon the presumed extent of phosphodiesterase inhibition in that the largest changes in levels are observed in response to the largest multiples of I_{50} concentrations applied. Cyclic GMP levels, however, are less responsive to large multiples of I_{50} concentrations than are cyclic AMP levels. For instance, 500 μM MIX (1) is 128 times the I_{50} concentration of this agent for inhibition of *in vitro* cyclic AMP hydrolysis and 238 × I_{50} for inhibition of cyclic GMP hydrolysis. Yet, at this concentration, MIX causes a fivefold increase in cyclic AMP but only a twofold increase in cyclic GMP levels.

DISCUSSION

The data reported in this study demonstrate that the ability of xanthine derivatives to increase tension of directly stimulated rat hemidiaphragms is not related to the ability of these agents to inhibit cyclic nucleotide phosphodiesterase activities. The order of abilities to increase tension by 20% (Table 3) does not resemble the order of potency to inhibit cyclic AMP or cyclic GMP hydrolysis (Table 1). In addition the dissociation between phosphodiesterase inhibition and increased tension is demonstrated in Fig. 1. Here a wide range of increases in tension was found in response to agents at $5 \times I_{50}$ concentrations of each compound for inhibition of *in vitro* cyclic AMP hydrolysis and also at $5 \times I_{50}$ for inhibition of *in vitro* cyclic GMP hydrolysis. Presumably, the extent of phosphodiesterase inhibition was the same for all agents under these conditions. Therefore, if there were a relationship between increased twitch tension and phosphodiesterase inhibition, the twitch tension responses to all drugs should have been the same. The dissociation is further confirmed by the observation that in response to xanthines, changes in total tissue cyclic AMP and cyclic GMP levels (Table 4) were independent of changes in tension. At concentrations that caused 20% increases in tension two of the agents [360 μM caffeine (9) and 100 μM 7-phenethyl MIX (6)] caused no significant changes in cyclic nucleotide levels while two other agents [500 μM MIX (1) and 1050 μM 8-methoxymethyl MIX (7)] caused significant increases in levels of cyclic AMP and cyclic GMP. Conversely, concentrations of MIX (1) (20 μM) and caffeine (9) (3415 μM) ($5 \times I_{50}$ values for inhibition of cyclic AMP hydrolysis) that caused similar increases in cyclic AMP levels and no significant changes in cyclic GMP levels had very different effects on tension (Fig. 1). Thus, all approaches to the comparison of the abilities of xanthines to increase tension with their abilities to inhibit cyclic AMP and cyclic GMP hydrolysis revealed no association between these parameters.

These results are in sharp contrast with those found in our study of relaxation of pig coronary arteries by a similar series of xanthine derivatives where a strong association was found between the potencies to cause relaxation and the potencies to inhibit both cyclic AMP and cyclic GMP hydrolysis (24). On the other hand no association could be discerned between increased tension of paced rabbit atrial strips and phosphodiesterase inhibition by a similar series of xanthines (25). There was, however, a strong association between the ability of these xanthines to reduce the 90% relaxation time of these atrial strips and the ability to inhibit atrial cyclic AMP phosphodiesterase activities. Preliminary studies indicate no effect of these agents on 90% relaxation time of the rat diaphragm (data not shown). Thus, while in the cardiac and smooth muscle systems an association between alteration of at least some contractile parameter and phosphodiesterase inhibition by xanthines was found, no such association was found with the rat diaphragm.

The approach in this study has been to compare the relative abilities of a series of xanthine derivatives to inhibit *in vitro* phosphodiesterase activities with their abilities to increase twitch tension in the rat diaphragm. The data in Table 4 are consistent with the idea that the magnitudes of changes in tissue levels of cyclic AMP and cyclic GMP in response to agents are related to the abilities of the agents to inhibit *in vitro* phosphodiesterases. The significance of this observation, however, is dependent upon the assumption that whole tissue cyclic nucleotide levels are indicative of levels at the physiologically important site of action. This assumption cannot

be tested with presently available techniques. The results from this study are not equivocal, however, and appear to lead directly to the conclusion that in the rat hemidiaphragm preparation there is no relationship between the ability of xanthines to inhibit phosphodiesterase and their ability to increase tension.

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