8-Chloroadenosine Mediates 8-Chloro-Cyclic AMP-induced Down-Regulation of Cyclic AMP-dependent Protein Kinase in Normal and Neoplastic Mouse Lung Epithelial Cells by a Cyclic AMP-independent Mechanism

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ABSTRACT

The 8-chloro analogue of the regulatory molecule, cyclic AMP (cAMP), modulates the intracellular concentrations of cAMP-dependent protein kinases (PKA) and inhibits both in vitro and in vivo growth of several neoplastic cell types. Because 8-chloro-cyclic AMP (8-Cl-cAMP) can be converted to 8-chloroadenosine (8-Cl-adenosine) by serum enzymes contained in cell growth media, we tested whether 8-Cl-cAMP effects were mediated by its adenosine metabolite in normal and neoplastic cell lines of mouse lung epithelial origin. 8-Cl-adenosine, directly added to cells or derived from exogenously applied 8-Cl-cAMP, specifically decreased the intracellular concentration of the type I isozyme of cAMP-dependent protein kinase (PKA I). 8-Cl-adenosine and 8-Cl-cAMP were equipotent at inhibiting cell growth, and elicited similar changes in the proportion of cells in the G0/G1, S, and G2/M phases of the cell cycle. The presence of 8-Cl-adenosine deaminase, which converts 8-Cl-adenosine to 8-chloroinosine, completely prevented growth inhibition by 8-Cl-cAMP and the concomitant diminution of PKA I. 8-Cl-cAMP had no discernible effect on cells when its conversion into 8-Cl-adenosine was prevented by 3-isobutyl-1-methyl-xanthine, an inhibitor of phosphodiesterase. 6-(p-Nitrobenzyl)thioinosine, an inhibitor of adenosine uptake, protected cells from cytostasis, indicating that 8-Cl-adenosine acts intracellularly. 8-Cl-adenosine greatly decreased RI (regulatory subunit of PKA I) and PKA catalytic (C) subunit protein concentrations without affecting RII (regulatory subunit of the PKA type II isozyme) or intracellular CAMP levels. Northern blot analysis of PKA subunit mRNAs following treatment of each cell line with 8-Cl-adenosine demonstrated decreased Cα mRNA expression, increased RIα mRNA, and no change in RIIα mRNA abundance. Our results indicate that 8-Cl-adenosine inhibits lung cell growth and induces PKA I down-regulation via a CAMP-independent mechanism.

INTRODUCTION

Many extracellular signals affect physiology by changing the intracellular concentration of CAMP. This ubiquitous regulatory molecule exerts bidirectional effects on the proliferation of mammalian cells, inhibiting the growth of several cell types (1, 2) while stimulating others (3). Opposing responses to CAMP may result from cell-specific differences in the concentration, types, and/or localization of cAMP receptors, the regulatory subunits of cAMP-dependent protein kinases. Indeed, most of the diverse actions of cAMP in animal cells result from activation of PKA and subsequent enhancement of endogenous protein phosphorylation (4). PKA exists as an inactive tetramer composed of an R subunit dimer and two monomeric catalytic subunits. In the holoenzyme form (R-C2), the C subunits are inhibited by their association with R subunits. Binding of two molecules of cAMP to each R subunit promotes the dissociation of C subunits, which are then catalytically active (5).

Most tissues contain two PKA isozymes, type I (PKA I) and type II (PKA II), whose R subunits, RI and RII, differ while their C subunits are identical (4). RI and RII exhibit divergent amino terminal domains, are antigenically distinct (6), and have different affinities for the C subunit in vivo (7). Distinct genes exist for each PKA subunit, encoding α and β isoforms of both RI and RII, and α, β, and γ isoforms of C subunits (8, 9). Expression of these isoforms is tissue specific (8) and hormonally regulated (10–12). Changes in the ratio of PKA I to PKA II associated with ontogenesis, differentiation, and neoplastic growth provide clues to the possible physiological function of each isozyme (reviewed in Ref. 13), but no consistent role in these processes has been clearly assigned to either isozyme.

Variations in isozymic expression can arise upon cellular perturbation. The 8-chloro analogue of CAMP is reported to be unique among CAMP analogues in its ability to inhibit proliferation, induce differentiation, and modulate PKA subunits in several human cancer cell lines at micromolar concentrations (reviewed in Ref. 13). Recently, a debate has ensued over the possibility that the growth inhibitory effects of this analogue may be mediated solely by its adenosine metabolite (14–16). Mouse lung epithelial cell lines are well characterized with respect to basal and chemically modulated PKA isozyme expression at the activity, protein, and mRNA levels (17–20). The great advantage of this system is the existence of non-tumorigenic cell lines of the same histological origin as tumorigenic cell lines (21, 22). We recently described reduced PKA I expression in several independently derived neoplastic mouse lung epithelial cell lines (17). Herein, the effects of 8-Cl-cAMP and its adenosine metabolite on growth and PKA subunit levels in these cells are examined. The cytostasis induced by both 8-Cl-cAMP and 8-Cl-adenosine is associated with a selective decrease in Cα mRNA and PKA I (RI and C) subunit protein concentrations. All effects of 8-Cl-cAMP on these cells are mediated by its adenosine metabolite, 8-Cl-adenosine. We initially examined the biological activity of 8-Cl-adenosine: PKA down-regulation by this agent is then demonstrated. This represents the first report of a CAMP-independent modulatory effect of 8-Cl-adenosine on PKA subunit protein and mRNA concentrations.

MATERIALS AND METHODS

Materials. 8-Br-cAMP, calf intestinal mucosal ADA, and NBTI, were purchased from Sigma. Prior to use, ADA was dialyzed against 2 liters of PBS for 12 h to remove ammonium sulfate and then filter sterilized. 8-Cl-cAMP was kindly provided by Dr. Yoon S. Cho-Chung (National Cancer Institute, Bethesda, MD). 8-Cl-adenosine was purchased from Biolog Life Sciences Inc. (Bremen, Germany).

Cell Culture. The nontumorigenic, immortal cell line (hereafter referred to as “normal”), Nal 1A C1C10 (C10), and the tumorigenic spontaneous transplant cell line Nal 1A C4E9 (E9) were derived from normal adult BALB/CJ lung epithelium (21–23). PCC4 is a tumorigenic cell line derived from a papillary tumor in BALB/CJ mice (24). C10 and E9 cells were maintained in CMRL 1066 (Gibco) and PCC4 cells in McCoy's 5A (Gibco) media on Corning
tissue culture plates supplemented with 10% fetal bovine serum. Cells were grown in a humidified atmosphere of 5% CO₂/95% air, and harvested prior to confluence.

**Growth Curves.** Cells were plated at a density of 50,000 viable cells/60-ml or 100-ml culture dish. Eight-12 h after plating and every 48 h thereafter the growth media were replaced with fresh media, and cultures were treated with 5 μM 8-Cl-cAMP, 100 μM 8-Br-cAMP, or 3 μM 8-Cl-adenosine, or 30-50 μM NBTI, or 3 units/ml ADA. The number of cells in duplicate cultures was determined by using a hemacytometer following trypantrypsinization. (0.25% trypsin in PBS), cell pelleting by centrifugation for 5 min at 135 × g, resuspending in 1 ml PBS; counting was continued until control cultures became confluent. For experiments using 8-Cl-cAMP and ADA, the fetal bovine serum used to supplement growth media was heated for 30 min at 65°C to inactivate the activity of serum phosphodiesterases and 5'-nucleotidases (14). Cell viability was determined by trypan blue exclusion (viable cells fail to take up the dye) and by direct examination (dead cells appear dark gray when viewed under a hemacytometer). Dead cells are easily distinguished from viable cells in culture because they detach from the surface of the culture dish.

**Analysis of Cell Cycle Distribution by Flow Cytometry.** The procedure used to stain DNA with propidium iodide was modified from Braylan et al. (25). Cells were removed from 100-ml culture dishes by trypsinization, pelleted by centrifugation for 5 min at 135 × g, washed once in 2-3 ml of serum-free media, and resuspended in 1 ml of this medium. An equal volume of absolute ethanol was added slowly to each tube during 1 min of agitation on a vortex mixer; tubes were then incubated for 1 h at 4°C. Cells were washed once with 2-3 ml of serum-free medium, and cell pellets containing 1-3 × 10⁶ cells were resuspended in 250 μl of 1.1% sodium citrate buffer (pH 8.4) containing 500 units/ml RNase A (Sigma), and then incubated for 30 min at 37°C. Citrate buffer (25 μl) containing 50 μg/ml propidium iodide (Sigma) was added to each tube; tubes were gently mixed and stored overnight at 4°C. To dissociate cell clumps, each sample was pipetted vigorously immediately before aspiration onto an Epics Profile (Coulter) flow cytometer. Cell cycle distribution analysis was performed by using the Mycycle (Phoenix Flow) program.

**Preparation of Cell Extracts.** Cells were washed twice with cold saline, scraped into fresh saline, pelleted by a 5-min, 135- × g-centrifugation, and homogenized in 5 volumes of buffer [0.32 M sucrose-10 mM potassium phosphate (pH 7.4)-1 mM EDTA-50 mg/ml leupeptin-2 mM fluoromethylsulfonyl fluoride]. Supernatant (cytosolic) fractions were prepared by a 30-min, 20,000- × g-centrifugation, and the recovered pellet (particulate fraction) was resuspended in the original volume of buffer. Protein concentrations were determined by the method of Lowry et al. (26).

**8-N3-[32P]cAMP Photolabeling.** The content of PKA R subunits in cytosolic and particulate fractions of cell extracts was determined by photolabeling with 8-N3-[32P]cAMP essentially as described (27), except that the final concentration of 8-N3-[32P]cAMP (ICN Biochemicals) was 1 μM.

**Western Immunoblot Analysis.** Cytosolic fractions containing 30 μg of protein were mixed with an equal volume of sodium dodecyl sulfate stop solution (28), boiled for 5 min, subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to nitrocellulose filters overnight at 0.6 A. Blots were incubated in blotto for 1 h at room temperature and then at 4°C overnight in antisera prepared against recombinant CHO cell Cox (generously provided by Dr. Marilyn Gosse, National Cancer Institute, Bethesda, MD) that had been previously diluted 1:500 in blotto. After 3 washings with PBS-0.1% Tween 20 and once with PBS, for 10 min each, blots were incubated for 1 h at room temperature with [ γ-32P]ATP, 7000 Ci/mmol specific activity (ICN Biochemicals) diluted 1-3 μl/ml in blotto. These blots were then washed as before, allowed to dry, and exposed to X-ray film at ~70°C for 1-3 days.

**PKA Activity.** The PKA activity in a 15-μg aliquot of cytosolic protein was assayed by measuring incorporation of the γ-phosphate from [ γ-32P]ATP, 7000 Ci/mmol specific activity (ICN Biochemicals), into the oligopeptide substrate, Kemptide, as described (29). Most of the PKA-specific activity in mouse lung epithelial cell lines is present in cytosolic fractions of cell extracts (17).

**Preparation of Total RNA and Northern Analysis.** The procedures used for preparing cDNA probes, isolating total cellular RNA, and Northern blotting were described by Shannon et al. (30). Expression vectors containing mouse Rla, Rllα, and Ca cDNA inserts were kindly provided by Dr. G. Stanley McKnight (University of Washington, Seattle, WA). Human β-actin cDNA insert was generously supplied by Dr. John Shannon (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO).

**RESULTS**

**Effects of cAMP Analogues on Growth of Normal and Neoplastic Lung Cells.** Micromolar concentrations of 8-Cl-cAMP inhibit the growth of several human cancer cell lines (31), and this analogue has been considered for clinical development. Since little is known about the effects of 8-Cl-cAMP on normal tissues, we applied this analogue to both normal and neoplastic lung cells. C10 is a nontumorigenic cell line derived from normal mouse lung epithelium (21); tumorigenic E9 cells represent spontaneous in vitro transformants of cells similar to C10 (22), and PCC4 cells were derived from a lung tumor (24). Five μM 8-Cl-cAMP severely inhibited the growth of both C10 and PCC4 cells for 48-72 h posttreatment and no cytotoxicity was observed (Fig. 1, A and C). Concentrations of 8-Cl-cAMP as high as 100 μM produced cytostasis without cytotoxicity (data not shown). In E9 cells, however, 8-Cl-cAMP concentrations as low as 1 μM were lethal, with cytotoxicity evident after 72 h of treatment (Fig. 1B).

8-Br-cAMP has similar affinity and identical selectivity for the cAMP-binding sites on PKA R subunits as does 8-Cl-cAMP (13, 14). In addition, 8-Br-cAMP is a more effective activator of PKA than cAMP itself, and is resistant to phosphodiesterase-mediated degradation (31). However, this analogue is a less potent growth inhibitor than

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*4 M. Gosse, R. Fleischmann, M. Marshall, N. Wang, S. Garges, and M. M. Gottesman, manuscript submitted.*
8-Cl-cAMP is now known to be metabolized to 8-Cl-adenosine by phosphodiesterases and 5'-nucleotidases contained in the fetal bovine serum used to supplement cellular growth media. Since 8-Cl-cAMP inhibits lung cell growth at very low concentrations (Fig. 1), unlike comparable cAMP analogues such as 8-Br-cAMP, its growth inhibitory and/or lethal effects could be mediated by its adenosine metabolite. Van Lookeren Campagne et al. (14) found that heat inactivation of serum reduced this conversion, but at least 11–25% of the total 8-Cl-cAMP was still hydrolyzed to 8-Cl-adenosine over a period of 72 h. HPLC analysis of lung cell growth media containing heat-inactivated serum indicated 90% conversion of 8-Cl-cAMP to 8-Cl-adenosine under similar conditions (data not shown). The growth of C10 and PCC4 cells was inhibited by 8-Cl-cAMP in media containing heat-inactivated serum (Fig. 2, A and C). E9 cells also remained highly sensitive to this treatment and cytotoxicity resulted in fewer viable cells (Fig. 2B). Heat inactivation apparently reduces the activity of the low Km form(s) of serum phosphodiesterase, but does not fully attenuate the activity of the high Km form(s) (16). ADA converts 8-Cl-adenosine into 8-Cl-inosine without affecting 8-Cl-cAMP; 8-Cl-inosine is nontoxic and does not inhibit cell growth (14). C10, E9, and PCC4 cells were treated with 8-Cl-cAMP in the presence and absence of ADA in media containing heat-inactivated serum. Addition of 3 units/ml ADA to 8-Cl-cAMP-treated cultures completely protected all three cell lines from the growth inhibitory or lethal effects of 8-Cl-cAMP (Fig. 2). Because heat inactivation of serum is only partially effective at preventing the formation of biologically significant concentrations of 8-Cl-adenosine, cells were treated with 8-Cl-cAMP in the presence and absence of the phosphodiesterase inhibitor, IBMX, to block 8-Cl-cAMP formation. IBMX alone was nontoxic and caused no cytostasis. 100–150 μM IBMX completely rescued all three cell lines from 8-Cl-cAMP-induced growth inhibition or cytotoxicity (data not shown). The growth inhibitory effects of 8-Cl-cAMP therefore appear to be mediated solely by its adenosine metabolite.

The human colon cancer cell line, LS-174T, is growth inhibited by both 8-Cl-cAMP and 8-Cl-adenosine, but purportedly by different mechanisms (33). When 8-Cl-cAMP was removed from the growth medium, LS-174T cells resumed their normal, untreated growth rate. These cells failed to recover from 8-Cl-adenosine treatment, however, and eventually died. To test the reversibility of 8-Cl-adenosine action and whether 8-Cl-cAMP and 8-Cl-adenosine affect lung cells via similar mechanisms, lung cells were treated for 72 h with either 5 μM 8-Cl-cAMP or 5 μM 8-Cl-adenosine. The cells were then washed and the spent media replaced with fresh media containing no nucleotide supplements. 8-Cl-adenosine caused exactly the same degree of growth inhibition or cytotoxicity as did 8-Cl-cAMP in each cell line (Fig. 3; compare first data point at 72 h). Upon release from these treatments, all three cell lines resumed rapid growth rates to reach the number of cells present in untreated 72-h cultures. The rate of recovery from both 8-Cl-cAMP and 8-Cl-adenosine was much faster in C10 cells than in E9 or PCC4 cells, probably because the degree of growth inhibition was less severe in the normal cells than in their neoplastic counterparts (Fig. 3; compare 72-h data points to level of dashed line; note difference in scales). Recovery was slightly less rapid in PCC4 cells and in the remaining viable E9 cells that had been treated with 8-Cl-adenosine than in cells treated with 8-Cl-cAMP. This is most likely attributable to the lower concentration of adenosine metabolite in 8-Cl-cAMP-treated cultures than in cultures given 8-Cl-adenosine directly. Thus, in this system, the effects of 8-Cl-cAMP cannot be separated from those of 8-Cl-adenosine, which acts through a reversible process.

8-Cl-cAMP treatment did not appreciably change the cell cycle distribution of LS-174T cells, while 8-Cl-adenosine increased the percentage of cells in G1 and reduced the size of the S phase population (33). To test whether 8-Cl-cAMP and 8-Cl-adenosine differentially affect cell cycle progression of mouse lung epithelial cells, C10, E9, and PCC4 cells were treated with 8-Cl-cAMP or 8-Cl-adenosine for 72 h, and the percentage of cells in each phase of the cell cycle was determined by propidium iodide DNA staining and flow cytometric analysis (Table 1). In both C10 and PCC4 cells, treatment with either 8-Cl-cAMP or 8-Cl-adenosine markedly increased the percentage of cells in G1, while proportionately decreasing the number of cells in S phase. However, treatment with either agent produced the opposite result in E9 cells; the viable cells remaining after exposure to 8-Cl-cAMP or 8-Cl-adenosine exhibited a decreased G1 phase and an increased S phase relative to untreated controls, indicating that these cells were growth inhibited prior to their death. While both agents produced the same trend in each cell line, the effect of 8-Cl-adenosine was greater than that of 8-Cl-cAMP. Again, this is probably a result of the lower concentration of adenosine metabolite formed in 8-Cl-cAMP-containing cultures as compared to direct addition of 8-Cl-adenosine. Since E9 cells behave differently than C10 and PCC4 cells with respect to cell cycle progression following exposure to 8-Cl-cAMP or 8-Cl-adenosine, the observed cytotoxicity in E9 cells may be attributed to the lower concentration of adenosine metabolite compared to other cell lines.
every 48 h. and fresh untreated medium was added; spent medium was replaced with fresh medium every 24 h, thereafter.

The results represent the means of triplicate measurements. The variation was less than 5%.

**Fig. 3.** Recovery of normal and neoplastic lung cells from 8-C1-CAMP- and 8-C1-adenosine-mediated growth inhibition and cytotoxicity. Cultures of C10 (A), E9 (B), and PCC4 cells (C) were treated with either 5 μM 8-C1-cAMP (○) or 5 μM 8-C1-adenosine (■) for 72 h. The medium was then removed, cultures were washed twice with normal saline, and fresh untreated medium was added; spent medium was replaced with fresh medium every 48 h. Duplicate cultures were counted at the time of medium removal (72 h) and every 24 h, thereafter. – – – – , number of cells present in untreated 72-h cultures. Data represent the mean number of cells in duplicate cultures ± the range from a typical experiment (bars).

**Table 1.** Cell cycle distribution following 8-C1-cAMP or 8-C1-adenosine treatment

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>% of cell population in C1</th>
<th>S</th>
<th>G2-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>C10</td>
<td>Control</td>
<td>54.2 ± 0.4</td>
<td>37.0 ± 0.4</td>
<td>8.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>8-C1-cAMP</td>
<td>67.4 ± 0.7</td>
<td>23.5 ± 1.3</td>
<td>9.1 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>8-C1-adenosine</td>
<td>73.4 ± 0.6</td>
<td>16.6 ± 0.8</td>
<td>9.9 ± 0.3</td>
</tr>
<tr>
<td>E9</td>
<td>Control</td>
<td>49.3 ± 0.6</td>
<td>28.3 ± 0.4</td>
<td>22.4 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>8-C1-cAMP</td>
<td>43.6 ± 0.3</td>
<td>34.5 ± 0.1</td>
<td>21.8 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>8-C1-adenosine</td>
<td>39.2 ± 0.1</td>
<td>38.6 ± 0.1</td>
<td>21.5 ± 0.3</td>
</tr>
<tr>
<td>PCC4</td>
<td>Control</td>
<td>45.7 ± 0.2</td>
<td>31.9 ± 0.5</td>
<td>22.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>8-C1-cAMP</td>
<td>50.4 ± 2.4</td>
<td>22.7 ± 2.4</td>
<td>17.9 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>8-C1-adenosine</td>
<td>63.5 ± 0.5</td>
<td>18.5 ± 0.3</td>
<td>18.2 ± 0.9</td>
</tr>
</tbody>
</table>

*Cells were treated with or without 5 μM 8-C1-cAMP or 8-C1-adenosine for 72 h, and the percentage of cells in each phase of the cell cycle was estimated by flow cytometric analysis following propidium iodide DNA staining, as described in “Materials and Methods.” The results represent the means of triplicate measurements ± SE.*

occur via a different biochemical mechanism(s) than that eliciting cytostasis in C10 and PCC4 cells.

**Cellular Uptake of 8-C1-Adenosine.** 8-C1-adenosine that is formed from extracellular 8-C1-cAMP could induce cytostasis or cytotoxicity by binding to adenosine receptors located on the exterior surface of the plasma membrane or by entering the cell via a nucleoside transporter mechanism. Once inside the cell, 8-C1-adenosine could act on intracellular targets to exert its inhibitory or lethal effects. Eukaryotic cells do not transport charged cyclic nucleotides or 5'-nucleotides, but efficiently take up nucleosides via a nonspecific, energy-free, facilitated diffusion transport mechanism. To test whether 8-C1-adenosine actually entered lung cells, cultures were exposed to 8-C1-cAMP for 3 days in the presence or absence of NBTI, an inhibitor of nucleoside uptake (34). Fifty μM NBTI alone had little effect on C10 and PCC4 cell growth and was nontoxic, while E9 cell viability was sensitive to 30 μM NBTI (Fig. 4). Addition of 30–50 μM NBTI to cultures containing 5 μM 8-C1-cAMP protected all three cell lines from the growth inhibitory or lethal effects observed with 8-C1-cAMP alone, suggesting that 8-C1-adenosine acts intracellularly. To ensure that 8-C1-adenosine was not altering intracellular cAMP concentrations via modulation of adenosine receptor-coupled adenylate cyclase activity, C10 cells were treated with 5 μM 8-C1-adenosine and cAMP concentrations were determined by radiommmunoassay. A time course following 8-C1-adenosine addition indicated no change in the basal intracellular level of cAMP (data not shown).

**Down-Regulation of PKA 1 Subunit Protein Concentrations by 8-C1-Adenosine.** 8-C1-cAMP treatment decreased the concentration of RI-subunits in both C10 and E9 cells, but had no effect on RII subunit levels (18). To test whether this decreased RI was actually caused by 8-C1-adenosine derived from 8-C1-cAMP via enzymes...
present in the growth media, C10, E9, and PCC4 cells were treated with 8-Cl-cAMP in the presence or absence of ADA for 72 h in media containing heat-inactivated serum, and R subunit concentrations were estimated by 8-N$_3$[P$^{32}$]cAMP photolabeling (Fig. 5A). Photolabeling and immunoblotting techniques yield similar results in these cells (17, 18). Consistent with our earlier findings (using unmodified serum), treatment with 8-Cl-cAMP alone markedly decreased RI subunit concentrations in all three cell lines, with negligible effects on RII levels. Indeed, RI subunits in neoplastic E9 cells fell from their already constitutively low basal concentrations to nearly undetectable amounts. Addition of ADA along with 8-Cl-cAMP completely abolished RI subunit diminution.

To confirm that the observed decreases in RI subunit concentrations were mediated by 8-Cl-adenosine that had been derived from the metabolism of 8-Cl-cAMP, cells were treated with $5 \mu$m 8-Cl-adenosine for 72 h and R subunit levels were examined by 8-N$_3$[P$^{32}$]cAMP photolabeling (Fig. 5B). 8-Cl-adenosine selectively decreased RI subunit concentrations in all three cell lines; RII levels decreased slightly. A 48-h 8-Cl-adenosine exposure produced no appreciable change in R subunit concentrations (data not shown), consistent with the absence of cytotoxic or cytotoxic effects at this earlier time point.

Coregulation of both PKA I subunit proteins in other systems (7, 35, 36) suggested that C subunit levels might also be down-regulated by 8-Cl-adenosine. Therefore, C subunit protein concentrations were examined by Western immunoblot analysis following treatment of lung cells for 72 h with or without $5 \mu$m 8-Cl-adenosine (Fig. 6). Basal expression of C subunit is lower in neoplastic E9 and PCC4 cells compared to normal C10 cells (17, 19). 8-Cl-adenosine reduced the level of C subunit protein by 50–60% in each cell line, as indicated by densitometric analysis of autoradiograms. This corresponded with a reproducible 45% loss of PKA-specific activity in C10 cells, and 20–25% decreases in E9 and PCC4 cells (Table 2). Protein kinase catalytic activity fell less in neoplastic E9 and PCC4 cells in response to 8-Cl-adenosine than in normal C10 cells, possibly because the basal level of PKA I expression in these neoplastic cells is already low.

Modulation of PKA Subunit mRNA Expression by 8-Cl-Adenosine. To determine if 8-Cl-adenosine lowers PKA I subunit protein levels by perturbing PKA mRNA expression, C10, E9, and PCC4 cells were treated with or without $5 \mu$m 8-Cl-adenosine for 72 h and PKA mRNA content was examined by Northern blot analysis (Fig. 7). 8-Cl-cAMP-treated cells were also studied to discern possible analogue-specific effects on mRNA expression. Neoplastic E9 and PCC4 cells constitutively express less Ca and 3.2-kilobase RIIe mRNA than normal C10 cells, while the expression of RIIc mRNA is greater (17, 19). In all three cell lines, both 8-Cl-cAMP (Lane 2) and 8-Cl-adenosine (Lane 3) markedly reduced Ca mRNA levels relative to controls (Lane 1); densitometric analysis indicated a reproducible 40–60% decrease in Ca mRNA expression. Basal levels of the three species of RII mRNA remained essentially unchanged following treatment with either agent. However, RIIc mRNA expression increased 2- to 3-fold in both 8-Cl-adenosine (Lane 2) and 8-Cl-cAMP (Lane 3)-treated cells, as indicated by densitometric analysis of autoradiograms. The changes in Ca and RIIc mRNA expression induced by 8-Cl-adenosine are slightly greater than those induced by 8-Cl-cAMP. Northern blot analysis 24 or 48 h after 8-Cl-cAMP or 8-Cl-adenosine addition demonstrated no change in PKA mRNA expression (data not shown). Similar to RIIc mRNA, β-actin mRNA

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**Fig. 5. A, 8-N$_3$[P$^{32}$]cAMP photolabeling of R subunits in 8-Cl-cAMP- and ADA-treated cells.** Normal C10 and neoplastic E9 and PCC4 cells were maintained in media containing heat-inactivated serum and treated for 72 h with (+) or without (-) $5 \mu$m 8-Cl-cAMP in the presence or absence of 3 units/ml ADA. R subunits in cytosolic (C) and particulate (P) subcellular fractions were photolabeled as described in “Materials and Methods,” subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and visualized by autoradiography. 8-Cl-cAMP treatment decreased RI subunit concentrations in each cell line without affecting RII levels. Addition of ADA to 8-Cl-cAMP-treated cells abolished the effects of 8-Cl-cAMP. B, 8-N$_3$[P$^{32}$]cAMP photolabeling of R subunits in 8-Cl-adenosine-treated cells. The same protocol was done by using 5 $\mu$m 8-Cl-adenosine, and this selectively decreased RI subunit levels, while having no effect on RII.
decreased C subunit levels in each cell line. Trace amounts of C subunit were visible in treatment. CI0, E9, and PCC4 cells were treated with (+) or without (-) 5 μM 8-CI-adenosine for 72 h and C subunits in cytosolic fractions of cell extracts were visualized by Western immunoblot analysis as described in "Materials and Methods." 8-Cl-adenosine decreased C subunit levels in each cell line. Trace amounts of C subunit were visible in particular fractions, and this was unaffected by 8-Cl-adenosine (data not shown).

| Table 2: Decreased PKA-specific activity in 8-Cl-adenosine-treated cells |
|-----------------------------|-----------------------------|
| Cell line | Treatment | PKA activity (pmol/min/mg) |
| C10 | Control | 6947 ± 169 |
| E9 | 8-Cl-adenosine | 3801 ± 62 |
| PCC4 | Control | 3909 ± 67 |
| | 8-Cl-adenosine | 2941 ± 28 |

* Cytosolic fractions of cell extracts were assayed following treatment of cells for 72 h with or without 5 μM 8-Cl-adenosine as described in "Materials and Methods." The results represent the means of triplicate measurements ± SE.

DISCUSSION

In both normal mouse lung cells and their neoplastic derivatives, the effects of 8-Cl-cAMP on cell proliferation and PKA subunit protein and mRNA expression are mediated by its adenosine metabolite. 8-Cl-adenosine most likely lowers PKA I subunit protein concentrations by initially reducing C subunit mRNA expression. The resultant decrease in C subunit protein causes an excess of free, and thus highly labile, RI subunits (37), which are then degraded. This interpretation is consistent with the finding that RI binds to the C subunit with a lower affinity than does RII in vivo (7). In mouse cells, competition between RI and RII for a limited pool of the C subunit resulted in preferential assembly of PKA II; the PKA I holoenzyme was constructed only when the amount of C subunit exceeded that of RII (7). Kin-49 mutants which lack detectable C subunits contain low RI, probably because of enhanced degradation of free RI-subunits (35). Elevation of C-subunits in NIH-3T3 cells increased the level of RI but not RII protein, suggesting that RI is stabilized in the holoenzyme complex by the C subunit (36). The 8-Cl-adenosine-mediated increase in RIIα mRNA expression observed herein is not reflected by elevated amounts of RII protein, which actually decreased slightly in response to this treatment (Fig. 5B), probably also due to enhanced degradation in the absence of free C subunit. Long-term treatment with the adenylyl cyclase-activating agent, forskolin, enhanced RIIα mRNA expression but had no effect on Cα or RIIα mRNA abundance in normal C10 cells; these changes in PKA subunit mRNA expression did not produce parallel changes in protein concentrations (18). 8-Cl-adenosine simultaneously decreased Cα and increased RIIα mRNA expression. Several tumorigenic mouse lung epithelial cell lines, including E9 and PCC4, which express deficient basal Cα mRNA, also constitutively contain more RIIα mRNA relative to nontumorigenic lines (38). This inverse relationship between Cα and RIIα mRNA expression implies that these messages are coregulated. Decreased Cα mRNA and the subsequent down-regulation of C-subunit protein may trigger RIIα gene expression.

The high potency and long latency period between application of 8-Cl-adenosine and its first observable effects may provide clues to its mechanism of action. The 8-Cl-adenosine-induced reduction of growth rate or viability, and the concomitant modulation of PKA subunit protein and mRNA levels are observable by 72 h of exposure to this agent. Perhaps 8-Cl-adenosine is further metabolized inside the cell and/or initially becomes incorporated into cellular nucleotide pools, nucleic acids, or other macromolecules. Consistent with this hypothesis, HPLC analysis failed to detect 8-Cl-adenosine or 8-Cl-cAMP in cell extracts prepared from 100 μM 8-Cl-cAMP-treated C10 cells (data not shown). Given that the endogenous concentration of ATP in cells is on the order of 200 μM, it is unlikely that 8-Cl-adenosine interferes with ATP-dependent pathways. 8-Cl-adenosine exerts its effects on mouse lung cells at extremely low concentrations, suggesting a specific interaction with its intracellular target(s) that necessitates a relatively high binding affinity. Identification of this target(s) and the structural requirements for this association are subjects currently under investigation.
The possible involvement of 8-Cl-adenosine in mediating the growth inhibitory effects of 8-Cl-cAMP was first examined by Tagliaferri et al. (33). 8-Cl-adenosine was not detected by HPLC analysis of LS-174T colon cancer cell extracts or growth medium after treatment of intact cells with 50 μM 8-Cl-cAMP. 8-Cl-cAMP reduced c-Myc and p21 Ras protein levels, while 8-Cl-adenosine did not affect the expression of these oncoproteins (39). In contrast, 8-Cl-adenosine decreased K-Ras oncprotein expression in C10 and E9 cells, as demonstrated by Western immunoblot analysis. Van Lookeren Campagne et al. (14) examined the growth inhibitory effects of 8-Cl-cAMP on mutant CHO cell lines having either deficient PKA activity or elevated cAMP-phosphodiesterase. These mutants were completely resistant to mM concentrations of 8-Br-cAMP, but remained highly sensitive to ~μM Molt-4 lymphoblasts from growth inhibition by 8-Cl-cAMP, while dipyrindamole, an inhibitor of nucleoside uptake, abolished 8-Cl-cAMP-mediated growth inhibition of Molt-4 cells but was lethal to CHO cells (14).

There are both similarities and differences between the results of these studies and the present data. Treatment of mouse lung cells with 8-Cl-adenosine selectively decreased RI and C subunit concentrations with minimal effects on RII levels (Figs. 5 and 6). 8-Cl-cAMP also reduced RI subunit concentrations in several human and animal cell lines, but RII subunits increased (reviewed in Ref. 39); the effects of 8-Cl-adenosine on PKA subunit proteins or mRNAs were not examined in those studies. ADA, IBMX, and inhibitors of nucleoside uptake such as NBTI, protected mouse lung cells from all of the effects of 8-Cl-cAMP, in common with the results obtained by using CHO mutants and Molt-4 cells (14). In contrast to our results showing complete recovery of mouse lung cell growth from both 8-Cl-cAMP and 8-Cl-adenosine (Fig. 3), LS-174T cells failed to recover from 8-Cl-adenosine exposure, but resumed growth following 8-Cl-cAMP treatment (33). Additionally, 8-Cl-adenosine-treated LS-174T cells underwent cell cycle distribution changes (33) similar to those observed in C10 and PCC4 cells treated with either 8-Cl-cAMP or 8-Cl-adenosine (Table 1). However, 8-Cl-cAMP did not significantly alter the cell cycle distribution of 8-Cl-cAMP-treated LS-174T cells, although slight changes are evident (33). These apparent contradictions may be explained by differences in the concentration of 8-Cl-adenosine when added directly versus its concentration when derived from 8-Cl-cAMP. Several of the experiments presented herein (Table 1; Figs. 3, 5, and 7) demonstrate that 8-Cl-adenosine is much more potent in producing its effects than similar concentrations of 8-Cl-cAMP, which must first undergo metabolism. Variation in the concentrations of metabolizing enzymes contained in different preparations of serum used to supplement cellular growth media as well as cell type-specific differences in sensitivity to 8-Cl-adenosine may explain why some cells recover from treatment with this agent while others do not.

Differences in response to 8-Cl-cAMP and/or 8-Cl-adenosine may ultimately depend on the cell-specific complement of PKA isozymes and isoforms. Cho-Chung et al. (39) propose that 8-Cl-cAMP added to neoplastic cells does not act via PKA activation per se, but instead preferentially binds to RIβ-subunits which then become stabilized, translocate to the nucleus, and restore the gene transcription pattern characteristic of normal cells. We have been unable to detect protein representing the β isoforms of PKA subunits in normal or neoplastic mouse lung cells (17). Perhaps those effects of 8-Cl-cAMP on cells which cannot be attributed to the action of 8-Cl-adenosine, such as certain changes in oncprotein expression (39), result from altered RIβ subunit expression. Exposure of HL-60 leukemia cells to RIβ-antibody oligodeoxynucleotide decreased basal and cAMP analogue (8-Cl and Aβ-2)-induced levels of RIβ protein, and reduced the degree of growth inhibition and differentiation induced by these analogues (40). Exposure of the same cells to RIα antisense oligonucleotide decreased RIα protein, increased RIβ protein, and induced growth inhibition and differentiation in the absence of cAMP analogues (41). An important similarity between these studies and the results described herein is that certain changes in PKA concentrations (i.e., decreased RIα protein content) clearly accompany growth inhibition. PKA most likely does not mediate changes in growth directly, but may play an indirect role in modulating the activity of oncogenes and other factors that can affect tumor cell growth. It would be interesting to test whether the effects of 8-Cl-adenosine on mouse lung cell growth and PKA I subunit concentrations are mimicked by exposure to RIα antisense oligonucleotides.

This is the first demonstration of a modulatory effect of 8-Cl-adenosine on PKA subunit mRNA and protein concentrations. 8-Cl-adenosine does not alter intracellular cAMP levels (i.e., via adenosine receptor stimulation), and is unrelated to cAMP in that it lacks the cyclic phosphate moiety, a requirement for R-subunit binding (4). This result is surprising, considering that PKA subunit protein and mRNA levels generally undergo changes only in response to cAMP or cAMP-elevating agents (10, 42, 43). Indeed, the only other example (to our knowledge) of a cAMP-independent decrease in RI subunit protein concentrations is that which follows treatment of murine neuroblastoma cells for 1 or more days with retinoic acid (44). The mechanism by which intracellular 8-Cl-adenosine decreases C subunit mRNA expression and whether this is causally related to growth inhibition or cytotoxicity is being investigated. It will be interesting to test whether other reported effects of 8-Cl-cAMP, such as increased binding of nuclear factors to the cAMP-responsive element consensus sequence (45, 46), or decreased transforming growth factor α mRNA expression (46, 47), are also mediated by its adenosine metabolite.

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8-CH-ADENOSINE 3',5'-MONOPHOSPHATE MEDIATES PKA DOWN-REGULATION


8-Chloroadenosine Mediates 8-Chloro-Cyclic AMP-induced Down-Regulation of Cyclic AMP-dependent Protein Kinase in Normal and Neoplastic Mouse Lung Epithelial Cells by a Cyclic AMP-independent Mechanism

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