Loss of Nuclear Cyclic AMP Binding in Cyclic AMP-unresponsive Walker 256 Mammary Carcinoma*

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A marked increase of cyclic AMP-binding and protein kinase activities occurs in the nuclei of N6,O2'-dibutyryl adenosine 3':5'-monophosphate (Bt,cAMP)-responsive Walker 256 mammary carcinoma (W256) following incubation of the tumor slices with CAMP in vitro. The macromolecular fraction containing [3H]cAMP in the nuclei can be extracted with 1.0 M KCl and identified by acrylamide gel electrophoresis. Cytoplasmic origin of these increased nuclear CAMP-binding and protein kinase activities is suggested by the following observations: (a) cytoplasmic and nuclear CAMP-binding and protein kinase activities are inversely related during the CAMP stimulation of tumor slices; (b) the sequential transfer of CAMP-binding proteins and protein kinase to the nuclei is a temperature-dependent process; and (c) an initial interaction of CAMP with cytoplasm is an absolute prerequisite for the nuclei binding in vitro.

The nuclear translocation of CAMP-binding proteins and protein kinase is greatly diminished in the other type (Bt,cAMP-unresponsive) of W256, which grows during the administration of Bt,cAMP in vivo. The experiments using a cell-free system show that cytoplasmic CAMP-binding protein ·CAMP complex from responsive W256 binds to isolated nuclei from both responsive and unresponsive tumors; whereas the complex from the unresponsive tumor binds neither nuclei. These results suggest that the lack of nuclear accumulation of CAMP-binding proteins and protein kinase observed in unresponsive W256 could have been due to a defect in cytoplasmic CAMP-binding proteins which fail to interact with nuclear components. Cyclic AMP-binding proteins of unresponsive W256 also fail to respond to endogenously generated CAMP; e.g. when tumor slices are incubated with prostaglandin E, (PGE,) in vitro CAMP-binding proteins in unresponsive W256 do not respond to the PGE, stimulus as do the binding proteins in responsive W256, despite a significant elevation of the CAMP level in the tumor slices. These results suggest that a molecular lesion in CAMP-binding proteins can be a cause of Bt,cAMP unresponsive of one cell population of Walker 256 mammary carcinoma.

Injection of N6,O2'-dibutyryl adenosine 3':5'-monophosphate (Bt,cAMP) into rats bearing Walker 256 mammary carcinoma (W256) produces regression of one type of W256 (Bt,cAMP-responsive) and CAMP-binding proteins appeared to play a major role in this regression (1). It has also been shown (2) that during Bt,cAMP treatment in vivo, CAMP-binding proteins and protein kinase located in the cytoplasm accumulated in the nuclei of the regressing tumor but not in the nuclei of the nonregressing tumor (Bt,cAMP-unresponsive).

The present studies explore further the mechanism of Bt,cAMP unresponsiveness of a W256 cell population in both an in vitro system of tumor slices and a cell-free system. Results suggest that the lack of nuclear binding in Bt,cAMP-unresponsive W256 is due to a defect in cytoplasmic CAMP-binding proteins.

EXPERIMENTAL PROCEDURES

Materials—Prostaglandin E, (PGE,) was a gift from Dr. John Pike, Upjohn CO., Kalamazoo, Mich. Other materials were the same as those described in the preceding papers (2, 3).

Cyclic AMP-binding Assay—The binding of CAMP to proteins was measured by a modification of the membrane filtration method of Gilman (4) as previously described (3). For routine assay, binding was measured at CAMP exchange conditions (3, 5–7) of 25°C for 3 h, pH 6.5, with 10–9 M [3H]·CAMP = 10–9 M nonradioactive CAMP.

Protein Kinase Assay—Protein kinase was assayed by measuring 32P incorporation from γ-labeled ATP into histone as described in detail in the preceding paper (3).

Preparation of Tumor Cytosol and Nuclear Extract—All procedures were performed at 0–4°C. Tumors were removed from animals and homogenized in a Teflon-glass homogenizer with 5 volumes of 10 mM Tris/HCl buffer, pH 7.5. The homogenates were centrifuged at 105,000 × g for 60 min and the supernatants were used as cytosols. The pellets centrifuged at 770 × g were rehomogenized in the original volume of Buffer A (0.25 mM sucrose, 2 mM MgCl2, 1 mM CaCl2, 10 mM KCl, 20 mM Tris/HCl, pH 7.5). These homogenates were passed through three layers of gauze and centrifuged at 770 × g for 10 min. The pellets were resuspended and homogenized in the same volume of Buffer A, then centrifuged at 770 × g for 10 min. These crude nuclear pellets were suspended in 2.2 mM sucrose (8) in Buffer A (6 ml/
g of original tissue) and centrifuged at 2° for 45 min in a Beckman SW 25.1 rotor at 24,000 rpm. The highly purified nuclear pellets thus obtained were suspended in 10 mM Tris/HCl, pH 7.5 (0.5 ml/g of original tissue), added to equal volumes of 2.0 M KCl in 10 mM Tris/HCl, pH 7.5, and extracted at 0° for 90 min. The suspensions were centrifuged at 105,000 × g for 45 min and the clear supernatants were used as nuclear extracts.

Other Methods – Disc gel electrophoresis on 7% acrylamide gel followed the method of Ornstein (9) and Davis (10) with a minor modification as previously described (2). Protein concentration was determined by the method of Lowry et al. (11). Glucose-6-phosphate dehydrogenase was assayed by the method of Glock and McLean (12). Dibutyryl cAMP-responsive and -unresponsive W256 were maintained in Sprague-Dawley female rats (3 to 4 months old, 200 g average body weight) as previously described (3).

RESULTS

Distribution of cAMP-binding Proteins and Protein Kinase between Cytoplasmic and Nuclear Cell Compartments

Uptake of [3H]cAMP – Results previously obtained with Bt,cAMP administration in vivo (13) showed a nuclear accumulation of cAMP-binding proteins during tumor regression (3). To examine the interaction of cAMP with cytoplasmic and nuclear binding molecules under more defined conditions in vitro, tumor slices from Bt,cAMP-responsive and -unresponsive W256 were incubated with [3H]cAMP and the intracellular distribution of [3H]cAMP was followed. The temporal sequence of radioactivity uptake by the cytoplasm and the crude nuclear fraction during incubation is shown in Fig. 1. When responsive tumor slices were incubated at 30°, the tritium was initially present almost exclusively in the cytoplasm and relatively little was detected in the nuclear fraction. Upon continued incubation, there was a progressive decrease in cytoplasmic [3H] and an increase in nuclear [3H], suggesting that the cAMP was sequentially transferred from the cytoplasm to the nucleus. Such changes were not observed when Bt,cAMP-unresponsive tumor slices were incubated under the same conditions, although the maximum cytoplasmic [3H] was about 55% of that found in the responsive tumor slices. At 0° incubation, both responsive and unresponsive tumor slices incorporated the radioactivity continuously into the cytoplasm but not appreciably into the nucleus. This suggests that the increase of [3H]cAMP into nucleus may be a temperature-dependent process.

Accumulation of cAMP-binding Proteins from Bt,cAMP-responsive W256 Cytoplasm to Nucleus in Vitro – To determine whether the intracellular distribution of [3H]cAMP requires and involves the entire [3H]cAMP-binding protein complex, responsive tumor slices were incubated in vitro, in the same manner as described in the legend to Fig. 1, and the labeled cytoplasmic and nuclear binding components were identified by electrophoresis on polyacrylamide gel (2). As shown in Fig. 2, at 0° incubation for 30 min (left), [3H]cAMP has entered the cell and formed a complex with the binding protein in the cytoplasm but very little of the complex was detected in the nucleus. At 30 min after incubation at 30° (right), an increased amount of [3H]cAMP was bound to the nuclear components, while the amount of [3H]cAMP bound to the cytoplasm decreased. The difference in apparent mobility between cytoplasmic and nuclear cAMP-binding components shown in Fig. 2 suggests a cAMP effect in vitro similar to that of Bt,cAMP in vivo (2).

Kinetics of Protein Kinase Distribution between Cytoplasmic and Nuclear Compartments – Dibutyryl cAMP treatment in vitro (13) resulted in the accumulation of cytoplasmic protein kinase into the nucleus during the regression of Bt,cAMP-responsive W256 (2). The intracellular distribution of protein kinase in the cytoplasm and the nucleus was examined during
Under the same conditions nuclear protein kinase activity in the unresponsive tumor slices did not increase and cytoplasmic result in only a small increase of nuclear kinase activity. This sequential transfer of cytoplasmic protein kinase into the nuclear fraction was shown to be a CAMP-dependent process since the incubation of tumor slices in the absence of CAMP resulted in an active decrease in cytoplasmic protein kinase activity and an increase of nuclear protein kinase activity, suggesting that protein kinase was subsequently transferred to the nucleus. The isolated nuclei from Bt,cAMP-responsive tumor slices incubated with [3H]cAMP at 30° was added when indicated, in the absence of [3H]cAMP. Incubation at 30° was terminated as described in the legend to Fig. 1 at the times indicated and cytosol and crude nuclear extracts were prepared as described under "Experimental Procedures." Protein kinase activity in 10 μl (50 μg of protein) and 20 μl (40 μg of protein) of cytosol and nuclear extract, respectively, was determined by the measurement of 32P incorporation from γ-labeled ATP into histone as previously described (3) in the absence of CAMP. The ordinate is expressed as counts per min per assay. Values are mean ± range of duplicate incubations per point, two determinations per incubation.

incubation of tumor slices with cAMP. Tumor slices were incubated at 30° in the presence or absence of unlabeled cAMP and the enzyme activity was measured in the isolated cytosols and nuclear extracts (Fig. 3). In both responsive and unresponsive tumor slices, protein kinase activity was present mainly in the cytoplasm with relatively little activity in the nuclei at zero time of incubation. Upon incubation of responsive tumor slices in the presence of CAMP, there was a progressive decrease in cytoplasmic protein kinase activity and an increase of nuclear protein kinase activity, suggesting that protein kinase was subsequently transferred to the nucleus. This sequential transfer of cytoplasmic protein kinase into the nuclear fraction was shown to be a CAMP-dependent process since the incubation of tumor slices in the absence of CAMP resulted in only a small increase of nuclear kinase activity. Under the same conditions nuclear protein kinase activity in the unresponsive tumor slices did not increase and cytoplasmic kinase activity remained the same. The examination of substrate specificity and effect of protein kinase inhibitor protein (15) on the increased nuclear protein kinase of responsive tumor slices (data not shown) suggested that the increased enzyme is probably the catalytic subunit derived from the cytoplasmic CAMP-dependent protein kinase (16-18) as shown.

Whether the increased kinase activity in the nuclei following incubation may be due to an experimental artifact was examined as follows. The isolated nuclei from Bt,cAMP-responsive tumor slices (incubated with 10^-5 M CAMP at 30° for 30 min) were added to the homologous cytosol (10 volumes), rehomogenized in 10^-5 M CAMP, then nuclei were reisolated. The specific activity of protein kinase in the reisolated nuclear extract was similar to that in the original nuclear extract, suggesting that the increased nuclear kinase activity is not due to nonspecific sticking of cytoplasmic protein kinase subunits.

In Vitro Incubation of [3H]cAMP-labeled Cytosol with Nuclei—To examine more precisely the transfer of cytoplasmic CAMP-binding proteins into the nucleus, studies were carried out with cytosol and whole purified nuclei prepared from Bt,cAMP-responsive and -unresponsive W256. Cytosol, preincubated with [3H]cAMP, were incubated further with whole nuclei, either at 0° or 23°, and the macromolecular-bound radioactivity recovered in the nuclear extract was identified by electrophoresis. Experimental results are shown in Fig. 4. The macromolecular-bound radioactivity peak (exhibiting a similarity as the nuclear CAMP-binding components shown in Fig. 2) was found in the responsive tumor nuclear extract but not in the nuclear extract of the unresponsive tumor. The radioactivity peak in the nuclear extract of the responsive tumor was higher when the nuclei were incubated at 0° rather than at 23°, and the incubation at 30° resulted in a marked decrease of the radioactive component (data not shown). By contrast, the [3H]cAMP-binding protein complex was readily extracted from the nuclei after incubation of whole tumor slices with [3H]cAMP at 30° (Fig. 2). This discrepancy may be due to the susceptibility of the cAMP-binding protein complex to proteolytic attack under the conditions of the cell.
free system in vitro. Incubation of nuclei with [$^{3}H]cAMP and buffer alone produced no extractable binding protein from the nuclei at any temperature. The data in Fig. 4 indicate that cell-free nuclear binding of the cAMP-binding protein complex is greatly diminished in Bt,cAMP-unresponsive W256.

Binding of cAMP-binding Protein Complex to Nuclei from Bt,cAMP-responsive and -unresponsive W256 — We next examined whether the failure of cAMP-binding proteins to bind to the nuclei of unresponsive tumor was due to defective cytoplasmic binding proteins or to a defect in the nuclear acceptor sites of these proteins. Cyclic AMP-binding protein [$^{3}H]cAMP complex derived from each responsive and unresponsive tumor cytosol was incubated with homologous or heterologous nuclei and the specifically bound [$^{3}H]cAMP in the washed nuclei was determined (Table I). The [$^{3}H]cAMP specifically bound to nuclei was greatly diminished when the nuclei and cAMP-binding protein complex were derived from unresponsive tumor; the radioactivity bound in the nuclei was as low as that found when [$^{3}H]cAMP was preincubated with Buffer A and then incubated with nuclei of both responsive or unresponsive tumors. However, nuclei isolated from unresponsive tumor accepted cAMP-binding protein complex from responsive tumor. In contrast, the cAMP-binding protein complex from unresponsive tumor did not bind nuclei from responsive tumor. Thus the defect in Bt,cAMP-unresponsive W256 seems to lie in the cytoplasmic cAMP-binding proteins. To investigate the possibility that the cytosol of unresponsive tumor contains an inhibitor of the nuclear binding reaction, experiments were performed with mixed cytosols from both responsive and unresponsive tumors. However, it can be seen that there is no inhibition of nuclear binding in the presence of unresponsive cytosol (Table I). The lesion in unresponsive tumor therefore lies in the cytoplasmic cAMP-binding molecules themselves.

Effect of Prostaglandin E$_{1}$ (PGE$_{1}$) in Vitro

Cyclic AMP-binding in Bt,cAMP-responsive and -unresponsive W256 Tumor Slices Incubated with PGE$_{1}$ in Vitro — It was previously shown that 6 days of PGE$_{1}$ treatment (1 mg/day 200 g rat subcutaneously) stimulated an accumulation of cAMP approximately 2-fold in both Bt,cAMP-responsive and -unresponsive W256, but produced regression of only the responsive tumor (19). To investigate whether the effect of PGE$_{1}$ on W256 could be related to the response of cAMP-binding proteins, tumor slices were incubated with PGE$_{1}$ in vitro and cAMP-binding activity in the cytosol was measured during the incubation period. Fig. 5 shows the binding activity and cAMP content of the tumor slices. The incubation of tumor slices with PGE$_{1}$ at $30^\circ$ resulted in a significant decrease of cAMP binding in responsive tumor cytosol, but the binding activity in unresponsive tumor slices did not change (Fig. 5B). The decrease in cAMP binding in responsive tumor slices may be due to an increase in endogenous binding of unlabeled cAMP which decreases the exogenous [$^{3}H]cAMP binding. This possibility was examined by performing the binding assay at $23^\circ$ in order to enhance the cAMP exchange (5) by which the total binding sites (free sites and sites endogenously bound) could be measured.

### Table I

Specific binding of [$^{3}H]cAMP by Bt,cAMP-responsive and -unresponsive W256 cell nuclei in a cell-free system

| R, dibutyryl cAMP-responsive W256; U, dibutyryl cAMP-unresponsive W256; R + U cytosol, one half of R and U cytosols; Buffer A, see "Experimental Procedures." Pools of six tumors, responsive and unresponsive, respectively, were homogenized in Buffer A and cytosols and purified nuclei were prepared as described in the text. The cytosol was incubated with $1 \times 10^{-7} \mu$M [$^{3}H]cAMP (27 Ci/mmol) $\pm$ unlabeled cAMP $10^{-4} \mu$M for 60 min at $0^\circ$. Following this preincubation, specific cytosol binding of [$^{3}H]cAMP was determined as previously described (3). The preincubated cytosol was treated with dextran coated charcoal, as described in the legend to Fig. 4, to remove unbound cAMP. Five-tenths milliliter ($2.5 \mu$g of protein) of the cytosol (from 0.1 g of tumor) containing [$^{3}H]cAMP and unlabeled cAMP was added to the nuclei suspension (in Buffer A, from 0.1 g of tumor) at $23^\circ$. In parallel incubations, nuclei were exposed to cytosol containing [$^{3}H]cAMP and $10^{-4} \mu$M unlabeled cAMP as a competitor for specific binding (14). As indicated below, nuclei were also incubated with mixed cytosols from both responsive and unresponsive tumors or with Buffer A containing the same amount of [$^{3}H]cAMP as the cytosol. After 1 h, incubation mixtures were chilled at $0^\circ$ for 10 min and the nuclei were sedimented. The nuclear pellets were washed five times with Buffer A, solubilized in 1% sodium dodecyl sulfate, and assayed for radioactivity and protein. The values are specific binding (see the legend to Fig. 1) and represent one of three similar experiments.

<table>
<thead>
<tr>
<th>Incubation mixture composition</th>
<th>[$^{3}H]cAMP (in)</th>
<th>Nuclei (from)</th>
<th>Cytosol-bound [$^{3}H]cAMP</th>
<th>Nuclei-bound [$^{3}H]cAMP</th>
<th>cpn/mg protein</th>
</tr>
</thead>
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<tr>
<td>R-cytosol</td>
<td>R</td>
<td>2900</td>
<td>1200</td>
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<tr>
<td>U-cytosol</td>
<td>U</td>
<td>2000</td>
<td>150</td>
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<tr>
<td>R + U-cytosol</td>
<td>R</td>
<td>2900</td>
<td>1220</td>
<td></td>
<td></td>
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<tr>
<td>R + U-cytosol</td>
<td>U</td>
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<tr>
<td>Buffer A</td>
<td>U</td>
<td>0</td>
<td>120</td>
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</table>
The objective of this study was to assess the mechanism of
Bt+AMP unresponsiveness of one type of W256 mammary
carcinoma by comparing it to a different type of W256 which is
responsive to Bt+AMP treatment. The results obtained with
Bt+AMP-responsive W256 support the hypothesis that the
nuclear translocation of cAMP binding proteins and protein
kinase is greatly diminished in Bt+AMP-unresponsive
W256. Since unresponsive W256 continues to grow during
Bt+AMP treatment, a correlation can be made between the
nuclear translocation of cAMP-binding proteins and protein
kinase and cAMP-induced tumor regression. Dibutyryl cAMP
unresponsive W256 has been shown (1-3) to contain altered
cAMP-binding proteins as compared to those in responsive
W256, although the unresponsive tumor exhibits a binding
activity equivalent to ~70% of that in the responsive tumor
(Table I). It is conceivable that the altered cAMP-binding
proteins in unresponsive W256 are unable to mediate the
binding of cAMP to the nucleus, thus causing Bt+AMP unre-
responsiveness. Results of experiments using a cell-free system
support this possibility. It was found that cytoplasmic cAMP-binding
protein-cAMP complex from responsive tumor binds to isolated nuclei from both responsive and unresponsive
tumors, whereas the complex from the unresponsive tumor binds
to neither nuclei. Thus it appears that the defect of the unre-
sponsive tumor cannot be in the nucleus, but in the cytoplas-
mic binding protein system. As indicated by the experiment
using mixed cytosols from both responsive and unresponsive
tumors, the failure of nuclear binding in the unresponsive
tumor is not due to a diffusible inhibitor present in the cyto-
plasm. Thus, the lesion in the unresponsive tumor lies in the
binding molecules themselves. If the nuclear association
of cAMP-binding protein-cAMP complex must be preceded by
activation of the complex and penetration into nucleus, the
altered binding molecule must theoretically be unable to carry
out any of these processes. However, it is difficult to examine
whether activation or binding itself is actually impaired in
unresponsive W256, since no assay is yet available for deter-
mining an activation reaction independent of nuclear binding.

The above data are consistent with the following model of
CAMP action presented schematically in Fig. 7. Exogenously
supplied or endogenously generated CAMP (N) binds to the
cytoplasmic binding proteins (holoenzyme) of protein kinase,
R,C, or R,C2, consisting of two asymmetrical regulatory sub-
units with two globular catalytic subunits (28). This binding
may induce the separation of catalytic subunits from the regu-
lar subunits, producing active protein kinase, C (32-39).
A different feature of this model is the introduction of a hypo-
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of the complex which is translocated into the nucleus. The
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**Fig. 6. Time course of total cAMP binding in Bt+AMP-respon-
sive and -unresponsive W256 exposed to PGE, in vitro. Tumor slices
were incubated either at 30° for 10 min or 4° for 19 h. The incubation
medium and homogenization were the same as those described in the
legend to Fig. 5. Total cAMP binding (cAMP binding in vitro to both
free binding sites and those sites endogenously bound by the
exchange (3, 5-7) to tumor cytosol (105,000 g, 1 h) was performed
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**Legend to Fig. 5. Total CAMP binding in vitro to both free binding sites and those sites endogenously bound by the exchange (3, 5-7) to tumor cytosol (105,000 g, 1 h) was performed at 23° for 4 and 16 h, respectively, as described under "Experimental Procedures." Values are the mean of triplicate determinations.
Fig. 7. Early steps of cAMP action in tumor regression. The symbols used are: AC = adenylate cyclase; N = cyclic AMP (nucleotide); C = catalytic subunit of protein kinase; R and R' = regulatory subunits of protein kinase (cAMP-binding proteins) of Bt2cAMP-responsive and -unresponsive tumors, respectively. A = nuclear acceptor sites; →, indicates the sequence of events only. Since pure protein kinase was not isolated from the tumors, the structural identification of the enzyme was deduced from the enzyme of normal tissues (28-31). The alternative possibility of the nuclear entry by the holoenzyme or R and C subunits is not shown in the scheme, since the data of these studies suggest that this is a less likely possibility in these tumors.

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