that the thyroid gland has regulatory effect on the enzyme which is independent of the previously demonstrated effects of a reduction in androgens on the enzyme.

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Xanthine accumulation by normal and SV40-transformed WI-38 fibroblasts*

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The human diploid fibroblast WI-38 and an SV40-transformant of WI-38 (VA13) have been used extensively as model systems for studying the regulation of cyclic nucleotide metabolism [1–9]. Both cell types were observed to respond to catecholamines and prostaglandins with dramatic increases in intracellular cyclic AMP levels. Concentrations of methylxanthines which potentiated the effects of agonists on WI-38 cyclic AMP content were, nevertheless, found to have no significant effects on the pattern of cyclic AMP accumulation by hormone-stimulated VA13 cultures [2, 4, 9]. In addition, recent work with MIX† and three MIX analogs, i.e. 7-benzyl MIX, 8-*t*-butyl MIX, and 1-isoamyl-3-isobutylxanthine, has shown that the ability of the xanthines to alter the cyclic AMP responses of WI-38 and VA13 cells cannot be predicted on the basis of their potencies as inhibitors of fibroblast phosphodiesterase activities [9]. In view of the observation of Barber and Butcher that the turnover constant for cyclic AMP is greater in WI-38 than in VA13 cultures [6], it seems likely that the different sensitivities of the fibroblasts to the xanthines are a function of higher phosphodiesterase activity in the normal cells. However, discrepancies between the potencies of the xanthines as inhibitors of fibroblast phosphodiesterase activity and their effects on cyclic AMP accumulation by intact WI-38 or VA13 cells cannot be readily explained. It is conceivable that phosphodiesterase activities in fibroblast supernatant fractions differ significantly from the enzyme activities of the intact cells (see Ref. 10). Alternatively, the effects of some of the xanthines on WI-38 and/or VA13 cyclic AMP content may be limited by slow penetration of fibroblast membranes. The experiments described in this report were conducted to evaluate the latter hypothesis by determining the capacities of intact WI-38 and VA13 fibroblasts for the uptake of radiolabeled theophylline, 7-benzyl MIX [11], 8-*t*-butyl MIX [12], and 1-isoamyl-3-isobutylxanthine [12] from incubation media.

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† Abbreviations: MIX, 1-methyl-3-isobutylxanthine; PGE_1, prostaglandin E_1; DMSO, dimethyl sulfoxide; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; and MEM, minimum essential medium.

Table 1. Xanthine accumulation by PGE_1-stimulated WI-38 and VA13 fibroblasts*

Cell type	Time (min)	Theophylline	MIX	7-Benzyl MIX	8-*t*-Butyl MIX	1-Isoamyl-3-isobutylxanthine
		cpm/μl intracellular H_2O / cpm/μl extracellular H_2O				
WI-38	5	0.91 ± 0.13 (11)	1.5 ± 0.1 (7)	8.1 ± 0.3 (7)	6.1 ± 0.2 (7)	24 ± 1 (8)
	25	0.70 ± 0.08 (11)	3.2 ± 0.5 (7)	11 ± 1 (7)	7.8 ± 0.7 (7)	29 ± 1 (12)
VA13	5	0.97 ± 0.10 (14)	2.0 ± 0.1 (7)	8.5 ± 0.4 (7)	8.4 ± 0.2 (7)	14 ± 1 (7)
	25	0.84 ± 0.11 (10)	2.0 ± 0.2 (7)	8.3 ± 0.5 (7)	7.0 ± 0.7 (7)	14 ± 1 (7)

* Intact WI-38 and VA13 cells were prepared as described under Materials and methods. MEM containing 20 mM Hepes buffer (pH 7.4) was added to each culture dish. The fibroblasts were then incubated with 5.7 μM PGE_1, [^{3}H]- or [^{14}C]inulin (approximately 1×10^6 cpm/ml) and [^{14}C]urea (approximately 1×10^6 cpm/ml) or [^{3}H]theophylline (20 nM; approximately 1×10^6 cpm/ml), [^{3}H]MIX (25 nM; approximately 3×10^6 cpm/ml), [^{3}H]7-benzyl MIX (2.5 nM; approximately 3×10^5 cpm/ml), [^{3}H]8-*t*-butyl MIX (7.4 nM; approximately 1×10^6 cpm/ml), or [^{3}H]1-isoamyl-3-isobutylxanthine (18 nM; approximately 1×10^6 cpm/ml). DMSO, the vehicle for the xanthines, was present in all cultures at a final concentration of 5%. Incubations were terminated at the end of 5 and 25 min by aspirating the media. Values are means ± S.E. for the number of experiments indicated in parentheses. WI-38 intracellular H_2O: 5 min, 10.2 ± 1.4 (6) μl/mg cell protein; 25 min, 9.28 ± 0.80 (23) μl/mg cell protein. VA13 intracellular H_2O: 5 min, 7.57 ± 0.36 (6) μl/mg cell protein; 25 min, 7.93 ± 0.30 (18) μl/mg cell protein. WI-38 intracellular H_2O in the presence of 5.7 μM PGE_1 and DMSO: 5 min, 9.45 ± 0.31 (35) μl/mg cell protein; 25 min, 6.18 ± 0.44 (51) μl/mg cell protein. VA13 intracellular H_2O in the presence of 5.7 μM PGE_1 and DMSO: 5 min, 5.96 ± 0.39 (44) μl H_2O/mg cell protein; 25 min, 5.57 ± 0.42 (51) μl H_2O/mg cell protein.

Materials and methods

[Methoxy-^{14}C]inulin-methoxy (5–20 mCi/g), [methoxy-^{3}H]inulin-methoxy (100–500 mCi/g), [^{14}C]urea (2–10 mCi/mmole), [8-^{3}H]theophylline (15–30 Ci/mmole), and Biofluor were purchased from the New England Nuclear Corp., Boston, MA. [^{3}H]MIX (5.9 Ci/mmole), [^{3}H]8-*t*-butyl MIX (6.8 Ci/mmole), and [^{3}H]1-isoamyl-3-isobutylxanthine (2.8 Ci/mmole) were synthesized by catalytic tritiation of the corresponding methallyl analogs. [^{3}H]7-Benzyl MIX (5.9 Ci/mmole) was prepared by benzylation of [^{3}H]MIX.* DMSO (100%) was used as the diluent for the xanthines.

WI-38 and VA13 cultures were originally obtained from Drs. Leonard Hayflick and Anthony Girardi respectively. The fibroblasts were propagated from frozen stock on 35 mm plastic dishes as described previously [2]. After the growth media had been removed by aspiration, the cell sheets were rinsed three times with 1 ml of serum-free, bicarbonate-free Eagle's MEM buffered at pH 7.4 with 20 mM Hepes and incubated under air for 30 min at 37°. Additions, i.e. [^{3}H]- or [^{14}C]inulin plus [^{14}C]urea, [^{3}H]MIX, [^{3}H]7-benzyl MIX, [^{3}H]8-*t*-butyl MIX, or [^{3}H]1-isoamyl-3-isobutylxanthine, were then made as required by experimental protocols. The incubations were subsequently continued for appropriate periods of time. Experiments were terminated by aspirating the incubation media. Radioactivity was extracted into 2 ml of 5% trichloroacetic acid at room temperature and quantified using a liquid scintilation counter with Biofluor as the scintillant. Cell residues were solubilized in 2 ml of 0.4 N NaOH and analyzed for protein as described by Lowry *et al.* [13] with the aid of a Technicon AutoAnalyzer. Data were corrected for the recoveries (90–100%) of the radiolabeled compounds into the trichloroacetic acid and the amount of xanthine present in the inulin space. Intracellular H_2O was calculated by subtracting the inulin space from the urea space.

For studies to determine the extent to which the retention of xanthines by WI-38 and VA13 cultures might be a function of binding of the phosphodiesterase inhibitors to cell membranes, fibroblasts were harvested from 100 mm plastic dishes by scraping with a rubber policeman and were homogenized in 3 ml of 10 mM Tris–HCl buffer (pH 7.4) with a Polytron PT10 high velocity metal homogenizer. Urea and the xanthines were added to 2 ml aliquots of the WI-38 and VA13 homogenates which were vortexed and centrifuged at 27,000 *g* for 20 min at 4°. Radioactivity in the resulting supernatant and pellet fractions was extracted into trichloroacetic acid. The acidified fractions were then centrifuged at 1000 *g* for 15 min to remove precipitated proteins. The trichloroacetic acid precipitates were assayed for protein content as described earlier. Data were again corrected for the recoveries (90–100%) of the radiolabeled compounds into the trichloroacetic acid.

Results

Table 1 summarizes the results of experiments to examine the uptake of xanthines by WI-38 and VA13 fibroblasts. The data are presented as the ratios of xanthine cpm/μl intracellular H_2O to xanthine cpm/μl extracellular H_2O. A value of 1 consequently indicates that a particular xanthine is equally distributed between the intra- and extracellular spaces. Values greater than 1 arise when the concentration of xanthine is higher within the cell than in the extracellular space.

The patterns of xanthine accumulation by WI-38 and VA13 fibroblasts were qualitatively similar, i.e. 1-isoamyl-3-isobutylxanthine > 7-benzyl MIX > 8-*t*-butyl MIX >> MIX > theophylline (Table 1). There were, however, temporal as well as quantitative differences in the uptake of the xanthines by the normal as compared to the transformed cells. For example, MIX, 7-benzyl MIX, 8-*t*-butyl MIX, and 1-isoamyl-3-isobutylxanthine equilibrated more rapidly into the transformed cells. WI-38 and VA13 fibroblasts showed comparable abilities to accumulate theo-

* G. L. Kramer and J. N. Wells, unpublished data.

Table 2. Distribution of urea and 7-benzyl MIX between fibroblast pellet and supernatant fractions*

Cell type	Fraction	% total counts	
		Urea	7-Benzyl MIX
WI-38	Pellet	7.0 ± 0.3 (20)	10 ± 1 (5)
	Supernatant	93 ± 1 (20)	88 ± 1 (5)
VA13	Pellet	8.3 ± 0.5 (20)	12 ± 1 (5)
	Supernatant	92 ± 1 (20)	88 ± 1 (5)

* Intact WI-38 and VA13 cells were prepared as described under Materials and methods. [^{14}C]Urea (approximately 3.5×10^4 cpm) and [^{3}H]7-benzyl MIX (approximately 3.5×10^4 cpm) were added to 2 ml aliquots of fibroblast homogenates containing 30 μM 7-benzyl MIX. The cell-free preparations were then vortexed vigorously and centrifuged at 27,000 *g* for 20 min at 4°. Radioactivity in the resulting pellet and supernatant fractions was extracted into trichloroacetic acid. Values are the means ± S.E. for the number of experiments indicated in parentheses. Distribution of WI-38 protein: pellet fraction, 54 ± 2% (20); supernatant fraction, 45 ± 2% (20). Distribution of VA13 protein: pellet fraction, 50 ± 3% (20); supernatant fraction, 52 ± 3% (20).

phylline, but uptake of 1-isoamyl-3-isobutylxanthine by the former cells clearly exceeded that of the latter cells ($P < 0.01$). At the end of the 5-min incubation period, VA13 cultures had accumulated more MIX and 8-*t*-butyl MIX than their WI-38 counterparts ($P < 0.01$). Yet, the MIX and 7-benzyl MIX levels of the WI-38 fibroblasts were significantly above those of the VA13 cells ($P < 0.05$ and < 0.01 respectively) after 25 min.

As shown in Table 2, the distribution of 7-benzyl MIX between the 27,000 *g* pellet and supernatant fractions of WI-38 and VA13 homogenates paralleled that of urea. Similar results were obtained with MIX, 8-*t*-butyl MIX and 1-isoamyl-3-isobutylxanthine (data not shown). In other words, radioactivity in trichloroacetic acid extracts of WI-38 as well as VA13 cultures appeared to be derived primarily from radiolabeled xanthines that were taken up into the cytosol as opposed to being bound to plasma membranes.

Discussion

The results reported here are consistent with the idea that more rapid turnover of cyclic AMP in WI-38 as compared to VA13 fibroblasts rather than slower diffusion of theophylline, MIX, and the MIX analogs across the plasma membranes of the latter cells is responsible for the more pronounced effects of these phosphodiesterase inhibitors on the cyclic AMP metabolism of the normal cultures. In fact, two of these compounds, i.e. MIX and 7-benzyl MIX, were initially taken up more rapidly by VA13 cells. The observation that neither theophylline nor MIX was accumulated by the normal fibroblasts to the same extent as 7-benzyl MIX or 8-*t*-butyl MIX indicates that the greater potencies of the latter xanthines in intact WI-38 cells [8] could be a function of both the rapidity with which they penetrate plasma membranes and their efficacies as phosphodiesterase inhibitors.

Data from studies with 1-isoamyl-3-isobutylxanthine were of particular interest. This compound is a relatively ineffective inhibitor of the form of phosphodiesterase, i.e. peak I_b (calmodulin-independent), which appears to be responsible for the bulk of cyclic AMP degradation in WI-38 cells at high intracellular levels of the nucleotide [8, 9]. However, at micromolar concentrations, it caused marked changes in the cyclic AMP metabolism of the normal fibroblasts [9]. From Table 1, it is evident that WI-38 intracellular levels of 1-isoamyl-3-isobutylxanthine exceeded those of the other inhibitors that were tested. It therefore seems likely that the somewhat surprising effects of 1-isoamyl-3-isobutylxanthine on WI-38 cyclic AMP content are due to the remarkable capacity of the normal fibroblasts to accumulate this xanthine.

DEAE-cellulose chromatography has revealed different profiles of phosphodiesterase activity in WI-38 and VA13 supernatant fractions [8]. Specifically, enzyme activities corresponding to WI-38 peak I_a (calmodulin-dependent) and peak II (calmodulin-independent), but not peak I_b, have been identified in the VA13 preparations. Both 7-benzyl MIX and 8-*t*-butyl MIX were approximately twenty times more potent than 1-isoamyl-3-isobutylxanthine as inhibitors of VA13 supernatant fraction and peak I_a phosphodiesterase activities [8]. Yet, 1-isoamyl-3-isobutylxanthine was the only one of these three compounds that enhanced the cyclic AMP responses of prostaglandin in E_1-stimulated VA13 fibroblasts [8]. The transformed cells accumulated 1-isoamyl-3-isobutylxanthine to slightly less than twice the extent that they accumulated 7-benzyl MIX or 8-*t*-butyl MIX. This being the case, the effects of 1-isoamyl-3-isobutylxanthine on VA13 cyclic AMP levels appear to involve factors in addition to the ability of the transformed fibroblasts to accumulate substantial amounts of the inhibitor. Unlike either 7-benzyl MIX or 8-*t*-butyl MIX, 1-isoamyl-3-isobutylxanthine is a potent and relatively selective inhibitor of VA13 phosphodiesterase activity which is not stimulated by calmodulin (peak II) [8]. Significant changes in VA13 cyclic AMP accumulation in the presence of 1-isoamyl-3-isobutylxanthine consequently imply that the calmodulin-dependent form of phosphodiesterase may not be a major determinant of cyclic AMP hydrolysis by the transformed cells during exposure to PGE_1.

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