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Inhibition of Fibroblast Cyclic AMP Escape and Cyclic Nucleotide Phosphodiesterase Activities by Xanthines

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SUMMARY

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The relationship between the ability of a compound to antagonize the escape of cyclic AMP from cultured cells and its cyclic 3′:5′-nucleotide phosphodiesterase (EC 3.1.4.17) inhibitory activity was investigated using 1-methyl-3-isobutylxanthine (MIX) and three MIX analogs, i.e., 7-benzyl MIX, 8-*t*-butyl MIX, and 1-isoamyl-3-isobutylxanthine. The addition of 30 μM MIX, 7-benzyl MIX, 8-*t*-butyl MIX, or 1-isoamyl-3-isobutylxanthine to the incubation media of WI-38 fibroblasts exposed to 5.7 μM prostaglandin E_1 (PGE_1) increased the intracellular cyclic AMP levels and decreased the release of cyclic AMP from the cells after 6 and 24 min. Concentration–response studies showed that cyclic AMP escape from PGE_1-stimulated WI-38 fibroblasts was most sensitive to inhibition by 7-benzyl MIX and 8-*t*-butyl MIX. At 30 μM, the highest concentration tested, only 1-isoamyl-3-isobutylxanthine potentiated the effects of 5.7 μM PGE_1 on cyclic AMP accumulation in SV40-transformed WI-38 (VA13) cells. None of the compounds at 30 μM affected cyclic AMP escape from PGE_1-treated VA13 cultures. WI-38- and VA13-soluble and DEAE-cellulose peak I phosphodiesterase activities were most effectively inhibited by 7-benzyl MIX and 8-*t*-butyl MIX when either 1 μM cyclic AMP or cylcic GMP was the substrate. In contrast, 1-isoamyl-3-isobutylxanthine was the most potent inhibitor of WI-38 and VA13 DEAE-cellulose peak II phosphodiesterase activities. These results suggested that there are similar structural requirements of the xanthines for antagonism of cyclic AMP escape from WI-38 cultures and inhibition of the fibroblast peak I phosphodiesterase activities. The data also appear to indicate that the ability of the xanthines to alter cyclic AMP metabolism in VA13 cells cannot be predicted solely on the basis of their potencies as phosphodiesterase inhibitors.

INTRODUCTION

Intact WI-38 and SV40-transformed WI-38 (VA13) cells dispose of cyclic AMP by intracellular hydrolysis and by release into the culture media (1–6). The escape of cyclic AMP from hormone-stimulated WI-38 and VA13 fibroblasts could not be attributed to cell injury (4, 5). Moreover, the extrusion process was sensitive to temperature, pH, and a variety of drugs including some phosphodiesterase inhibitors (2–6). Recent work by Kelly *et al.* (5) suggested that there was a proportionality between the ability of a compound to interfere with cyclic AMP escape from WI-38 cells and its potency as an inhibitor of phosphodiesterase activity. These data could be interpreted as indicating that WI-38 phosphodiesterase and a cyclic AMP transport system associated with the normal fibroblasts possessed similar binding sites for the nucleotide. However, the results were also compatible with a role for phosphodiesterase in the cyclic AMP escape mechanism. The observation that only a small fraction of the total cyclic AMP formed in response to hormones appeared in the media of cell lines which showed low phosphodiesterase activity, e.g., VA13 (2), offered some support for the latter hypothesis. The studies described in the present report were designed to distinguish between these two possibilities by comparing

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the potencies of MIX[1] and three MIX analogs, i.e., 7-benzyl MIX, 8-*t*-butyl MIX, and 1-isoamyl-3-isobutylxanthine, as inhibitors of WI-38 and VA13 cyclic AMP escape, supernatant fraction phosphodiesterase activities, and partially purified phosphodiesterase activities. These four xanthines were chosen because they were previously found to be relatively selective inhibitors of cyclic AMP or cyclic GMP hydrolysis by pig coronary artery phosphodiesterase activities (7, 8). A preliminary account of some of the experiments discussed here has been published elsewhere (9).

EXPERIMENTAL PROCEDURES

Synthesis of MIX analogs. 7-Benzyl MIX, 8-*t*-butyl MIX, and 1-isoamyl-3-isobutylxanthine were synthesized as outlined by Garst *et al.* (7) and Kramer *et al.* (8). DMSO (100%) was used as the diluent for MIX and the three MIX analogs.

Cell cultures. WI-38 and VA13 cultures were originally obtained from Drs. Leonard Hayflick and Anthony Girardi, respectively. The fibroblasts were propagated from frozen stock as described previously (2).

Intact cell studies. A [^{3}H]adenine prelabeling method was used to estimate intracellular and media cyclic AMP levels in WI-38 and VA13 cultures (10–12). Normal and transformed fibroblasts grown on 35-mm plastic dishes (Costar) were incubated at 37°C under 95% air:5% CO_2 for 1 h with 10 μCi of [^{3}H]adenine in 1 ml of complete growth medium. At the end of this incubation period, the media were aspirated and the cell sheets were washed three times with 2 ml of serum-free, bicarbonate-free Eagle's minimal essential medium buffered at pH 7.4 with 20 mM Hepes. Following the addition of 1 ml of the serum-free medium to the culture dishes, fibroblasts were incubated under air for 20 min at 37°C. PGE_1 and one of the four xanthines or DMSO were then added as required by experimental protocols and the incubations were continued for appropriate lengths of time. DMSO was present in the incubation media of control and xanthine-treated WI-38 and VA13 cultures at a concentration of 1%. The experiments were terminated by transferring the incubation media to tubes containing aproximately 10,000 dpm of cyclic [^{14}C]AMP in 1 ml of cold 0.2 N HCl. ATP and cyclic AMP were extracted from WI-38 and VA13 fibroblasts which remained on the culture dishes with 0.6 ml of cold 5% trichloroacetic acid solution supplemented with approximately 5000 dpm of [^{14}C]ATP and 10,000 dpm of cyclic [^{14}C]AMP. The acid extracts were taken up with Pasteur pipets and the precipitated proteins from the WI-38 and VA13 cells were solubilized in 1 and 2 ml of 0.2 N NaOH, respectively.

[^{3}H]ATP and cyclic [^{3}H]AMP in acidified cell and media samples were isolated by sequential chromatography on Dowex 50W-x4 (100 to 200 mesh) and neutral alumina (13, 14). Unlabeled ATP in Dowex 50W-x4 eluates was determined by a modification of the luciferase assay developed by Stanley and Williams (15). Data were corrected for the recoveries of [^{14}C]ATP (approximately 70%) and cyclic [^{14}C]AMP (approximately 60%) from the chromatography columns. Radioactivity was quantified using a liquid scintillation counter and tT-21 as the scintillant (16). The protein concentration of NaOH-solubilized fibroblast residues was measured as proposed by Lowry *et al.* (17) with the aid of a Technicon AutoAnalyzer.

Phosphodiesterase preparations. The procedures used to obtain WI-38 and VA13 supernatant fractions and partially purified phosphodiesterases were outlined in a recent report from this laboratory (18). Briefly, WI-38 and VA13 fibroblasts were homogenized in cold 10 mM Tris–HCl buffer (pH 7.5) containing 1 mM DTT. $MgCl_2$ was added to the cell homogenates to give a final concentration of 2 mM Mg^{2+}, and these preparations were centrifuged at 100,000*g* for 70 min in an IEC Model B-60 ultracentrifuge. The resulting fibroblast supernatant fractions (16 to 40 ml containing 22 to 77 mg of protein) were assayed immediately for phosphodiesterase activity and applied to 10 × 0.9-cm columns of DEAE-cellulose equilibrated at 4°C with 20 mM Tris–HCl buffer (pH 7.5) containing 1 mM DTT and 2 mM $MgCl_2$ (Tris/DTT/$MgCl_2$) (19). Phosphodiesterase activity was eluted from DEAE-cellulose columns with a linear gradient of 0.0 to 0.5 M $(NH_4)_2SO_4$ in Tris/DTT/$MgCl_2$ at a flow rate of 0.5 ml/min (19). Column fractions which represented separate peaks of phosphodiesterase activity were pooled, concentrated to approximately 15% of their original volumes by ultrafiltration through Amicon CF50A membrane cones at 1000*g* for 30 min, and dialyzed at 4°C for 24 h against 100 vol (two changes) of the Tris/DTT/$MgCl_2$ equilibration buffer. Following concentration and dialysis, enzyme preparations were rechromatographed on 10 × 0.9-cm columns of DEAE-cellulose to remove overlapping peaks of phosphodiesterase activity. Appropriate column fractions from the rechromatography step were again pooled, concentrated, and dialyzed prior to storage at −70°C.

Phosphodiesterase assay. Phosphodiesterase activity in WI-38 and VA13 enzyme preparations was estimated at 30°C using a radioisotope method and cyclic AMP or cyclic GMP as the substrate (20). *Crotalus atrox* venom was included in the assay system to convert [^{3}H]AMP and [^{3}H]GMP to the corresponding tritiated nucleosides. In some experiments, sample tubes were boiled for 5 min before the addition of the snake venom. Tritiated adenosine or guanosine was isolated by chromatography on Dowex 1-x2 (200 to 400 mesh) and quantified with a liquid scintillation counter and tT-21 as the scintillant (16). Recoveries (85 to 95%) of the ^{3}H-labeled nucleosides from anion-exchange columns were monitored with [^{14}C]adenosine or [^{14}C]guanosine. Phosphodiesterase assay data were corrected for the recoveries of the nucleosides from the chromatography columns. Enzyme dilutions were made with Tris/DTT/$MgCl_2$ buffer containing 1 mg/ml bovine serum albumin and were chosen to ensure that 3 to 20% of the substrate was hydrolyzed during the 30-min assay period. Under these conditions, adenosine or guanosine formation was linear with respect to time and to enzyme concentration. All phosphodies-

[1] Abbreviations used: MIX, 1-methyl-3-isobutylxanthine; DMSO, dimethyl sulfoxide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PGE_1, prostaglandin E_1; DTT, dithiothreitol; DEAE cellulose, DE52 diethylaminoethyl cellulose.

terase determinations were performed in duplicate or triplicate. The protein content of WI-38 and VA13 phosphodiesterase preparations was measured as described by Lowry *et al.* (17).

Concentrations of MIX, 7-benzyl MIX, 8-*t*-butyl MIX, and 1-isoamyl-3-isobutylxanthine which inhibited the hydrolysis of 1 μM substrate by 50% (I_{50}) were estimated from concentration versus percentage inhibition curves using 10^{-7} to 10^{-4} or 10^{-3} M levels of the xanthines. These concentrations of MIX and the MIX analogs did not affect the 5′-nucleotidase activity of the *Crotalus atrox* venom. DMSO was present in all phosphodiesterase assay samples including the blank at a concentration of 3%.

Materials. PGE_1 was generously provided by Dr. John Pike of the Upjohn Co., Kalamazoo, Mich. *Crotalus atrox* venom was purchased from the Ross Allen Reptile Institute, Inc., Silver Spring, Fla.; Dowex 1-x2 (200 to 400 mesh) was from Sigma Chemical Co., St. Louis, Mo.; Dowex 50W-x4 (100 to 200 mesh) was from Bio-Rad Laboratories, Richmond, Calif.; cellulose was from Whatman, Ltd., Maidstone, England; MIX was from the Aldrich Chemical Co., Milwaukee, Wis.; and [8-^{3}H]adenine (18.9 Ci/mmol), [U-^{14}C]ATP (501 mCi/mmol), cyclic [8-^{14}C]AMP (53.1 mCi/mmol), cyclic [G-^{3}H]AMP (20 to 50 Ci/mmol), cyclic [G-^{3}H]GMP (5 to 10 Ci/mmol), [8-^{14}C]adenosine (40 to 60 mCi/mmol), and [8-^{14}C]guanosine (>400 mCi/mmol) were from New England Nuclear, Boston, Mass. Tritiated cyclic AMP and cyclic GMP were purified by ion-exchange chromatography on Dowex 1-x2 (200 to 400 mesh) before inclusion in phosphodiesterase substrate mixtures.

RESULTS

Effects of xanthines on cyclic AMP accumulation in intact fibroblasts. In the experiments presented in Tables 1 and 2, PGE_1 and MIX, 7-benzyl MIX, 8-*t*-butyl MIX, or 1-isoamyl-3-isobutylxanthine were added simultaneously to WI-38 and VA13 cultures. The incubations were terminated at 6 or 24 min because these time points coincided with different phases of cyclic AMP accumulation in PGE_2-stimulated WI-38 fibroblasts in the absence of phosphodiesterase inhibitors (1, 2). Intracellular cyclic AMP levels in these cells peaked 5 to 10 min after the addition of PGE_1, but fell to a plateau value by 24 min. In contrast, the cyclic AMP content of VA13 cultures exposed to PGE_1 reached a maximum after 20 min and declined slowly thereafter (2).

Regardless of the length of the incubation period, all of the xanthines at a concentration of 30 μM elevated cellular and total WI-38 cyclic AMP accumulation above the levels seen with PGE_1 alone (Table 1). After both 6 and 24 min, total cyclic AMP was significantly higher ($P < 0.05$) in PGE_1-stimulated WI-38 cultures exposed to MIX than in fibroblasts incubated with 7-benzyl MIX or 8-*t*-butyl MIX. 1-Isoamyl-3-isobutylxanthine also increased the total cyclic AMP in hormone-treated WI-38 cultures to levels which were above those seen in the presence of 7-benzyl MIX ($P < 0.05$ at 6 and 24 min) or 8-*t*-butyl MIX ($P < 0.05$ at 6 min). Each of the four compounds used in this study additionally reduced the proportion of total cyclic AMP which was released into WI-38 incubation media (Table 1). 7-Benzyl MIX and 1-isoamyl-3-isobutylxanthine were the most effective inhibitors of cyclic AMP escape from the normal fibroblasts at 6 min. However, by 24 min the decrease in cyclic AMP extrusion was most pronounced in WI-38 cells which had been incubated with either 7-benzyl MIX or 8-*t*-butyl MIX.

The addition of 1-isoamyl-3-isobutylxanthine to WI-38 cultures resulted in a small, but significant, rise in the ATP content of WI-38 fibroblasts (Table 1). The reason for this is unclear. Bogdanov and Bally (21) have observed that ATP levels in platelets were decreased by 70% in the presence of millimolar concentrations of MIX.

TABLE 1

The effects of xanthines on ATP levels, intracellular cyclic AMP, and cyclic AMP escape in PGE_1-stimulated WI-38 cells

Intact WI-38 fibroblasts were obtained as described under Experimental Procedures. PGE_1 (5.7 μM) and DMSO or the xanthines (30 μM) were added to the cells simultaneously and the incubations were continued for the indicated time periods. The final concentration of DMSO in the media of control and xanthine-treated WI-38 cultures was 1%. Values are means ± SE for determinations on three different preparations. Cyclic AMP accumulation in WI-38 cultures at the start of the incubation period: cellular, 36 ± 4 pmol/mg protein; media, 28 ± 3 pmol/mg protein.

Additions to incubation media	Length of Incubation	Cellular ATP	Cellular cyclic AMP	Media cyclic AMP	Total cyclic AMP	Media cyclic AMP/total cyclic AMP
	min	*nmol/mg protein*	*pmol/mg protein*	*pmol/mg protein*	*pmol/mg protein*	
PGE_1 + DMSO	6	45.3 ± 2.7	1678 ± 183	528 ± 54	2206 ± 237	0.24 ± 0.00
	24	44.1 ± 1.8	810 ± 76	1946 ± 83	2756 ± 146	0.17 ± 0.01
PGE_1 + MIX	6	49.3 ± 1.3	3190 ± 188*	674 ± 21**	3864 ± 206*	0.17 ± 0.00*
	24	41.9 ± 1.6	3167 ± 199*	4460 ± 22*	7627 ± 265*	0.59 ± 0.02*
PGE_1 + 7-benzyl MIX	6	48.2 ± 1.6	2958 ± 190*	269 ± 2***	3227 ± 188*	0.084 ± 0.005*
	24	46.6 ± 1.2	3137 ± 148*	2054 ± 57	5191 ± 175*	0.40 ± 0.01*
PGE_1 + 8-*t*-butyl MIX	6	52.5 ± 4.6	2744 ± 128*	357 ± 21**	3101 ± 149***	0.11 ± 0.01*
	24	45.8 ± 2.1	3065 ± 157*	2598 ± 91*	5663 ± 217*	0.46 ± 0.01*
PGE_1 + 1-isoamyl-3-isobutylxanthine	6	52.8 ± 1.9**	3954 ± 73*	392 ± 6**	4346 ± 77*	0.090 ± 0.001*
	24	51.9 ± 1.8**	3166 ± 361*	3509 ± 209*	6675 ± 529*	0.53 ± 0.02*

* Significantly different from PGE_1 + DMSO, $P < 0.01$.
** Significantly different from PGE_1 + DMSO, $P < 0.05$.
*** Significantly different from PGE_1 + DMSO, $P < 0.02$.

TABLE 2

The effects of xanthines on ATP levels, intracellular cyclic AMP, and cyclic AMP escape in PGE_1-stimulated VA13 cells

Intact VA13 fibroblasts were obtained as described under Experimental Procedures. PGE_1 (5.7 μM) and DMSO or the xanthines (30 μM) were added to the cells simultaneously and the incubations were continued for the indicated time periods. The final concentration of DMSO in the media of control and xanthine-treated VA13 cultures was 1%. Values are means ± SE for determinations on three different preparations. Cyclic AMP accumulation in VA13 cultures at the start of the incubation period: cellular, 27 ± 2 pmol/mg protein; media, 13 ± 6 pmol/mg protein.

Additions to incubation media	Length of incubation	Cellular ATP	Cellular cyclic AMP	Media cyclic AMP	Total cyclic AMP	Media cyclic AMP/total cyclic AMP
	min	*nmol/mg protein*	*pmol/mg protein*	*pmol/mg protein*	*pmol/mg protein*	
PGE_1 + DMSO	6	35.1 ± 2.3	2319 ± 130	192 ± 11	2511 ± 138	0.076 ± 0.003
	24	31.9 ± 1.8	3639 ± 260	1143 ± 174	4782 ± 401	0.24 ± 0.02
PGE_1 + MIX	6	28.6 ± 2.5	2202 ± 172	175 ± 14	2377 ± 185	0.074 ± 0.002
	24	30.5 ± 2.3	4080 ± 262	1302 ± 45	5382 ± 217	0.24 ± 0.02
PGE_1 + 7-benzyl MIX	6	32.1 ± 3.3	2043 ± 234	153 ± 17	2196 ± 250	0.070 ± 0.000
	24	31.3 ± 3.8	3231 ± 365	843 ± 189	4074 ± 480	0.21 ± 0.03
PGE_1 + 8-*t*-butyl MIX	6	30.9 ± 3.0	2043 ± 221	171 ± 15	2214 ± 235	0.077 ± 0.002
	24	34.3 ± 1.9	4675 ± 453	964 ± 43	5639 ± 490	0.17 ± 0.07
PGE_1 + 1-isoamyl-3-isobutylxanthine	6	31.0 ± 1.8	2520 ± 227	189 ± 19	2709 ± 244	0.070 ± 0.002
	24	35.3 ± 0.5	4959 ± 276*	1286 ± 54	6245 ± 168**	0.21 ± 0.01

* Significantly different from PGE_1 + DMSO, $P < 0.02$.

** Significantly different from PGE_1 + DMSO, $P < 0.05$.

However, neither MIX nor 7-benzyl MIX or 8-*t*-butyl MIX altered the ATP levels of WI-38 cells under the conditions of the study summarized in Table 1. The inhibitory effects of the xanthines on cyclic AMP escape from WI-38 fibroblasts can therefore not be attributed to ATP depletion.

Cyclic AMP metabolism in PGE_1-treated VA13 fibroblasts was relatively insensitive to MIX and the MIX analogs (Table 2). Only 30 μM 1-isoamyl-3-isobutylxanthine at 24 min altered cellular and total cyclic AMP accumulation in the transformed cultures. Release of cyclic AMP into VA13 incubation media was not affected by 30 μM concentrations of any of the four xanthines at either 6 or 24 min. Kelly *et al.* (2) have previously observed that 2 mM theophylline produced only minor changes in the pattern of cyclic AMP accumulation in PGE_1-stimulated VA13 cells. Concentrations of MIX which were higher than those used in this study, i.e., 0.1 to 2 mM, have been found to inhibit cyclic AMP escape from the transformed fibroblasts (4). In the absence of PGE_1, 30 μM MIX, 7-benzyl MIX, 8-*t*-butyl MIX, or 1-isoamyl-3-isobutylxanthine had only minimal effects on WI-38 and VA13 cyclic AMP accumulations (data not shown). Phase-contrast microscopy revealed no dramatic morphological changes in WI-38 and VA13 cells which were exposed to PGE_1 and the xanthines under the same conditions as the cultures used in the experiments summarized in Tables 1 and 2.

Antagonism of cyclic AMP escape from WI-38 fibroblasts by MIX and the MIX analogs was examined more closely in a series of concentration–response experiments (Fig. 1). In an attempt to ensure complete equilibration of the xanthines into the WI-38 cells, cultures were incubated for 24 min with 5.7 μM PGE_1 and varying concentrations of MIX, 7-benzyl MIX, 8-*t*-butyl MIX, or 1-isoamyl-3-isobutylxanthine. The data which were obtained indicated that the cyclic AMP extrusion process in WI-38 cells was most sensitive to inhibition by 7-benzyl MIX (Fig. 1B) and 8-*t*-butyl MIX (Fig. 1C). Increasing the concentration of either of these two inhibitors from 3.3 to 30 μM in WI-38 cultures caused a rise in intracellular cyclic AMP in conjunction with a progressive reduction in media levels of the nucleotide. On the other hand, the increase in media cyclic AMP remained roughly proportional to the increase in intracellular cyclic AMP content in WI-38 fibroblasts which were exposed to PGE_1 and MIX (1.1–30 μM) (Fig. 1A) or 1-isoamyl-3-isobutylxanthine (1.1–10 μM) (Fig. 1D).

Inhibition of WI-38 and VA13 supernatant fraction phosphodiesterase activity. In order to determine whether or not the ability of xanthines to interfere with cyclic AMP escape from cultured cells was causally related to their phosphodiesterase inhibitory activity, work with intact cells was extended to broken-cell preparations of WI-38 and VA13 fibroblasts. For these studies, WI-38 and VA13 homogenates were separated into pellet and supernatant fractions by centrifugation at 100,000*g* for 70 min. Since WI-38 and VA13 supernatant fractions contained the bulk of the phosphodiesterase activity of the whole homogenate (18), these soluble enzyme preparations were used in this series of experiments. The complex kinetic behavior of WI-38 and VA13 supernatant fraction phosphodiesterase activities with either cyclic AMP or cyclic GMP as the substrate (data not shown) made it difficult, if not impossible, to estimate meaningful K_I values for the xanthines by graphical analysis of enzyme assay data. The inhibitory potency of a xanthine was therefore expressed as I_{50}, i.e., the concentration of a compound which decreased hydrolysis of 1 μM cyclic nucleotide by 50%. Since 1 μM substrate is in or below the K_m range of the WI-38 and VA13 supernatant fraction phosphodiesterases (data not shown), it was possible to detect changes in enzyme activity which were the result of inhibitor effects on K_m as well as on V_{max}.

From Table 3 it is evident that 7-benzyl MIX and 8-*t*-butyl MIX were the most potent inhibitors of soluble WI-38 phosphodiesterase activity with either 1 μM cyclic AMP or 1 μM cyclic GMP as the substrate. MIX was equipotent with 1-isoamyl-3-isobutylxanthine as an inhibitor of WI-38 cyclic AMP hydrolysis, but was more

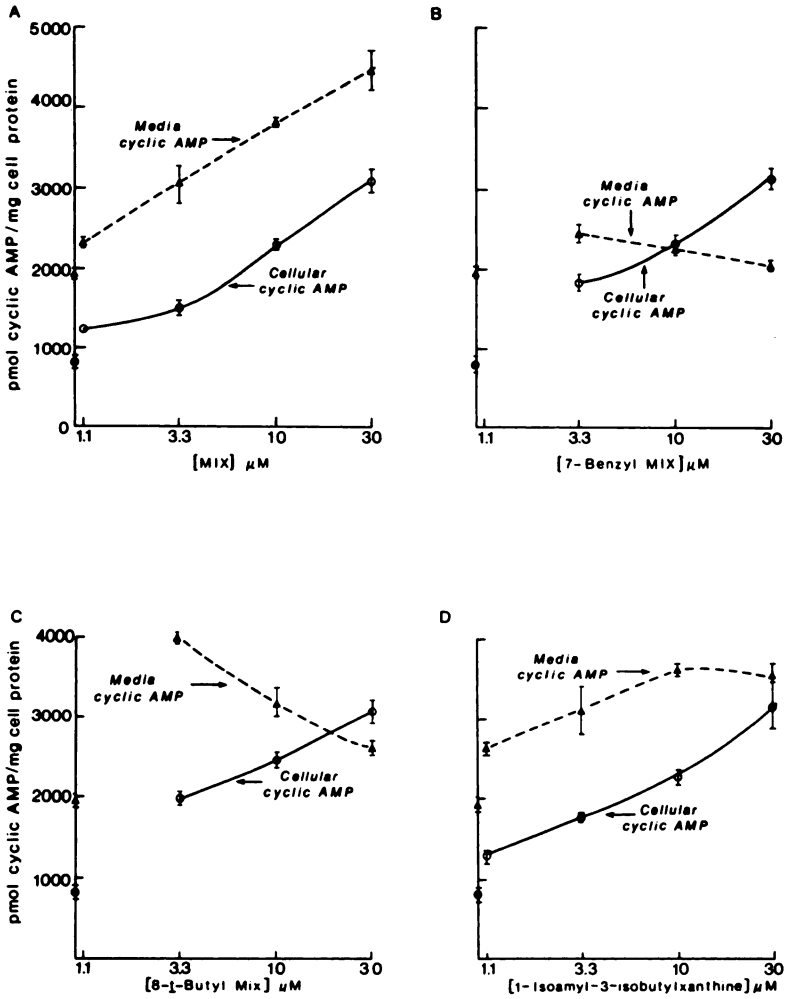

FIG. 1. *Cellular and media cyclic AMP in WI-38 cultures in response to PGE$_1$ and various xanthine concentrations*

Intact WI-38 cells were obtained as described under Experimental Procedures. PGE$_1$ (5.7 μM) and DMSO or the indicated concentrations of the xanthines were added simultaneously and the incubations were continued for 24 min. The final concentration of DMSO in the media of control and xanthine-treated cultures was 1%. Values are the mean ± SE for determinations on three different preparations. (●) Cellular cyclic AMP in control fibroblasts; (○) cellular cyclic AMP in xanthine-treated fibroblasts; (▲) cyclic AMP in the media of control fibroblasts; (△) cyclic AMP in the media of xanthine-treated fibroblasts.

potent than this compound as an inhibitor of WI-38 cyclic GMP degradation. 7-Benzyl MIX and 8-*t*-butyl MIX were also the most effective inhibitors of cyclic AMP and cyclic GMP phosphodiesterase activities in VA13 supernatant fractions (Table 3). The inhibitory potency of 7-benzyl MIX was similar for WI-38 and VA13 supernatant fractions when cyclic AMP was the substrate. In contrast, 8-*t*-butyl MIX was a less potent inhibitor ($P < 0.05$) of cyclic AMP hydrolysis by supernatant preparations from the transformed cells. Nearly identical concentrations of 7-benzyl MIX or 8-*t*-butyl MIX were required to inhibit WI-38 and VA13 supernatant fraction cyclic GMP hydrolysis by 50%. It is noteworthy that although 1-isoamyl-3-isobutylaxanthine was a poor inhibitor of VA13 soluble phosphodiesterase activity, it caused significant changes in VA13 cyclic AMP accumulation (see Table 2).

Inhibition of partially purified WI-38 and VA13 phosphodiesterase activities. As reported in a recent publication (18), DEAE-cellulose chromatography revealed different profiles of phosphodiesterase activity in WI-38 and VA13 supernatant fractions. It was therefore conceivable that the dissimilar cyclic AMP responses in WI-38 and VA13 cultures exposed to PGE$_1$ and the xanthines were related to qualitative and/or quantitative differences in the phosphodiesterase activities of the two cell strains. In order to evaluate this possibility, the inhibitory effects of MIX, 7-benzyl MIX, 8-*t*-butyl MIX, and 1-isoamyl-3-isobutylxanthine on partially purified WI-38 and VA13 phosphodiesterase activities were examined. The WI-38 and VA13 phosphodiesterase activities which were isolated for this series of experiments showed anomalous kinetic behavior with either cyclic AMP or cyclic GMP as the substrate (data not shown). Consequently, inhibitor potencies were again expressed in terms of I_{50}. WI-38 peak I appeared to reflect the presence of at least two phosphodiesterase activities. WI-38 peak I$_a$ phosphodiesterase activity could be stimulated by boiled, dialyzed fibroblast homogenates and was eluted from DEAE-cellulose columns at the same ionic strength as VA13 peak

TABLE 3

Inhibition of cyclic nucleotide phosphodiesterase activity in supernatant fractions from WI-38 and VA13 cells

WI-38 and VA13 supernatant fractions were obtained and assayed for phosphodiesterase activity as described under Experimental Procedures. The concentration of DMSO in all phosphodiesterase assay samples including controls and blanks was 3%. I_{50}, the concentration of inhibitor which was required to produce a 50% decrease in the hydrolysis of 1 μM substrate, was determined graphically. Values are means ± SE for determinations on the number of different preparations indicated in brackets. 1-Isoamyl-3-isobutylxanthine was insoluble above 100 μM. Therefore, percentage inhibition at 100 μM is shown in parentheses if the I_{50} for this inhibitor is greater than 100 μM.

Inhibitor	$I_{50\ \mu M}$			
	WI-38		VA13	
	Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP
MIX	23 ± 8 [9]	14 ± 4 [4]	86 ± 22 [3]	65 ± 7 [3]
7-Benzyl MIX	2.7 ± 1.0 [6]	3.5 ± 0.4 [4]	4.3 ± 0.9 [3]	3.7 ± 0.6 [3]
8-*t*-Butyl MIX	1.9 ± 0.8 [6]	4.8 ± 2.0 [4]	5.0 ± 1.0 [3]	4.4 ± 0.8 [3]
1-Isoamyl-3-isobutylxanthine	11 ± 4 [6]	50 ± 17 [4]	>100 [3] (32 ± 2%)	>100 [3] (42 ± 5%)

I phosphodiesterase activity. WI-38 peak I_b had approximately 15-fold greater activity with 1 μM cyclic GMP as the substrate than with 1 μM cyclic AMP. Phosphodiesterase activity with the characteristics of WI-38 peak I_b was not isolated from VA13 supernatant fractions. Attempts to separate WI-38 peak I phosphodiesterase activities were unsuccessful because of the limited amounts of enzyme which could be obtained from even large numbers of fibroblast cultures.

In addition to being the most potent inhibitors of 1 μM cyclic AMP and cyclic GMP hydrolysis by WI-38 and VA13 supernatant fractions (see Table 3), 7-benzyl MIX and 8-*t*-butyl MIX were also the most effective inhibitors of the fibroblast peak I phosphodiesterase activities with these substrates (Tables 4 and 5). 1-Isoamyl-3-isobutylxanthine was a weak inhibitor of WI-38 and VA13 peak I phosphodiesterase activities. Yet, this compound was by far the most effective inhibitor of 1 μM cyclic AMP degradation by the fibroblast peak II phosphodiesterase activities. The potencies of MIX and the MIX analogs were not altered by the inclusion of phosphodiesterase activator preparations in the enzyme assay systems (data not shown). The marked inhibition of peak II phosphodiesterase activity by 1-isoamyl-3-isobutylxanthine probably accounts for the potency of this compound as an inhibitor of 1 μM cyclic AMP hydrolysis by WI-38 supernatant fractions. At this substrate concentration, peak II phosphodiesterase activity makes up a relatively greater proportion of the total cyclic AMP phosphodiesterase activity in WI-38 supernatant fractions than in the comparable VA13 preparations (18).

DISCUSSION

On the basis of the studies described here, it appears that there are similar structural requirements of xanthines for inhibition of WI-38 and VA13 peak I phosphodiesterase activities and antagonism of cyclic AMP escape from the normal fibroblasts. This conclusion is supported by three observations. First, the most potent inhibitors of WI-38 and VA13 peak I phosphodiesterase activities, i.e., 7-benzyl MIX and 8-*t*-butyl MIX, were also the most effective antagonists of cyclic AMP extrusion from the WI-38 cultures when incubation conditions were most likely to permit equilibration of the xanthines into the fibroblasts. Second, MIX was equipotent with 7-benzyl MIX and 8-*t*-butyl MIX as an inhibitor of WI-38 and VA13 peak II phosphodiesterase activities, but had less dramatic effects on WI-38 cyclic AMP escape than either of the two other xanthines. Third, only a high concentration of 1-isoamyl-3-isobutylxanthine, a relatively selective inhibitor of WI-38 and VA13 peak II phosphodiesterase activities, caused a decrease in the release of cyclic AMP into the WI-38 incubation media. Garst *et al.* (7) and Kramer *et al.* (8) have previously reported that 7-benzyl MIX and 8-*t*-butyl MIX were potent inhibitors of calmodulin-sensitive coronary artery phosphodiesterase activity (peak I). The same compounds were found to be weak inhibitors of coronary tery phosphodiesterase activity which was characterized by a low K_m for cyclic AMP (peak II). This situation was reversed with 1-isoamyl-3-isobutylxanthine (8). Consequently, the MIX analogs seemed to display parallel selectivities as inhibitors of WI-38, VA13, and coronary artery peak I or peak II phosphodiesterase activities.

Data which revealed that neither 7-benzyl MIX nor 8-*t*-butyl MIX at 30 μM had any significant effect on the pattern of cyclic AMP accumulation in VA13 fibroblasts indicated that the effects of the xanthines on cyclic AMP metabolism in the transformed cells cannot be predicted solely from information about their potencies as phos-

TABLE 4

Inhibition of partially purified WI-38 cyclic nucleotide phosphodiesterase activities

WI-38 Peak I and Peak II phosphodiesterase activities were obtained as described under Experimental Procedures. The concentration of DMSO in all phosphodiesterase assay samples including controls and blanks was 3%. I_{50}, the concentration of inhibitor which was required to produce a 50% decrease in the hydrolysis of 1 μM substrate, was determined graphically. Peak I phosphodiesterase activity was determined in the absence of added activator. Values are means ± SE for determinations on the number of different preparations indicated in brackets. 1-Isoamyl-3-isobutylxanthine was insoluble above 100 μM. Therefore, percentage inhibition at 100 μM is shown in parentheses if the I_{50} for this inhibitor is greater than 100 μM. WI-38 Peak II phosphodiesterase activity with 1 μM cyclic GMP as the substrate was too low to determine.

Inhibitor	$I_{50\ \mu M}$		
	Peak I		Peak II
	Cyclic AMP	Cyclic GMP	Cyclic AMP
MIX	23 ± 4 [3]	13 ± 6 [4]	34 ± 7 [3]
7-Benzyl MIX	4.0 ± 1.4 [3]	2.4 ± 0.5 [4]	52 ± 11 [3]
8-*t*-Butyl MIX	3.2 ± 1.4 [3]	2.9 ± 0.7 [4]	42 ± 10 [3]
1-Isoamyl-3-isobutylxanthine	>100 (45 ± 7%) [3]	48 ± 17 [4]	4.2 ± 1.9 [3]

TABLE 5

Inhibition of partially purified VA13 cyclic nucleotide phosphodiesterase activities

VA13 Peak I and Peak II phosphodiesterase activities were obtained as described under Experimental Procedures. The concentration of DMSO in all phosphodiesterase assay samples including controls and blanks was 3%. I_{50}, the concentration of inhibitor which was required to produce a 50% decrease in the hydrolysis of 1 μM substrate, was determined graphically. Peak I phosphodiesterase activity was determined in the absence of added activator. Values are means ± SE for determinations on the number of different preparations indicated in brackets. 1-Isoamyl-3-isobutylxanthine was insoluble above 100 μM. Therefore, percentage inhibition at 100 μM is shown in parentheses if the I_{50} for this inhibitor is greater than 100 μM. VA13 peak II phosphodiesterase activity with 1 μM cyclic GMP as the substrate was too low to determine.

Inhibitor	$I_{50\ \mu M}$		
	Peak I		Peak II
	Cyclic AMP	Cyclic GMP	Cyclic AMP
MIX	86 ± 14 [4]	39 ± 4 [3]	29 ± 3 [3]
7-Benzyl MIX	5.2 ± 1.6 [4]	3.3 ± 1.0 [3]	34 ± 6 [3]
8-*t*-Butyl MIX	4.2 ± 0.7 [4]	2.9 ± 0.9 [3]	30 ± 4 [3]
1-Isoamyl-3-isobutylxanthine	>100 (28 ± 3%) [4]	>100 (36 ± 4%) [3]	2.2 ± 0.4 [3]

phodiesterase inhibitors. The very different cyclic AMP responses seen in intact WI-38 and VA13 cells during exposure to PGE_1 and 7-benzyl MIX or 8-*t*-butyl MIX could be related to slower diffusion of the xanthines into the transformed fibroblasts. In other words, WI-38 and VA13 plasma membranes might pose dissimilar barriers to the movement of xanthines from the incubation media into the intracellular space. Experiments to test this hypothesis are currently in progress. The finding of essentially unchanged cyclic AMP levels in PGE_1-stimulated VA13 cells incubated with 30 μM 7-benzyl MIX or 8-*t*-butyl MIX is also compatible with inhibition of phosphodiesterase activity which was not a major determinant of cyclic AMP hydrolysis at elevated substrate levels.

Both the intact cell studies discussed in this report and a separate series of *in situ* turnover experiments[2] demonstrated that cyclic AMP hydrolysis in WI-38 fibroblasts was more sensitive than cyclic AMP escape to inhibition by xanthines. For example, 3.3 μM MIX caused nearly a twofold increase in the cyclic AMP content of PGE_1-exposed WI-38 cells without significantly affecting the escape of the nucleotide from the same fibroblasts (see Fig. 1). It therefore seems highly unlikely that some form of phosphodiesterase is directly involved in the cyclic AMP extrusion process. The presence of phosphodiesterase activity with a high affinity for cyclic GMP in WI-38 supernatant fractions, i.e., peak I_b (18), raised the possibility that antagonism of cyclic AMP escape from the normal fibroblasts by 7-benzyl MIX and 8-*t*-butyl MIX was related to decreased cyclic GMP degradation. That is, inhibition of WI-38 cyclic GMP hydrolysis by 7-benzyl MIX or 8-*t*-butyl MIX might permit the guanine nucleotide to compete with cyclic AMP for transport into the culture media. This idea nevertheless proved to be inconsistent with the observed effects of MIX and 1-isoamyl-3-isobutylxanthine on WI-38 cyclic AMP extrusion and cyclic GMP phosphodiesterase activity. Specifically, 1-isoamyl-3-isobutylxanthine was a slightly more effective antagonist of WI-38 cyclic AMP escape than MIX, but MIX was more potent than 1-isoamyl-3-isobutylxanthine as an inhibitor of cyclic GMP degradation by WI-38 supernatant fraction and peak I phosphodiesterase activities.

In summary, the relationship between a xanthine's ability to interfere with the escape of cyclic AMP from WI-38 cells and its potency as an inhibitor of fibroblast peak I phosphodiesterase activity seems to be that the enzyme and a WI-38 cyclic AMP transport system have similar binding sites for the nucleotide. The VA13 cyclic AMP extrusion process may be insensitive to xanthine inhibition or the experimental conditions used in this study may not have allowed adequate partitioning of MIX and the MIX analogs across the plasma membranes of the transformed cells.

[2] R. Barber and R. W. Butcher, unpublished observations.

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Erratum

Volume 18, No. 1 (1980), in the article, "Inhibition of Fibroblast Cyclic AMP Escape and Cyclic Nucleotide Phosphodiesterase Activities by Xanthines," by Georgina M. Nemecek, Jack N. Wells, and Reginald W. Butcher, pp. 57–64: the last sentence of column two, page 59 should read:

> Bogdanov and Bally (21) have observed that ATP levels in platelets were decreased to approximately 70% of the control value in the presence of 10 mM concentrations of MIX.