Studies on the Phosphorylation and Synthesis of Type I Regulatory Subunit of Cyclic AMP-dependent Protein Kinase in Intact S49 Mouse Lymphoma Cells*

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Phosphorylation and synthesis of type I regulatory subunit (R₁) of cAMP-dependent protein kinase were studied using two-dimensional polyacrylamide gel electrophoresis of [³²P]methionine-labeled proteins from intact S49 mouse lymphoma cells. [³²P]Phosphate association rates for cAMP and different relative affinities for a variety of analogs of cAMP (6).

Two general classes of cAMP-dependent kinases, designated types I and II, have been characterized and appear to differ primarily in their R₁ subunits (7). S49 cells have...
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(23), so RI phosphorylation might be a quite general phenomenon. In the case of RI from bovine and rat skeletal muscle, Gahalen and Krebs have shown recently that the charge heterogeneity results from phosphorylation (20). RI can be phosphorylated in vitro by cGMP-dependent protein kinase (24), but there is no evidence that cGMP-dependent kinase is involved in in vivo phosphorylation.

In this report we present data confirming that the isoelectric point heterogeneity of S49 cell RI results from phosphorylation and suggesting that a single serine residue is phosphorylated. We analyze the kinetics and extent of RI phosphorylation in intact cells and assess the effect of endogenous kinase activation on RI phosphorylation. In view of the reduction in R subunit activity in kin- cells (11), we assess the effects of kinase activation on RI synthesis and compare rates of RI synthesis in wild type and kin- cells.

Since activation of kinase involves complex formation between R and cAMP (or one of its analogs), labeling or purification of R with cAMP affinity reagents is complicated, if not excluded, as an approach for visualization and quantification of RI in cells stimulated with analogs or inducers of cAMP. To avoid this problem and to minimize possible artifacts of differential extraction, purification, or lability of the two forms of RI, we have extracted cells directly in a strongly denaturing solvent and subjected this total cell extract to high resolution two-dimensional gel electrophoresis (25). Since resolution of RI is not always complete in gel patterns of whole cell extracts, we have developed and used a procedure of computer-assisted densitometry and image enhancement to quantify the radioactivity in RI spots from autoradiographs of the two-dimensional gel patterns (26).

EXPERIMENTAL PROCEDURES

Materials

Dibutyryl-cAMP, 8-bromo-cAMP, methyl isobutylxanthine, n-isoproterenol. Tris base, and 4-morpholinoethanesulfonic acid were purchased from Sigma; Ampholines were from LKB; electrophoresis grade acrylamide and N,N,N',N'-tetramethylethylenediamine were from Bio-Rad and Eastman; sodium dodecyl sulfate (specially pure) was from BDH; Nonidet P-40 was from The Albright Corp., urea (ultrapure) and cholaer enterotoxin (a gift from Dr. C. Johnson) were from Schuchardt/Mann; trypsin (tosylphenylalanine chloromethyl ketone-treated) was from Worthington; Staphylococcus aureus strain V8 protease was from Miles; and RO-20-1724 (4-(3-hutoxy-4-methoxybenzyl)-2-imidazolodindone) was from H. Sheppard of Hoffman-La Roche. N(2)-Aminoethyl)-cAMP-Sepharose was prepared as described by Dills et al. (27) and control Sepharose was prepared by reacting CNBr-activated Sepharose 4B (Pharmacia) with ethanolamine (10). [35S]Methionine (>800 Ci/mmol) and scintillation mixtures (PCS and ACS) were from Amersham; [32P]Orthophosphate (carrier-free in water) was from New England Nuclear. X-ray film and photographic chemicals were from Kodak and intensifying screens from Dupont. Cellulose thin layer plates (0.1 mm thickness) were from E. Merck. Other chemicals were reagent grade and used without further purification.

S49 mouse lymphoma cells (28) were grown in suspension culture as described previously (11). Wild type cells were from subclone 24.3.2 (11, 29), kin- cells (subclone 24.6.1) from a spontaneous kinase-negative mutant clone (11, 29), and kin- cells (subclone 1200.19) from a kinase-negative clone which arose after mutagenesis with the frameshift mutagen ICH 191 (11, 30).

Methods

Radiolabeling and Preparation of Cell Extracts—Cells were labeled with [35S]methionine or [32P]Orthophosphate after preincuba-

1 Cyclic AMP-binding proteins from mouse neuroblastoma cells have two-dimensional gel mobilities extremely similar to the two forms of RI cell Rb, but the isoelectric point heterogeneity has been attributed, thus far, to peptide differences, rather than to phosphorylation (23).

Fig. 1 illustrates the heterogeneity in isoelectric point of RI isolated from wild type S49 cells. Fig. 1a shows a portion of a two-dimensional gel pattern of [35S]methionine-labeled proteins purified from S49 cells by affinity chromatography on N(2)-(2-aminoethy1)-cAMP-Sepharose; spots designated by arrows have been shown to correspond to RI (10). Fig. 1b and c shows the same portions of gel patterns from extracts of cells...
labeled with [\(^{32}\)P]phosphate which were either affinity-puriﬁed (Fig. 1c) or mock-puriﬁed on a control Sepharose column (Fig. 1b); the spot designated by the right-hand arrow in Fig. 1c was the only [\(^{32}\)P]labeled species speciﬁcally puriﬁed by the cAMP afﬁnity resin. Fig. 1d shows that this [\(^{32}\)P]labeled species co-migrates with the more acidic form of [\(^{35}\)S]methionine-labeled Rg.

Figs. 2 and 3 show strong similarities in peptide maps of the two forms of [\(^{35}\)S]methionine-labeled Rg and the species labeled with [\(^{32}\)P]phosphate. The maps for both these ﬁgures were generated by a modiﬁcation of the partial proteolysis procedure of Cleveland et al. (33) in which proteins separated in a ﬁrst dimension isoelectric focusing gel are subjected to proteolysis in a second dimension sodium dodecyl sulfate gel. Fig. 2a shows peptides from the Rg region of a gel from a preparation similar to that shown in Fig. 1a, but from cells labeled for only 2 h to give increased relative labeling of the more basic spot (see below); Fig. 2b shows peptides from a control Sepharose-puriﬁed preparation to allow discrimination between peptides from the two Rg spots and those from contaminating proteins. (Several contaminating peptides are indicated by arrowheads to aid in alignment.) The two forms of Rg have similar, but nonidentical, maps: most of the differences appear to involve peptides from the more acidic form which have slightly slower mobilities than corresponding peptides from the more basic form. Comparable results were also obtained using papain to generate peptides (not shown). Fig. 3 compares peptides from the more acidic form of Rg labeled with either [\(^{35}\)S]methionine (Fig. 3a) or [\(^{32}\)P]phosphate (Fig. 3b); peptides from a mixture of [\(^{35}\)S]methionine- and [\(^{32}\)P]phosphate-labeled material are shown in Fig. 3c. The near identity of these peptide patterns conﬁrms the view that the same protein is labeled by both isotopes. Furthermore, many of the misaligned peptides between the basic and acidic forms of [\(^{35}\)S]methionine-labeled Rg, seen in Fig. 2a are those containing phosphate label, suggesting that the nonidentity of maps of the two forms of [\(^{35}\)S]methionine-labeled Rg results from an effect of the phosphate on sodium dodecyl sulfate gel mobility. Taken with the genetic evidence reported previously (10), the results of the peptide mapping experiments of Figs. 2 and 3 provide strong conﬁrmation that the two isoelectric forms of S49 cell Rg are non-phosphorylated and phosphorylated forms of the same protein; these will be referred to as RgN and RgP, respectively, throughout the rest of this paper.

To ascertain the nature of the phosphate in S49 cell Rg, [\(^{32}\)P]phosphate-labeled Rg was excised from two-dimensional gels (similar to that of Fig. 1c), hydrolyzed with hydrochloric acid, and subjected to electrophoresis as described under “Experimental Procedures.” Unlabeled phosphoserine and phosphothreonine markers were run in parallel lanes. In two experiments using slightly different hydrolysis conditions, a single labeled anodic species was observed which migrated identically to phosphoserine (not shown—free orthophosphate was run off the electrophoretograms). RgN and RgP differ by a single charge unit (10) which is consistent with a difference of one and only one serine phosphate. Fig. 4a shows that when gel-puriﬁed [\(^{32}\)P]phosphate-labeled Rg is digested exhaustively with trypsin, a single [\(^{32}\)P]-labeled peptide is found. Fig. 4a shows tryptic peptides from [\(^{35}\)S]methionine-labeled Rg to illustrate the resolution obtained in this chromatograph: Fig. 4a shows a more intense exposure of the track shown in Fig. 4b; the faster migrating, faintly labeled peptide observed in this track is a product of incomplete digestion. Preliminary results using partial proteolysis of native S49 kinase with
thermolysin or chymotrypsin suggest that the phosphorylation site is about 8,000 to 11,000 daltons from the NH₂ terminus of R₁.

Figs. 5 and 6 show results from an experiment investigating the time course of R₁ phosphorylation in intact S49 cells. Cells were pulse-labeled with [³⁵S]methionine, washed, and resuspended in media with or without Bt_cAMP and RO 20-1724, an inhibitor of phosphodiesterase. At various times after resuspension, cells were harvested by centrifugation and frozen for later analysis; initial samples were taken at about 30 min after the addition of label. Duplicate samples were taken from the drug-free control culture and either extracted under non-denaturing conditions for affinity purification of R₁ or dissolved in gel sample buffer for whole extract electrophoresis. Drug-treated samples were analyzed only by the second method since R₁ from treated cells did not bind efficiently to the affinity resin. Fig. 5 shows the R₁ portions of two-dimensional gel patterns from these samples and Fig. 6 shows densitometric data from these autoradiograms. Autoradiograms from purified R₁ samples (left-hand column in Fig. 5) were scanned manually with a Joyce-Loebel microdensitometer while those from crude cell extracts (middle and right-hand columns in Fig. 5) were scanned with a Syntax computer-assisted microdensitometer; an image enhancement procedure was used to separate optical density in R from that in contaminating spots for the crude extract patterns (26). The two methods for estimating the extent of R₁ phosphorylation gave similar results (Fig. 6); the small differences at early times may result from exclusion of R-cAMP complexes from the affinity-purified material. In control cells, phosphorylation proceeded with a half-time of about 25 min until about 95% of R₁ label was in R₁. In cells treated with Bt_cAMP, the proportion of R₁ in R₁ reached only about 75%. The kinetics of R₁ phosphorylation in the presence of Bt_cAMP cannot be determined from this experiment because it takes nearly 15 min for exogenously added Bt_cAMP to exert its full effect on endogenous protein kinase (31). Label-chase experiments carried out over longer time courses suggest that the differences in extent of R₁ phosphorylation seen in the experiment of Figs. 5 and 6 reflect steady state differences; in two experiments in which Bt_cAMP was added to cultures labeled for 1 h in its absence, the proportion of labeled R₁ in R₁ dropped from more than 80% to about 75% after 6-7 h of chase with only slight degradative loss of total R₁ label. Portions of these same cultures untreated with the analog maintained about 90% of their labeled R₁ in R₁ for more than 10 h of chase.

The experiment of Fig. 7 uses labeling of intact cells with [³²P]phosphate and two-dimensional gel electrophoresis of whole cell extracts to confirm the Bt_cAMP-mediated inhibition of R₁ phosphorylation. Wild type cells labeled in the absence of Bt_cAMP exhibited relatively strong labeling of R₁ (Fig. 7a) compared with cells labeled in the presence of the analog (Fig. 7b). There was only slight labeling of R₁ in kinase-negative cells (Fig. 7c) consistent with previous results using affinity-

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Fig. 5. Rf patterns from cells labeled for 15 min with [35S]methionine and chased in the presence and absence of 8Bt2cAMP. Wild-type cells labeled for 15 min with 250 μCi/ml of [35S]methionine were washed by centrifugation through a layer of horse serum and resuspended in conditioned growth medium with or without 1 mM 8Bt2cAMP and 30 μM RO 20-1724. At the times indicated to the right of the figure (in minutes), cells were harvested and subjected to isoelectric focusing and autoradiography. The left-hand column shows two-dimensional gel patterns from affinity-purified material from drug-free control cells; the center column shows patterns from whole cell extracts of these control cells; and the right-hand column shows patterns from whole cell extracts of cells exposed to 8Bt2cAMP and RO 20-1724 during the chase. 2.5 × 10^6 cpm of the affinity-purified material and 10^6 acid-precipitable cpm of the whole cell extracts were loaded per gel and autoradiographic exposures were for 10 days and 4 days, respectively. Arrows indicate the positions of Rf1 and Rf2.

purified Rf1 from these mutants (11). Reduced phosphorylation of Rf1 in kin- cells has also been confirmed by quantitation of label in the positions of Rf1 and Rf2 in gels of whole extracts of kin- cells pulse-labeled with [35S]methionine (see below, Table I). Rf spots from the gels of Fig. 7 were excised and subjected to tryptic mapping (see Fig. 4); all three preparations gave identical phosphopeptides (not shown).

Table 1 presents data from several experiments in which wild-type or kin- cells were pulse-labeled with [35S]methionine for 10 to 30 min after preincubation with or without 8Bt2cAMP. Equal amounts of acid-precipitable radioactivity from whole cell extracts were subjected to two-dimensional gel electrophoresis and radioactivity in Rf1 and Rf2 was quantified from the resulting autoradiograms using computer-assisted densitometry and image enhancement. In cells labeled without drugs, the proportion of Rf1 in Rf2 varied with labeling time in a manner consistent with the 25-min phosphorylation half-time determined above (Fig. 6). 8Bt2cAMP treatment caused a 50 to 80% reduction in the relative labeling of Rf2; the greater inhibitions were seen in cells treated for several hours with the analog. In the experiments of Table I, labeling durations were sufficiently short that phosphorylation was well below its steady state levels. Therefore, the proportion of Rf1 in Rf2 is largely a reflection of the phosphorylation rate. 8Bt2cAMP is thus seen to slow Rf phosphorylation by as much as 4- to 5-fold. In kin- cells, Rf phosphorylation is very low and not significantly affected by 8Bt2cAMP.

The data of Table I also show the effects of 8Bt2cAMP and kin- mutations on the rate of synthesis of Rf1 relative to total cellular protein synthesis. Kinase activation by 8Bt2cAMP (Table I) or a variety of other agents (see below, Fig. 8) had no significant immediate effect on Rf1 synthesis, but after several hours of drug treatment an increase in Rf1 synthesis was seen consistently. The extent of induction has been quite variable, ranging from about 10% to almost 2-fold (Table I, Fig. 8). In kin- cells, which lack functional C subunits (11), the relative rate of Rf1 synthesis was about 50% of that in wild type cells and was unaffected by 8Bt2cAMP.

Although 8Bt2cAMP has invariably inhibited Rf1 phosphorylation in more than a dozen experiments, such inhibition was not observed with 8-Br-cAMP or isoproterenol in an experiment performed to compare different agonist effects on

![Fig. 6. Time course of Rf phosphorylation in intact S49 cells and the effect of 8Bt2cAMP on this phosphorylation. Radioactivity in Rf1 and Rf2 was quantified from autoradiographs of the gels shown in Fig. 5 using the densitometric procedures described under "Experimental Procedures" and the data were expressed as the proportion of total Rf1 in Rf2. For the 0- and 5-min time points of affinity-purified material, additional gels were run and quantified because of slight artifacts in the gels of Fig. 5. Δ—Δ, affinity-purified Rf1 from drug-free control cells; ○—○, whole extract Rf1 from control cells; ●—●, whole extract Rf1 from cells treated with 8Bt2cAMP and RO 20-1724 during the chase.](image-url)
TABLE I  
Effects of BtCAMP and kin- mutations on synthesis and phosphorylation of R1

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Subline</th>
<th>Drug pretreatment</th>
<th>Labeling duration</th>
<th>A in R1 spots</th>
<th>Rf</th>
<th>Σ Rf</th>
<th>Rf/Σ Rf</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Wild type</td>
<td>None</td>
<td>10 min</td>
<td>0.80</td>
<td>0.20</td>
<td>1.00</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BtCAMP + RO, 2 h</td>
<td></td>
<td>1.43</td>
<td>0.09</td>
<td>1.52</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>None</td>
<td></td>
<td>0.59</td>
<td>&lt;0.04</td>
<td>0.63</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BtCAMP + RO, 2 h</td>
<td></td>
<td>0.58</td>
<td>&lt;0.03</td>
<td>0.61</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>II</td>
<td>Wild type</td>
<td>None</td>
<td>15 min</td>
<td>0.71</td>
<td>0.29</td>
<td>1.00</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BtCAMP, 6 h</td>
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<td>1.03</td>
<td>0.09</td>
<td>1.12</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>None</td>
<td></td>
<td>0.39</td>
<td>&lt;0.04</td>
<td>0.43</td>
<td>&lt;0.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BtCAMP, 6 h</td>
<td></td>
<td>0.44</td>
<td>&lt;0.04</td>
<td>0.48</td>
<td>&lt;0.08</td>
</tr>
<tr>
<td>III</td>
<td>Wild type</td>
<td>None</td>
<td>10 min</td>
<td>0.84</td>
<td>0.16</td>
<td>1.00</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BtCAMP, 5 min</td>
<td></td>
<td>1.05</td>
<td>0.09</td>
<td>1.14</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BtCAMP, 6.33 h</td>
<td></td>
<td>1.51</td>
<td>0.05</td>
<td>1.56</td>
<td>0.03</td>
</tr>
<tr>
<td>IV</td>
<td>Wild type</td>
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<td></td>
<td>0.52</td>
<td>0.48</td>
<td>1.00</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BtCAMP, 5 min</td>
<td></td>
<td>0.67</td>
<td>0.26</td>
<td>0.93</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BtCAMP, 5.5 h</td>
<td></td>
<td>1.14</td>
<td>0.14</td>
<td>1.28</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>None</td>
<td>30 min</td>
<td>0.47</td>
<td>0.04</td>
<td>0.51</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BtCAMP, 5 min</td>
<td></td>
<td>0.51</td>
<td>0.06</td>
<td>0.57</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BtCAMP, 5.5 h</td>
<td></td>
<td>0.62</td>
<td>0.06</td>
<td>0.68</td>
<td>0.09</td>
</tr>
</tbody>
</table>

"See "Experimental Procedures" for cell lineages.

BtCAMP at 1 mM and RO 20-1724 at 30 μM were added during preincubation in low methionine medium and remained present throughout labeling; preincubations were for 2, 6, 6.33, and 5.5 h, respectively, for experiments I, II, III, and IV.

Cells were labeled with [35S]methionine at concentrations ranging from 100 to 250 μCi/ml as described under "Experimental Procedures."

For each experiment, equal numbers (0.75 to 2 × 106) of acid-precipitable counts per min from whole cell extracts were subjected to two-dimensional gel electrophoresis and radioactivity in R1 spots was quantified by computer-assisted densitometry of the resulting autoradiograms as described under "Experimental Procedures." Data for each experiment have been normalized to the total radioactivity in R1 from drug-free wild type cells.

Fig. 7. Effects of BtCAMP and a kin- mutation on labeling of R1 with [35P]phosphate. Wild type or kin- cells were labeled for 3% h with [35P]phosphate at 4 μCi/ml in the presence or absence of 1 mM BtCAMP, washed once with ice-cold phosphate-buffered saline, and 2 × 105 acid-precipitable cpm from whole cell extracts were subjected to two-dimensional gel electrophoresis as described under "Experimental Procedures." Autoradiographs were exposed for 4% h without screens. a, wild type cells without drug; b, wild type cells with BtCAMP; c, kin- cells without drug. Arrows indicate the position of Rf.

endogenous substrate phosphorylations (31): this same experiment showed inhibition of R1 phosphorylation with both BtCAMP and cholera toxin, but not with sodium butyrate. Fig. 8 shows gel patterns of labeled R1 from an experiment in which these controls were repeated and extracted. Cells were labeled for 30 min with [35S]methionine after short or long treatments with analogs or inducers of cAMP; long treatments were for 6 h plus the labeling interval and short treatments varied from 5 min for isoproterenol to 90 min for cholera toxin to give a maximal response at the time of labeling (31, 34, 35). Drug-free control cells exhibited nearly equal labeling of Rf and Rf in 30 min (Table I, Fig. 8a and b). As seen previously, BtCAMP inhibited phosphorylation relative to that in control cells and the inhibition was greater in cells treated for 6 h before labeling (Fig. 8d) than in those treated for only 15 min (Fig. 8e). 8-Br-cAMP had no apparent effect on the relative proportion of Rf and Rf (Fig. 8e, f, and j) although the dose used is physiologically equivalent to the 1 mM BtCAMP used for Fig. 8a, c and d (34). Cholera toxin inhibited R1 phosphorylation with short treatment (Fig. 8g), but not after longer treatment (Fig. 8h). Isoproterenol in combination with the phosphodiesterase inhibitor methyl isobutyxanthine behaved similarly to cholera toxin (Fig. 8, k and l). Methyl isobutyxanthine alone had no effect on R1 phosphorylation (Fig. 8, i and j). Judging by electrophoretic shifts in endogenous substrate proteins (31, 35), all agents except methyl isobutyxanthine alone caused complete activation of endogenous kinase during the short treatment protocols (not shown): methyl isobutyxanthine caused a significant, but incomplete, response. The relative phosphorylations of endogenous substrates were lower after long treatments with all agents, but the effects of BtCAMP, 8-Br-cAMP, and cholera toxin were approximately equivalent and nearly maximal. Isoproterenol plus methyl isobutyxanthine-treated cells exhibited a somewhat lower relative phosphorylation of endogenous substrates after long treatment and cells treated for 6 h with methyl isobutyxanthine alone exhibited an even smaller, but still significant, response. As mentioned above, prolonged treatment with all of these agents led to a significant inhibition of R1 synthesis (Fig. 8, d, f, h, j, k, and l).

Studies on the effects of cAMP analogs on two-dimensional gel patterns from a variety of cultured cell types (35) provide comparative data on R1 metabolism. Three additional murine cell lines of lymphoid origin (two lymphomas and a myeloma, Ref. 35) were virtually identical with S49 cells in relative

R. A. Steinberg, unpublished results
generated from the two [\(^{35}S\)]methionine-labeled forms can be attributed to effects of the phosphate group on peptide mobilities in the sodium dodecyl sulfate gel system. Pulse-labeling and labelf-lace experiments indicate that R\(_1\) phosphorylation is a post-translational modification; interconversion of the two [\(^{35}S\)]methionine-labeled forms of R\(_1\) has been observed in both phosphorylation and dephosphorylation directions. Phosphorylation appears to be on a single serine residue located in the NH\(_2\) terminal quarter of the protein. R\(_1\) phosphorylation proceeds to an extent of 90 to 95% with a half-time of about 25 min in drug-free wild type cells and is inhibited by some, but not all, treatments causing endogenous activation of cAMP-dependent protein kinase. R\(_1\) phosphorylation is also greatly reduced in kin\(^{\pm}\) mutants of S49 cells which lack detectable C subunit activity (11).

The difference in steady state phosphorylation of R\(_1\) in basal and Bt\(_2\)cAMP-treated cells can be explained fully by a 3- to 6-fold inhibition of the rate of phosphorylation assuming that phosphorylation and dephosphorylation are first order (or pseudo-first order) processes; this is about the range of inhibition seen in pulse-labeling experiments (Table 1). These same assumptions predict that the half-time for phosphorylation and dephosphorylation of R\(_1\) during treatment with Bt\(_2\)cAMP should be 2.5 to 5 times greater than the half-time for phosphorylation in the absence of the analog. Consistent with this prediction, label-lace experiments in which Bt\(_2\)cAMP was added to cells with more than 80% of labeled R\(_1\) in R\(_F\) revealed a dephosphorylation half-time on the order of 1 to 2 h.\(^{\text{a}}\) The relatively small difference in steady state phosphorylation of R\(_1\) under basal and activated conditions and the slow dephosphorylation rate in the presence of Bt\(_2\)cAMP render unlikely the notion that R\(_1\) phosphorylation plays an important role in modulating the rapid dissociation or reassociation of kinase subunits in S49 cells. Such a role has been postulated for R\(_1\) phosphorylation on the basis of studies with purified kinase (12, 19), but evidence is lacking for the rapid cycling of R\(_1\) phosphate in vivo as required by this model. Whether or not phosphorylation of R\(_1\) affects parameters for the activation of kinase remains unknown; the phosphate appears to be located in a region of the molecule which has been implicated in R-C interaction (36), so an effect on this interaction would not be surprising. If this is indeed the case, the differences in relative R\(_1\) phosphorylation observed in different cell types may reflect a fine-tuning mechanism in cellular responsiveness to cAMP elevation.

The reduction of R\(_1\) phosphorylation in kin\(^{\pm}\) sublines of S49 cells strongly suggests that C subunit plays a role in the phosphorylation, but the nature of that role remains unclear. Four possibilities we have considered are that C is catalytic in an autophosphorylation, that C activates another kinase which, in turn, phosphorylates R\(_1\), that C inactivates a phosphatase active against R\(_F\), or that C plays a structural role facilitating the phosphorylation of R\(_1\) by another kinase. The second and third of these possibilities are apparently ruled out by the inhibition of R\(_1\) phosphorylation under some conditions which activate kinase; catalytic cascade or phosphatase inhibition mechanisms predict greater phosphorylation in activated cells. Some phosphorylation of R\(_1\) by another kinase is suggested by the low, but detectable, phosphorylation of R\(_1\) in kin\(^{\pm}\) cells at the same site which is phosphorylated in wild type cells. A model in which C facilitates R\(_1\) phosphorylation by another kinase would easily accommodate this observation as well as the failure of purified type I kinase to undergo autophosphorylation (8). On the other hand, the

\(^{\text{a}}\) A similar, but more dramatic, effect of phosphorylation on protein mobility in this gel system has been reported for bovine R\(_1\) (15).

\(^{\text{b}}\) R. A. Steinberg and D. A. Agard, unpublished results.
proportion of \( R_\text{I} \) in labeled \( R_\text{I} \) from kin* cells remains low even after several hours of labeling or in a chase of several hours (11) in contrast to the significant phosphorylation of \( R_\text{I} \) in wild type cells labeled in the presence of \( \text{Br}_{2}\text{cAMP} \); this result suggests that phosphorylation of "free" \( R_\text{I} \) is greater in wild type than in kin* cells and thus implicates C in a catalytic role.

Studies on the autophosphorylation of type II kinase indicate that \( R_\text{II} \) phosphorylation is unimolecular in the absence of cAMP and bimolecular in its presence (37), presumably as a result of \( \text{cAMP} \)-mediated subunit dissociation. If C subunit plays a catalytic role in \( R_\text{I} \) phosphorylation, this sort of behavior could explain the inhibition of phosphorylation by agents which activate kinase. It should be noted, however, that inhibition of \( R_\text{II} \) phosphorylation by \( \text{cAMP} \) has been reported only at very low concentrations of kinase (37) or in the presence of \( \text{Zn}^{2+} \) (13). If C plays a structural role facilitating \( R_\text{I} \) phosphorylation, inhibition of phosphorylation upon kinase activation is expected so long as activation proceeds by subunit dissociation.

Although \( R_\text{I} \) phosphorylation has been inhibited by \( \text{Br}_{2}\text{cAMP} \), choleterin, and isoproterenol plus methyl isobutylxanthine, such inhibition is not an obligatory result of intracellular kinase activation. 8-Br-cAMP had no apparent effect on \( R_\text{I} \) phosphorylation and the effects of choleterin and isoproterenol were limited to times when their effects on intracellular cAMP levels were maximal. These results suggest that, in intact cells, kinase can distinguish between analogs of cAMP, and, perhaps, between physiological and superphysiological levels of cAMP. A basis for such discrimination is provided by the presence of two cAMP binding sites per \( R_\text{I} \) monomer (4, 5); recent studies of Rannels and Corbin suggest that these sites differ in their affinities for cAMP analogs (6). To explain our results with choleterin and isoproterenol plus methyl isobutylxanthine, one must postulate that the two sites also differ in their affinities for cAMP; purified C exhibits apparently equal cAMP binding to the two sites (6), but the situation in holoenzyme has not yet been investigated. If the inhibition of \( R_\text{I} \) phosphorylation by \( \text{Br}_{2}\text{cAMP} \) or high levels of intracellular cAMP results from subunit dissociation as discussed above, the absence of inhibition by 8-Br-cAMP or submaximal levels of intracellular cAMP suggests that kinase can be activated without dissociation. Such a possibility has been entertained previously (2, 38), but, to our knowledge, there is no evidence in its support. Alternatively, activation might always involve subunit dissociation, but the conformation and, hence, phosphorylability of the released \( R_\text{I} \) might depend upon the agent used to activate. Both of these models predict that binding of butyryl-cAMP and 8-Br-cAMP to \( R_\text{I} \) result in different conformations, and this prediction is supported by differences in the affinity of \( R_\text{I} \) cells activated with \( \text{Br}_{2}\text{cAMP} \) or 8-Br-cAMP (39). Nevertheless, we cannot at present rule out alternative explanations for our results involving effects of cAMP metabolites on \( R_\text{I} \) phosphorylation or effects mediated by some unknown system affected differentially by various analogs and inducers of cAMP.

We have reproducibly observed a moderate induction of \( R_\text{I} \) synthesis in S49 cells and in a variety of other cell types after several hours of treatment with analogs or inducers of cAMP; the variability in magnitude of this effect has prevented our obtaining good kinetic data for the response. Two- to five-fold increases in \( R_\text{I} \) have been reported in mouse neuroblastoma cells (23) and in hybrids between mouse neuroblastoma and rat glioma cells (40), after prolonged incubation in \( \text{Br}_{2}\text{cAMP} \). At least part of the increase of \( R_\text{I} \) in neuroblastoma cells has been attributed to increased synthesis (41). Kin* S49 cells exhibit reduced synthesis of \( R_\text{I} \) which is opposite to the effect of "freeing" \( R_\text{I} \) by kinase activation. The reduced synthesis of \( R_\text{I} \) in kin* cells and the induction of \( R_\text{I} \) by kinase activation can be reconciled by assuming either a direct or indirect role of C in modulating \( R_\text{I} \) gene expression where both basal and activated kinase have positive effects, but the activated enzyme is more effective. The regulation of cAMP phosphodiesterase in S49 cells is similar in that it is induced by kinase activation and its basal expression is reduced about 2-fold in kin* cells (29, 34). The 2-fold reduction of \( R_\text{I} \) synthesis in kin* cells is insufficient in explaining the 5- to 7-fold decrease in cAMP-binding activity found in extracts of these cells (11); we have results suggesting that degradation of \( R_\text{I} \) is also affected in these mutants (39).

Acknowledgments—We would like to thank Drs. Gary Johnson and Lloyd Waxman for generous advice on these studies and the resulting manuscript. Dr. Johnson, also, for his gift of choleterin, Dr. Robert M. Stroud for making available the equipment for computer-assisted densitometry, Mrs. Marian Retttenmeyer for her skillful photographic assistance, and Miss Liz Jean for her help in preparation of the manuscript.

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