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Role of High Affinity cAMP Phosphodiesterase Activities in the Response of S49 Cells to Agonists

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SUMMARY

Quantitative analysis of a drug or hormone action expressed through increased rates of cAMP synthesis is dependent on the activities and affinities of the phosphodiesterases (PDEs) that hydrolyze the cAMP. It is shown herein that, on both theoretical and experimental grounds, significant contribution to hydrolysis by high affinity PDE activity would lead to striking departures from proportionality of the relationships between rates of cAMP synthesis and intracellular cAMP accumulations. Simulations of cAMP decay curves, cellular concentration-response curves to agonists, and cAMP time courses were used to predict the consequences of a high affinity PDE in an intact cell experimental system. Specifically, the simulations predicted 1) an upward convexity in the cAMP log-decay curve, 2) an upward concavity in the concentration-response curve, and 3) the amplification of small differences in initial rates resulting in large differences in accumulation at long times. S49 WT lymphoma cells demonstrated properties that fitted the predictions of the simulations in an experimental system. We conclude that the presence of significant high affinity PDEs has a profound effect on the nature of the response of cells to agonists and antagonists of the adenylate cyclase system. In addition, intracellular rates of hydrolysis were compared with PDE activities measured in cell-free systems. The data showed that cell-free estimates of PDE activity seriously overestimated the intracellular rates of cAMP hydrolysis.

In hormone or neurotransmitter actions mediated by cAMP, elimination is a major determinant of cellular levels of the nucleotide (1-3). Elimination of cAMP is comprised of hydrolysis by the PDEs and by egress of the cyclic nucleotide from the intracellular to extracellular milieu. In those mammalian cells where rate constants for egress and hydrolysis have been measured, the latter was quantitatively much more important (4, 5). Thus, accurate quantitation of cellular cAMP metabolism requires a thorough understanding of cAMP hydrolysis.

Several classes of cyclic nucleotide PDEs have been described and characterized by kinetic parameters, substrate specificity, and immunospecificity (6). Kinetically, the PDEs have been classified into the broad categories of high and low affinity (6, 7). Typically, high affinity PDEs have had K_m values of around 1 μM and low affinity PDEs have K_m values in the region of 100 μM (6, 7).

If the intracellular K_m for the high affinity enzymes is similar to that measured in cell free preparations, then there should be important consequences reflected in cellular cAMP accumulations. This is especially so when basal concentrations of cAMP in many intact cells are less than the K_m for the high affinity enzymes. If a hormonal stimulation caused cAMP accumulation within a cell to attain a concentration that was many times the K_m of a PDE, then that PDE would be effectively saturated and operating at its maximum rate. A further increase in the rate of cAMP synthesis could then cause a disproportionately large increase in accumulation unless there were substantial quantities of low affinity PDE present. High affinity forms of PDE are widely distributed (6, 7), have been extensively studied in cell-free preparations, and have been reported to be under hormonal control in some cells (8-10). It was, therefore, important to determine if the high affinity kinetics demonstrable in purified preparations are relevant to intact cells. Discussions of the consequences of high affinity PDE on cAMP accumulations have been published (11, 12). However, there have not been reports demonstrating whether such consequences can be observed *in situ*. In the present communication we describe three diagnostic tests for the operation of high affinity PDE in intact cells. These tests were applied to S49 lymphoma cells, cell free preparations which have been shown to contain a substantial fraction of high affinity PDE (13). These experiments have demonstrated that a high affinity cAMP hydrolytic process is operative in intact S49 cells.

Experimental Procedures

Materials. *l*-Epinephrine bitartrate was purchased from K & K Biochemicals (Plainview, NY) and forskolin from Calbiochem (San

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ABBREVIATIONS: PDE, cAMP phosphodiesterase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IBMX, 3-isobutyl-1-methylxanthine.

Diego, CA) [2,8-^{3}H]adenine (35 Ci/mmol), [8-^{14}C]adenine (55.6 mCi/mmol), [U-^{14}C]ATP (501 mCi/mmol), and [8-^{14}C]cAMP (52.1 mCi/mmol) were purchased from ICN Radiochemicals (Irvine, CA). Other chemicals were of the highest grade commercially available. S49 wild type cells were kindly provided by Dr. Henry Bourne of the University of California at San Francisco.

Cell culture. S49 cells were propagated as described by Bourne *et al.* (14). The cells were grown in 1-liter roller bottles with antibiotic supplement (GIBCO) to the Dulbecco's modified Eagle's medium, which was supplemented by 5% horse serum. The population density was maintained between 1.5 and 2 × 10^6 cells/ml by daily addition of fresh media.

Experimental incubations. Cellular cAMP accumulation was measured as described previously (1) with minor modifications being necessitated by the use of S49 cells. Briefly restated, the adenine ribonucleotide pool was labeled with [2,8-^{3}H]adenine, and the percentage conversion of [^{3}H]AXP to [^{3}H]cAMP was expressed as cAMP/(cAMP + AXP), where AXP is radioactivity in those fractions containing ATP and ADP. After 1-hr incubation with [2,8-^{3}H]adenine, the cells were washed three times in fresh medium and incubated for another hour without additions. Each experimental point contained 10 × 10^6 cells in 0.5 ml. The incubation medium was serum-free Dulbecco's medium without $NaHCO_3$, with additional NaCl (37 mM), and with buffering capacity increased by the addition of HEPES (20 mM). Since the S49 cells were grown in suspension, the experiments were terminated after centrifuging for approximately 10 sec in an Eppendorf microfuge. The medium was rapidly removed by aspiration, the pellet loosened by agitation on a vortex stirrer, 0.5 ml of the "killing" solution containing radioactive tracers to monitor cAMP recovery and 5% trichloroacetic acid added, and the tubes placed in ice. After centrifugation, the [8-^{3}H]AXP and [8-^{3}H] cAMP in the cell extract supernatants were isolated as previously described. In experiments in which catecholamines were employed, 0.1 mM ascorbate and 1.0 mM thiourea (final concentrations) were present in all samples. The validity of cellular cAMP measurements made by the preincubation method described above was established by occasional use of a radioimmunoassay for cAMP (15) with cells treated identically.

Phosphodiesterase enzyme assay. Cells were harvested by centrifugation and washed twice (to remove growth medium) in 50 μl of ice-cold 0.25 M sucrose in 20 mM Tris-HCl (pH 7.4) with 7 mM β-mercaptoethanol. They were then suspended in 20 mM Tris-HCl (pH 7.4), containing 2 mM $MgCl_2$, 0.4 mM phenylmethanesulfonyl fluoride, 5 μM leupeptin, 25 μg/ml trypsin inhibitor, 2 mM benzamidine, 5 mM β-mercaptoethanol, and 0.25 M sucrose, to give approximately 250 × 10^6 cells/ml. They were lysed after equilibration for 20 min in a Parr bomb at 500 p.s.i. The lysate was assayed without further purification.

The assay was based on the method described by Thompson and Appleman (16). The standard reaction mixture had a total volume of 250 μl and contained 40 mM Tris-HCl (pH 7.4), 5 mM $MgSO_4$, 5 mM β-mercaptoethanol, and 1 mg/ml bovine serum albumin. The cAMP used in the assay contained 1 × 10^6 dpm [^{3}H]cAMP that was purified once a month with anion exchange chromatography.

The assay was started by the addition of 100 μl of the enzyme to 150 μl of the assay mixture prewarmed to 30°. The enzyme solution was diluted to keep the substrate conversion at <10%. The reactions were terminated after 10 min at 30° with the addition of 50 μl of 3 mM cAMP followed by heating for 1 min in a vigorously boiling water bath. The samples were cooled on ice before the second incubation with 100 μl of a solution containing 1 mg Crotalus atrox venom/ml H_2O. This incubation was at 30° for 15 min and was stopped with the addition of 600 μl of 20 mM ammonium formate (pH 7.4) with 15 mM EDTA and 10^{-4} M adenosine (17).

Adenosine and other dephosphorylated products derived from cAMP were separated from cAMP by column chromatography with QAE-Sephadex A25, formate form (18). The eluate from the 1-ml sample and two additional 1-ml samples of 20 mM ammonium formate were collected from the QAE-A25 columns and counted as a gel in 10 ml of Liquiscint (National Diagnostics). When [^{14}C]adenosine or [^{14}C]cAMP was included in the sample, more than 95% of the adenosine and no cAMP were recovered in the first 3-ml fraction collected.

Simulations and calculations. Runge Kutta numerical solutions to differential equations were calculated on an IBM AT using the calculus program published by John Wiley and Sons (New York). Error bars in the graphs represent standard error of the mean, defined as

$$\sqrt{\sum(X - \bar{X})^2/((n-1)n)}$$

where X is the variable and n is number of estimations.

Theoretical Considerations

Basis for nonlinearity of cAMP decay curves. The potential effect of a high affinity phosphodiesterase on cAMP accumulation has been discussed in some detail by Fell (12) and invoked by some earlier authors (3, 11). In any system where high and low K_m enzymes are active in destroying cAMP, the destruction of cAMP can be described by the following equation:

$$\frac{-d\,[\text{cAMP}]}{dt} = \frac{V_H\,[\text{cAMP}]}{K_H + [\text{cAMP}]} + \frac{V_L\,[\text{cAMP}]}{K_L + [\text{cAMP}]} + \frac{V_E\,[\text{cAMP}]}{K_E + [\text{cAMP}]} \quad (1)$$

where V is the maximal rate for the enzyme or process, H is high affinity enzyme, L is low affinity enzyme, and E is escape process. At cAMP concentrations lower than the K_m for the low affinity enzyme or the escape process, those two processes will be effectively first order. Therefore,

$$\frac{-d\,[\text{cAMP}]}{\text{dt}} = \frac{V_H\,[\text{cAMP}]}{K_H + [\text{cAMP}]} + \text{k}_l\,[\text{cAMP}] \quad (2)$$

where $k_l = (V_L/K_L) + (V_E/K_E)$. When elimination of cAMP is a first-order process, the decay of cAMP accumulation after complete cessation of hormonal stimulation is given by:

$$\frac{d\,[\text{cAMP}]}{\text{dt}} = -k_{\text{decay}}\,[\text{cAMP}] \quad (3)$$

where k_{decay} is the decay parameter. When k_{decay} is invariant with cAMP accumulation a plot of $\ln([\text{cAMP}] - [\text{cAMP}]_\infty)$ against time gives a straight line with slope equal to $-k_{\text{decay}}$. This condition will hold for cells that do not possess significant high affinity components for either hydrolysis or egress of cAMP. Eq. 2 shows that when there is a significant high affinity process present, k_{decay} must be replaced by the term:

$$\frac{V_H}{K_H + [\text{cAMP}]} + k_l \quad (4)$$

Thus, k_{decay} and [cAMP] would be inversely related. Consequently, a plot of $\ln([\text{cAMP}] - [\text{cAMP}]_\infty)$ against time would be convex upward. Fig. 1 presents hypothetical curves calculated from Eq. 2 with K_H set arbitrarily at 1.0 unit concentration and k_l at 0.1 min^{-1}. V_H was given the values indicated in Fig. 1, and the initial concentration of cAMP was 25 units. When $V_H = 0$, cAMP decay was first order, and the plot was a straight line. Increasing values for V_H, corresponding to increasing activities of a high affinity process, caused increasing departures from linearity, which appeared as an upward convexity. Note that the same general argument would apply if there were more than one type of high affinity PDE or if the high affinity

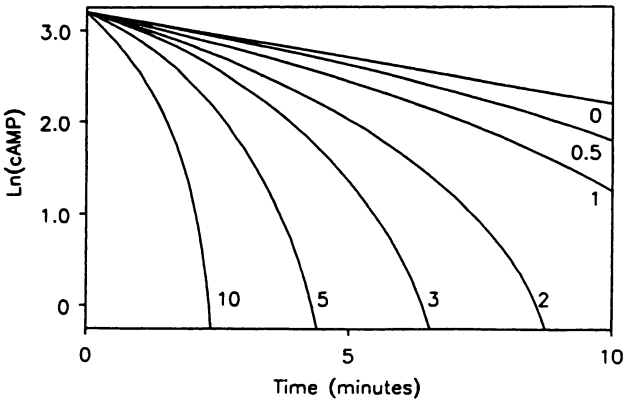

Fig. 1. The effect of high affinity on cAMP decay in intact cells. Curves were calculated by substituting term 4 for k_{decay} in Eq. 3 and solving analytically. The numbers associated with each curve give the value V_{max} for the high affinity enzyme used in the calculation of that curve.

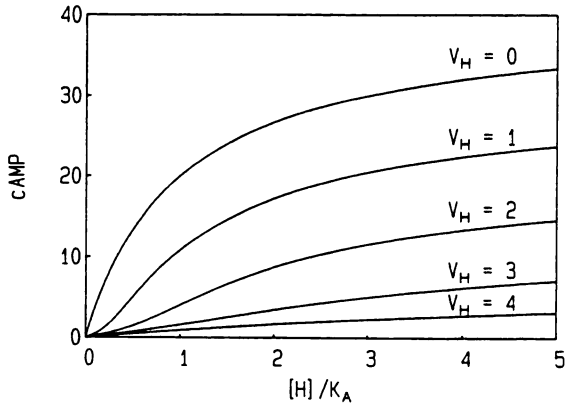

Fig. 2. The effect of increasing amounts of high affinity phosphodiesterase on cAMP accumulation in intact cells. The curves were calculated from equation 6 with k_s = 4 units/min, K_H = 1 unit concentration, and k_i = 0.1 min^{-1}.

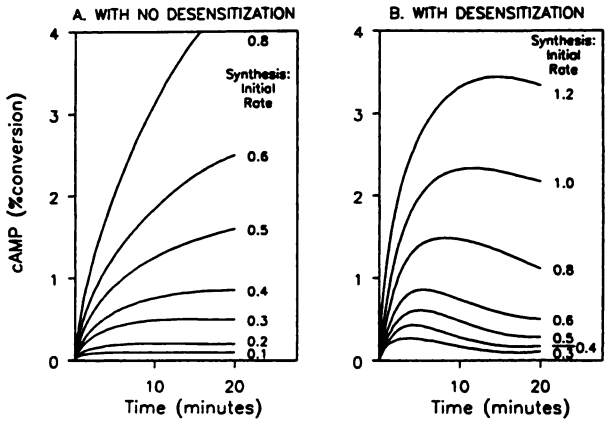

Fig. 3. Theoretical time courses for accumulation of cAMP in cells with an effective high affinity phosphodiesterase. A, the calculation assumed that cAMP was subject to hydrolysis by a high affinity enzyme with V_{max} = 0.42 units/min and a K_m of 0.23 units and by a low affinity enzyme with effectively first order kinetics and a rate constant of 0.07 min^{-1}. The constant rates of synthesis for each curve are given. B, the calculation was as in panel A, but the rate of synthesis was assumed to decline with time as described in the text.

PDE were itself negatively cooperative. The important point is that there be a cAMP concentration term in the denominator for k_{decay} (Eq. 4). This will remain true whether PDE is Michaelian or negatively cooperative.

Theoretical basis for pseudo-cooperative kinetics. Erneux *et al.* (19) have suggested that a sufficiently potent high affinity phosphodiesterase would result in hormone concentration-cAMP accumulation curves in intact cells that were concave upward on a linear plot. In this section we calculate the numerical values of the enzyme parameters that are necessary to produce an upward concavity in the concentration-response curve.

At a steady state of cAMP accumulation, the rate of synthesis (v_s) is equal to the rate of removal (by hydrolysis plus egress); therefore:

$$v_s = \frac{V_H\,[\text{cAMP}]}{K_H + [\text{cAMP}]} + k_i\,[\text{cAMP}] \qquad (5)$$

When v_s is described by a rectangular hyperbola in hormone concentration (h) (i.e., when the Hill coefficient of the relationship is unity) we can write:

$$v_s = \frac{k_{s(MAX)}h}{K_A + h} = \frac{V_H\,[\text{cAMP}]}{K_H + [\text{cAMP}]} + k_i\,[\text{cAMP}] \qquad (6)$$

where $k_{s(MAX)}$ is the maximum rate of synthesis at high hormone concentrations and K_A is the hormone concentration that produces half maximal response. The curves illustrated in Fig. 2 were calculated from Eq. 6 by assigning arbitrary values to $k_{s(MAX)}$, V_H, K_H, and k_i. They represent the steady state accumulation of cAMP in cells with various activities of high affinity phosphodiesterase present. Where removal of cAMP is first order (i.e., when $V_H = 0$), the concentration-response curve is a rectangular hyperbola (i.e., it also has a Hill coefficient of unity) with accumulation proportional to the rate of synthesis. However, increasing the contribution of the high affinity enzyme (increasing values for V_H) relative to the total rate of hydrolysis produced increasing degrees of upward concavity at the lower hormone concentrations.

Time course of cAMP accumulation. The effect of a high affinity PDE on the time course of cAMP accumulation is given by the solution to the following differential equation:

$$\frac{d\,[\text{cAMP}]}{dt} = v_s - k_i\,[\text{cAMP}] - \frac{V_H\,[\text{cAMP}]}{(K_H + [\text{cAMP}])} \qquad (7)$$

where v_s is the rate of cAMP synthesis. The rationale for the terms that describe the removal of cAMP (i.e., k_i[cAMP] + V_H[cAMP]/(K_H + [cAMP])) have been described above. The equation may be solved by using the Runge-Kutta numerical technique. In the present case this was done by an IBM PC using a commercially available calculus program. Fig. 3A shows simulations of a set of time courses with constant rates of synthesis as indicated. Other values used in the calculations were V_H = 0.42 units/min, K_H = 0.23 units, and k_i = 0.07 min^{-1}. These values were not completely arbitrary, for they were consistent with the values that must be assumed to explain the experimental data described in Results (where 1 unit corresponds to 1% conversion of ATP). Fig. 3B shows a similar set of simulations with rates of synthesis that decrease with time. These calculations with decreasing synthetic rates were designed to simulate the effects of desensitization. In all cases the rates of synthesis as a function of time were given by $v_s = 0.5v_s^0(1 + e^{-0.2t})$, where v_s^0 is initial rate of synthesis. This corresponded to a reduction of the rate of synthesis at long times to one half the initial value. The value of 0.2 min^{-1} in the exponent was the rate constant for the desensitization

process and was equivalent to a half-time for desensitization of ln2/0.2 = 3.5 min. The salient point of both figures was that there was a range of cAMP accumulations (between ~0.5 and 1.0% conversion) where a modest increase in the rate of synthesis led to a very large increase in the extent of accumulation. This range of cAMP accumulations is encountered at values slightly larger than the value of K_m for the high affinity PDE chosen for this simulation. Over this range of cAMP concentrations the contribution of the low affinity PDE to the hydrolysis was small, and the high affinity PDE was more than 50% saturated. Consequently, increases in cAMP accumulation did not produce proportional increases in hydrolysis.

Results

Bourne *et al.* (13) showed that the bulk of the PDE activity in wild type S49 cells was of the high affinity type. We have fractionated cell free extracts of wild type S49 cells on DEAE cellulose using the procedure of Erneux *et al.* (17). More than 80% of the activity (assayed at 1 μM cAMP) was in the peak that corresponded to the high affinity enzyme (data not shown). The identification of the PDE activity with the high affinity enzyme was supported by its lack of sensitivity to EGTA or to added Ca^{2+} and calmodulin. These data were in agreement with those published previously (13). Therefore it seemed that these cells would be useful for testing the suitability of the criteria of high affinity PDE action.

Nonlinear cAMP decay in S49 cells. Fig. 4 shows the decay in cAMP levels in wild type S49 cells after the abrupt cessation of cAMP synthesis in epinephrine-stimulated cells. Cells were treated with 100 nM epinephrine for 20 min at 30° to raise cAMP accumulation to about 12% conversion, then propranolol (10 μM) was added to terminate cAMP synthesis. An incubation temperature of 30° was used to allow direct comparison with the cell free PDE activities described below. The slope of the graph became greater at low cAMP accumulation as was predicted by Eq. 4 for a system in which the high affinity PDE played a significant role in cAMP hydrolysis. Estimates for the fractional turnover constant from the data in Fig. 4 are 0.076 min^{-1} at 5% conversion, 0.127 min^{-1} at 1.5% conversion, 0.177 min^{-1} at 0.5% conversion, and 0.246 min^{-1} at 0.15% conversion. These estimates were made by determining the slopes of the curve in Fig. 4 at the above concentrations of cAMP. The equivalent cAMP concentrations are given in Table 1. The amount of radioactive ATP in these cells was essentially constant during the entire experiment, indicating that the specific radioactivity of ATP did not change significantly during that time.

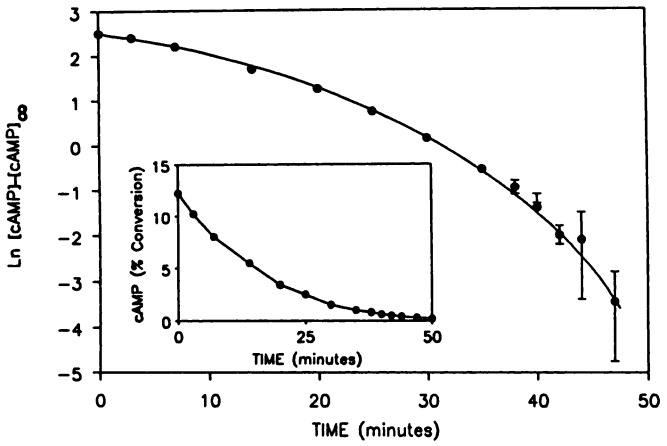

Fig. 4. Decay of cAMP accumulation in intact wild type S49 cells at 30°C after termination of hormonal stimulation. The graph plots the function ln([cAMP] − [cAMP]∞) against time. *Inset*, untransformed data.

Comparison of cell-free with intracellular PDE activities. PDE activities were determined in unfractionated lysates of S49 WT cells to compare intracellular and cell free PDE activities. Table 1 shows the PDE activities in S49 lysates measured over a range of cAMP concentrations and the intracellular PDE activities calculated from the decay data in Fig. 4 for comparison. The intracellular rates of hydrolysis were calculated using a value of 18.8% (w/v) for the intracellular protein concentration (determined using the [^{3}H]inulin technique) (20). It is clear from the data that the intracellular rates of hydrolysis were consistently lower than those measured in lysates. These differences were greatest at lower cAMP concentrations. The intracellular data are consistent with a single high affinity enzyme behaving with Michaelian kinetics. The lysate data cannot be explained so simply but are consistent with a negatively cooperative enzyme, as has been found in many other cell types (21).

cAMP decay was measured in concentrated homogenates (lysates) of S49 cells. In these experiments cells were lysed in a suspension containing HEPES buffer (20 mM) and $MgCl_2$ (3 mM) to a final protein concentration of around 3%. The protease inhibitors phenylmethanesulfonyl fluoride (0.4 mM), leupeptin (5 mM), and trypsin inhibitor (25 μg/ml) were also present. PDE activity was determined by an assay procedure analogous to that used in intact cells, i.e., by addition of [^{3}H]cAMP, withdrawal of samples at a series of times, and determination of the concentration of cAMP remaining in the samples. cAMP was isolated and estimated using the same column procedure as described above for experiments with intact cells. Decay curves of cAMP starting with initial concentrations of 200, 20, and 2 μM and followed over appropriate times are shown in Fig. 5. Fractional cAMP decay was greater at low cAMP concentrations, again suggesting that a high affinity component contributed significantly to the decay process. The initial slopes for the decay curves were 0.016 min^{-1} at 200 μM, 0.114 min^{-1} at 20 μM, and 0.45 min^{-1} at 2 μM cAMP. These slopes correspond to the decay constants for cAMP in those lysates at the indicated cAMP concentrations. The concentration of cell protein in the lysates was 3.72% compared with 18.8% for the intact cells, and therefore, the decay constants

TABLE 1

Comparison of intracellular and cell-free PDE activities

In the lysate assay the raw data were for exogenously added cAMP (column 1) and PDE activities (column 3); the equivalent concentrations (column 2) and decay constants (column 4) were calculated as described in the text. For the intact cells the %conversion (column 2) and the decay constants (column 6) were determined directly; the equivalent concentrations (column 1) and PDE activities (column 5) were calculated as described in the text.

cAMP		PDE in lysates		PDE in intact cells	
		Activity	k_{decay}	Activity	k_{decay}
μM	% converted	nmol/min · mg protein	min^{-1}	nmol/min · mg protein	min^{-1}
100	5	0.0510	0.096	0.040	0.076
30	1.5	0.0302	0.189	0.020	0.127
10	0.5	0.0288	0.54	0.009	0.177
3	0.15	0.0188	1.18	0.004	0.246
1	0.05	0.0144	2.71		
0.1	0.005	0.0034	6.39		

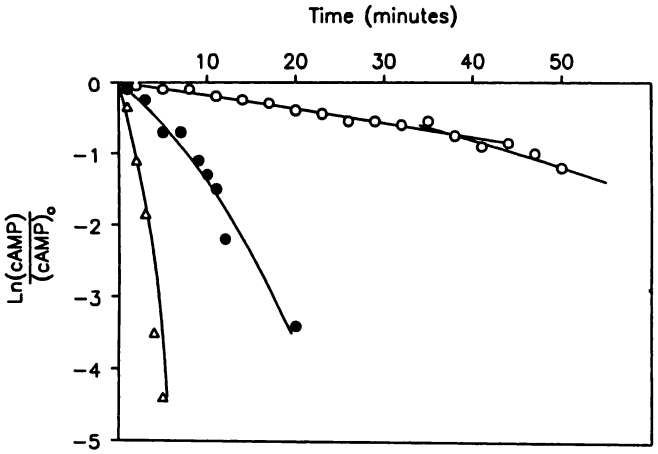

Fig. 5. Decay of cAMP accumulation in concentrated homogenates of wild type S49 cells at 30°. cAMP was added after rupture of the cells The initial concentrations of the exogenously added cAMP follow: ○, 200 μM; ●, 20 μM; and △, 2 μM.

corrected for the difference in PDE concentration were 0.081, 0.576, and 2.27 min^{-1} at 200, 20, and 2 μM cAMP, respectively. These data closely resemble those found with the PDE activities determined by conventional assay and displayed in Table 1. Again, the cell lysate PDE activity was consistently greater than that found in intact cells.

Upward concavities in concentration-response experiments. In the theoretical section it was demonstrated that the action of a dominant high affinity PDE would cause concentration-response curves to be upwardly concave at low hormone concentrations. There was an experimental complication which was not considered in that section. This was the uncertainty that arises from desensitization when cells are treated with an agonist until a steady state of cAMP accumulation is attained. To avoid that complication in the experiments described here, the cells were stimulated for only 1 min. Although this procedure has the advantage of minimizing desensitization, it reduced the effect of the cellular PDEs on measured cAMP accumulation. Thus the test was made very stringent under these conditions, and a modest high affinity PDE activity might not be detected. Fig. 6 shows four separate concentration response curves for S49 wild type cells measured on separate occasions. There is no significance to the pairings in parts A and B; data are presented in two figures solely for the sake of clarity. All data points were measured of 1-min accumulations. Three time courses showed a clear upward concavity, whereas the fourth (Fig. 6A) did not. In fact different preparations of S49 cells varied considerably in their basal cAMP accumulations and in their response to hormones.

To accentuate the role of PDE in cAMP accumulation at short times the experiment was performed using the high PDE S49 variant K_{30a} (13). The data from this experiment are shown in Fig. 7. As expected for a variant containing a high concentration of high affinity PDE, there was a pronounced upward concavity in the graph. Also included in Fig. 7 are concentration-response data obtained in the presence of the drug IBMX, which is a potent PDE inhibitor. The elimination of the upward concavity by the PDE inhibitor is good evidence that that feature was in fact a consequence of PDE action. One would not have expected IBMX to have had such an action if the upward concavity were the result of a positively cooperative

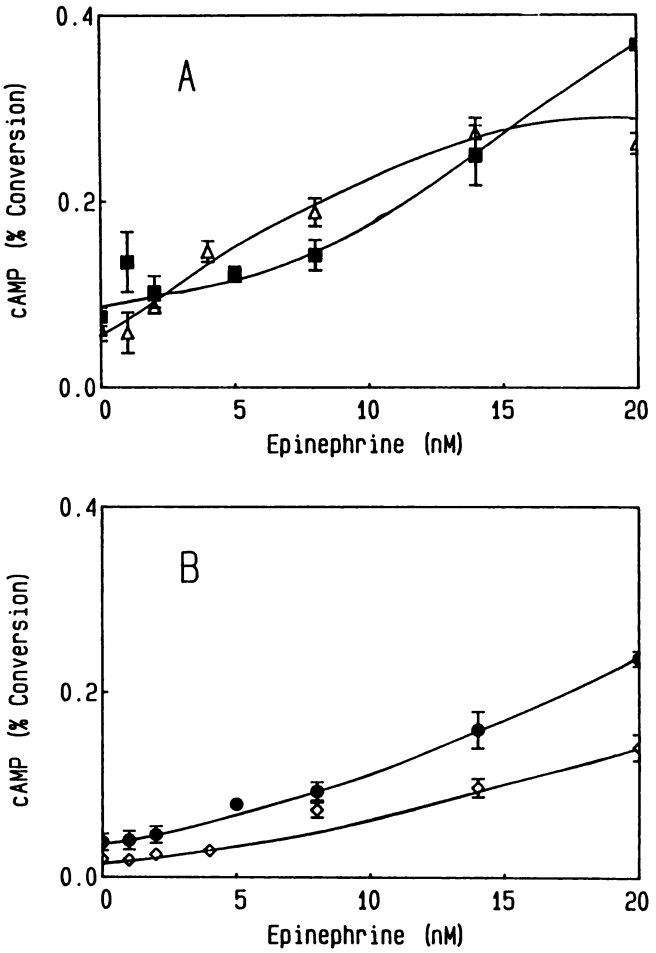

Fig. 6. The concentration response of wild type S49 cells to epinephrine stimulation at 37°. The data from four separate experiments are shown. Incubation times were 1 min.

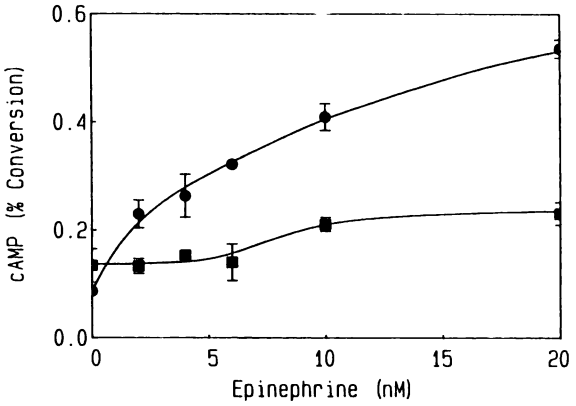

Fig. 7. The concentration response of the S49 high PDE variant K_{30a} to epinephrine. Stimulation was for 1 min at 37°: ■, no PDE inhibitor; ●, plus 1 mM IBMX.

stimulation of the cyclase by the hormone. To test the effect of a change in stimulating agent the experiment was repeated using S49 cyc^- cells stimulated by forskolin. It had been shown that (unlike wild type S49 cells) the adenylate cyclase from cyc^- cells responds to forskolin with a Hill coefficient of around unity (22). That is, the rate of synthesis as a function of forskolin concentration is accurately given by Eq. 6. Moreover, since there has been little sign of desensitization to forskolin

in these cells we could use 20-min incubations. The results of this experiment are shown in Fig. 8. The upward concavity in the concentration response curve reinforces the existence of the phenomenon and shows that its demonstration is not dependent on stimulation by any one type of agonist of the adenylate cyclase system.

Time courses of cAMP accumulation. Time courses for cAMP accumulation in cells stimulated by 10, 30, 100, and 300 nM epinephrine at 30°C are shown in Fig. 9. These data illustrate the salient points of the simulations shown in Fig. 3. First, small differences in the initial rate of accumulation produced by two different concentrations of agonist can be magnified at longer times. Second, there is a range of cellular cAMP concentrations over which a modest increase in agonist concentration (and hence adenylate cyclase activity) leads to a very large increase in cAMP accumulation. In the present case this is most noticable when the 10 and 30 nM epinephrine stimulations are compared. Third, the "peak" in the cAMP accumulation only occurs for the 10 mM epinephrine stimulation; at higher epinephrine concentrations there were no peaks during the 48-min time courses.

Discussion

Taken together, these data demonstrate that there is high affinity hydrolysis of cAMP in the intact cell. The most direct evidence comes from the decay data described in Fig. 4. Since the measurements were made after cessation of adenylate cyclase stimulation, the data are difficult to explain by a mechanism involving cAMP synthesis. Therefore, this provides strong support for a nonlinear cAMP decay, that is, for the action of a high affinity PDE. However, although the main features of the high affinity system are maintained in the intact cell, a detailed quantitative comparison with cell free data indicates that the action of the high affinity enzyme is considerably blunted there. Table 1 showed that although cell free measurements were reasonable predictors for intracellular cAMP destruction at high cAMP concentrations, at lower cAMP concentrations intracellular hydrolyses were much less than the values predicted by the cell free measurements. In fact it was in the range of concentrations where the high affinity enzyme would be most effective (below 3 μM) that the disparity was the greatest. Moreover, double-reciprocal plots of hydrolytic rates and cAMP concentrations show negative cooperativity for the lysate but not the intact cell rates (graphs not shown). Possible explanations for these disparities follow. 1) The high affinity enzyme is activated as the result of cell rupture, and the cell free measurements are artifactually high. This could be the result of the dilution of an intracellular inhibitor, a changed interaction of the enzyme with cell organelles, or simply because of the buffer used in the cell free assay. The assay conditions for PDE that have evolved were presumably developed with the intention on the part of the investigators to maximize and preserve activity. It would not be surprising, therefore, if the popularly used assay systems supported greater enzyme activities than the intracellular milieu. 2) There is compartmentalization within the cell (23, 24). 3) Some cAMP is bound to other macromolecules in the cell and is therefore unavailable to the PDE for hydrolysis. One can be quite sure that this effect will be present to some extent, and in the case of S49 cells it is probably significant. N. Prashad (personal communication) has estimated the concentration of cAMP-dependent protein kinase regulatory subunit in these cells at 1.9 μM. One would therefore expect a high fraction of the cAMP in the μM range to be bound to the kinase and unavailable to the PDE for hydrolysis (25, 26). All three explanations could contribute to the observed discrepancy between the cell free and the intact cell data. The second two proposed mechanisms would also have the effect of increasing the apparent K_m of the high affinity PDE determined intracellularly.

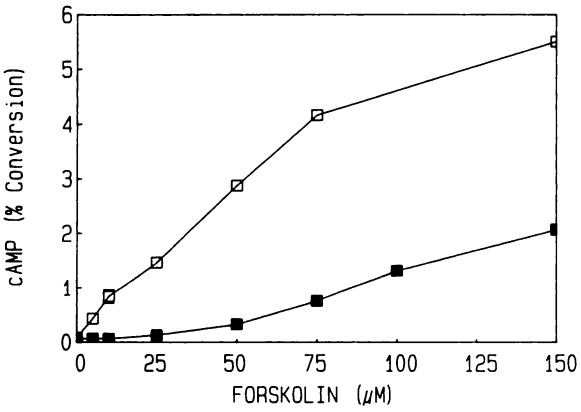

Fig. 8. The concentration response to forskolin of the S49 variant cyc⁻. Incubations were for 20 min at 24°; ■, no PDE inhibitor; □, plus 1 mM IBMX.

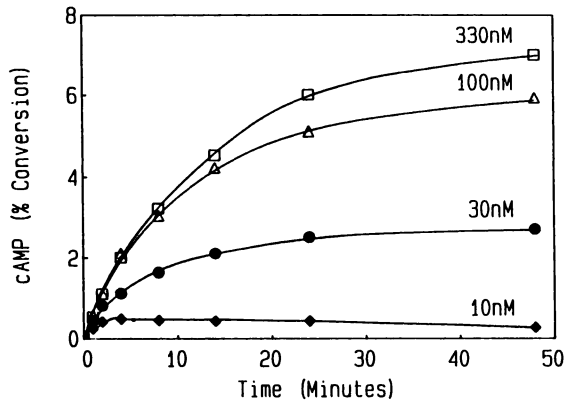

Fig. 9. Time courses of cAMP accumulation in wild type S49 cells in response to epinephrine stimulation at 30°. Epinephrine concentrations are shown.

Some aspects of intracellular concentration response profiles have been discussed previously by Erneux *et al.* (19). Those authors argued for theoretical reasons that a high affinity PDE should result in a Hill coefficient for response as a function of hormone concentration of greater than unity, i.e., that there should be positive cooperativity in response to the hormone. We have not adopted this approach in the present communication because it does not allow for an easy comparison of cell free with intact cell data. It is, however, less stringent than the linear plots we have used, and it should be possible in principle to demonstrate Hill coefficients of greater than unity from data that do not shown an obvious upward concavity on linear plots.

The time courses of cAMP responses as shown in Fig. 9 are probably the most relevant to understanding the effects that the high affinity PDE has on the response of cells to hormones that act via the cAMP mechanism. However, it is those data that are most difficult to compare with cell free PDE activities

and the simulations calculated in Theoretical Considerations are intended to describe the experiments only in very general terms. What both the experimental results and the simulations show is that there is a range of hormone concentrations over which a relatively small increase in the amount of hormone results in a greater than proportional increase in cAMP accumulation, and that departure from proportionality increases with time. This has the potential for, at least to some extent, an "all or nothing" response to the hormone.

This communication contains intact cell experiments that demonstrated the effects of high affinity PDEs made in the presence of epinephrine (Figs. 6, 7, and 9) and where epinephrine stimulation has been terminated (Fig. 4). If epinephrine had a short term effect on PDE activity, the data from those classes of experiment might not be directly comparable. This does not seem to be a problem over the time courses considered in these experiments. We have shown (27) that the cAMP decay constant determined in the absence of epinephrine and the cAMP fractional turnover constant determined in its presence do not differ significantly in S49 cells. Therefore, in the short term, epinephrine appears to have no significant effect on the PDE activity in these cells.

The data presented in this communication go some way toward explaining the presence of two major (kinetic) classes of PDEs in many cells (6, 7). The high affinity PDE in S49 wild type cells has a K_m of around 1 μM. Therefore, when cAMP accumulations exceed 1 μM by a few-fold a further increase in accumulation would not result in a much greater increase in cAMP hydrolysis by the high affinity PDE. This in turn would allow a massive build-up of cAMP unless there were a low affinity hydrolytic capacity also present to react to the high concentrations. Stimulation by the appropriate hormone of many cell types results in cAMP accumulations equivalent to tens or even hundreds of micromolars. It is not surprising, therefore, that low affinity PDEs should be widespread. Indeed, we have shown that in some cell types (e.g., fibroblasts WI-38 and VA13) the major PDE is of the low affinity type (1).

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