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The Quantitative Relationship between Intracellular Concentration and Egress of Cyclic AMP from Cultured Cells

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SUMMARY

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To test the hypothesis that cyclic AMP egress was a function of intracellular concentration, the relationship between those quantities was investigated. It was found that there was a linear relationship between intracellular concentration and the rate of egress of the nucleotide in the four cultured cell lines tested (WI-38, VA13, MRC-5, and IMR-90). The ability to relate egress to intracellular concentration for these cells allowed a quantitative approach to several long-standing questions on the role and mechanism of cyclic AMP egress. Thus, the effects of 3-isobutyl-1-methylxanthine and meclofenamate on egress were determined. In addition, this approach has been used to show that egress is not dependent either on free diffusion or on a passive carrier mechanism. It is, therefore, presumably energy-dependent. Finally, in conjunction with previously published data, it was shown that egress of cyclic AMP is not a major factor in the control of intracellular accumulation of the nucleotide in these cells. Egress accounted for no more than 15.5% of the total turnover of cyclic AMP in WI-38 cells and 18% in VA13 cells.

INTRODUCTION

The escape of cyclic AMP from intact animal cells was first described by Davoren and Sutherland (1), who reported the appearance of considerable amounts of cyclic nucleotide in the medium of pigeon erythrocytes stimulated by *l*-epinephrine. Since that time, the escape of cyclic AMP from intact tissues, a variety of cultured cells (2–7), and a variety of lower forms including *Dictyostelium discoideum* and bacteria (8) has been described.

In a recent publication, we described the general properties of cyclic AMP escape from intact human diploid fibroblasts, including WI-38 and an SV40-transformant of WI-38, VA13 (6). In these cells, cyclic AMP efflux was a concomitant of stimulation of the cells with prostaglandins or catecholamines. Increased cyclic AMP levels in medium were detectable as quickly as increased cellular levels, and escape was maintained for up to 24 hr after stimulation (6, 9). A variety of compounds, including several different types of phosphodiesterase inhibitors, reduced the rate of escape. King and Mayer (10) have demonstrated that efflux of cyclic AMP from pigeon erythrocytes was sensitive to vinblastine, colchicine, papaverine, and several other chemical agents, most of which could bind to microtubal proteins. More recently, Brunton and Mayer (11) reported that certain prostaglandins were effective and irreversible inhibitors of catecholamine-stimulated efflux. They also provided strong evidence for the ATP dependence of egress from pigeon erythrocytes and showed that under a variety of circumstances the rate of escape was proportional to intracellular cyclic AMP levels.

The earlier publications dealing with escape from mammalian cells have been largely phenomenological. In this publication, we report complete or partial answers to three mechanistic questions: (a) Is the efflux of cyclic AMP from cultured human diploid fibroblasts proportional to intracellular cyclic AMP levels, the activities of adenylate cyclase or of the phosphodiesterases, or none of the above? (b) Is the movement of cyclic AMP across the mammalian cell membrane vectorial? (c) Is egress a significant component in the regulation of cellular cyclic AMP levels?

METHODS

The prelabeling technique used for determination of intracellular accumulation has been described previously

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(12). Medium cyclic [^{3}H]AMP was isolated using a slight modification of the method of Jakobs *et al.* (13). The 2-ml medium samples were washed with 10 ml of water. The sample tubes were washed with 1 ml 0.5 M perchloric acid and the wash was added to the column. When the samples had run through, the columns were washed with 10 ml of water. The alumina columns were then placed over 0.7 × 4 cm Dowex 50W-X8, 100–200 mesh which had been prepared by washing with 10 ml of 0.1 N HCl followed by 20 ml of water. Cyclic [^{3}H]AMP was eluted from the alumina columns onto the Dowex-50 columns with 3.0 ml of ammonium formate (0.2 M, pH 6.1). The columns were separated, and the Dowex-50 columns were washed with 5.0 ml water; the cyclic [^{3}H]AMP eluted with an additional 5.0 ml of water. Aliquots (3.0 ml) were counted for recovery and for cyclic [^{3}H]AMP in 15 ml of toluene/Triton X-100 (2:1) containing 2,5-diphenyloxazole and 2,2′-*p*-phenylenebis(4-methyl-5-phenyloxazole). Cellular and medium cyclic AMP were expressed as counts per minute per milligram of protein under circumstances where the specific activities of cyclic AMP and ATP could reasonably be assumed to be identical.

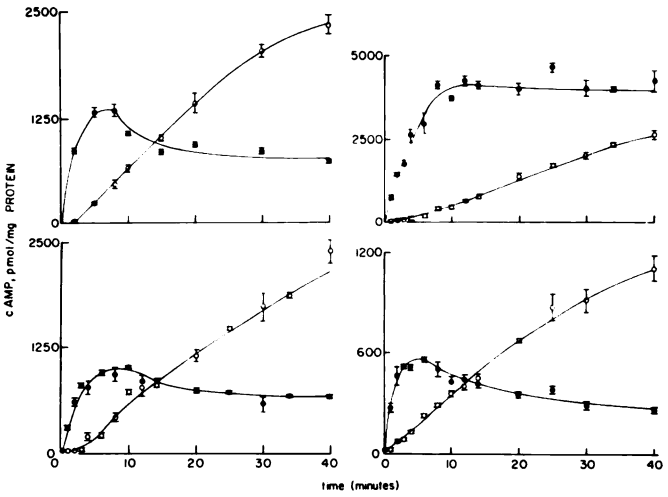

FIG. 1. *Time courses for cyclic AMP accumulation and release for WI-38 cells (upper left), VA13 cells (upper right), IMR90 cells (lower left), and MRC5 cells (lower right)*

In all cases the intracellular accumulation is indicated by ● and extracellular accumulation by ○. The stimulating hormone was 5.7 μM PGE_1.

RESULTS

The rate of escape is determined by cyclic AMP levels. When WI-38, IMR-90, MRC-5, or VA13 cells were stimulated with 5.7 μM PGE_1,[3] cyclic AMP appeared in the medium as quickly as intracellular levels were elevated (Fig. 1). However, whereas cellular levels reached a steady state within the time frame of the experiments, escape continued at a high rate throughout the experiments.

The observation that the greatest rates of escape occurred when intracellular concentrations of cyclic AMP were greatest suggested that the rate of escape was directly proportional to the intracellular concentration. If this were true, then extracellular and intracellular accumulations of cyclic AMP would be related by the following equation

$$a = k \int_0^T c\, dt$$

where a is the amount of cyclic AMP in the medium as picomoles per milligram of cell protein, c is the intracellular concentration of cyclic AMP (in the same units), k is the first-order rate constant for escape, and t is time. The term $\int_0^T c\, dt$ is given by the area under the curve that represents the time course of intracellular accumulation of cyclic AMP between $t = 0$ and $t = T$. A plot of the amount of cyclic AMP that has escaped into the medium against that term should, therefore, give a straight line with slope equal to k. The results of such a treatment for representative experiments on WI-38, VA13, MRC-5, and IMR-90 cells are plotted in Fig. 2. The linearity of the resulting plots argues strongly for the proposed dependence of escape on cellular cyclic AMP levels and militates against a relationship between egress and adenylate cyclase activity, which is known to decrease rapidly with desensitization (14, 15). This finding, coupled with the demonstrated dissociations between efflux and phosphodiesterase activity in pigeon erythrocytes (11) and in WI-38 cells (16), leaves little doubt that intracellular cyclic AMP levels determine escape.

Escape constants. The slopes of the plots of extracellular cyclic AMP versus $\int_0^T c\, dt$ shown in Fig. 2 provide a quantitative expression of ability of the cells to move cyclic AMP to the medium. The escape constants for the cells were as follows: WI-38, 0.068 min^{-1}, VA13, 0.018 min^{-1}, MRC-5, 0.073 min^{-1}; and IMR-90, 0.076 min^{-1}.

As mentioned above, over the time course of stimulations presented, there were no changes in the escape

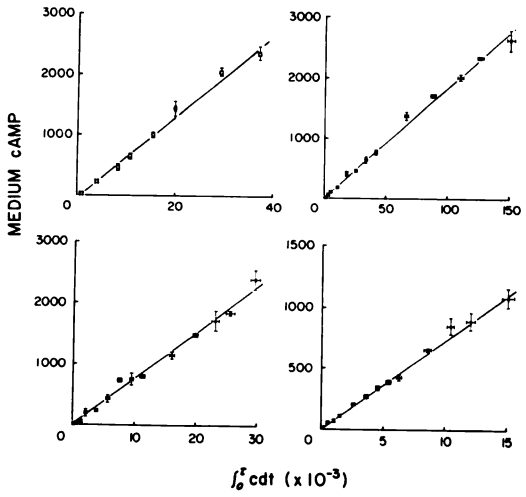

FIG. 2. *The relationship between extra- and intracellular accumulation of cyclic AMP*

Cyclic AMP present in the medium at a series of time points was plotted as ordinate against the time integral of intracellular cyclic AMP to the same time point (as abscissa). The panels show the data for WI-38 cells (*upper left*), VA13 cells (*upper right*), IMR90 cells (*lower left*), and MRC5 cells (*lower right*).

[3] The abbreviations used are: PGE_1, prostaglandin E_1; MIX, 3-isobutyl-1-methylxanthine; MEM, Eagle's minimal essential medium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TCN-P, 1,4,5,6,8-pentazaacenaphthylene, 3-amino-1,5-dihydro-5-methyl-1-β-D-[^{14}C]ribofuranosyl.

constants as opposed to the nonlinearity in cellular cyclic AMP levels and the decreasing activity of adenylate cyclase. This conclusion was confirmed by showing that the escape constant was the same for naive and desensitized WI-38 cells (Fig. 3), where the rate of escape fell in concert with intracellular accumulation.

Effect of MIX on k. Although the total effects of MIX on the cyclic AMP accumulation system are complex, when the data are viewed in terms of a rate constant relating intracellular accumulation to escape the effect of the drug in this respect is easy to quantitate. Figure 4 relates the quantity of cyclic AMP in the medium to the time integral of intracellular concentration for WI-38 cells incubated with PGE_1 (5.7 μM) and MIX (0.5 mM). Although the amount in the medium is increased over the case where only PGE_1 (5.7 μM) was used as stimulant, the intracellular levels are increased much more and the rate constant for escape was reduced to 0.038 min^{-1}, i.e., a reduction of approximately 44%.

Effect of medium composition on k. Substitution of 0.154 M NaCl buffered with 20 mM Hepes (pH 7.4) for the usual MEM-Hepes did not cause any substantial change in either accumulation or rate of escape of cyclic AMP from WI-38 cells. However, the effects of a 0.154 M KCl with Hepes were profound. The time course of accumulation of cyclic AMP in cells incubated in this latter medium was somewhat variable, but in all cases the escape constant was reduced to approximately 30% of its value in NaCl solution or complete medium. Experiments in media containing 75 mM each of KCl and NaCl gave results very similar to those in media containing only KCl.

Directionality of cyclic AMP movement. Many studies of cyclic AMP escape have been based on the assumption that the process was vectorial (i.e., unidirectional). However, this assumption has not been verified, but rather has relied on the often repeated inability of exogenous cyclic AMP to mimic the physiological effects of hormones. Experiments to examine the ingress of cyclic AMP into cells have shown that, while radiolabel from cyclic AMP was taken up, little if any of the intracellular label was cyclic AMP (e.g., see ref. 17).

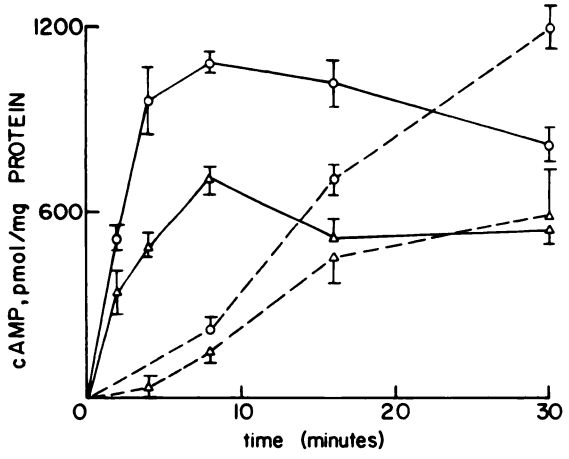

FIG. 3. *Release of cyclic AMP from naive and desensitized WI-38 cells*

Naive cells, on stimulation with 5.7 μM PGE_1, gave the time courses for intracellular (——) and extracellular (- - -) cyclic AMP illustrated by ○. Cells previously desensitized by treatment with 5.7 μM PGE_1 followed by washout of the hormone and a further incubation for 5 min gave the time courses for intracellular (——) and extracellular (- - -) cyclic AMP illustrated by △.

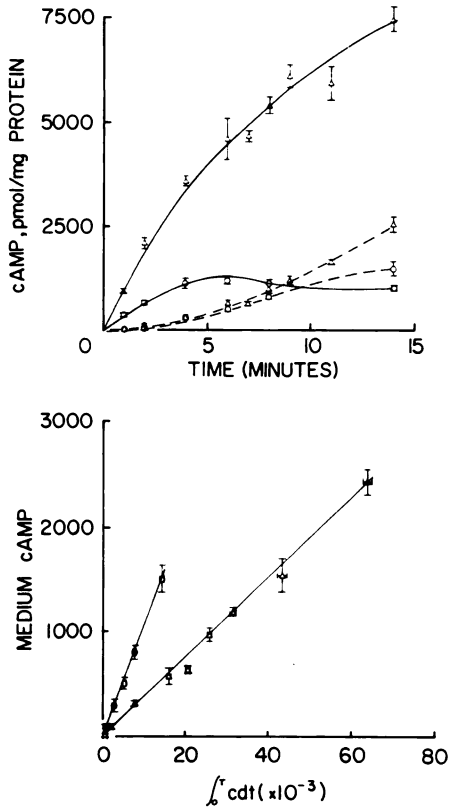

FIG. 4. *The effect of MIX on cyclic AMP release from WI-38 cells*

Upper panel, the time course for accumulation of cyclic AMP associated with cells treated with 5.7 μM PGE_1 and 0.5 mM MIX (△); ——, intracellular accumulation; - - -, extracellular accumulation. Also in the *upper panel,* the time course for accumulation of cAMP associated with cells treated with 5.7 μM PGE_1 (○); ——, intracellular accumulation; - - -, extracellular accumulation. *Lower panel,* data from the *upper panel* transformed and plotted as described in legend to Fig. 2.

Definitive proof that cyclic AMP transport through the cell's plasma membrane is truly vectorial requires that several criteria be met. It is not sufficient to show, as did Davoren and Sutherland (1), that after prolonged incubation of loosely packed cells with hormone there was a greater concentration of cyclic AMP in the medium, because from considerations of membrane potential alone, it might be supposed that freely exchanging cyclic AMP pools inside and outside the cell would be in chemical equilibrium when the extracellular concentration was greater. It is also insufficient (by itself) to show that cyclic AMP enters the cells at a much lower rate than that at which it leaves the cell. For a facilitated but not energy-requiring diffusion process, the Haldane equation relates maximal rates of transport out of V_1 and into V_2, the cell with the Michaelis constants for those processes (K_a and K_p, respectively), and the equilibrium constant K_{eq}; i.e., $V_1/K_a = K_{eq}\ (V_2/K_p)$. If K_p were very small as compared with K_a (i.e., if extracellular cyclic AMP bound more tightly to the carrier than did intra-

cellular cyclic AMP), then V_2 would be correspondingly smaller and the transport could be effectively unidirectional at experimental concentrations. To eliminate the possibility of such a system, it was necessary to show that the rate of ingress of cyclic AMP was much less than egress for a similar concentration gradient and to show that externally applied cyclic AMP had no effect on egress in this system.

For the present experiments, it was assumed that any cyclic AMP entering the cells was effectively trapped, since it would be rapidly converted to AMP and subsequently ATP. Also, since adenine and adenosine are formed from cyclic AMP in a milieu that contains cells, high concentrations of those (unlabeled) compounds were included in the incubation medium so as to inhibit transfer of labeled adenine and adenosine into the cells.

Cells were incubated with cyclic [^{3}H]AMP in various concentrations of adenine and/or adenosine (Table 1). In both WI-38 and VA13 cells, the uptake of label was severely inhibited in the presence of the purine and the nucleoside. The slope of the plot for WI-38 cells shown in Fig. 2 gives $k_{(escape)}$, and escape is given by the time-concentration integral × 0.068. Assuming WI-38 cells to be 15% protein, at an external cyclic [^{3}H]AMP concentration of 0.76 μM, ingress should be equal to 20.67 pmoles/mg. However, in the presence of 1 mM adenine and 10 mM adenosine, the uptake value was 0.13 ± 0.03 pmole/mg. The same treatment can be applied to VA13 cells, wherein the amount of escape is given by the time concentration integral × 0.018. At an extracellular cyclic AMP concentration of 0.76 μM, 5.48 pmoles/mg of protein would be expected to have entered the cells. In fact, only 0.23 ± 0.04 pmole/mg were found. Table 2 summarizes the effects of adenine and adenosine on egress of cyclic AMP from these cells. As neither agent caused any significant change in rate constant, the difference in rates observed above cannot be explained in terms of these agents acting as general inhibitors.

If this slow rate of ingress were due to a saturation of high affinity sites associated with a low V_{max}, then a similar concentration applied to the exterior of the cells should inhibit escape from the interior, and the observed differences could be explained in terms of the Haldane relationship. In fact, a concentration of 10^{-5} M cyclic AMP in the medium had no significant effect on escape of cyclic [^{3}H]AMP from WI-38 cells (Table 3). Free diffusion (whether or not facilitated) is thereby ruled out as a mechanism.

Cyclic AMP egress is not a major factor in determining cellular levels of the nucleotide. The escape constant quantitates the contribution of escape to the control of cellular levels of cyclic AMP. Total elimination of cyclic AMP by intact cells was the sum of hydrolysis by the phosphodiesterase and egress. We have recently determined fractional turnover constants for WI-38 cells (12) and VA13 cells (18, 19) and so could easily estimate the contributions of egress to turnover. In the case of WI-38 cells, the fractional turnover constant was 0.44 min^{-1} and the escape constant was 0.068 min^{-1} under the same conditions. Thus, escape could account for only 15.5% of total elimination. In VA13 cells, the constants were 0.10 min^{-1} and 0.018 min^{-1}, and 18% was the maximal contribution of egress to elimination.

These percentages were so small that it did not appear

TABLE 1

Uptake of ^{3}H from extracellular cyclic [^{3}H]AMP by WI-38 and VA13 cells in the presence of exogenous adenine and adenosine

Cells were grown as described under Methods. The experiment was begun by changing the medium to 1 ml of MEM-Hepes containing the desired concentration of adenine and/or adenosine. After 30 minutes of equilibration, cyclic [^{3}H]AMP was added. The incubation was terminated 1 h after the addition of cyclic [^{3}H]AMP by washing the cells four times with MEM-Hepes (containing the same concentration of adenine and/or adenosine used in the incubations) and then adding 1 ml of 5% trichloroacetic acid to the cell sheets. The entire acid extract was then counted as described in text.

Addition		Apparent ingress of cyclic AMP	
Adenine	Adenosine	WI-38 cells	VA13 cells
mM	*mM*	*pmoles/mg protein/hr*	
0	0	17.97 ± 1.4	
1	0	12.96 ± 1.4	8.55 ± 0.51
1	1	0.39 ± 0.08	0.83 ± 0.11
1	10	0.13 ± 0.03	0.23 ± 0.04
0	1	1.20 ± 0.08	1.01 ± 0.07

TABLE 2

Effect of adenine and adenosine on cyclic AMP egress from WI-38 and VA13 cells

Intra- and extracellular accumulations of cyclic AMP were determined 30 and 60 min after stimulation with PGE_1 and the indicated additions. The escape constants were calculated as the increase in medium accumulation between 30 and 60 min divided by 30 times the mean intracellular accumulation during that period.

Addition		Rate constant for escape	
Adenine	Adenosine	WI-38 cells	VA13 cells
mM	*mM*		
0	0	0.061 ± 0.005	0.021 ± 0.003
1	0	0.069 ± 0.008	0.023 ± 0.004
0	10	0.066 ± 0.011	0.023 ± 0.002

TABLE 3

Effect of medium cyclic AMP on cyclic AMP egress from WI-38 cells

WI-38 cells were prelabeled with adenine as described under Methods. The incubations were carried out as described, except that cold cyclic AMP (in 100 μM Tris, pH 7.4) was added, as indicated, simultaneously with PGE_1. Egress values are means ± standard error of the mean.

Cyclic AMP in incubation media,	PGE_1, 5.7 μM	Incubation time	Cyclic AMP	
			Cellular	Medium
M		*min*	*pmoles/mg protein*	
0	−	0	20 ± 4	21 ± 5
0	−	8	21 ± 1	32 ± 4
0	+	8	634 ± 15	403 ± 13
0.1	+	8	608 ± 41	416 ± 31
1.0	+	8	659 ± 31	504 ± 17
10.0	+	8	575 ± 20	499 ± 10
0	−	30	28 ± 3	74 ± 14
0	+	30	517 ± 15	2146 ± 50
0.1	+	30	482 ± 27	2090 ± 106
1.0	+	30	538 ± 20	1970 ± 57
10.0	+	30	491 ± 25	2162 ± 274

likely that escape could be a primary determinant of cyclic AMP levels in cultured human cells, as has been shown for some bacteria (8, 20) and found to be the case in turkey erythrocytes.

Furthermore, escape could be reduced without accompanying gross changes in the cellular concentration of the nucleotide. For example, the prostaglandin antagonist meclofenamate, when included in incubation of WI-38 cells with isopropylnorradrenalin, produced such effects (Fig. 5). When the quantity of cyclic AMP in the medium was plotted against $\int_0^T c\,dt$ in the same manner as described for Fig. 2, the escape constant in the presence of meclofenamate was 0.0134 min^{-1}, or only about 20% of the value in the absence of the drug. On the other hand, estimates of the turnover constant suggest that meclofenamate did not significantly alter that rate.

DISCUSSION

The quantitation of cyclic AMP efflux presented in this communication allows considerable insight into the process itself and into studies on the effect of environmental parameters on the process. The proportionality between intracellular accumulation and rate of escape persisted up to the highest levels of accumulation generated. In other words, there was no sign that the transport system was saturated at these levels, which was also the case in pigeon erythrocytes (11). When WI-38 cells were stimulated with PGE_1 (5.7 μM) plus MIX (0.5 mM), cyclic AMP levels within the cells reached over 7 nmoles/mg of protein. Assuming that the cells are 15% protein, this figure corresponded to a concentration of 0.47 mM. However, there was no sign of a break in the proportionality between accumulation and rate of escape. These data, taken by themselves, would have argued in favor of simple diffusion as an explanation for the escape process. However, the vectorial nature of the process (demonstrated in both WI-38 and VA13 cells) was much better evidence that the process is mediated by specific carriers and in an energy-dependent manner.

The studies by Plagemann and Erbe (3) are especially important for comparison with the present studies. Although these authors were studying the egress of a different compound, the phosphate derivative of TCN-P from mouse L-cells, there were many similarities in their system to cyclic AMP egress from WI-38 cells, and the same molecular apparatus might well be involved. Plagemann and Erbe (3) found that the egress of the TCN-P was, like that of cyclic AMP, a first-order process dependent on the intracellular concentration of the nucleotide at concentrations up to 200 μM. They determined that its escape constant from mouse L-cells was 0.021 min^{-1}, a value comparable to those found here for cyclic AMP escape. It is interesting that very high concentrations of TCN-P were necessary before saturation of the transport system was apparent. This is consistent with our finding of first-order kinetics of egress of cyclic AMP even at the highest intracellular levels measured.

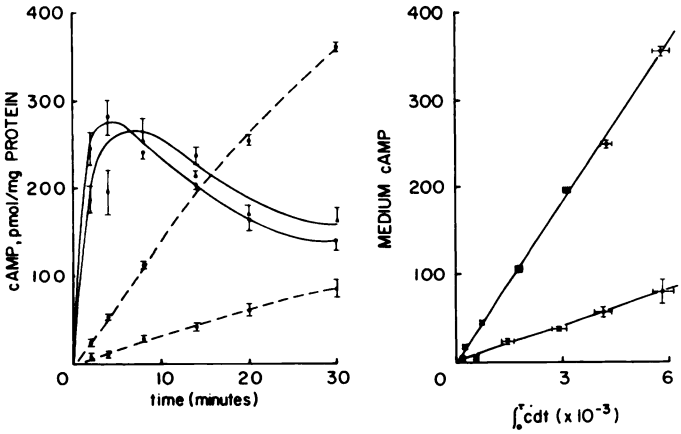

FIG. 5. *The effect of meclofenomate on cyclic AMP release from WI-38 cells*

The *left panel* shows the time course of accumulation (——) and release (- - -) of cyclic AMP for WI-38 cells, in the presence (○) and absence (●) of 10 μM meclofenamic acid. The *right panel* shows the same data transformed as described in legend to Fig. 2.

The experimental data presented here for the time course of the escape of cyclic AMP from WI-38 cultured fibroblasts differ from those of Rindler *et al.* (5) in that we detect no appreciable delay in the appearance of cyclic AMP in the medium. By contrast, Rindler *et al.* (5), using what appears to be the same system stimulated by a similar concentration of the same hormone, reported a considerable delay before efflux became apparent. At present we can offer no suggestion as to the reason for this fundamental difference between experimental data. The point is clearly an important one, since a delay in the escape process after the addition of hormone would imply that a time-variable system is responsible.

It has been suggested by others that the egress of cyclic AMP from cells could be important in controlling cyclic AMP levels within the cells (4). In WI-38 and VA13 cells, escape was responsible for only 15% and 18%, respectively, of the cyclic AMP turnover. As it has been shown that the turnover as a whole is a first-order process, a complete absence of escape would by itself result in a maximal 15% increase of intracellular levels in WI-38 cells. Therefore, we must conclude that, at least in these cells, escape is only a minor determinant in the control of intracellular levels of cyclic AMP.

It has been previously suggested that cyclic AMP movement across cell membranes was vectorial (5), although in mammalian systems the evidence has been mostly circumstantial. The efflux of cyclic AMP against a moderate concentration gradient cannot prove such a contention. Furthermore, a variety of earlier drug studies purported to show that cyclic AMP escape was energy-mediated (8, 11). However, those studies, which demonstrated a positive correlation between inhibition of cellular energy generation and inhibition of escape, could not be themselves be regarded as conclusive, since ATP is required not only as the source of energy but as the precursor for cyclic AMP.

Brunton and Mayer (11) have reported convincing evidence that escape is energy-dependent in pigeon erythrocytes. The fractional turnover constant in these cells is low as compared with that in WI-38 cells, so they could "load" the cells with cyclic AMP by exposing them to a catecholamine. The catecholamine was then washed away and the rate of efflux could be studied in the absence of adenylate cyclase stimulation. Under such

conditions, experiments using a variety of inhibitors of ATP generation showed a positive correlation between cellular ATP levels and the rate of escape. Energy dependence of the escape system also appears likely in bacteria. Saier *et al.* (8) have shown that the addition of metabolizable sugars to cyclic AMP-loaded cells resulted in rapid efflux, whereas nonmetabolizable sugars did not show this result.

Two major questions about egress still elude complete answers. The first asks whether the process is or is not ATP-dependent. Although unequivocal evidence is lacking, the vectorial nature of the process makes an affirmative answer seem likely. Furthermore, the demonstration of the energy dependence of the system in pigeon erythrocytes (11) adds some confidence that a similar requirement obtains in mammalian cells. The second question concerns the functions of escape, which are presently unclear. A major role in the regulation of cellular levels of cyclic AMP seems to have been eliminated by this report. Likewise, the use of cyclic AMP as an extracellular signal has little support by way of evidence in mammals. This possibility clearly deserves attention. However, it seems most likely that cyclic AMP may be involved in important (if obscure) aspects of membrane function.

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