Comparative Studies on Cyclic AMP Binding and Protein Kinase in Cyclic AMP-responsive and -unresponsive Walker 256 Mammary Carcinomas

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N\textsuperscript{6},O\textsuperscript{2}'-Dibutyryl cyclic adenosine 3':5'-monophosphate (Bt,cAMP) treatment in vivo inhibits growth of one type of Walker 256 mammary carcinoma (W256) transplants (Bt,cAMP-responsive) but does not affect growth of other W256 transplants (Bt,cAMP-unresponsive). Cyclic AMP-binding proteins and protein kinases are present in the cytosol of both responsive and unresponsive W256 but have qualitative and quantitative differences. Scatchard plots for the binding of cAMP have been compared at pH 4.5 and 6.5. At both pH values, the responsive tumor shows two types of cAMP binding, one with a higher affinity ($K_d < 10^{-4}$ M) for the nucleotide, the other with a lower affinity ($K_d > 10^{-7}$ M). The unresponsive tumor shows mainly lower affinity binding ($K_d > 10^{-7}$ M) at pH 4.5 and higher affinity binding ($K_d < 10^{-7}$ M) at pH 6.5. The maximum binding is significantly greater in the responsive tumor at pH 6.5 and in the unresponsive tumor at pH 4.5. Under all conditions tested, the unresponsive tumor shows a decreased binding compared to the responsive tumor. The binding equilibrium is reached faster and the peak binding lasts longer in the responsive tumor cytosol than in the unresponsive tumor cytosol. The greater instability of binding by the cytosol of the unresponsive tumor is also shown in cAMP exchange reaction carried out at 0° and 23°, respectively. Preincubation at 50° for 15 min decreases the higher affinity binding of the unresponsive tumor by about 50%, but has no effect on the binding of the responsive tumor. Protein kinase activity in the cytosol of the responsive tumor is stimulated about 5-fold by cAMP, but the enzyme in the unresponsive tumor is stimulated only 2-fold. Cyclic AMP causes a decrease in the $K_m$ of the responsive tumor enzyme for ATP but has no effect on the affinity of the unresponsive tumor enzyme for ATP. The catalytic properties of the kinase from both responsive and unresponsive W256, however, are similar in the absence of cAMP.

The difference in "responsiveness" to Bt,cAMP of two types of Walker 256 mammary carcinoma, therefore, appears to be associated with the qualitative differences of their cAMP-binding proteins, the regulatory subunits of the cAMP-dependent protein kinases.

Cyclic AMP is well known as an intracellular "second messenger" for many hormones and plays an important role in the regulation of cell growth. However, the molecular mechanism of cAMP action, especially that involving the control of tumor growth in vivo, is not clear. We have reported the selection of two cell populations from Walker 256 rat mammary carcinoma (W256), one type regressing (Bt,cAMP-responsive) and the other type growing (Bt,cAMP-unresponsive) under Bt,cAMP treatment (1). We have also presented evidence that these cell populations may prove useful in defining the role of cAMP in the regulation of tumor growth in vivo (2). Recently, we reported a correlation between Bt,cAMP unresponsiveness in vivo and altered cAMP binding by tumor cytosol (3). One function of cAMP-binding proteins in many eukaryotic systems is to serve as regulatory subunits for cAMP-dependent protein kinases (4-10). Thus, the determination of protein kinases may be helpful for the elucidation of cAMP action in its "growth regulatory function."

This paper presents a further analysis of the cAMP-binding reaction as well as the determination of protein kinases in Bt,cAMP-responsive and Bt,cAMP-unresponsive W256.

EXPERIMENTAL PROCEDURES

Materials—Theophylline, cAMP, [\textsuperscript{3}H]cAMP (27 Ci/mmol) and [\textsuperscript{14}C]cAMP (49.2 Ci/mmol), and Bt,cAMP were obtained from Schwarz/Mann, Orangeburg, N.Y.; cyclic AMP, ATP, UTP, GTP, ADP, adenosine, d-ribose, 5'-AMP, calf thymus histones (type II) were purchased from Sigma Chemical Co., St. Louis, Mo.; DEAE-cellulose (DE52) was from Whatman Ltd., Springfield Mill, Maidstone, Kent, England; Econofluor was from New England Nuclear Corp., Boston, Mass.; [\textsuperscript{32}P]ATP (15.5 Ci/mmol) was from ICN Pharmaceuticals, Inc., Irvine, Calif. (\textsuperscript{32}P)ATP was used since it has a half-life 2 times longer than \textsuperscript{3}P and emits a radiation energy similar to that of \textsuperscript{14}C.)

1 The abbreviations used are: cAMP, adenosine 3':5'-monophosphate; W256, Walker 256 mammary carcinoma; Bt,cAMP, N\textsuperscript{6},O\textsuperscript{2}'-dibutyryl adenosine 3':5'-monophosphate; cGMP, guanosine 3':5'-monophosphate; cIMP, inosine 3':5'-monophosphate; $K_d$, apparent equilibrium dissociation constant.

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**Characterization of cAMP Binding**

**pH Optimum** — The pH dependence of the binding of responsive and unresponsive tumor cytosols is shown in Fig. 1. The binding of unresponsive tumor cytosol had a pH optimum of about 4.5 for both high and low concentrations of cAMP. At the lower concentration of cAMP, the responsive tumor cytosol also showed optimal binding at pH 4.5. However, the pH-binding curve of the responsive tumor cytosol at the higher nucleotide concentration showed a pH optimum of 6.5.

**Temperature Dependence of cAMP Exchange** — The effect of temperature on the extent of cAMP exchange was compared between cytosols of responsive and unresponsive W256 (Fig. 2). Tumor cytosol was first saturated with \(^{14}C\)CAMP at 0° for 4 h and then exchanged with \(^{3}H\)CAMP (10^{-6} M) at 0° or 23°. The completion of exchange was followed for 18 h after the addition of \(^{3}H\)CAMP. At 0° (Fig. 2, left) the exchange between \(^{14}C\)CAMP and \(^{3}H\)CAMP in both cytosols proceeded at about the same rate for the first 3 h after the addition of \(^{14}C\)CAMP, and then stopped in the unresponsive tumor and continued in the responsive tumor. At 18 h, the \(^{3}H\)CAMP exchange was about 3-fold higher in the responsive tumor than in the unresponsive tumor. At 23° the exchange was much more rapid and by 3 h \(^{3}H\)CAMP binding exceeded the peak of \(^{14}C\)CAMP binding in both tumors (Fig. 2, right). The peak \(^{3}H\)CAMP binding in the unresponsive tumor declined rapidly, however, and no detectable binding was observed at 18 h; whereas the peak \(^{14}C\)CAMP-binding activity in the responsive tumor remained constant up to 18 h. No significant effect of the MgATP complex (18-20) was observed in the exchange reaction, probably because crude cytosol instead of a purified preparation was used in the reaction.

**Binding Equilibrium** — Since the cAMP exchange reaction suggested a difference in the stability of binding between the responsive and unresponsive tumors, it was important to characterize the binding reactions under a variety of conditions, i.e. temperature, pH, and cAMP concentration (Fig. 3). Since the cAMP exchange was completed within a few hours at 23° by the cytosols of both responsive and unresponsive tumors (Fig. 2), the total cAMP binding (binding to free sites as well as to those sites endogenously bound) was measured at 23° and compared with the binding at 0°. The binding equilibrium was tested over a wide range of cAMP concentrations (10^{-4} - 10^{-10} M) and at pH 4.5 and 6.5 (see Fig. 1). At 0° the binding activity in the responsive tumor reached 80% of its maximum within 2 h and continued to increase for 16 h when maximum binding was reached at all cAMP concentrations and pH values tested. In the unresponsive tumor, 80% of the maximum binding was reached only after 3 to 4 h and never reached the...
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FIG. 2. Cyclic AMP exchange by cytosols of responsive (○, △) and unresponsive (●, ▲) W256. Binding of ['QcAMP to tumor cytosol was carried out at 0°, pH 4.5. Incubation conditions were as described in the text, except for the addition of 10^{-7} M ['QcAMP (49.2 mCi/mmol) (instead of [3H]cAMP). At the times indicated the reaction was stopped with cold acetate buffer, pH 4.5. left graph, cyclic AMP exchange was measured by adding 10^{-6} M [3H]cAMP (16 Ci/mmol) to the reaction mixtures 4 h after ['QcAMP was added, then following the incorporation of [3H]cAMP and the disappearance of ['QcAMP at the times indicated. right graph, a duplicate set of reaction mixtures was maintained at 0° for 1 h after the addition of [3H]cAMP, then shifted to 23°. Six pooled tumors in each group were used in the experiment. A duplicate set of determinations was in good agreement with these results.

FIG. 3. Temperature, cAMP concentration, and pH dependence of cAMP-binding equilibrium in responsive and unresponsive W256 cytosols. Time course of binding was determined at cAMP concentrations of 10^{-7} M (○, ●), 10^{-8} M (△, ▲), 10^{-9} M (○, △), 10^{-10} M, and 10^{-11} M. Data on the last two concentrations are not shown. Binding is shown at the two optimum pH values (see Fig. 1) of 4.5 (open symbols) and 6.5 (closed symbols), and at two temperatures (0° and 23°). Incubation conditions were as described in the text and the reaction was stopped at the times indicated. Six pooled tumors in each group were used in the experiment. The values represent replicate experiments.

maximum binding observed at 23°. At 23° the responsive tumor reached peak binding within 1 h and maintained high levels for at least 16 h. The unresponsive tumor, on the contrary, reached peak binding after 3 h, showed a decrease in binding at 4 h, and lost a significant amount of binding activity after 16 h. For all cAMP concentrations tested, the binding capacity for the unresponsive tumor at 64 h was zero as compared to 50% (of the peak binding) for the responsive tumor. The difference in the binding between responsive and unresponsive tumors based on pH dependence, shown in Fig. 1, was reproduced in the binding equilibrium at both temperatures. The binding in the unresponsive tumor was higher at pH 4.5 than at pH 6.5 regardless of the cAMP concentration. In contrast, the binding in the responsive tumor was higher at pH 6.5 than at pH 4.5 at higher cAMP concentrations of 10^{-6} M and 10^{-7} M; whereas at cAMP concentrations lower than 10^{-8} M, the binding was higher at pH 4.5.

Scatchard Plots for cAMP Binding—The binding components in the responsive and unresponsive tumors were further analyzed by Scatchard plots (21). Results of such experiments, compared at two temperatures and pH values, respectively, are shown in Fig. 4. In the responsive tumor, the Scatchard plots at both pH 4.5 and 6.5 showed two major types of binding sites: a higher affinity binding (Kd = 1.5 × 10^{-9} M) was predominant at pH 4.5 and a lower affinity binding (Kd = 1.3 × 10^{-7} M) was predominant at pH 6.5. The pH optimum for high and low binding affinity was revealed in the maximum binding capacity. The amount of higher affinity binding, estimated by extending the upper curve of the Scatchard plot to the "bound" axis, was greater at pH 4.5 while the amount of lower affinity binding (the intercept on the bound axis minus the extended intercept due to higher affinity binding) was greater at pH 6.5. The unresponsive tumor, unlike the responsive tumor, showed mainly a single type of binding. The major binding component revealed at pH 4.5 exhibited a Kd of 1.3 × 10^{-9} M which was identical with that of the lower affinity binding.
binding present in the responsive tumor. The upper binding curve, however, showed a small upward concavity which indicated a higher affinity binding ($K_d = 3.0 \times 10^{-4}$ M) that became more apparent at pH 6.5. The maximum binding by both higher and lower affinity sites in the unresponsive tumor was greater at pH 4.5 than at pH 6.5. This reverse pH optimum of the lower affinity binding in the unresponsive tumor might be due to the instability of the binding at pH 6.0 (see Fig. 3). The temperature of the binding reaction did not influence the shape of the binding curves but did influence markedly the intercept on the bound axis, the maximum binding. The maximum binding in both tumors at both pH values was significantly greater at 23° than at 0°.

**Heat Stability of cAMP Binding** - The difference in the binding stability between responsive and unresponsive tumors was further evidenced when tumor cytosols were heated at several temperatures. The binding activities of the heated cytosols were compared with unheated controls (Fig. 5). At low (10^{-8} M) and high (10^{-6} M) cAMP concentrations, binding by the responsive tumor was more stable than binding by the unresponsive tumor. Greater heat stability was shown by the responsive cytosol, particularly at 50° for 15 min. when 95% of the binding activity of the unheated control was retained; under the same conditions, the unresponsive tumor reduced its binding capacity to 30%. This occurred at the low cAMP concentration which detects high affinity binding (16). The heat stability test on mixed cytosols from both responsive and unresponsive tumors gave additive results. The heat stability of cAMP-binding proteins can be influenced by the intracellular cAMP concentrations which may have a protective effect on the binding sites during heating. However, the heat lability of the unresponsive tumor binding was not due to a lower cAMP concentration since the cAMP concentrations in both responsive and unresponsive tumors were shown to be similar (2). Moreover, 8 mM β-mercaptoethanol decreased the heat lability of the binding in the unresponsive tumor but had little or no effect on the heat stability of the responsive tumor binding.

The Scatchard plots of the binding data from heated and unheated tumor cytosols are shown in Fig. 6. In the responsive tumor, heating resulted in about a 50% loss of the maximum binding (the intercept on the bound axis), while the amount of higher affinity binding (estimated by extending the upper curve to the bound axis) showed less than a 10% loss. Thus selective stability of the higher affinity sites from the heat inactivation was found in the responsive tumor. Heat treatment in the unresponsive tumor resulted in a decrease of the maximum binding to 30% of the unheated control. The slope of the binding curve of the heated cytosol was similar to that of the upper limb of the control curve and the apparent $K_d$ was 4.2 $\times$ 10^{-8} M. Thus the unresponsive tumor also showed a selective stability of the higher affinity sites from the heat inactivation. Protection of the higher affinity sites from heat denaturation, however, was significantly lower in the unresponsive tumor which lost 50% of the estimated amount of higher affinity sites after heat treatment.

The results of sucrose gradient centrifugation further demonstrated the greater loss of binding components due to heat treatment in the unresponsive tumor (data not shown). After 15 min at 50°, 75% of the radioactivity of the complex with proteins from the unresponsive tumor had dissociated to give free cAMP; whereas only 10% of the complex with proteins from the responsive tumor had dissociated to give free cAMP.

**Identity of cAMP-binding Proteins** - In many different mammalian tissues the cAMP-binding protein is a regulatory subunit that controls the activity of a protein phosphokinase (4–10). Experiments were performed to determine whether this is the case in W256. A homogenate of responsive W256 cytosols was assayed for cAMP binding at pH 4.5 as described in the legend to Fig. 5, except that a wide range of cAMP concentrations was used in the reaction to construct a Scatchard plot.

![Fig. 5. Temperature stability of cAMP-binding proteins in responsive (C, △) and unresponsive (O, ▼) W256. Five pooled tumors were homogenized and cytosols were prepared as described in the text. Protein concentrations of cytosols were adjusted to 2 mg/ml with the homogenizing buffer. The cytosols were placed in glass tubes (12 x 75 mm), heated (in duplicate) in a water bath for 15 min at the temperatures indicated with gentle stirring with a glass rod, then cooled immediately to 0°. The unheated and heated cytosols were assayed for cAMP binding at cAMP concentrations of 10^{-8} M (at pH 6.5) and 10^{-6} M (at pH 4.5), respectively. Incubation conditions were as described in the text, except that the amount of cytosol was 100 µl (200 µg of protein). The reaction was stopped after 90 min at 0°. Specific binding activity was expressed as the per cent of activity of the unheated control. Tumors frozen at −70° for up to 2 weeks gave reproducible results. The values are the average of triplicate determinations of each duplicately heated sample and represent replicate experiments. Mean standard errors of values were within ±10%.

![Fig. 6. Scatchard plots for cAMP binding by responsive and unresponsive W256 cytosols before and after heat treatment. The unheated and heated (50° for 15 min) cytosols were assayed for cAMP binding at pH 4.5 as described in the legend to Fig. 5, except that a wide range of cAMP concentrations was used in the reaction to construct a Scatchard plot.

DEAE-cellulose chromatography (DE52) of cytosols from both B2cAMP-responsive and -unresponsive W256 tumors showed a single major peak of cAMP-dependent protein kinase activity (80% of the total cAMP-dependent protein kinase activity) eluted at a conductivity of 6 mmho (protein kinase, Peak II, Ref. 23).
gradient centrifugation of responsive and unresponsive cytosols also showed the identity between the cAMP-binding proteins and the cAMP-activated kinase (24). It appears, therefore, that in both tumors, the cAMP-binding protein is associated with cAMP-dependent protein kinase.

Properties of Protein Kinase

Effect of Incubation Time, Amount of Enzyme, and pH - In the presence or absence of CAMP, the enzyme activity was proportional to the reaction time for 7 min and proportional to the amount of protein up to 120 μg per reaction mixture (data not shown). The pH optimum of the enzyme was about 7.5, although the activity decreased only slightly at more alkaline pH values up to 8.5 (data not shown). The effect of incubation time, amount of enzyme, and pH optimum for the kinase was similar for both responsive and unresponsive tumors.

Apparent $K_m$ for CAMP - The relationship between enzyme activity and CAMP concentration is shown in Fig. 7. From this plot (Fig. 7) and from other similar experiments, the concentrations of CAMP required to give half-maximal stimulation were $4.0 \times 10^{-8}$ M for the kinase of the responsive tumor and $1.5 \times 10^{-7}$ M for the enzyme in the unresponsive tumor. This nucleotide exerted a cooperative effect on the kinase from the responsive tumor but not on the enzyme from the unresponsive tumor. With the kinase in the responsive tumor, reciprocal plot, as shown, is nonlinear and a Hill coefficient of 1.4 was obtained (Fig. 7).

Apparent $K_m$ for ATP - The effect of ATP concentrations on histone phosphorylation in the presence and absence of CAMP is shown in Fig. 8. It is clear that CAMP causes a great decrease in the $K_m$ of the responsive tumor enzyme for ATP. Double reciprocal plots showed that the apparent $K_m$ of the enzyme for ATP was $5.0 \times 10^{-5}$ M and $1.8 \times 10^{-4}$ M, respectively. In addition to the pronounced effect on the $K_m$ of the enzyme for ATP, CAMP also caused a 5-fold increase in the $V_{max}$ of the responsive tumor enzyme. On the contrary, however, CAMP did not affect the affinity of the unresponsive tumor enzyme for ATP. The $K_m$ of the enzyme for ATP was $2.0 \times 10^{-4}$ M either in the presence or absence of CAMP. The $V_{max}$ of the enzyme was increased 2-fold by CAMP.

Apparent $K_m$ for Histone - The effect of varying histone concentrations on enzyme activity in the presence and absence of CAMP was studied for the kinases from responsive and unresponsive tumors (data not shown). The amount of histone needed for half-maximal activity was not affected appreciably by the addition of CAMP. The apparent $K_m$ for histone of both enzymes was 0.9 mg/ml in the presence and absence of CAMP. Addition of CAMP increased the $V_{max}$ of the enzyme of the responsive tumor 5-fold, whereas the $V_{max}$ of the kinase from the unresponsive tumor increased only 2-fold.

Effect of Adenosine and Derivatives - Inhibition of enzyme activity by adenosine and some structurally related compounds is shown in Table II. In general, the ability of a...
Our present studies have revealed several differences in the properties of CAMP-binding proteins and CAMP-dependent protein kinase between Bt,cAMP-responsive and -unresponsive W256. An analysis of the CAMP binding by the two tumor cytosols produced Scatchard plots of different shapes. The responsive tumor showed higher and lower affinity binding at both pH values of 4.5 and 6.5. The unresponsive tumor showed mainly lower affinity binding at pH 4.5 and higher affinity binding at pH 6.5. Our preliminary studies (3) showed the presence of only the higher affinity binding in the responsive tumor under standard conditions (16) of binding assay. At 0°C, the binding equilibrium in the responsive tumor reached its maximum after 16 h of assay (see Fig 3); therefore, it seems that the lower affinity binding was not detected after 75 min of binding assay. A different interpretation is possible for the binding curve of the responsive tumor which is curvilinear with an upward concavity. Rather than attribute it to the presence of two classes of binding sites with different fixed affinities, it might be due to the presence of a single class of binding sites showing negative cooperativity. The binding sites might be present as a homogenous class with a high affinity for CAMP ligand when unoccupied, but are then switched to a conformation with a low affinity for the ligand when occupancy increases. Such a model has been proposed for insulin-receptor interaction (25). On the other hand, both negative cooperativity and the dissociation model can give rise to a hyperbolic shape with upward concavity in the Scatchard plot, so that these two models become indistinguishable (26).

Thus, despite its sensitivity, the Scatchard plot may result in an ambiguous interpretation. The difference in the binding curves between responsive and unresponsive tumors, therefore, seems to be due to the apparent difference in pH optimum or pH stability of the binding proteins rather than to the difference in the type of binding sites. The difference in pH stability of the binding proteins from responsive and unresponsive tumors was also shown by their maximum binding capacity. The maximum binding in the responsive tumor was higher at pH 6.5 than at pH 4.5; whereas the maximum binding in the unresponsive tumor was higher at pH 4.5 than at pH 6.5. Altered physical properties of CAMP binding proteins for cultured mouse lymphosarcoma cells have also been reported by Daniel et al. (27). Cells resistant to the killing effect of Bt,cAMP possessed CAMP-binding proteins that have different CAMP binding and pH optimum than those in sensitive cells.

The difference of stability of the binding proteins between responsive and unresponsive tumors became quite substantial after tumor cytosol was heated. The binding by the unresponsive tumor was more sensitive to temperature than that by the responsive tumor. This correlation between the heat stability of binding proteins and "responsiveness" in vivo to exogenous Bt,cAMP was also noted recently in other tumor models (28). The observation that the presence of β-mercaptoethanol decreased the heat lability of the binding in the unresponsive tumor but had no effect on the stability of responsive tumor binding suggests a difference in the structure or conformation of the binding proteins between responsive and unresponsive tumors. A different temperature sensitivity of CAMP-binding proteins for cultured neuroblastoma cells has also been reported by Simantov and Sachs (29). Cells resistant to the cytotoxic effect of Bt,cAMP had CAMP-binding proteins more sensitive to temperature than nonresistant cells. The heat stability of the binding proteins from normal rat tissues is sensitive to temperature than nonresistant cells. The heat stability of the binding proteins from normal rat tissues is shown in Fig. 9. Binding proteins from brain and heart are the most heat-stable; those from intestine, uterus, and skeletal muscle are the least stable; and liver and mammary gland appear to have moderately stable binding proteins. The stabil-
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Cyclic AMP binding proteins are the regulatory subunits of CAMP-dependent protein kinases. The other subunits of these enzymes are catalytic ones that phosphorylate various proteins, including membrane proteins (30, 31), histones (32, 33), ribosomal proteins (34), and various enzymes (35, 36). The mechanism by which CAMP induces regression of Bt-CAMP-responsive W256 is unknown. Dibutyl cAMP treatment produced an increase in CAMP concentration in both responsive and unresponsive W256, but tumor regression occurred in only one of the cell populations (2). Thus a simple endocellular change in the CAMP concentration is probably not a determining factor in the regulation of tumor growth in vivo.

Kuo and Greengard (5) proposed that all CAMP effects in animal cells are modified through protein kinase. Our results confirm their proposal, and further elucidation of the causal relationship between Bt-cAMP responsiveness and the behavior of CAMP-binding proteins will be presented in two subsequent papers (24, 37).

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