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Selective Phosphodiesterase Inhibition and Alterations of Cardiac Function by Alkylated Xanthines

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SUMMARY

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This study identified a series of alkylated xanthines with a range of potencies and selectivities as inhibitors of cardiac cyclic AMP and cyclic GMP phosphodiesterase activities. The potencies of these xanthines to inhibit phosphodiesterase activities of the rabbit atrial supernatant fraction were compared with the potencies of these agents to alter (a) spontaneous right atrial rate, (b) duration of left atrial contraction, and (c) contractile force of electrically paced left atria. Although the abilities of xanthines to increase heart rate or to shorten the duration of contraction were predictable from cyclic AMP phosphodiesterase inhibition data, abilities of xanthines to alter contractile force were not. No simple correlations were observed between inhibition of cyclic GMP phosphodiesterase in tissue extracts and alterations of any of the atrial functions. Failure to observe correlations in the present study, however, did not stem from a failure of xanthines to inhibit phosphodiesterase of intact cell preparations. Xanthines altered cyclic nucleotide contents of intact, functioning atria as predicted from their abilities to inhibit phosphodiesterase activities of the supernatant fraction. In addition, all xanthines potentiated contractile responses to isoproterenol, suggesting that the xanthines were capable of inhibiting phosphodiesterase activities relevant to contractile function. Thus, the present results, whule pointing to phosphodiesterase inhibition as the mechanism by which xanthines increase heart rate and shorten the duration of contraction, raise questions concerning the importance of phosphodiesterase inhibition in the effects of the xanthines on the force of contraction.

INTRODUCTION

It is well known that xanthines can inhibit cyclic nucleotide phosphodiesterase and can alter cardiac function (1). If xanthines exert their pharmacological effects exclusively by inhibiting the hydrolysis of cyclic AMP, they should be expected to mimic the effects of other interventions that act via cyclic AMP. In many studies, the effects of xanthines on cardiac functions resembled those of *beta*-adrenergic agonists, dibutyryl cyclic AMP, or cyclic AMP itself (e.g., ref. 2). It is clear, however, that xanthines do not produce all of their cardiac effects by impeding the hydrolysis of cyclic AMP (3). In fact, some effects of xanthines, such as myocardial contracture (4) or prolongation of cardiac contraction (3), are opposite to those expected from a cyclic AMP-mediated response.

It has been proposed that cyclic GMP may counteract or modify cardiac effects of cyclic AMP (5). This idea is of obvious concern in a study of xanthines, since they inhibit the hydrolysis of cyclic GMP as well as cyclic AMP. To be sure, xanthines that have been previously studied in heart, such as caffeine, theophylline, or MIX[2] are relatively nonselective as inhibitors of cardiac cyclic GMP and cyclic AMP phosphodiesterase activities (6). Recently, several alkylated xanthines, with a wide range of potencies and selectivities as inhibitors of cyclic AMP and cyclic GMP hydrolysis, have been synthesized (7, 8). Some of these compounds preferentially altered cyclic AMP and cyclic GMP concentrations in pig coronary artery strips (9) and in rat hemidiaphrams (10). In the former study, a significant positive correlation was observed between relaxation of the smooth muscle strips and cyclic nucleotide phosphodiesterase inhibition,

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[2] The abbreviations used are: MIX, 1-methyl-3-isobutylxanthine; 8-methoxymethyl MIX, 1-methyl-3-isobutyl-8-methoxymethylxanthine; 8-methyl MIX, 1-methyl-3-isobutyl-8-methylxanthine; 8-*t*-butyl MIX, 1-methyl-3-isobutyl-8-*t*-butylxanthine; DF, developed force; 90% RT, 90% relaxation time; max *dF/dt*, maximal rate of force development; SA, sinoatrial.

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whereas in the latter study, no relationship was observed between contraction of the skeletal muscle preparation and phosphodiesterase inhibition.

The present study utilized a series of alkylated xanthines, with a wide range of potencies and selectivities as inhibitors of cyclic AMP or cyclic GMP hydrolysis, to determine if changes in cardiac muscle function *in vitro* correlated with inhibition of cyclic AMP or cyclic GMP phosphodiesterase.

MATERIALS AND METHODS

Animals. New Zealand White rabbits of either sex, weighing 2–3 kg (4–6 months old), were supplied by Hilltop Rabbit Ranch (Columbia, Tenn.).

Materials. Drugs purchased for use in this study included isoproterenol (Sterling-Winthrop Research Laboratories, Rensselaer, N. Y.), propranolol (Ayerst Laboratories New York, N. Y.), caffeine (Merck and Company, Inc., Rahway, N. J.), and theophylline (Mallinckrodt Inc., St. Louis, Mo.). The synthetic xanthines, MIX, 8-methoxymethyl MIX, 8-methyl MIX, and 8-*t*-butyl MIX, were prepared by the methods of Kramer *et al.* (8).

Cyclic AMP and cyclic GMP (Sigma Chemical Company, St. Louis, Mo.) were used without further purification, but tritiated cyclic nucelotides (New England Nuclear Corporation, Boston, Mass.) were purified on Dowex-50 cation exchange resins (11), prior to use.

Cardiac mechanical studies. Rabbits were stunned by a blow to the neck and the heart was removed rapidly and rinsed with oxygenated Krebs-bicarbonate buffer, pH 7.4, consisting of (millimolar) NaCl, 127; KCl, 2.3; KH_2PO_4, 1.3; $MgSO_4$, 0.61; $CaCl_2$, 2.5; $NaHCO_3$, 25; and glucose, 5.6. Buffer (20–40 ml) was flushed through the heart, until both the effluent and tissue appeared free of blood. The left atrium was divided into two strips, each approximately 4 × 13 mm. Atrial preparations were suspended to contract isometrically in a constant temperature bath (30°) containing Krebs-bicarbonate buffer that was aerated with 95% O_2-5% CO_2. Each left atrial strip was stimulated through punctate electrodes, 30 times per min, by square wave pulses (3 msec in duration) that were 10% above the threshold voltage. Right atria were allowed to beat spontaneously. Both left and right atrial preparations were stretched until the resting force was 0.5 *g*. A higher resting force (1 *g*), however, was used in freeze-clamp studies (see below).

Contractile force was measured with a Statham isometric force transducer (Model UC2) and recorded on a Gould oscillographic recorder. Values for the measured variables were determined from high speed oscillographic tracings (chart speed, 100 mm/sec) and represent the averages of data obtained from three successive contractions. The following left atrial parameters were monitored: peak DF, duration of contraction (approximated as the time interval from the onset of contraction until DF decreased by 90%; 90% RT), and the maximal rate of force development (max dF/dt). Right atrial rate was determined with a Biotach preamplifier or by counting the number of spontaneous contractions in a 30-sec period. Atrial preparations were allowed to equilibrate for 2–3 hr. Baths were then washed three times in succession. Left atrial strips were discarded if DF changed by more than 10% within a 30-min period following the final wash. Right atria were not used if their spontaneous rate varied by more than 10% over the 1-hr period preceding the experiment. Immediately before each experiment, drugs were dissolved in Krebs-bicarbonate buffer. To study the concentration-response relationship of each atrial preparation, cumulative concentrations of a test drug were added to the muscle bath (complete concentration-response relationships could not be obtained with some xanthines because of their low solubilities).

Atrial cyclic AMP and cyclic GMP concentrations. After equilibrating left atrial strips for 2–3 hr, the muscle bath was emptied and refilled with 25 ml of the appropriate xanthine solution. (In some experiments, microliter volumes of isoproterenol were added to the bath 12 min later.) After 15 min of exposure to the xanthine, the muscle was clamped with Wollenberger tongs which had been cooled in liquid nitrogen. The frozen tissue samples were pulverized as described by Kramer and Wells (9). The pulverized tissue was suspended in 4 ml of ethanol (−79°) containing 0.1 M perchloric acid and tracer amounts of tritiated cyclic AMP and cyclic GMP. The suspension was centrifuged (28,000 × *g*) at 2° and the cyclic nucleotides in the supernatant fraction were separated and purified by the methods of Schultz *et al.* (12). The protein that was solubilized by heating the pellet at 100° for 30 min in 1 N NaOH was determined by the method of Lowry *et al.* (13).

Cyclic nucleotide concentrations were determined by radioimmunoassay (14) and each value was adjusted to account for losses during homogenization and purification steps. No cyclic AMP or cyclic GMP could be detected by radioimmunoassay when atrial extracts of control (buffer)- or xanthine-treated preparations were incubated with phosphodiesterase prior to assay.

Phosphodiesterase assay. Both right and left atria were removed from the rabbit heart and washed by immersion in ice-cold homogenization buffer (20 mM Tris, pH 7.5; 2 mM magnesium acetate; and 1 mM dithiothreitol). Four to six atria were pooled, blotted on filter paper, and minced with scissors. Buffer (14 ml/g wet weight) was added to the mince. The tissue was homogenized (4°) for three 25-sec periods using a precooled Ultra-Turrax homogenizer (Jahnke and Kunkel, Staufen, Germany). The homogenate was centrifuged (0°) at 48,000 × *g* for 30 min. Phosphodiesterase activities of the supernatant and particulate fractions were assayed by the method of Keravis *et al.* (15) using 1 μM substrate concentrations. The assays were run at dilutions of the enzymes that hydrolyzed 10–20% of the substrate in the absence of inhibitors. Phosphodiesterase activities were linear for at least 30 min and were proportional to the enzyme concentration. The abilities of xanthines to inhibit phosphodiesterase activities were assessed in the 48,000 × *g* supernatant fraction, since this fraction contained more than 90% of the cyclic AMP or GMP hydrolytic activity of the whole homogenate. The concentration of each xanthine that inhibited the hydrolysis of 1 μM cyclic nucleotide by 50% (I_{50}) was determined by interpolation of concentration-percentage inhibition curves (3–5 points per curve). Since these curves were parallel for all agents studied, the relative potencies of

xanthines as phosphodiesterase inhibitors were determined by comparing I_{50} values. None of the xanthines altered the efficacy of the nucleotidase reaction or subsequent steps in the assay.

Statistical analyses. Treatments were assigned to atrial strips according to either a completely randomized design or a Latin square design. One-way analysis of variance was used to compare means among treatment groups. Homogeniety of variance was determined by using Cochran's *C*-test, and heteroscedasticity, when observed, was eliminated by logarithmic transformation of data prior to analysis. Duncan's new multiple range test (groups of equal size) or the Student-Newman-Keals procedure (unequal sample sizes) were used to statistically compare treatment group means. Multiple regression analysis was used to assess the relationship of potency to inhibit phosphodiesterase and potency to alter spontaneous atrial rate. A 2 × 2 factorial analysis was used to test for interactions between the effects of xanthines in the presence and absence of isoproterenol. The 5% level was chosen for statistical significance in all experiments.

RESULTS

Inhibition of phosphodiesterase activities of the atrial supernatant fraction. The most potent inhibitor of cyclic AMP hydrolysis was MIX, which has an I_{50} of 8 μM (Table 1). This xanthine was 100-fold more potent than caffeine, the least potent inhibitor in the series. The 8-substituted derivatives of MIX (8-methyl MIX, 8-methoxymethyl MIX, and 8-*t*-butyl MIX) had intermediate potencies as inhibitors of cyclic AMP phosphodiesterase. However, 8-methyl MIX and 8-*t*-butyl MIX were the most potent inhibitors of cyclic GMP phosphodiesterase, having I_{50} values of 2 μM. These MIX derivatives were 100-fold more potent than theophylline and almost 500-fold more potent than caffeine as inhibitors of cyclic GMP phosphodiesterase activity. The 8-substituted MIX derivatives were 10–16 times more potent as inhibitors of cyclic GMP phosphodiesterase than as inhibitors of cyclic AMP phosphodiesterase. In contrast, the xanthines employed most frequently in previous cardiac studies, i.e., caffeine, theophylline, and MIX, were relatively nonselective in terms of their abilities to inhibit cyclic AMP and cyclic GMP hydrolysis.

Effects of spontaneous right atrial rate. Isolated right atria beat spontaneously at rates of 100–150 beats/min in the absence of drugs. All xanthines studied increased heart rate (Fig. 1), and some were as effective in this regard as a maximally effective concentration of isoproterenol (increased rate by 80 ± 17 beats/min, $N = 4$). The xanthines studied had widely varying potencies as positive chronotropic agents. Some of the xanthines also exerted negative chronotropic effects at high concentrations (Fig. 1). With regard to increasing heart rate, MIX was the most potent of the xanthines studied, being about 50 times more potent than caffeine as a positive chronotropic agent. The selective inhibitors of cyclic GMP phosphodiesterase activity, 8-methyl MIX, 8-*t*-butyl MIX, and 8-methoxymethyl MIX, had intermediate potencies as positive chronotropic agents. Xanthines, at concentrations that inhibited cyclic AMP phosphodiesterase by 50% in tissue extracts, produced average increases in spontaneous atrial rate that ranged from 22–33 beats/min (Fig. 2). The increases in rates elicited by these agents were not statistically different, despite vast differences in amounts of xanthines present (compare 8 μM MIX with 733 μM caffeine) in the various treatment groups.

Furthermore, comparable increases in heart rate were elicited by concentrations of the xanthines that inhibited the hydrolysis of 1 μM cyclic AMP by 50% despite the fact that some of the agents were 10–16 times more potent than others as inhibitors of *in vitro* cyclic GMP phosphodiesterase activity (Fig. 2). At concentrations of xanthines that produced a 50% inhibition of GMP phosphodiesterase activity, mean increases in rate ranged from 5 beats/min (8-methoxymethyl MIX) to 46 beats/min (caffeine) and were statistically different ($p < 0.001$). Data were analyzed by multiple linear regression using a plot of the log concentration of xanthine that increased spontaneous atrial rate by 30 beats/min versus log I_{50} cyclic AMP and log I_{50} cyclic GMP concentrations. A significant regression was observed between potencies of xanthines as positive chronotropic agents and their potencies as inhibitors of cyclic AMP phosphodiesterase activity ($r = 0.998, p < 0.05$). The standard partial regression coefficients for cyclic AMP and cyclic GMP (0.96 versus 0.04) indicated that potencies of xanthines as inhibitors of cyclic AMP phosphodiesterase contributed

TABLE 1

Inhibition of phosphodiesterase activity of the rabbit atrial supernatant fraction

Compound	I_{50}[a]		Selectivity[b] (I_{50} cyclic AMP/I_{50} cyclic GMP)
	Cyclic AMP	Cyclic GMP	
	μM		
MIX	8.3 ± 0.7	6.8 ± 0.6	1.2
8-Methyl MIX	25.3 ± 5.1	2.4 ± 0.1	10.4
8-*t*-Butyl MIX	25.9 ± 0.6	2.1 ± 0.1	12.2
8-Methoxymethyl MIX	135.0 ± 20.2	8.5 ± 0.9	16.2
Theophylline	236.7 ± 8.8	225.0 ± 22.9	1.0
Caffeine	733.3 ± 52.1	963.0 ± 35.0	0.8

[a] I_{50} = the concentration (micromolar) of the agent required to inhibit by 50% the hydrolysis of cyclic AMP (1 μM) or cyclic GMP (1 μM). Values are the mean ± standard error of the mean from three preparations. Atria (four to six) were pooled for each preparation, and each preparation was assayed in triplicate.

[b] Selectivities are the values determined by dividing the mean value of I_{50} cyclic AMP by the mean value of I_{50} cyclic GMP for a given drug in three preparations.

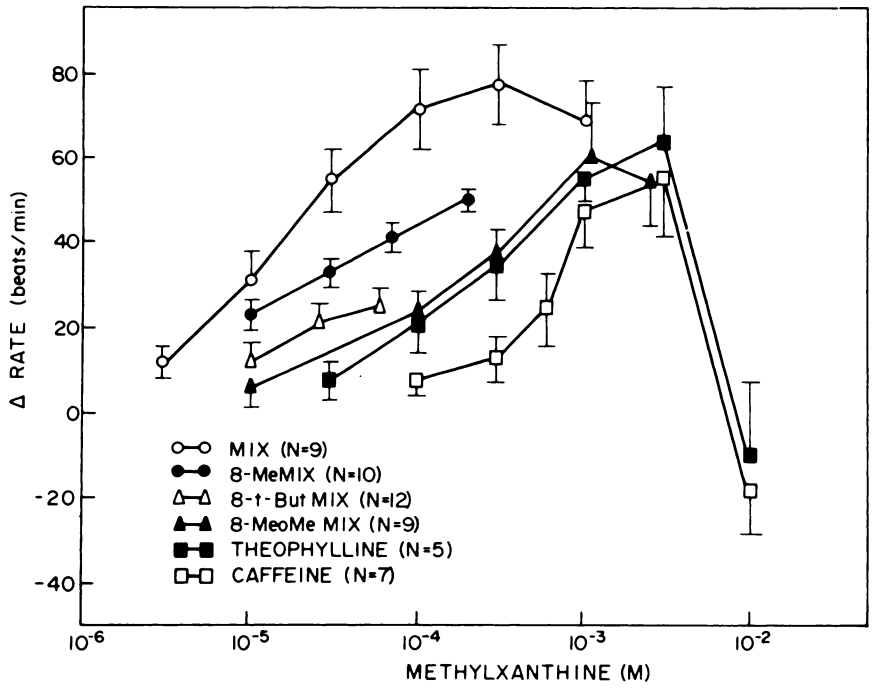

FIG. 1. *Concentration-related chronotropic effects of xanthines in right atria isolated from rabbits*

Mean spontaneous atrial rates ranged from 124–139 beats/min prior to addition of xanthines and did not differ statistically among treatment groups. Cumulative concentration-response curves were performed; the xanthine concentration in the bath was increased after the response to the previous concentration had stabilized (10–20 min). *Symbols* show mean changes from predrug spontaneous rates at various xanthine concentrations. *Vertical bars* show standard error of the mean, *N* = number of experiments. *8-MeMIX*, 8-methyl MIX; *8-t-But MIX*, 8-*t*-butyl MIX; *8-MeoMe MIX*, 8-methoxymethyl MIX.

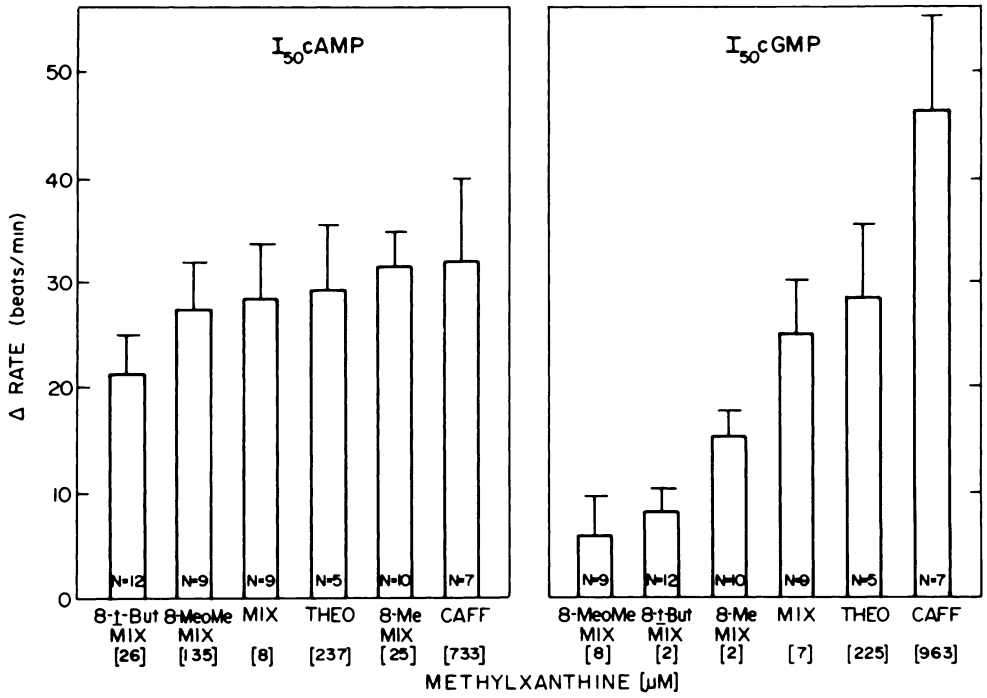

FIG. 2. *Chronotropic effects of xanthines in isolated, spontaneously beating, right atria from rabbits*

The xanthines were present at concentrations equal to those concentrations producing 50% inhibition of the hydrolysis of 1 μM substrate [cyclic AMP (*cAMP*) or cyclic GMP (*cGMP*)] by the 48,000 × *g* supernatant fraction of rabbit atria. The concentrations of xanthines are shown in *brackets*. *Bar heights* indicate mean changes in spontaneous atrial rates. *Vertical extensions on bars* show standard error of the mean. *N* = number of experiments. *8-t-But MIX*, 8-*t*-butyl MIX; *8-MeoMe MIX*, 8-methoxymethyl MIX; *THEO*, theophylline; *8-Me MIX*, 8-methyl MIX; *CAFF*, caffeine.

at least 20 times more to the regression than did their potencies as inhibitors of cyclic GMP phosphodiesterase activity; potency to inhibit cyclic GMP phosphodiesterase did not contribute significantly to the regression ($p < 0.75$).

Effects on the duration of contraction. It was difficult to accurately measure the total duration of left atrial contraction because the descending portion of the contraction curve asymptotically approached resting force on the time axis. Instead, 90% relaxation times (90% RT) were measured. In general, potencies of the xanthines to reduce the 90% RT paralleled the potencies of these agents as inhibitors of cyclic AMP phosphodiesterase activity. The most potent inhibitor of cyclic AMP phosphodiesterase, MIX, was the most potent at shortening the contractile duration, producing half of its maximal effect (EC_{50}) at 23 μM (Fig. 3). MIX was also the most efficacious xanthine in this respect, decreasing the 90% RT by 50 ± 5 msec at 300 μM. The second most potent inhibitor of cyclic AMP phosphodiesterase activity, 8-methyl MIX, was also the second most efficacious in shortening the contraction. This agent, near its solubility limit (300 μM), lowered the 90% RT by 33 ± 3 msec. Neither 8-methoxymethyl MIX (near its solubility limit, 2.5 mM) nor theophylline (1 mM) lowered the 90% RT by more than 10% of the control value, but the effect of each agent was nevertheless statistically significant ($p < 0.05$). Although 8-*t*-butyl MIX (50–100 μM) decreased the 90% RT, these decreases could not be quantitated because this agent, unlike the others, occasionally depressed DF to an extent that precluded accurate measurements of changes in 90% RT. At concentrations exceeding 3 mM, caffeine and theophylline each markedly increased the duration of the left atrial contraction. It is uncertain whether the other xanthines possess this capability, since they are not sufficiently soluble to be tested at millimolar concentrations.

Effects of xanthines on DF. Preliminary observations suggested that the magnitude of contractile responses to inotropic agents was greater when basal contractile forces were relatively low. Thus, atrial strips (loaded with 500 mg of force) were paced electrically at 30 beats/min (a frequency that produced relatively low basal contractile forces). Mean DF of treatment groups ranged from 126–179 mg, and mean max dF/dt ranged from 3.3–4.6 g/sec prior to addition of drugs. Xanthine-induced changes in

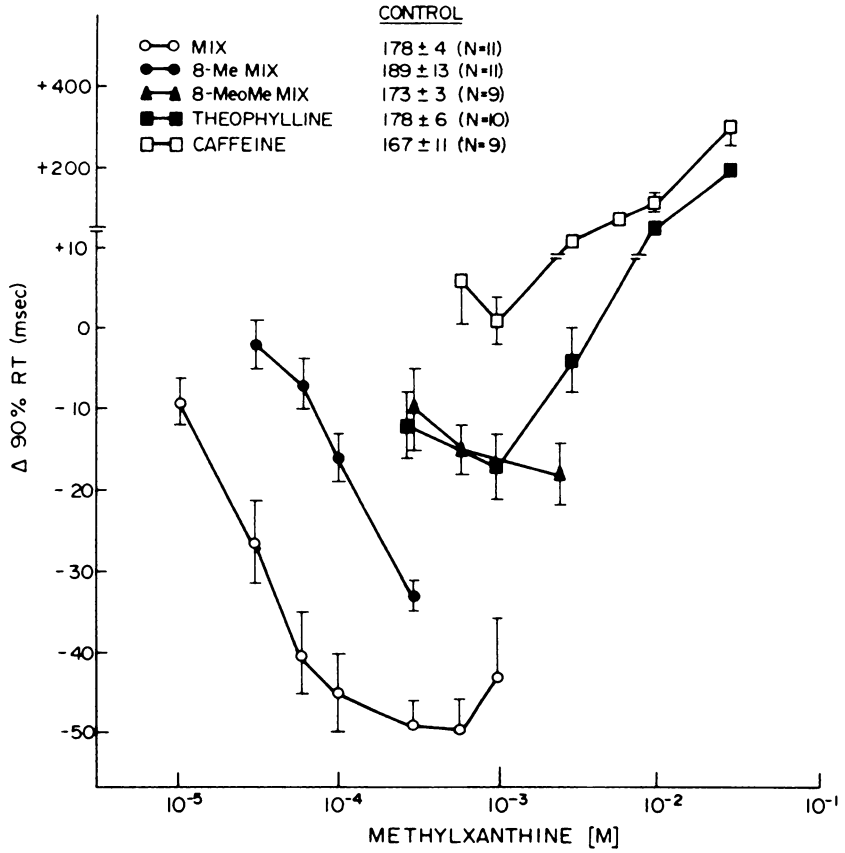

FIG. 3. *Concentration-related effects of xanthines on the duration of the left atrial contraction*

Left atrial strips of rabbits, loaded with 500 mg of force, were stimulated electrically at 0.5 Hz with a square wave pulse 3 msec in duration at 10% above threshold voltage. An approximation of the contractile duration, the 90% RT, was determined by measuring the time elapsed from the onset of contraction until peak DF declined by 90% of its maximal value for that contraction. Control gives mean 90% RT in milliseconds prior to addition of xanthines. These control values ranged from 167–189 msec. Cumulative concentration-response curves were performed; xanthines were added to give successively higher concentrations when the inotropic response (DF) to the previous concentration had stabilized (usually 10–20 min). *Symbols* show mean changes from the control 90% RT. *Vertical bars* show standard error of the mean. *N* = number of left atrial strips. Note the discontinuity of the ordinate at +15 msec. *8-Me MIX*, 8-methyl MIX; *8-MeoMe MIX*, 8-methoxymethyl MIX.

DF (top panel, Fig. 4) paralleled changes in max *dF/dt* (bottom panel, Fig. 4). Of the six xanthines studied, four significantly increased DF ($p < 0.001$). Under the conditions of this study, no xanthines increased DF to the extent observed with either 10 μM isoproterenol (1684 ± 182 mg, $N = 8$) or 100 μM tyramine (1556 ± 625 mg, $N = 2$).

The most potent inhibitor of atrial cyclic AMP phosphodiesterase, MIX, was also the most potent and most efficacious xanthine as a positive inotropic agent. The second and third most potent inhibitors of atrial cyclic AMP phosphodiesterase, 8-*t*-butyl MIX and 8-methyl MIX, failed to significantly increase contractile force at concentrations that exceeded 4 and 10 times, respectively, the concentrations required to inhibit by 50% the hydrolysis of 1 μM cyclic AMP. In fact, 8-*t*-butyl MIX (77 μM) decreased DF by 24 ± 9% of the control DF ($p <$ 0.05; see accompanying paper, ref. 16). The fourth most potent inhibitor of cyclic AMP phosphodiesterase activity, 8-methoxymethyl MIX, did not increase DF by more than 100 mg at a concentration that was 7-fold above the concentration required to inhibit by 50% the hydrolysis of 1 μM cyclic AMP. On the other hand, the least potent inhibitors of cyclic AMP phosphodiesterase, theophylline and caffeine, increased DF by more than 100 mg at concentrations only 3 times those required to inhibit by 50% the hydrolysis of 1 μM cyclic AMP.

Inotropic responses to the xanthines were compared at concentrations that were 3 times those required to inhibit by 50 % the hydrolysis of 1 μM cyclic AMP by the the tissue extracts (Fig. 5). Increases in DF by MIX (25 μM), caffeine (2200 μM), and theophylline (710 μM) were not significantly different. However, inotropic responses to theophylline or caffeine exceeded those to any of the 8-

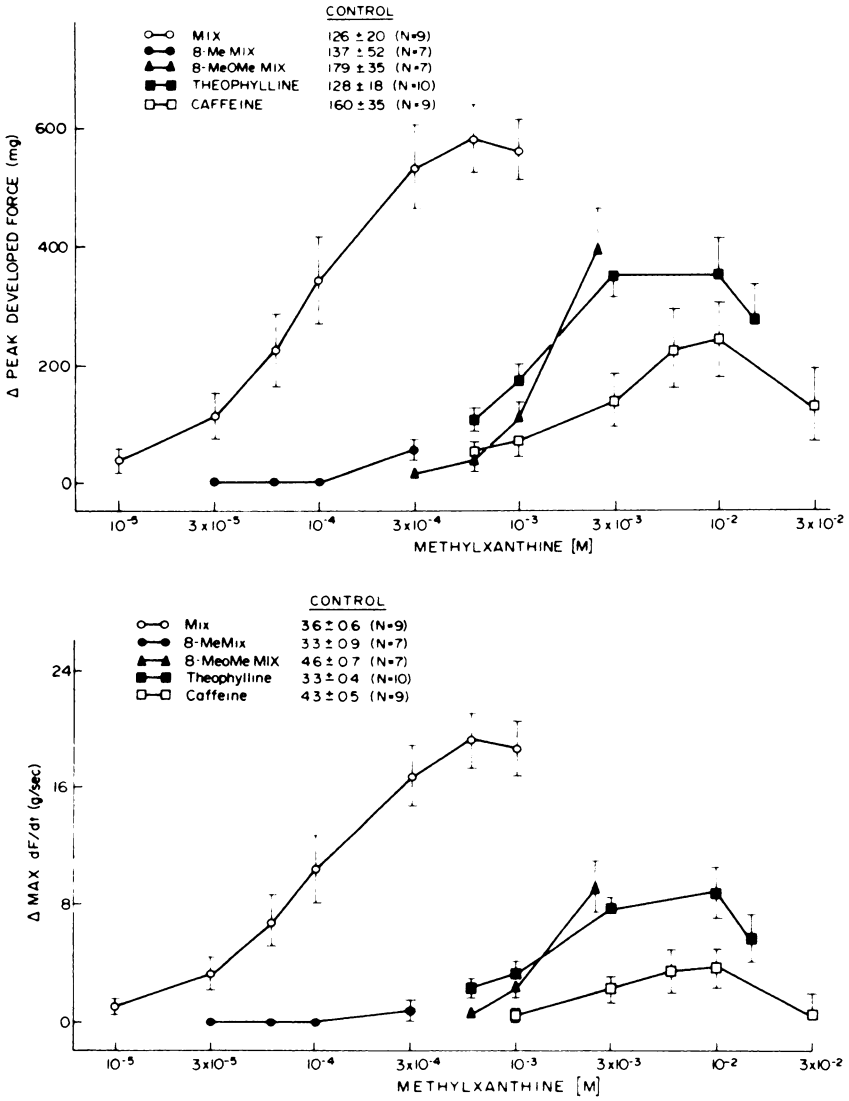

FIG. 4. *Concentration-related effects of xanthines on peak developed force and on dF/dt of isolated atrial strips of rabbits*

Atrial strips were treated as described in the legend to Fig. 3. Control shows mean peak DF (*top panel*) or mean max *dF/dt* (*bottom panel*) for treatment groups prior to addition of xanthines. *Symbols* show mean changes in DF (*top panel*) or mean changes in max *dF/dt* (*bottom panel*) at various xanthine concentrations. *Vertical bars* show standard error of the mean. N = number of atrial strips. *8-Me MIX*, 8-methyl MIX; *8-MeoMe MIX*, 8-methoxymethyl MIX.

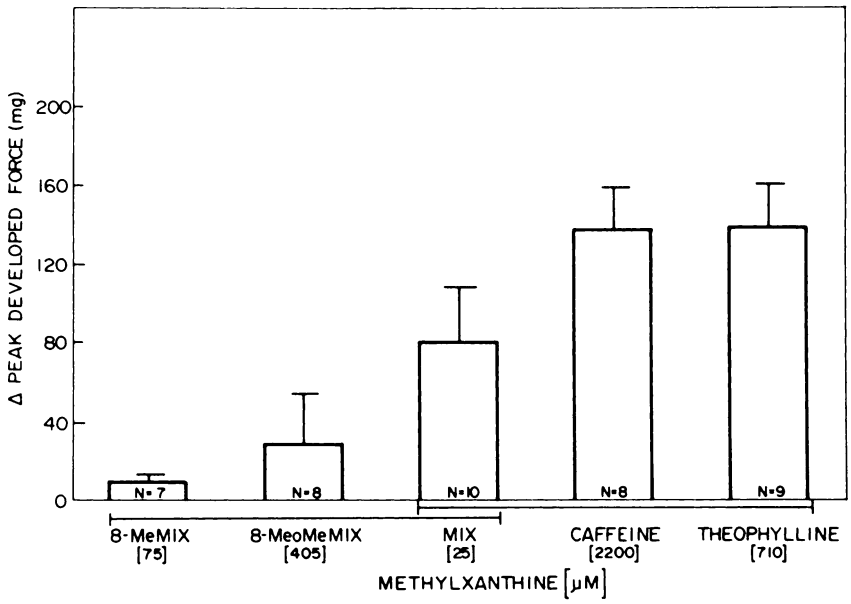

FIG. 5. *Changes of peak developed force in left atrial strips treated with xanthines at concentrations that produce equivalent inhibition of in vitro cyclic AMP hydrolysis*

Xanthine concentrations (3 times those required to inhibit by 50% the hydrolysis of 1 μM cyclic AMP by a tissue extract) are shown in *brackets*. *Bar heights* indicate mean changes in peak DF as determined by interpolation of concentration-response data in Fig. 4. *Vertical extensions on bars* show standard error of the mean. N = number of experiments. One-way analysis of variance was used to test for a difference among treatment group means. Means were then contrasted using Duncan's New Multiple Range Test. *Bars* not underscored by a common line were statistically different from one another ($p < 0.05$). Mean increases in DF with theophylline or caffeine, for example, were significantly greater than mean increases in developed force with 8-methyl MIX (*8-MeMIX*) or 8-methoxymethyl MIX (*8-MeoMe MIX*).

substituted MIX derivatives. It should be noted that the positive inotropic effects of xanthines (MIX, theophylline, and caffeine) may be attenuated at concentrations exceeding those producing ostensibly maximal inotropic effects.[3]

Effects of xanthines on contractile responses to isoproterenol. As in the previous experiments (Fig. 5), MIX (25 μM) and theophylline (170 μM) significantly increased DF (paried t-test; $p < 0.05$), whereas 8-methoxymethyl MIX (405 μM) and 8-methyl MIX (75 μM) failed to exert significant positive inotropic effects (data not shown). When the data were analyzed by making unpaired comparison, mean developed forces of groups treated with xanthines for 40 min did not differ statistically from the control groups. After a 40-min incubation in the presence or absence of xanthines, cumulative concentration-response curves to isoproterenol were generated. None of the xanthines altered the maximal positive inotropic response to isoproterenol, but each xanthine produced a significant leftward shift of the concentration-response curve to isoproterenol ($p < 0.05$; Fig. 6). The xanthines reduced the concentrations of isoproterenol required to produce half-maximal effects (EC_{50}) by 3- to 30-fold.

Effects of xanthines on atrial cyclic AMP and cyclic GMP concentrations. Preliminary experiments were conducted using MIX as a prototype xanthine. This xanthine produced time- and concentration-related increases in atrial cyclic nucleotide contents (data not shown). Both cyclic AMP and cyclic GMP responses (more than 300% of control values) appeared to be maximal after a 15-min exposure to MIX (600 μM) (i.e., no further increases were observed with MIX up to 40 min). Thus, effects of xanthines on atrial cyclic nucleotide contents were determined in strips freeze-clamped 15 min after exposure to the xanthine.

Because it has been suggested that methylxanthines may alter cardiac mechanical function (17, 18) or increase myocardial cyclic AMP contents (19) by releasing endogenous catecholamines, studies were conducted to determine whether the *beta*-adrenergic blocker, propranolol, would attenuate the effects of MIX on atrial cyclic nucleotide contents, DF, or 90% RT. After a 20-min exposure to propranolol (5 μM) or buffer, MIX (83 μM) or buffer was added to the baths; the strips were freeze-clamped 15 min later. Propranolol, by itself, produced a slight depression of contractile force without altering the duration of contraction or cyclic nucleotide contents of atrial strips (Table 2). More importantly, 5 μM propranolol failed to alter the effects of MIX on cyclic nucleotide contents, contractile force, or the duration of contraction. This same concentration of propranolol, however, completely blocked equivalent contractile effects of isoproterenol or tyramine (data not shown).

Thus, in subsequent experiments, effects of xanthines on cyclic nucleotide concentrations were determined in the absence of propranolol after strips had been exposed to a xanthine for 15 min. These experiments were conducted at a higher stimulation frequency (45 beats/min) to provide basal DF that were 75–100% greater than in earlier experiments. This was done to rule out the possibility that the failure of some xanthines to exert significant positive inotropic effects was related to the relatively depressed inotropic state of atria stimulated at 30

[3] P. Mushlin, R. C. Boerth, and J. N. Wells, unpublished observations.

TREATMENT GROUPS	N	PRE-ISO DF (mg $\bar{x}$ ± SEM)	MAX RESPONSE TO ISO ΔDF (mg, $\bar{x}$ ± SEM)
▽—▽ BUFFER + ISO	8	314 ± 79	1684 ± 182
●—● 8-Me MIX (75μM) + ISO	7	225 ± 82	2152 ± 217 #
▲—▲ 8-MeoMe MIX (405μM) + ISO	7	214 ± 41	1764 ± 153
■—■ Theophylline (710μM) + ISO	5	494 ± 130	1610 ± 119
○—○ MIX (25μM) + ISO	7	413 ± 140	1297 ± 115 #

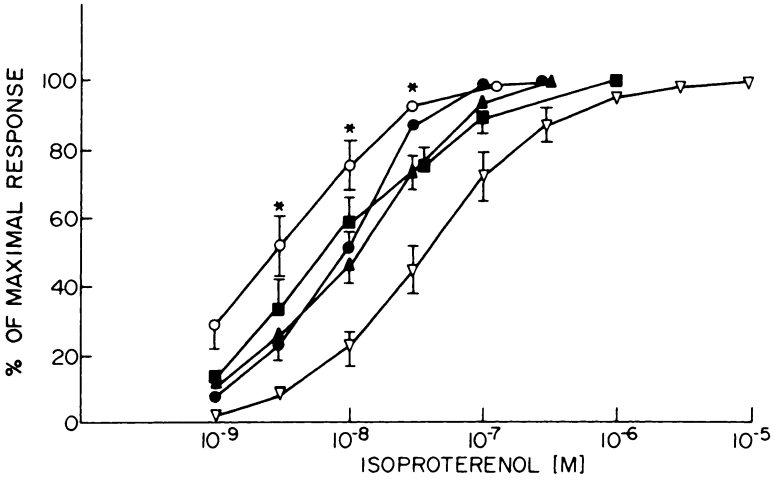

FIG. 6. *Potentiation of positive inotropic responses to isoproterenol (ISO) by xanthines*

Xanthines were present at concentrations that were 3 times those required to inhibit by 50% the hydrolysis of 1 μM cyclic AMP by tissue extracts. Left atrial strips, electrically paced at 30 beats/min and loaded with 500 mg of force, were randomly assigned to one of the five treatment groups shown above. A xanthine, (concentrations are given in parentheses), or buffer was added to the bath 40 min prior to addition of ISO. Pre-ISO DF gives mean peak developed force ± standard error of the mean immediately before adding ISO. The ISO concentration was successively increased after the inotropic response to the previous concentration had stabilized (between 1–8 min). Symbols show percentages of the maximal inotropic response in each treatment group at various ISO concentrations. *Vertical bars* show standard error of the mean. N = number of experiments. One-way analysis of variance and the Student-Newman-Keuls procedure indicated that contractile response to ISO (3 10, or 30 nM) was enhanced significantly by the presence of any of the xanthines (* = $p < 0.05$). These statistical procedures also indicated that the maximal inotropic response to ISO + 8-methyl MIX (*8-Me MIX*) (as DF in milligrams) was significantly greater than that of ISO + MIX (# = $p < 0.05$). *8-MeoMe MIX*, 8-methyoxymethyl MIX.

beats/min. Atria did appear more responsive to positive inotropic stimuli under the conditions of cyclic nucleotide content studies, but the relative potencies of xanthines as contractile agents were unaltered (data not shown).

Each xanthine was used at 3 or 10 times the concentration that inhibited the cyclic AMP phosphodiesterase activity of atrial extracts by 50%. In general, these xanthines produced concentration-related increases of both cyclic AMP or cyclic GMP contents of atrial strips (Table 3, compare values from Experiments 1 and 2). At the lower concentrations (Experiment 1), only 8-methyl MIX significantly increased the atrial cyclic AMP content ($p < 0.05$); however, all xanthines except theophylline ($p < 0.10$) significantly increased the cyclic GMP content

TABLE 2

Failure of propranolol to alter effects of MIX on peak developed force, duration of contraction, cyclic AMP content, or cyclic GMP content of atrial strips[a]

Treatment	Atrial cyclic nucleotide content		Peak DF[b]	90% RT[c]
	Cyclic AMP	Cyclic GMP		
	pmoles/mg protein		*% of control*	
None	8.0 ± 1.2 (8)	0.8 ± 0.1 (8)	100	100
Propranolol	8.0 ± 1.0 (8)	0.9 ± 0.1 (8)	85 ± 4 (7)	100 ± 3 (6)
MIX	15.2 ± 1.1 (8)[d]	1.8 ± 0.3 (8)[e]	230 ± 59 (4)[e]	83 ± 2 (5)[d]
Propranolol + MIX	16.0 ± 1.6 (8)[d]	1.9 ± 0.3 (8)[e]	230 ± 27 (6)[d]	80 ± 2 (7)[d]

[a] Cyclic nucleotide contents, changes in peak DF and changes in 90% RT were determined after a 20-min exposure to propranolol (5 μM) or buffer followed by a 15-min exposure to MIX (80 μM) or buffer. Table values are mean values ± standard error of the mean of the number of experiments in *parentheses*.

[b] Control indicates values of mechanical variables prior to treatment.

[c] 90% RT was determined by measuring the time elapsed from the onset of contraction until peak DF declined by 90% of its maximal value for that contraction.

[d] $p < 0.001$ as compared with basal values or values in the presence of propranolol alone.

[e] $p < 0.01$.

TABLE 3

Concentration-related effects of xanthines on cyclic AMP and cyclic GMP contents of atrial strips[a]

Agent	Experiment 1				Experiment 2			
	Concentration[b]	N[c]	Cyclic AMP	Cyclic GMP	Concentration[d]	N	Cyclic AMP	Cyclic GMP
	μM		*pmoles/mg protein*		μM		*pmoles/protein*	
Control[e]	—	8	8.0 ± 1.2	0.8 ± 0.1	—	16	9.0 ± 0.4	0.9 ± 0.1
Theophylline	710	10	9.8 ± 0.8	1.1 ± 0.1	2400	7	13.5 ± 1.2[f]	1.6 ± 0.2[f]
Caffeine	2200	10	11.6 ± 1.2	1.4 ± 0.3[f]	7300	8	14.0 ± 1.3[f]	1.4 ± 0.1[f]
8-Meome MIX[g]	400	14	10.3 ± 1.0	2.5 ± 0.4[f]	1400	8	12.2 ± 0.6[f]	3.5 ± 0.4[f]
8-Me MIX[h]	76	8	12.1 ± 1.0[f]	1.8 ± 0.2[f]	250	8	16.0 ± 1.7[f]	3.5 ± 0.7[f]
MIX	25	8	10.2 ± 0.9	1.3 ± 0.2[f]	83	8	15.2 ± 1.1[f]	1.8 ± 0.3[f]
8-*t*-But Mix[i]	78	9	8.0 ± 0.5	1.4 ± 0.1[f]	—	—	—	—

[a] Cyclic nucleotide contents are mean values ± standard error of the mean of atria 15 min after addition of xanthine or buffer to the bath.

[b] These concentrations are 3 times the concentration of agent that produces a 50% inhibition of the hydrolysis of 1 μM cyclic AMP by the 48,000 × *g* atrial supernatant fraction.

[c] *N* = number of experiments.

[d] These concentrations are 10 times the concentration of agent that inhibits by 50% the hydrolysis of 1 μM cyclic AMP by tissue extracts.

[e] Buffer, rather than xanthine, was added to the bath.

[f] $p < 0.05$ as compared with the control using one-way analysis of variance and the Student-Newman-Keuls procedure.

[g] 8-Meome MIX, 8-methoxymethyl MIX.

[h] 8-Me MIX, 8-methyl MIX.

[i] 8-*t*-But-MIX, 8-*t*-butyl MIX. 8-*t*-Butyl MIX was not sufficiently soluble to be studied in Experiment 2.

($p < 0.05$). At the higher concentrations (Experiment 2), all xanthine-treated groups contained significantly more ($p < 0.05$) cyclic AMP and cyclic GMP than the buffer-treated control group. Mean cyclic AMP contents of groups treated with xanthines in Experiment 2 were not statistically different from one another.

Xanthines that were nonselective as inhibitors of phosphodiesterase activities of the atrial supernatant fraction (i.e., MIX, caffeine, and theophylline, see Table 1) produced comparable relative elevations of cyclic AMP and cyclic GMP contents; they did not significantly alter the cyclic AMP to cyclic GMP ratio of atria (Table 3). Xanthines that preferentially inhibited cyclic GMP phosphodiesterase activity (i.e., 8-methyl MIX, 8-*t*-butyl MIX, and 8-methoxymethyl MIX), however, produced relatively larger increases of cyclic GMP than of cyclic AMP content, and thereby significantly decreased the cyclic AMP to cyclic GMP ratio of atria ($p < 0.05$). The most selective inhibitor of cyclic GMP phosphodiesterase, 8-methoxymethyl MIX, produced the greatest decline of the cyclic AMP to cyclic GMP ratio of functioning atrial strips.

DISCUSSION

This study addressed the question of whether or not xanthines alter cardiac function by inhibiting cyclic GMP or cyclic AMP phosphodiesterase activities. A strong correlation was observed between the abilities of xanthines to increase spontaneous right atrial rate and their potencies to inhibit the cyclic AMP phosphodiesterase activity of the atrial supernatant fraction. The abilities of xanthines to shorten the duration of the left atrial contraction also appeared to correlate with the potencies of xanthines to inhibit the hydrolysis of cyclic AMP. However, the abilities of xanthines to increase the contractile force of left atrial strips did not correlate with their abilities to inhibit phosphodiesterase activities. Also, xanthine-induced alterations of heart rate, duration of contraction, or contractile force could not be predicted from the potencies of xanthines to inhibit the cyclic GMP phosphodiesterase activity.

The correlative approach of the present study embodies two major assumptions. First, it was assumed that the potencies, efficacies, and selectivities of xanthines as inhibitors of the cyclic AMP and cyclic GMP phosphodiesterase activities of atrial extracts reflect the abilities of these agents to inhibit phosphodiesterases of the working atrium. Earlier studies attested to the validity of this assumption in smooth and skeletal muscle preparations where xanthine derivatives altered cyclic nucleotide contents of pig coronary artery strips (9) and of rat hemidiaphragms (10) in manners that generally paralleled their abilities to inhibit phosphodiesterase activities of the corresponding tissue extract. Similar results were obtained in the present study (Table 3). For example, only xanthines that selectively inhibited the cyclic GMP phosphodiesterase activity of atrial extracts significantly increased the cyclic GMP to cyclic AMP ratio in working atria (Table 3).

A second important assumption of this correlative approach is that subcellular effects of xanthines, unrelated to phosphodiesterase inhibition, do not obscure cardiac mechanical effects that may be due to phosphodiesterase inhibition. Testing this assumption is not simple, since xanthines exert many subcellular and mechanical effects that are apparently unrelated to phosphodiesterase inhibition (3, 20, 21). Xanthine-isoproterenol interaction studies demonstrated, however, that xanthines inhibit physiologically and pharmacologically relevant phosphodiesterase activity. Furthermore, the xanthine effects on heart rate and the duration of contraction indicate that xanthines are capable of producing cyclic AMP-like effects.

Increases in cyclic AMP levels in the heart are generally thought to give rise to positive chronotropic effects. Methylxanthines (primarily caffeine, theophylline, and MIX) have been observed to produce positive chronotropic effects in a variety of preparations (e.g., ref 22). Of

the xanthines presently studied, caffeine and theophylline were the least potent as positive chronotropic agents. A striking positive correlation was observed between the potencies of the xanthines to increase spontaneous right atrial rate and their potencies to inhibit cyclic AMP phosphodiesterase activity of atrial extracts. This observation strongly suggests that xanthines produce their positive chronotropic effects by inhibiting cyclic AMP phosphodiesterase in the SA node (i.e., in pacemaker cells), despite a report that the SA node contains forms of phosphodiesterase different from those of the rest of the atrium (23). Taniguchi and his co-workers did not study the comparative effects of phosphodiesterase inhibition on the SA nodal and extranodal enzyme activities (23), but the present data would indicate that xanthines exert similar inhibitory effects on the functionally important phosphodiesterase from both cell types. Several investigators have suggested that cyclic GMP produces negative chronotropic effects (24, 25) or antagonizes the positive chronotropic effects of cyclic AMP (26, 27). In the present study, however, two of the xanthines which were more potent as inhibitors of cyclic GMP phosphodiesterase than cyclic AMP phosphodiesterase (i.e., 8-methyl MIX and 8-methoxymethyl MIX), failed to produce negative chronotropic effects (Fig. 1), although each of these agents increased the ratio of cyclic GMP to cyclic AMP in atria by more than 300% of the control ratio (Table 3). Furthermore, xanthines, at concentrations producing an equivalent inhibition of cyclic AMP phosphodiesterase activity, were equally effective as positive chronotropic agents (Fig. 2), despite the fact that some xanthines were 10–16 times more potent than others as inhibitors of cyclic GMP phosphodiesterase. Therefore, it seems unlikely that elevations of cyclic GMP by xanthines alter the chronotropic effects of cyclic AMP. Thus, the present data, which strongly suggest a role for cyclic AMP in the chronotropic effect of xanthines, fail to suggest a role for cyclic GMP in these effects.

If cyclic AMP is positively inotropic as the literature strongly suggests (28, 29), it seems reasonable to surmise that at least a component of the effects of the xanthines on heart should be related to the abilities of xanthines to inhibit cyclic AMP phosphodiesterase. Blinks *et. al.* (3), after conducting a very thorough study on the mechanical effects of theophylline and caffeine (relatively weak phosphodiesterase inhibitors) in isolated kitten atria and papillary muscles, argued that abilities of xanthines to increase contractile force do not correlate with their abilities to inhibit phosphodiesterase. The present study, using a series of xanthines having a 100-fold potency range as inhibitors of cyclic AMP phosphodiesterase activity, also indicates that there is no correlation between phosphodiesterase inhibition and the positive inotropic effects of xanthines (Fig. 4). One of the xanthines, 8-*t*-butyl MIX, actually depressed contractile force at a concentration that was 3 times that required to inhibit by 50% the hydrolysis of 1 μM cyclic AMP by tissue extracts. These decreases in DF were accompanied by a significant elevation of cyclic GMP but not cyclic AMP (Table 3). Since the other selective inhibitors (8-methyl MIX and 8-methoxymethyl MIX) did not depress contractile function at concentrations 100–160 times those required to inhibit by 50% the hydrolysis of 1 μM cyclic GMP, it seems unlikely that the cardiac depressant effects of 8-*t*-butyl MIX are due to an inhibition of cyclic GMP phosphodiesterase. Thus, the present findings are in accord with earlier reports that elevations of atrial cyclic GMP content *per se* are not negatively inotropic (30, 31).

Watanabe and Besch (5) have suggested that cyclic GMP itself may not be negatively inotropic, but it may antagonize positive inotropic effects of agents that exert contractile effects via cyclic AMP. If cyclic GMP antagonizes positive inotropic effects of agents acting through cyclic AMP, then compounds that selectively inhibit the hydrolysis of cyclic GMP might be expected to attenuate contractile responses to isoproterenol. The results of the isoproterenol-xanthine interaction study, however, suggest that cyclic GMP is not "antiadrenergic," since xanthines that significantly increased the cyclic GMP to cyclic AMP ratio in atria were no less effective than the nonselective xanthines at potentiating isoproterenol-induced increases of contractile force (Fig. 5). Thus it seems unlikely that cyclic AMP and cyclic GMP act antagonistically as modulators of contractile force in the rabbit heart (see also accompanying paper, ref. 16).

The present data support the concept that cyclic AMP produces relaxant effects, since the potencies of xanthines to inhibit the activity of cyclic AMP phosphodiesterase generally paralleled their potencies to shorten the duration of contraction (Table 1; Fig. 3). All xanthines studied (except caffeine) shortened the atrial contraction by at least 10%. These responses were less variable than contractile force responses to xanthines. For example, MIX (600 μM) invariably shortened the duration of contraction but failed to increase contractile force in some preparations. Moreover, 8-methyl MIX (300 μM) decreased the duration of the contraction by 20% without significantly altering contractile force. The literature generally supports the idea that xanthines produce relaxant effects, presumably by inhibiting the hydrolysis of cyclic AMP (2, 32). The effects of xanthines on cyclic AMP metabolism, however, cannot account for the well established observation that certain methylxanthines prolong the duration of systole (3). This prolongation, also observed in the present study with theophylline and caffeine, appears to require millimolar concentrations (Fig. 3). In all likelihood, xanthines prolong the contraction by mechanisms unrelated to phosphodiesterase inhibition. It is easy to see how the tendency of xanthines to prolong the contraction could modify or mask relaxant effects expected in the presence of increased concentrations of cyclic AMP. Thus, it is not surprising that caffeine and theophylline in some studies (e.g., ref. 3) fail to shorten systole, considering their relative lack of potency to inhibit cyclic AMP phosphodiesterase.

In conclusion, the present data indicate that inhibition of cyclic GMP phosphodiesterase (and therefore cyclic GMP) probably does not play a role in cardiac responses to the xanthines. Inhibition of cyclic AMP phosphodiesterase (and therefore cyclic AMP), however, is responsible, in a large part, for the positive chronotropic and relaxant effects of xanthines. Therefore, any lack of cor-

relation between phosphodiesterase inhibition and cardiac effects of xanthines probably does not arise from a failure of xanthines to inhibit physiologically or pharmacologically important phosphodiesterases; instead, it more likely points to cyclic nucleotide-independent effects of xanthines.

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