Turnover of Adenosine 3′,5′-Monophosphate in WI-38 Cultured Fibroblasts†

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ABSTRACT: We have sought to determine the turnover of adenosine 3′,5′-monophosphate in WI-38 cultured fibroblasts subjected to hormonal stimulation. To this end, both mathematical analysis of the accumulation time course for the cyclic nucleotide and tracer techniques have been used. For the mathematical analysis, a model for the desensitization process is necessary. Use of the model with experimental data allows estimates to be made for the rate of removal of cAMP from the cells (by hydrolysis and escape) and for the rate of synthesis of cAMP at all points during the time course. The time course for cAMP accumulation is expressed as an equation with four fixed parameters: the rate constant for synthesis of cAMP (v_s^0), the rate constants for inactivation and reactivation of adenylate cyclase (respectively k_m and k_n), and the rate constant for removal of cAMP (k_e). With this approach, it is possible to produce an adequate description of the time course of cAMP accumulation under PGE_1 stimulation in WI-38 cultured cells. Independent estimations of the turnover of cAMP, of the rate of inactivation of adenylate cyclase, and of the rate of reactivation of the adenylate cyclase have been made using a tracer technique in which the specific radioactivity of the ATP precursor pool is increased continuously. Estimations of the kinetic parameters of cAMP accumulation by both approaches are in excellent agreement. A major conclusion that may be derived from these data is that the rate of decay of cAMP accumulation in WI-38 cells after removal of the hormone greatly overestimates turnover in the presence of hormone, since while hormone is present, phosphodiesterase activity is greatly reduced.

In general, the time course of cAMP[1] accumulation in intact cells continuously stimulated by an appropriate agonist follows a characteristic pattern (Chlapowski et al., 1975; Robison et al., 1971). Intracellular cAMP reaches a maximum a short time after stimulation and thereafter declines to a lower, relatively steady level, which in some cases is only a little above basal. The changes in intracellular cAMP seen in such experiments obviously reflect the relative activities of adenylate cyclase and at least two processes involved in the elimination of cellular cAMP, hydrolysis by cyclic nucleotide phosphodiesterases (PDE) and the escape of the nucleotide from the intracellular to the extracellular compartment (Rindler et al., 1978; Kelly et al., 1978). However, the spike and plateau shape of cAMP time courses cannot be described by any combination of simple time-invariant activation mechanisms (Su et al., 1976a,b; Barber et al., 1978). It has become obvious that a major factor directing the shape and magnitude of the time course is a process called desensitization or refractoriness. Desensitization can most easily be seen by challenging stimulated intact cells with fresh agonist after cAMP levels have peaked and come to the relative plateau. Under such circumstances the agonist produces a much diminished response or even no additional increase in cAMP levels. Since the first publication on the desensitization phenomenon by Kakiuchi & Rall (1968) with brain slices, its generality has been demonstrated by work with a number of cell types (Franklin & Foster, 1973; Kelly et al., 1974; Axelrod, 1974; de Vellis & Brooker, 1974; Ho & Sutherland, 1971; Manganiello & Vaughan, 1972; Schultz et al., 1972).

While a great body of literature exists pertinent to the characterization of adenylate cyclase (Howlett et al., 1979) and phosphodiesterase (Wells & Hardman, 1977) in cell-free systems, it is well recognized that a major problem in understanding the regulation of cyclic AMP levels in living cells is due to the lack of a valid quantitative method for measuring the activities of these enzymes in the context of living cells (Su et al., 1976b). The reason for this gap in our knowledge comes from the difficulty of deducing the activities of the different enzymes solely from the cAMP accumulation data of whole cells.

Further, the use of broken cell systems introduces a new set of uncertainties when an attempt is made to extrapolate the results back to the whole cell system. While broken cell determinations of specific enzymes might be a reliable indication of actual activities within the cell, a priori this is by no means certain. Further, to date desensitization has only been demonstrable as a result of hormone interactions with intact cell preparations. That is, changes in both adenylate cyclase and PDE activities have been reported in cell-free systems only when prepared from desensitized intact cells (Terasaki et al., 1978). But, in no case has there been a convincing demonstration of desensitizations of cell-free systems exposed to hormones.

Thus, investigations into the control of cellular cAMP levels in hormone-stimulated cells have been seriously handicapped by the inability to measure the turnover of the nucleotide. Obviously, accurate turnover measurements at various times after the onset of hormone stimulation would provide a clear definition of the relative activities of an adenylate cyclase and the cAMP removal systems. Further, knowing the relative rates of cAMP synthesis and destruction would make it possible to determine the extent (and hence the rate) of desensitization.

Our interests have been largely in the area of cAMP turnover, using cell culture systems as models. It became obvious after a variety of fruitless approaches that to quantitate the contributions of synthesis and degradation to cellular cAMP levels, an adequate definition of the system was required.

In this communication the fractional turnover constant for cAMP in intact WI-38 cells is determined by three different

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[1] Abbreviation used: cAMP, adenosine 3′,5′-monophosphate.

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methods. All three methods give good agreement for the numerical value of that parameter. Thus, it is possible to describe in numerical detail the time course of accumulation of cAMP in terms of the activities of adenylate cyclase and phosphodiesterase and the rate of escape during stimulation of prostaglandin E_1. This is true in spite of the complications caused by the process of desensitization.

Materials and Methods

Materials. PGE_1 was a gift from Dr. John Pike of the Upjohn Co. [8-^{3}H]Adenine (18.0 Ci/mmol), [U-^{14}C]ATP (501 mCi/mmol), and [8-^{14}C]cAMP (53.1 mCi/mmol) were purchased from the New England Nuclear Co. Other chemicals were of the greatest grade commercially available.

Cell Culture. As described previously, WI-38 cells were grown in Eagle's minimal essential medium (MEM) with Earle's salts supplemented with 10% fetal calf serum (Barber et al., 1977). Stock cultures from T150 flasks (Falcon) were seeded into 35-mm plastic dishes (Falcon) at a concentration of 5×10^5 cells per dish. The cells were allowed to grow for 3 days before the experiment, unless otherwise specified, at which time a confluent monolayer of cells results.

Experimental Incubations. Cellular cAMP accumulation was measured by prelabeling (Kuo & Dill, 1968; Kuo & deRenzo, 1969; Shimizu et al., 1969) the adenine ribonucleotides of the cells by incubating the dishes with 10 μCi of [8-^{3}H]adenine in 1 mL of complete growth medium for exactly 1 h at 37 °C. The media were aspirated and the cell sheets were washed 3 times with 2 mL of serum-free MEM–Hepes without $NaHCO_3$. Subsequent steps were carried out at 37 °C in air. The cells were incubated for 20 min, and then the hormones were added or other additions were made. The incubations were terminated by aspirating off the media and adding 0.6 mL of an ice-cold solution containing 5000 dpm [U-^{14}C]ATP, 10000 dpm [^{14}C]cAMP, and 5% trichloroacetic acid to the cell sheets, which were then placed on ice. [8-^{3}H]cAMP was isolated by using the methods described by Salomon et al. (1974). [8-^{3}H]ATP in the cell extracts was isolated during the first column (Dowex 5 W-X4) step. After sample application (0.6 mL), the columns were allowed to run dry and an additional 2.4 mL of H_2O was added to each. The entire 3.0 mL was collected in tubes containing 0.25 mL of 3 M $KHCO_3$. These ATP-containing fractions were assayed for endogenous ATP by a modification of the method of Stanley & Williams (1969), and 1-mL aliquots diluted with 2 mL of H_2O were counted in 15.0 mL of tT21 (Green, 1970) for [8-^{3}H]ATP and [U-^{14}C]ATP. ATP recovery was routinely ~70%, and the adenine nucleotide in the fraction was 90% ATP and the remainder was ADP, as judged by paper chromatography. The Dowex 50 columns were then washed with an additional 0.6 mL of H_2O, and [8-^{3}H]cAMP was eluted in 3.0 mL of H_2O directly onto neutral alumina columns and the fractionation was completed as described (Salomon et al., 1974). Recoveries of [8-^{14}C]cAMP were routinely ~60%. The entire final extracts (3 mL) were counted in 15 mL of tT21. For experiments in which the cells were incubated with [8-^{14}C]adenine for short times (a few minutes), an additional purification step was necessary. Prior to counting, the assay samples were lyophilized. After lyophilization, water (3 mL) is added to redissolve the samples, which are then counted as described above.

To estimate recovery from desensitization, we incubated WI-38 cells for 40 min with PGE_1 (5.7 μM) separated from the hormone by rapid washing 3 times with fresh medium. The incubation was continued in fresh medium with no hormone present. At a series of times subsequent to the washout, fresh PGE_1 to a final concentration of 5.7 μM and [8-^{3}H]adenine (20 μCi) were added. Three minutes later the incubations were terminated by removal of medium and addition of 5% Cl_3-AcOH. The radioactivity in the cAMP and ATP was determined as described above.

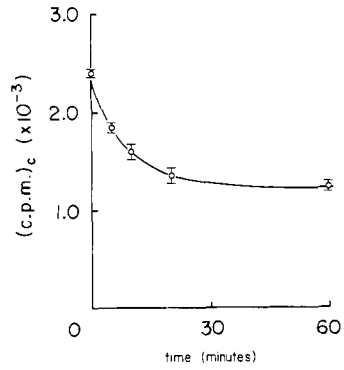


FIGURE 1: Accumulation of radioactivity into the cAMP of WI-38 cells during 2-min exposures to [8-^{3}H]adenine.

Experiments in which cellular and medium cAMP levels were measured by the [^{3}H]cAMP method and by the endogenous cAMP methods (Barber et al., 1977; Harper & Brooker, 1975) showed that the values obtained were superimposable under a wide variety of experimental conditions. All values reported are the means ± SEM from a minimum of three individually incubated, fractionated, and assayed dishes.

Results

Relative Rate of cAMP Synthesis in WI-38 Cells. When the ATP that is the precursor of cAMP is made increasingly radioactive during a short time period, the amount of radioactivity incorporated into cAMP is directly proportional to its rate of synthesis during the period of the pulse if the fractional turnover constant is invariant. Figure 1 shows the incorporation of tritium label into the cAMP in WI-38 cells stimulated with 5.7 μM PGE_1. [8-^{3}H]Adenine was added to the system at various times after stimulation of the cells by PGE_1 as indicated on the abscissa. The incubations were terminated 2 min after the addition of [8-^{3}H]adenine. Assuming that the incorporation of [8-^{3}H]adenine into [8-^{3}H]ATP was the same at all times, the decrease in the rate of synthesis of [8-^{3}H]-cAMP began immediately after addition of the hormone. This process of desensitization had a $T_{1/2}$ of ~4.5 min. The total amount of desensitization during the acute phase of stimulation of these cells corresponded to a loss of ~50% of the activity of the adenylate cyclase system. Broken cell data on adenylate cyclase activities were in substantial agreement with these figures (Clark & Butcher, 1979).

Turnover of cAMP in WI-38 Cells. ATP is the only known immediate precursor for cAMP within the cell, so any changes in the isotopic composition of the ATP should ultimately be reflected by similar changes in the cAMP. As a radioactive isotope is continuously added to the ATP, it should be intuitively clear that the isotope will eventually be found in the cAMP, but with a delay dependent on the rate of cAMP turnover. A slower turnover would cause a longer delay in the accumulation of the radioactivity into the cAMP and vice versa. The formal mathematical presentations of this idea, when cAMP accumulation is at a steady-state level, are straightforward and do not require unusual assumptions concerning the nature or kinetics of cAMP synthesis or removal (Appendix). The bulk of the mathematical manipulation given in the Appendix was obligatory only to obtain equations in which the fractional turnover constant (k_e) is

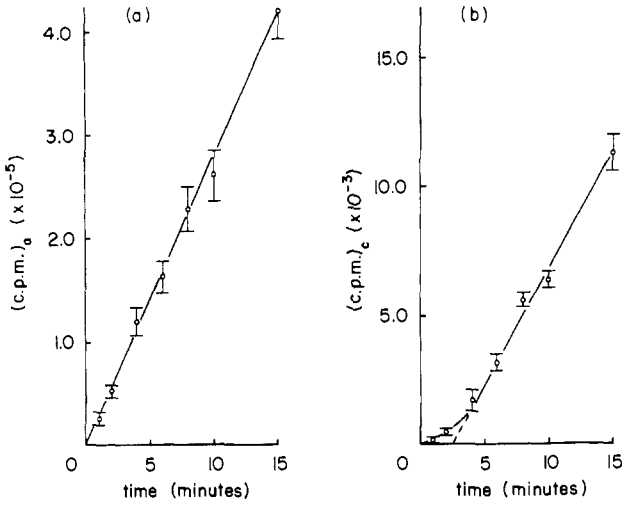


FIGURE 2: Incorporation of radioactivity into (a) the ATP and (b) the cAMP of WI-38 cells treated for the indicated times with [8-^{3}H]adenine.

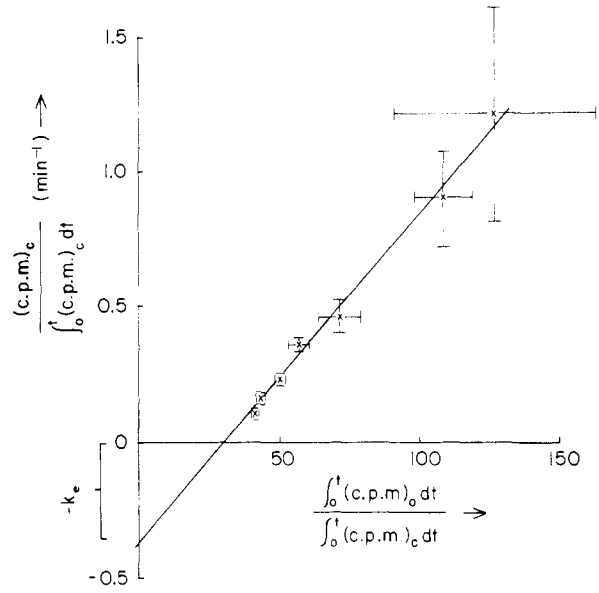


FIGURE 3: Determination of the fractional turnover constant for cAMP. An explanation of the ordinate and abscissa are given in the text.

expressed in terms of experimentally measurable quantities.

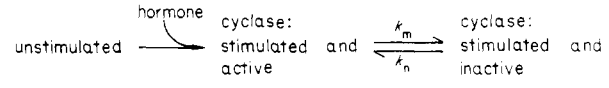
Figure 2a shows the incorporation of [8-^{3}H]adenine into the ATP of WI-38 cells. The cells had been stimulated with PGE_1 (5.7 μM) for 40 min previously. The zero time point on this graph marks the addition of [8-^{3}H]adenine to the incubation medium. The incorporation of label into cAMP is given in Figure 2b. As expected from the theoretical considerations discussed above and in the Appendix, incorporation into the cAMP pool was delayed relative to that into the ATP. There was no perceptible delay at the shortest time studied (1 min) for the buildup of radioactivity in the ATP pool.

These data were used to generate the integral expressions derived in the Appendix (eq A5). Figure 3 is a plot of $(cpm)_c/\int_0^t(cpm)_c\,dt$ against $\int_0^t(cpm)_a\,dt/\int_0^t(cpm)_c\,dt$, where $(cpm)_c$ and $(cpm)_a$ are respectively the counts per minute in the cellular cAMP and ATP. As anticipated by the derivation in the Appendix, the graph plotted as a straight line. k_e was equal to the negative intercept of the y axis. The value found was 0.38 min^{-1}.

cAMP Accumulation in Whole Cells. The previous two experiments gave a description of relative adenylate cyclase activity in WI-38 cells at various times during the PGE_1 stimulation and a measure of the fractional turnover constant of cAMP during the steady state. If it can be further assumed that the turnover constant is invariant over the range of intracellular cAMP concentrations achieved during hormonal stimulation, then it should be possible to deduce the form of actual time courses of cAMP accumulation from the data presented above. This could be done numerically without any additional assumptions or by devising a model, consistent with the above data, that is capable of describing the experimental time course of accumulation in detail.

The decrease in synthetic activity as the time of stimulation is extended as illustrated in Figure 1 can be described as a first-order process from the higher to the lower level of activity. That is, the rate of inactivation was proportional to whatever extent the earlier activity exceeded that of the final fully desensitized adenylate cyclase. There are several types of molecular mechanisms which could explain this operational description. The process could be one in which desensitization completely abolished the activity of a functional unit of adenylate cyclase. Synthetic activity did not reach zero because reactivation of inactivated units permits activity at a finite rate even in the continued presence of the stimulating hormone. Alternatively, the process could be one not involving recovery of the initial state of synthetic activity by the catalytic apparatus in the continued presence of hormone but one in which desensitization did not involve complete inactivation of each individual functional unit. Obviously, the process could be a mixture of the above two possibilities. It is the first possibility that has been chosen for development here, and the merit of this mechanism as opposed to the others is discussed later.

The specific assumptions in the formulation of a mathematical description of cAMP accumulation in these cells are therefore as follows. (1) The response of the adenylate cyclase to added hormone is extremely rapid. The rate of synthesis of cAMP reaches a maximum within seconds of the addition of hormone. (2) Sufficient hormone is added for complete activation of the adenylate cyclase. (3) The stimulated adenylate cyclase is transformed to a desensitized form with no synthetic activity by first-order kinetics. (4) Desensitized adenylate cyclase recovers activity by first-order kinetics. (5) Elimination of cAMP from the cell by hydrolysis and escape is a first-order process. (6) The rate constant for elimination of cAMP is invariant with time during the time periods used in these studies. Assumptions 1, 3, and 4 are illustrated in the diagram



Assumptions 5 and 6 relate to the properties of phosphodiesterase and of escape of cAMP from the cells.

A priori evidence that postulate 5 might be true comes from the first-order decay of cAMP levels in astrocytoma (Su et al., 1976b) and in cells used for the present experiments (Figure 6). It will be shown later that if this assumption is not true, estimates of the presumed fractional turnover constant by mathematical modeling would give smaller values than those of the labeling technique above. It will be shown that this is not the case.

With regard to postulate 6, it has been presumed by many authors (Kakuchi & Rall, 1968; Ho & Sutherland, 1971; Franklin & Foster, 1973; Su et al., 1976a,b) that desensitization of the cAMP accumulation system is primarily a function of changes in synthetic rather than elimination activities. The best evidence for this comes from the hormonal

specificity of desensitization in many systems, especially when relatively short times (30 min) of stimulation are used, whereas time-dependent changes in the parameters of elimination of cAMP might be expected to change accumulation equally whatever the stimulating hormone (Su et al., 1976a,b; Terasaki et al., 1978). Thus, it is not unreasonable in relating the above experiments to an actual time course of accumulation to assume that the kinetics of elimination do not change greatly in the time frame of the experiment.

Assumptions 2, 3, and 4 above can be expressed as equations as

$$\frac{dx}{dt} = -k_m x + k_n y \quad (1)$$

where x is the amount of stimulated and active cyclase and y is the amount of stimulated and inactive cyclase, and

$$x + y = x^0 \quad (2)$$

where x^0 is the total amount of cyclase. Substituting eq 2 into eq 1 and integrating with the initial condition that $x = x^0$ when $t = 0$, we have

$$x = x^0\left[\frac{k_n + k_m e^{-(k_m+k_n)t}}{k_m + k_n}\right] \quad (3)$$

The cyclase activity is proportional to the amount of active cyclase

$$\frac{v_s}{v_s^0} = \frac{x}{x^0} \quad (4)$$

where v_s is the rate of synthesis at time t and v_s^0 is the rate of synthesis at short times. Therefore

$$v_s = v_s^0\left[\frac{k_n + k_m e^{-(k_m+k_n)t}}{k_m + k_n}\right] \quad (5)$$

In general

$$\frac{dc}{dt} = v_s - v_e \quad (6)$$

where c is the accumulation of cAMP and v_e is its rate of elimination. From postulates 5 and 6, v_e can be written as $k_e c$. Consequently, we may write

$$\frac{dc}{dt} = v_s^0\left[\frac{k_n + k_m e^{-(k_m+k_n)t}}{k_m + k_n}\right] - k_e c \quad (7)$$

where k_e is the first-order rate constant for elimination of the cAMP. Equation 7 is solved by using the integration factor $e^{k_e t}$; then the initial condition that $c = 0$ when $t = 0$ eliminates the integration constant and the final expression is

$$c = v_s^0\left[\frac{k_n(1 - e^{-k_e t})}{(k_m + k_n)k_e} + \frac{k_m(e^{-(k_m+k_n)t} - e^{-k_e t})}{(k_m + k_n)(k_e - k_m - k_n)}\right] \quad (8)$$

Figure 4 shows the accumulation of cAMP with time in WI-38 cultured fibroblasts stimulated with PGE_1 (5.7 μM). The solid line is the numerical fit of eq 8 to these data with the following parameter assignments. $v_s^0 = 0.0105$ conversion/min, $k_n = 0.077$ min^{-1}, $k_e = 0.44$ min^{-1}, and $k_m = 0.073$ min^{-1}, or, alternatively, v_s^0 and k_n have the same values as above, but $k_e = 0.15$ min^{-1} and $k_m = 0.363$ min^{-1}.

When either of the above sets of values for the kinetic parameters is substituted into eq 4, the relationship between accumulation and time is given by

$$c = 0.01225 + 0.01762e^{-0.15t} - 0.02987e^{-0.44t} \quad (9)$$

Only the two sets of parameters quoted above are capable of giving eq 9, which is plotted as the continuous line in Figure 4.

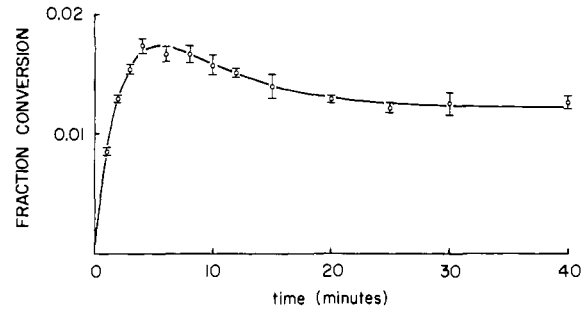


FIGURE 4: Time course of cAMP accumulation in WI-38 cells stimulated by PGE_1 (5.7 μM). The ordinate in this figure gives the fraction of cellular ATP that has been converted to cAMP. The alternative presentation of the ordinate values in terms of picomoles of cAMP per milligram of cell protein gives values with larger errors due to the error in determining cell protein. In effect, the fraction conversion used here gives cAMP accumulation in moles per mole of cellular ATP.

Thus, this technique of curve fitting of a model equation to accumulation data cannot give a unique estimate for either the fractional turnover constant (k_e) or the rate and extent of desensitization. However, it can limit each of those parameters to just two possible values.

Fitting the Model Equation to Experimental Results. It is clear from Figure 4 that correspondence between the model prediction and the experimental time course is quite good when suitable numerical values for the parameters are chosen. However, it is important to determine the range of parameter values that enable good fits of the model to the actual time course to be made. Given the quantity of data available in the experimental time course illustrated, the allowed variation is in fact quite small. In fitting numerical values of the equation, v_s^0 is very dominant for small values of t. A good estimate for v_s^0 is therefore available almost by inspection. k_e, k_m, and k_n are then chosen so that there is a minimum variation in k_s^0 in fitting all of the points. If k_e, k_m, and k_n are presumed to be respectively precisely 0.44 min^{-1}, 0.073 min^{-1} and 0.077 min^{-1}, then calculating v_s^0 for an exact fit to each experimental point gives a mean value of 0.01042 fraction conversion of ATP per min with a standard error of 0.0001. Significant changes in any one of the first three parameters even with compensating changes in the others increase the estimated standard error in v_s^0. If k_e is increased to 0.6 min^{-1} or reduced to 0.3 min^{-1}, the estimated standard error in v_s^0 is increased threefold, and, what is worse, in a plot of the equation with $k_e = 0.6$ min^{-1} and the best mean value for v_s^0, the predicted values are consistently high at short times and low at long times. With k_e set at 0.3 min^{-1}, the converse is true.

Correlations between the Experimental Results. The solid line drawn in Figure 1 was not specifically drawn to pass through the experimental points. It was the expression of $2415[[k_n + k_m e^{-(k_m+k_n)t}]/(k_m + k_n)]$, where $k_n = 0.077$ min^{-1} and $k_m = 0.073$ min^{-1} (the same values used to fit eq 8 to the experimental time course). It is not difficult to see that any great variation in k_m or k_n from the above values gives a much less satisfactory fit. For instance, if either parameter were set at 0.1 or 0.05 min^{-1}, then it was impossible to obtain a satisfactory fit with the experimental results regardless of how the other parameters were adjusted.

The value of the parameter k_e adduced from Figure 3 similarly allowed little variation within the terms of the accuracy needed for correlation of this result with those of the other experiments. A least-squares regression line gives an intercept

(i.e., a value for k_e) of 0.382 min^{-1} with a correlation coefficient of 0.996. It was not possible to draw a straight line that intercepted the y axis at values outside the range 0.28–0.55 min^{-1} without passing outside of the standard error bars of at least some of the results.

The results obtained from the three experimental approaches presented above were mutually supportive. From Figure 1 it can be seen that, at 60 min, the rate of synthesis of cAMP was reduced to 52% of its value at the onset of stimulation. From Figure 4, the initial rate of accumulation (i.e., the initial rate of synthesis) (v_s) was a fraction conversion of ~0.01 per min. The steady-state accumulation at 60 min was 0.0128 fraction conversion. Consequently, since in the steady state $dc/dt = 0$ and $v_s = k_e c$, $k_e = 0.52v_s^0/c = (0.52)(0.01)/0.0128 = 0.41$ min^{-1}. At the peak of the accumulation curve, dc/dt is equal to zero and again $v_s = k_e c$. This occurs close to 5 min from the start of stimulation, and at this time the cyclase activity was reduced to 77% (Figure 1) of its initial value, while c (Figure 4) was equal to 0.0173 fraction conversion. This gave a k_e of $(0.01)(0.77)/0.0173 = 0.44$ min^{-1}, in good agreement with the estimate from the 60-min time point. These estimates for k_e were not based on the specific assumptions used in the development of the model equations, yet they were consistent with the k_e values adduced from the modeling (0.44 min^{-1}) and from the independent estimation of the fractional turnover constant by labeling (0.38 min^{-1}).

The agreement between the value for k_e obtained from curve fitting (0.44 min^{-1}) and that obtained from continuous labeling (0.38 min^{-1}) was rather more important in defining the system since the quantity measured in those two approaches could be the same only if elimination of cAMP from the cells was a first-order process. A priori, the rate of elimination of cAMP is a function of its concentration; i.e., $r = f(c)$, where r is the rate of elimination. The determination of k_e using curve fitting of the model equation estimates k_e as the mean value for dr/dc over the range of cAMP concentrations attained during the time course. The labeling technique gives r/c as its estimate for k_e. If there were a substantial zero-order component to the elimination (i.e., if the cellular phosphodiesterase were approaching saturation), then the curve-fitting estimate for k_e would be less than the continuous labeling estimate. This was clearly not the case experimentally. We concluded, therefore, that the estimates were in good agreement and constituted evidence that cAMP elimination could best be described as a first-order process. There is other support for this contention; i.e., if there were a zero-order component, then the time course differential equation should be written as $dc/dt = v_s - z - k_e c$ (where z was the zero-order component of elimination). This could be rewritten as $dc/dt = v_x' - k_e c$ (where $v_x' = v_s - z$) which could be solved to give an equation identical in form with the one used here but in which v_s was throughout less than the actual value of the rate of synthesis by an amount equal to z. If this were true, the curve-fitting technique would have overestimated the fraction that the adenylate cyclase desensitized. The good agreement between the fraction of desensitization by curve fitting and by labeling (50% in each case) again implied that a zero-order component of elimination was not great.

Recovery from the Refractory State. The specific model of desensitization that developed in this communication implied that the adenylate cyclase system was constantly shuttling between an active state and an inactive state. The alternative model discussed earlier could be developed mathematically into a form that would require the same values for cAMP turnover and for rate and extent of inactivation of the adenylate cyclase

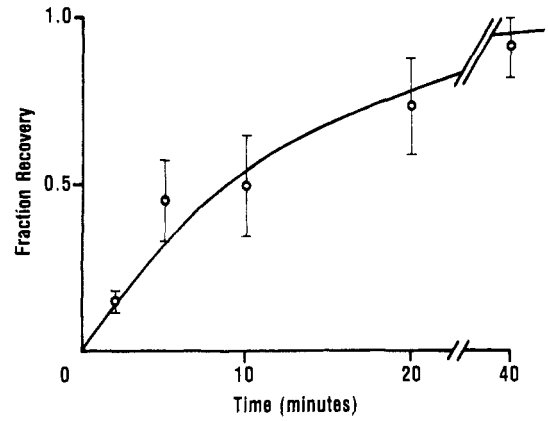


FIGURE 5: Recovery of adenylate cyclase from the desensitized state after removal of the hormone. The ordinate is defined as the fraction (measured cyclase activity – desensitized cyclase activity)/(naive cyclase activity – desensitized cyclase activity).

actually found. Thus, neither the curve fitting for the whole time course data nor the tracer techniques which enabled the independent estimation of k_e or of v_s as a function of time serve to discriminate between the alternatives. However, determination of the rate at which the enzyme system recovered after removal of the stimulating hormone allowed such a discrimination. From the value of k_n determined by curve fitting to the entire time course or by analysis of the data on rate and extent of desensitization, a clear prediction could be made for the rate of recovery.

If the fractional turnover rate were the same for all cells after readdition of hormone, the relative incorporation of radioactivity into cAMP for a 3-min period gives directly the relative adenylate cyclase activity. In Figure 5, the fraction of the adenylate cyclase activity that has recovered is plotted against the time between washout and readdition of the hormone, i.e., the time allowed for recovery. The continuous line is the theoretical curve for recovery, assuming that the recovery was a first-order process with a rate constant of 0.077 min^{-1} (i.e., the value previously assigned to k_n). While the errors are rather large, the data are consistent with the idea that recovery was a first-order process with a rate constant of ~0.077 min^{-1}.

Turnover. The fractional turnover constant is central to any numerical description of the dynamics of cAMP accumulation in intact cells, permitting the relative importance of hydrolysis and escape in the removal of cAMP to be determined, and, in conjunction with a complete time course, it allows the rate and extent of desensitization to be assessed. Obviously, and perhaps most importantly, a knowledge of k_e allows an assessment of the effects of agonists on phosphodiesterase activity in intact cells.

As discussed in the previous sections, a value of 0.38 min^{-1} was obtained for the fractional turnover constant by direct measurement in WI-38 cells. Moreover, only a value of about that magnitude was compatible with the estimates for turnover both from the desensitization data and by modeling. The rate of disappearance of cAMP in the absence of agonist can be determined by removing the agonist and following decay of cAMP with time. The results of such an experiment are shown in Figure 6. The straight-line relationship between the logarithm of the concentration and the time demonstrated that the decay was first order. The slope of the line gave the rate constant from the decay. If the process responsible for the removal of cAMP were unaffected by the hormone, the decay constant found by this experiment should have been identical with the fractional turnover constant of 0.38 min^{-1}. In fact,

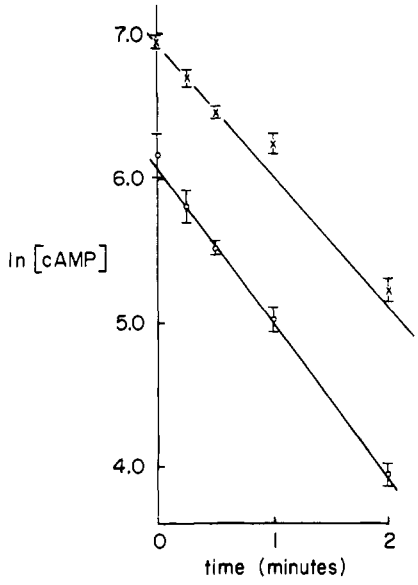


FIGURE 6: Decay of cAMP levels in WI-38 cells after removal of PGE_1. (×) PGE_1 removed after 2 min of stimulation. (○) PGE_1 removed after 40 min of stimulation.

the values of 0.91 min^{-1} after 2 min of stimulation and 1.08 min^{-1} after 40 min of stimulation were much too high (Figure 6). Very little of the cAMP that disappeared from the cells on washout of agonist appears in the medium. Thus, the conclusion that the rate of hydrolysis in the absence of agonist was ~2.5 times greater than that during stimulation was unavoidable. In other words, directly or indirectly, the hormone had a profound effect on the activity of phosphodiesterase in intact cells. Direct measurements of phosphodiesterase activity in broken cell systems from naive and desensitized cells have corroborated this view (Nemecek et al., 1979).

Turnover of cAMP in the Presence of Carbachol. The accumulation of cAMP in PGE_1-stimulated cells was greatly reduced in the presence of carbachol (Butcher, 1978). The observation that the maximum in the accumulation curve shifted to shorter times suggested that at least part of this reduction was due to increased phosphodiesterase activity (i.e., increased turnover) in these cells relative to those without the carbachol treatment.

To test this hypothesis, we determined turnover of cAMP in cells stimulated with PGE_1 (5.7 μM) in the presence and absence of carbachol (1 μM) using the labeling technique described above. Figure 7 is a representation of the function $(cpm)_c/\int_0^t(cpm)_c\,dt$ plotted against $\int_0^t(cpm)_a\,dt/\int_0^t(cpm)_c\,dt$ for both sets of data. As before, the fractional turnover constant can be read directly as the negative intercept of the y axis. In this experiment, the turnover constant in cells treated with PGE_1 was ~0.5 min^{-1}, while the turnover constant in the presence of PGE_1 and carbachol was 0.98 min^{-1}. Thus, the turnover constant for cAMP in WI-38 cells was increased by carbachol. The accumulation of total radioactivity (mainly as ATP) was not significantly different for either condition.

Discussion

The most significant observation resulting from these studies is that, at least in WI-38 cells, prostaglandin E_1 stimulation of intact cells resulted in a very rapid decrease in phosphodiesterase activity as well as the expected increase in the activity of adenylate cyclase. Activity of the adenylate cyclase was maximal in a very short time and rapidly fell due to desensitization. Desensitization was 95% complete after 20 min of stimulation.

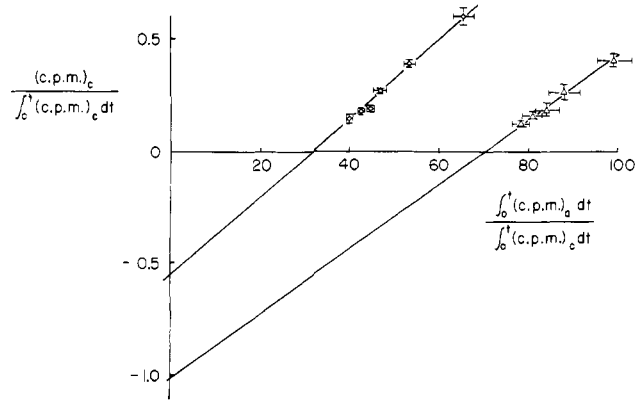


FIGURE 7: Determination of the fractional turnover constant for cAMP in the presence (△) and absence (○) of carbachol. An explanation of the ordinate and abscissa is given in the text.

The idea that hormones might affect phosphodiesterase activity is not new (Schwartz & Passoneau, 1974; Browning et al., 1976), and others have reported that prolonged treatment of intact cultured cells with a relevant agonist increased total phosphodiesterase activity as measured in broken cell preparations. Increases in phosphodiesterase activity have also been demonstrated in cells treated with phosphodiesterase inhibitors (Russell & Pastan, 1974). The present work does nothing to confirm or deny these previous observations but does show that in the early times of adenylate cyclase stimulation in intact cells, intracellular PDE activity was decreased. The much greater value obtained for the decay constant for cAMP determined by removal of the stimulating agonist (e.g., ~1.0 min^{-1}), as compared to the direct determination of the fractional turnover constant in the hormone-stimulated steady state (0.38 min^{-1}) [and the estimate of the fractional turnover constant deduced by curve fitting of the model equation (0.44 min^{-1}) as well], mandates strongly for the idea that in WI-38 cells, PGE_1 treatment has a profound and rapid effect on lowering phosphodiesterase activity. As mentioned above, supporting evidence for this idea has been obtained in experiments demonstrating decreases in phosphodiesterase activity in broken cell preparations from intact cells exposed to PGE_1 for short periods (Nemecek et al., 1979).

There is a potential problem for the general use of the tracer technique for the determination of fractional turnover constants. A serious error would be introduced if the bulk of the ATP synthesis took place in a pool separate from that serving as substrate for the adenylate cyclase (e.g., the mitochondria). The delay in incorporation of label into the cAMP pool would then reflect the rate constant for exchange of adenine nucleotides between the compartments as well as the turnover constant for cAMP. WI-38 cells seem to be largely anaerobic and dependent on glycolysis (R. W. Butcher and G. M. Nemecek, unpublished experiments). [^{14}C]Glucose metabolized almost exclusively to lactate with very little [^{14}C]CO_2 production. Further, after 3 h of incubation in an atmosphere of 95% nitrogen–5% CO_2, WI-38 cells responded to PGE_1 with levels of cAMP accumulation over a 60-min period which were identical with those seen in air–CO_2. Similar conclusions on the largely anaerobic nature of ATP generation in WI-38 cells have been reached by Rindler et al. (1978). Thus, it seems unlikely that our estimates for turnover were made artifactually low by the nature of the dynamics of the ATP pool.

For modeling purposes the specific assumptions have been made that the effects of PGE_1 on the activities of both adenylate cyclase and phosphodiesterase were instantaneous. The greater the actual lag time between addition of the prosta-

glandin and the response in terms of enzyme activation (or deactivation), the more inaccurate the model would have been in describing the early phases of the time course. This would have been especially true for the adenylate cyclase, as a delay in stimulation of synthesis would have caused the accumulation curve to be concave upward at short times. However, actual measurements of cAMP accumulation at times as short as 4 s after PGE_1 addition did not produce an upward concavity (R. W. Butcher and R. Barber, unpublished observation). A delay in hormonal effects on phosphodiesterase activity would have been less of a problem since the rate of hydrolysis was a function of both the properties of the enzyme and the concentration of cAMP (which was low at short times). Further, it has been demonstrated previously (Barber et al., 1978) that independently of any assumptions regarding specific models, the accumulation of cAMP in these cells at short times is incompatible with turnover constants of the magnitude of the decay constants estimated by cessation of stimulus (that is, by washout experiments). This suggested that the effect of PGE_1 on the turnover of cAMP was very rapid, at least within the time scale of this experiment.

It should be emphasized that the wide variety of experimental factors that influence desensitization in different cells and tissues implies that the mechanism of the process can be different in different cell types [see Terasaki et al. (1978) for a review]. Obviously, therefore, the first-order kinetics of desensitization demonstrated here for WI-38 cells at relatively short times need not be generally applicable.

Appendix

After 40 min of treatment with PGE_1, under our standard incubation the cAMP level in WI-38 cells has reached its steady-state value (Barber et al., 1978). At this stage its rates of synthesis and destruction are both invariant with time and are equal to each other. If the ATP pool that is the precursor of cAMP contains radioactive label, the rate of transfer of label to cAMP is given by $v_s a$, where v_s is the rate of cAMP synthesis in picomoles per milligram of cell protein per minute and a is the specific radioactivity of the ATP in cpm per picomole of ATP. The rate of removal of radioactivity from the cAMP pool is given by $k_e cb$, where k_e is the first-order rate constant for cAMP hydrolysis (plus escape), c is the cAMP accumulation (in picomoles per milligram of cell protein), and b is the specific radioactivity in cpm per picomole. The difference between the rate of addition to and subtraction of label from the cAMP pool gives its net change. Therefore, we can write

$$\frac{d(cb)}{dt} = v_s a - k_e cb \qquad (A1)$$

But cb is simply equal to the radioactivity in the cAMP pool per milligram of protein (i.e., the product of the amount of cAMP and its specific radioactivity). Therefore, we can write

$$\frac{d(\text{cpm})_c}{dt} = v_s a - k_e(\text{cpm})_c \qquad (A2)$$

where $(\text{cpm})_c$ is the radioactivity that is present in the cAMP pool. The above equation can be further modified to a form that allows direct experimental determination of k_e. Since a (the specific radioactivity of the ATP pool) is equal to the radioactivity in that pool $[(\text{cpm})_a]$ divided by the quantity of ATP in picomoles per milligram of cell protein ([ATP]), we can write

$$\frac{d(\text{cpm})_c}{dt} = \frac{v_s(\text{cpm})_a}{[\text{ATP}]} - k_e(\text{cpm})_c \qquad (A3)$$

After integration with respect to t

$$(\text{cpm})_c = \frac{v_s}{[\text{ATP}]}\int_0^t (\text{cpm})_a\, dt - k_e\int_0^t (\text{cpm})_c\, dt \qquad (A4)$$

and after rearrangement

$$\frac{(\text{cpm})_c}{\int_0^t (\text{cpm})_c\, dt} = \frac{v_s}{[\text{ATP}]}\frac{\int_0^t (\text{cpm})_a\, dt}{\int_0^t (\text{cpm})_c\, dt} - k_e \qquad (A5)$$

Thus, if there is an experimental technique available for varying the radioactivity in the ATP pool and the radioactivity in the ATP and cAMP pools can be determined experimentally at a series of time points, it should be possible to determine the parameters $v_s/[\text{ATP}]$ and k_e in eq A5. The quantity of radioactivity in the cAMP $[(\text{cpm})_c]$ is determined directly, and the integral expressions are determined by plotting $(\text{cpm})_c$ and $(\text{cpm})_a$ against time and measuring the areas under the curves.

A plot of $(\text{cpm})_c/\int_0^t(\text{cpm})_c\, dt$ against $\int_0^t(\text{cpm})_a\, dt/\int_0^t(\text{cpm})_c\, dt$ should therefore give a straight line with a slope equal to $k_s/[\text{ATP}]$ and a y intercept equal to $-k_e$.

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Interactions of Tris Buffer with Nucleotides: The Crystal Structure of Tris(hydroxymethyl)methylammonium Adenosine 5′-Diphosphate Dihydrate†

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ABSTRACT: The crystal and molecular structures of the Tris salt of adenosine 5′-diphosphate were determined from X-ray diffraction data. The crystals are monoclinic, space group $P2_1$, and $Z = 2$ with $a = 9.198$ (2) Å, $b = 6.894$ (1) Å, $c = 18.440$ (4) Å, and $\beta = 92.55$ (2)°. Intensity data were collected on an automated diffractometer. The structure was solved by the heavy-atom technique and refined by least squares to $R = 0.047$. The ADP molecule adopts a folded conformation. The conformation about the glycosidic bond is anti. The conformation of the ribose ring is close to a perfect C(2′)-endo–C(3′)-exo puckering. The conformation about C(4′)–C(5′) is gauche–gauche, similar to other nucleotide structures. The pyrophosphate chain displays a nearly eclipsed geometry when viewed down the P–P vector, unlike the staggered conformation observed in crystal structures of other pyrophosphates. The less favorable eclipsed conformation probably results from the observed association of Tris molecules with the polar diphosphate chain through electrostatic interactions and hydrogen bonds. Such interactions may play an important role in Tris-buffered aqueous solutions of nucleotides and metal ions.

The effect of the environment on the electronic and geometrical properties of nucleotides is important for understanding the reaction mechanisms of biological systems involving these molecules. The structure of the Tris [tris(hydroxymethyl)methylammonium] salt of adenosine 5′-diphosphate (ADP)[1] was investigated as a part of our crystallographic studies on the conformation of the ADP and ATP molecules as a function of external factors such as metal ions, buffering agents, and hydration. Other structures determined so far are those for the monorubidium salt of ADP (Viswamitra et al., 1976), ADP free acid (Viswamitra & Hosur, 1977), the potassium salt of ADP (Swaminathan & Sundaralingam, 1979), which is isostructural with the Rb–ADP structure, and the disodium salt of ATP (Kennard et al., 1971).

The Tris buffer is a widely used buffering agent in the pH range 7–9, important for studies of physiological media. The accurate dimensions of Tris and Tris-HCl in the crystalline state have been recently determined (Rudman et al., 1978, 1979). The possible formation of Tris–nucleotide complexes in solution was indicated in stability constant studies of Mg–ATP and Mg–ADP complexes using Tris buffer as the supporting electrolyte. Norby (1970) has shown that the stability constant of Mg–nucleotide complexes decreases with increasing ionic strength. O'Sullivan & Perrin (1964) have shown that the stability constant of the complex is considerably lower in Tris than in triethanolamine or *N*-ethylmorpholine buffers. The interactions between Tris and ADP molecules and their effect on the molecular conformation of the nucleotide might

Table I: Crystal Data

stoichiometry	$C_{10}H_{14}N_5O_{10}P_2 \cdot C_4H_{12}NO_3 \cdot 2H_2O$
space group	$P2_1$
Z	2
a	9.198 (2) Å
b	6.894 (1) Å
c	18.440 (4) Å
β	92.55 (2)°
d_c	1.65 g cm^{-3}
d_m	1.66 g cm^{-3}
μ (Cu Kα)	22.6 cm^{-1}

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[1] Abbreviations used: AMP, ADP, and ATP, adenosine 5′-mono-, 5′-di-, and 5′-triphosphate; NMR, nuclear magnetic resonance.

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