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Original Paper

8-Chloroadenosine 3′,5′-monophosphate (8-Cl-cAMP) Selectively Eliminates Protein Kinase A Type I to Induce Growth Inhibition in c-*ras*-transformed Fibroblasts

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8-Chloroadenosine 3′,5′-monophosphate (8-Cl-cAMP), a site-selective cyclic adenosine 3′,5′-monophosphate (cAMP) analogue, exhibits growth inhibition in a broad spectrum of cancer cell lines. We investigated the effect of 8-Cl-cAMP on c-*ras*-transformed mouse fibroblasts (MP3/3T3) which were established by transfection of Balb3T3 cells (Balb3T3) with the point-mutated c-*ras* gene [G12→V12]. 8-Cl-cAMP (2–5 μM) exerted over 80% growth inhibition by day 4 on MP3/3T3, while inhibiting parental Balb3T3 cell growth less than 40%. In order to distinguish the effect of 8-Cl-cAMP from that of 8-chloroadenosine (8-Cl-adenosine), we examined the effect of 8-Cl-cAMP in serum-free medium. 8-Cl-cAMP demonstrated a potent growth inhibition of MP3/3T3 cells cultured in serum-free medium, suggesting that the growth inhibitory effect of 8-Cl-cAMP was not due to its hydrolysed product, 8-Cl-adenosine. In addition, both Balb3T3 and MP3/3T3 contained cAMP phosphodiesterases mainly composed of isozyme IV which has previously been reported to be insensitive towards the hydrolysis of 8-Cl-cAMP. Non-transformed Balb3T3 cells contained only type II cAMP-dependent protein kinase (PKA), whereas transformed MP3/3T3 exhibited a marked increase in type I PKA. The growth inhibition of MP3/3T3 by 8-Cl-cAMP accompanied almost complete elimination of type I PKA without affecting type II PKA. Moreover, 8-Cl-cAMP induced an arrest in the G_0/G_1-phase of the cell cycle in MP3/3T3. 8-Cl-adenosine had little or no effect on the cell cycle kinetics of MP3/3T3 cells. These results show that 8-Cl-cAMP is a novel cAMP analogue which selectively eliminates type I PKA to induce growth inhibition in transformed fibroblasts.

Key words: cAMP phosphodiesterase, cell cycle, growth inhibition, protein kinase A, site-selective cAMP analogue
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INTRODUCTION

Cyclic adenosine 3′,5′-monophosphate (cAMP) plays a critical role in the regulation of cell growth and differentiation. Previous studies from our laboratory have shown that 8-chloroadenosine 3′,5′-monophosphate (8-Cl-cAMP), a site-selective cAMP analogue, exhibits growth inhibitory activity *in vitro* and *in vivo* in a broad spectrum of human carcinoma, fibrosarcoma and leukaemia cell lines without causing cytotoxicity [1–3]. 8-Cl-cAMP's potent growth inhibitory effect is a result of its ability to modulate selectively two isoforms of cAMP-dependent protein kinase (PKA-I and PKA-II). Specifically, 8-Cl-cAMP downregulates PKA-I with or without upregulating PKA-II [1, 4]. PKA-I and PKA-II share a common C subunit but contain different R subunits (RI and RII, respectively) that bind cAMP [5]. It has also been reported that 8-Cl-cAMP can be hydrolysed to 8-chloroadenosine (8-Cl-adenosine) by the serum enzymes, cAMP

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phosphodiesterases (PDEs) and nucleotidase and growth inhibition is caused by the adenosine metabolite, 8-Cl-adenosine, which has a strong cytotoxic effect [6, 7]. In contrast, 8-Cl-cAMP has been shown to be a poor substrate for cyclic nucleotide PDEs [8, 9]. Moreover, 8-Cl-adenosine is cytotoxic for non-transformed cells, whereas 8-Cl-cAMP exhibits little or no growth inhibition in normal cells [10].

ras p21 is a member of the small (guanosine 5′-triphosphate) GTP-binding protein superfamily and plays an important role in cell growth and differentiation [11, 12]. It can be converted to an active form by point mutations that block its intrinsic GTPase activities and lead to increased cell growth and proliferation. Recently, it has been shown that PKA inhibits the *ras* signalling pathway by phosphorylating *raf* kinase [13] and that the activated α subunit of the heterotrimeric guanine nucleotide binding proteins inhibits the proliferative signals from *ras* through cAMP and PKA [14]. These studies suggest a role of cellular cAMP and PKA, although there was no strict correlation between cAMP levels and growth inhibition. It has also been suggested that the blockade of transformation by the activated α subunit of guanine nucleotide binding proteins can be achieved without raising the intracellular cAMP level [14]. Thus, it is possible that 8-Cl-cAMP may directly inhibit the *ras* signalling pathway by its unique ability to modulate two isoforms of PKA.

The objective of this study was to investigate the effect of 8-Cl-cAMP on the growth of *ras*-transformed mouse fibroblasts and the parent normal fibroblasts and also to investigate the mechanism of 8-Cl-cAMP action on growth inhibition. Our study demonstrates that 8-Cl-cAMP exerts a potent growth inhibitory effect on *ras*-transformed fibroblasts but not on non-transformed parental cells, working through downregulation of PKA-I, the positive cAMP signal for cell proliferation and that such action of 8-Cl-cAMP is not mimicked by the cytotoxic 8-Cl-adenosine, the hydrolysed product of 8-Cl-cAMP.

MATERIALS AND METHODS

Chemicals

8-Cl-cAMP was obtained from the National Cancer Institute, Drug Synthesis and Chemistry Branch (Bethesda, Maryland, U.S.A.). 8-Cl-adenosine was obtained from Biology Life Science Institute (Bremen, Germany). Propidium iodide and RNase were from Sigma Chemical Co. (St Louis, Missouri, U.S.A.) and 8-N_3-[^{32}P]cAMP and [γ-^{32}P]ATP were from ICN Pharmaceuticals, Inc. (Irvine, California, U.S.A.).

Cell culture

MP3/3T3 cells, kindly provided by Dr Scott Abrams (National Cancer Institute, Bethesda, Maryland, U.S.A.), were established by transfection with the c-*ras* point-mutated gene [G12→V12] to Balb3T3 mouse fibroblasts. Balb3T3 and MP3/3T3 cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), streptomycin (500 μg/ml) and penicillin (50 units/ml). For cell growth experiments, cells were seeded at a density of 1×10^5 cells/60 mm dish and the cAMP analogue 8-Cl-cAMP or 8-Cl-adenosine added 16 h after seeding. The cells were harvested for cell counting at specified days. The percentage inhibition of cell growth was obtained by comparing the growth of treated cells with that of untreated control cells.

cAMP PDE assay

For the cAMP PDE assay, cells were resuspended in 1 ml of homogenisation buffer [10 mM (N-tris[hydroxymethyl]-methyl-2-aminoethanesulphonic acid; 2-([2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino)-ethanesulphonic acid) (TES) (pH 7.4), 5 mM $MgSO_4$, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM ethyleneglycol-aminoethyl-tetraacetic acid (EGTA), 1 mM benzamidine, 10 μg/ml of leupeptin, pepstatin A and aprotinin, 0.5 mM pefabloc and 10% glycerol], sonicated three times on ice for 20 sec each and centrifuged to obtain supernatant fractions. cAMP PDE activity was assayed by a modification of a previously described procedure [15]. Briefly, samples were incubated at 30°C for 10 min in a total volume of 0.3 ml containing 50 mM Hepes (pH 7.4), 0.1 mM EDTA, 8.3 mM $MgCl_2$ and 0.1 μM [^{3}H] cAMP (18 000 cpm). PDE III and PDE IV activity (pmol cAMP hydrolysed per min) was measured using the specific inhibitor 0.5 mM OPC 3689 (a specific inhibitor of PDE III [16]) and 10 μM Rolipram (an inhibitor of PDE IV).

Photoaffinity labelling of the R subunits of PKA

Cell extract preparation and the photoactivated incorporation of 8-N_3-[^{32}P]cAMP were performed as previously described [17, 18]. The samples containing 50 μg of proteins were subjected to sodium dodecylsulphate–polyacrylamide gel elecrophoresis (SDS–PAGE) and the proteins were transferred to nitrocellulose sheets. The sheets were dried and autoradiographed. The photoaffinity labelled samples were also immunoprecipitated using anti-RIα polyclonal antibody and were subjected to SDS–PAGE. The immunoprecipitation was performed as described previously [18, 19].

Diethyl-aminoethyldextran (DEAE) cellulose column chromatography

Cell extracts (10 mg protein) were loaded on to DEAE cellulose columns (1×10 cm) and fractionated with a linear salt gradient (0 to 0.35 M) [4]. PKA activity was measured by the method described previously [4].

Cell cycle analysis

Cells were cultured with 0.5% FBS for 2 days in order to synchronise cell cycles. The 0.5% FBS treatment led to differences in the cell numbers between MP3/3T3 and Balb3T3. In order to adjust cell numbers, cells were harvested and 3×10^6 cells were seeded on to 10 cm diameter plates with 10% FBS. 8-Cl-cAMP or 8-Cl-adenosine was added after cell attachment and the cells were harvested at 6, 12, 18, 24 and 48 h of culture time and fixed with ice-cold 70% ethanol for 30 min. After washing with Ca^{2+}/Mg^{2+}-free phosphate buffered saline (PBS), 50 μg/ml of propidium iodide with 1 mg/ml of RNase were added to 1×10^6 cells and incubated for 30 min at room temperature. The DNA content was analysed with flow cytometry (FACScan, Becton Dickinson, San Jose, California, U.S.A.) in duplicate as described previously [20]. Cell cycle data analysis was performed using Modifit software (Becton Dickinson).

Statistical analysis

Significant differences were determined by paired *t*-test or Wilcoxon signed-rank test. A P value of less than 0.05 was considered to be statistically significant.

RESULTS

Cell growth inhibition by 8-Cl-*cAMP*

The effect of 8-Cl-cAMP or 8-Cl-adenosine on the cell growth of MP3/3T3 and parental Balb3T3 was examined. The transformed cell MP3/3T3 showed a higher growth rate in monolayer culture as compared with the non-transformed parental cells. 8-Cl-cAMP exerted growth inhibition in MP3/3T3 cells in a dose- and time-dependent manner. Treatment with 8-Cl-cAMP at 2–5 μM for 96 h produced more than 80% growth inhibition in MP3/3T3, whereas the same treatment exerted only 36% growth inhibition in parental Balb3T3 cells (Figure 1). In contrast, 8-Cl-adenosine (2–5 μM for 96 h) produced over 80% growth inhibition in both cell lines (Figure 1). The growth inhibition of MP3/3T3 cells by 8-Cl-cAMP treatment accompanied changes in cell morphology. The 8-Cl-cAMP-treated cells exhibited an enlarged cytoplasm and a flat phenotype resembling that of the formerly described flat revertants [21]. In contrast, 8-Cl-adenosine treatment, especially at higher concentrations, produced shrinkage in the cell size without morphological change.

The effect of 8-Cl-*cAMP on cell growth in serum-free medium*

Because serum PDE and nucleotidase are responsible for the hydrolysis of 8-Cl-cAMP [6], we performed cell growth experiments using serum-free medium in the absence of serum substitutes (see Figure 2 legend). During the 48 h culture in serum-free medium, both MP3/3T3 and Balb3T3 grew exhibiting a logarithmic phase of growth, although Balb3T3 showed a slower rate of growth. The cell numbers at zero time and 48 h of culture were 5.0×10^5 and 16.5×10^5, respectively, in MP3/3T3 and 2.9×10^5 and 8.0×10^5, respectively, in Balb3T3. The cell viability determined using the trypan blue dye exclusion test showed that more than 90% of cells were viable after 48 h of culture in serum-free medium in both MP3/3T3 and Balb3T3 cells.

As shown in Figure 2, 8-Cl-cAMP treatment at 2 and 5 μM concentrations resulted in 34 and 42% growth inhibition, respectively, in MP3/3T3, while producing 5 and 16% growth inhibition, respectively, in parental cells ($P < 0.05$).

cAMP PDE activity in ras-*transformed cells*

It has been shown in HL-60/ADR leukaemia cells [8] and in HT-29 colon cancer cells [22] that 8-Cl-cAMP accumulates intracellularly primarily as nucleotide metabolites and 8-Cl-cAMP, but not as 8-Cl-adenosine. Furthermore, 8-Cl-cAMP accounted for approximately 30% of the intracellular nucleotides after 48 h of incubation [8], suggesting that 8-Cl-cAMP is a poor substrate for cyclic nucleotide PDEs. In fact, 8-Cl-cAMP was a poor substrate for several purified isoforms of PDEs. At high concentrations (100 μM), 8-Cl-cAMP is hydrolysed by all PDEs with the exception of PDE II. However, as 8-Cl-cAMP is unlikely to reach this concentration inside cells, the rate of hydrolysis at 1 μM is probably more significant. At this concentration, 8-Cl-cAMP is only significantly metabolised by PDE III at one-third of the rate of cAMP [9].

We measured PDE isozyme activities in MP3/3T3 and parental Balb3T3 cell extracts using OPC 3689 and Rolipram, the specific inhibitors for PDE III (isozyme III) and PDE IV (isozyme IV), respectively. As shown in Figure 3, Rolipram, which specifically inhibits PDE IV, decreased PDE activity by 90% in Balb3T3 and reduced PDE activity by 70% in MP3/3T3, whereas OPC, which selectively inhibits PDE III, reduced the PDE activity only 10% in both Balb3T3 and MP3/3T3 cell extracts (Figure 3). These results show that both Balb3T3 and MP3/3T3 cells contained PDE IV, to which 8-Cl-cAMP is a poor substrate, as the major form of PDE.

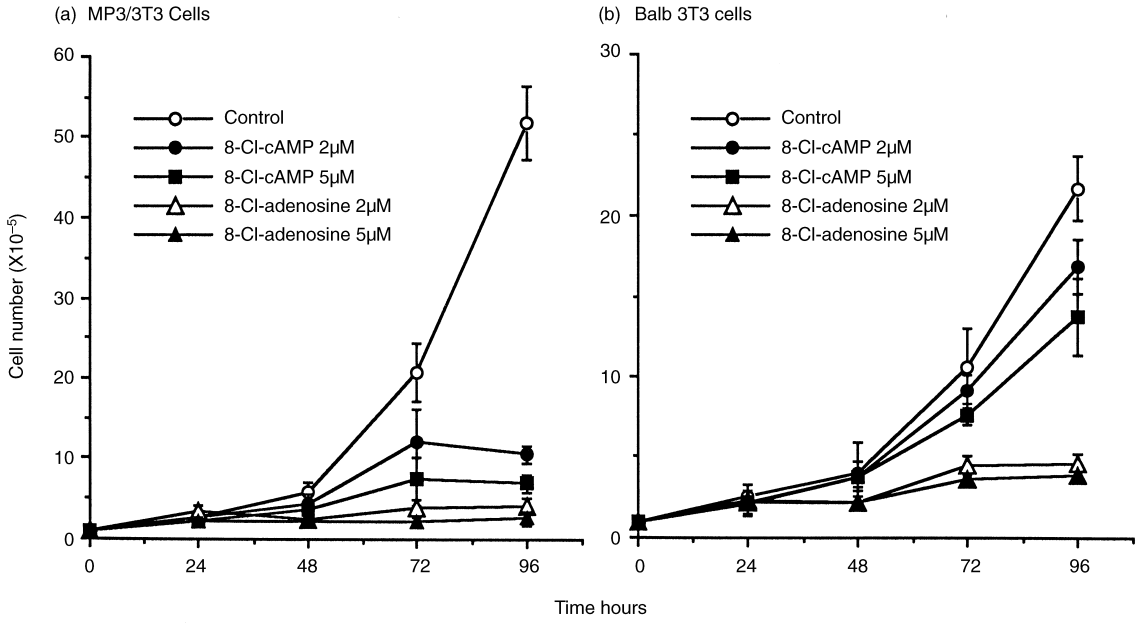

Figure 1. Effect of 8-chloroadenosine 3′,5′-monophosphate (8-Cl-cAMP) and 8-chloroadenosine (8-Cl-adenosine) on the growth of (a) MP3/3T3 and (b) Balb3T3 cells. Cells were cultured as described in Materials and Methods. Control and treated cells were harvested at days indicated and cell numbers were counted in triplicate by a Coulter counter (see Materials and Methods). Data represent mean ± standard deviation (S.D.) obtained from six independent experiments.

The effect of 8-Cl-*cAMP on PKA isozyme distribution in* ras-*transformed cells*

The expression of the regulatory subunits of PKA was determined using photoaffinity labelling with 8-N_3-[^{32}P]cAMP. The RIα level was further determined by immunoprecipitation with anti-RIα antiserum [18, 19] following photoaffinity labelling. As shown in Figure 4, Balb3T3 cells contained a greater amount of RIα than MP3/3T3 cells. Importantly, 8-Cl-cAMP treatment markedly reduced RIα levels in MP3/3T3 cells, whereas 8-Cl-adenosine treatment had a little or no effect on RIα levels in MP3/3T3 cells (Figure 4). 8-Cl-cAMP also reduced RIIα protein levels in MP3/3T3, whereas 8-Cl-adenosine had no effect on RIIα levels (Figure 4). This reduction in RIIα by 8-Cl-cAMP may indicate the effect of 8-Cl-cAMP on the free RIIα subunit. This is because 8-Cl-cAMP treatment did not affect the PKA-II holoenzyme level (Figure 5). We speculate that 8-Cl-cAMP treatment may have caused an increased turnover rate of RIIα present in an excess amount of the 'C' subunit of PKA. It has been shown that 8-Br-cAMP, isoproterenol and cholera toxin destabilise the R subunit in cells to an extent comparable with cells containing the 'kinase-negative' (lacking functional C subunit) mutation where the R subunit turnover rate increases at 10-fold over that of wild-type cells [23]. That the decrease of both RI and RII levels occurs in MP3/3T3 cells by 8-Cl-cAMP treatment but not by 8-Cl-adenosine treatment further supports that 8-Cl-cAMP acted upon MP3/3T3 as its intact molecule.

An interesting observation was that RIα levels in Balb3T3 cells were not altered by the treatment with either 8-Cl-cAMP or 8-Cl adenosine (Figure 4). These results suggest that in Balb3T3 cells, RIα may be present in its subunit form rather than being complexed with the C subunit to form PKA-I holoenzyme. If this is the case, the RIα in Balb3T3 cells will serve as a cAMP sink; thus, 8-Cl-cAMP will have no effect. We, therefore, examined the PKA isozyme distribution in Balb3T3 and MP3/3T3 cells before and after 8-Cl-cAMP treatment.

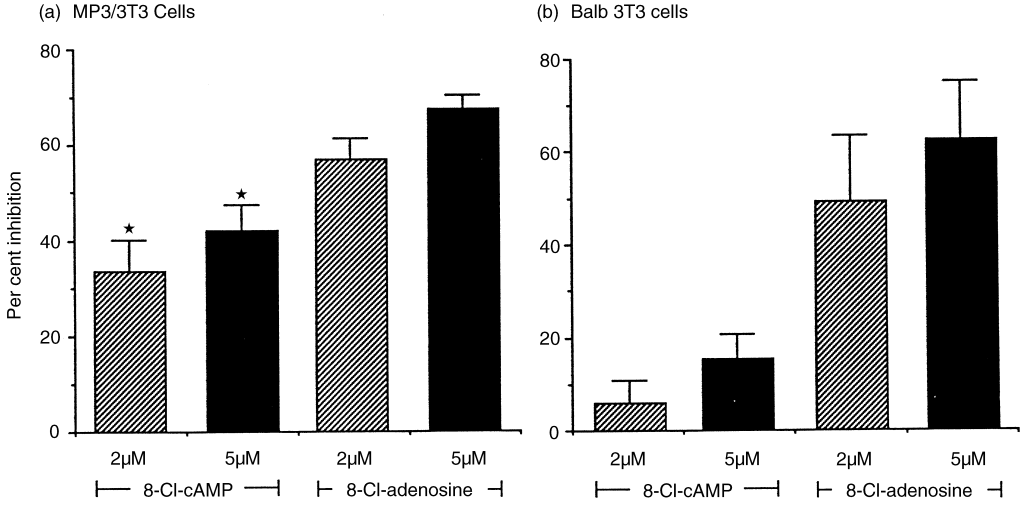

Figure 2. The effect of 8-chloroadenosine 3′,5′-monophosphate (8-Cl-cAMP) on cell growth in serum-free medium. (a) MP3/3T3 and (b) Balb3T3 cells were seeded at a density of 1×10^5 cells/ml in a 60 mm dish in medium containing serum. After 48 h, cells were washed twice with serum-free medium and culture continued in serum-free medium in the absence and presence of 2 μM (▨) or 5 μM (■) 8-Cl-cAMP or 8-chloroadenosine (8-Cl-adenosine) for 48 h. The percentage of growth inhibition was determined compared with untreated control cells (see Materials and Methods). Data represent mean ± S.D. obtained from three independent experiments (*$P < 0.05$, versus that of parental Balb3T3).

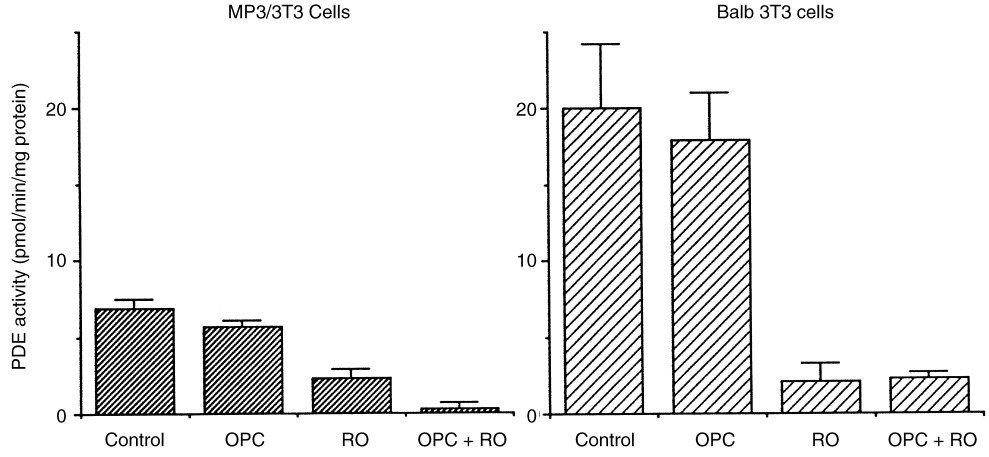

Figure 3. Phosphodiesterase (PDE) isozymes in *ras*-transformed and parental non-transformed cells. OPC 3689 (OPC) and Rolipram (RO) are the specific inhibitors of PDE isozymes, PDE III and PDE IV, respectively (see Materials and Methods). Data represent mean ± S.D. obtained from three independent experiments ($P < 0.05$ versus that of parental Balb3T3).

We added cell extracts to DEAE ion-exchange columns followed by elution with a linear salt gradient. Fractions were then assayed for kinase activity in the absence and presence of cAMP. Under these conditions, two major peaks of PKA activity were separated: PKA-I eluted between 50 and 100 mM NaCl and PKA-II eluted between 200 and 300 mM NaCl (Figure 5). Non-transformed Balb3T3 cells contained the PKA-II isozyme as the sole form of PKA holoenzyme as no detectable level of PKA-I was present (Figure 5). The transformed MP3/3T3 cells exhibited a marked increase in PKA-I level without changing the PKA-II level as compared with parental non-transformed Balb3T3 cells (Figure 5). This result confirms the previous report that the SV40 viral transformation was accompanied by an increase in PKA-I; normal Balb3T3 contained only PKA-II [24]. 8-Cl-cAMP (2 μM, 48 h) treatment resulted in almost complete elimination of PKA-I without affecting the PKA-II level in MP3/3T3 cells (Figure 5).

Cell cycle analysis

In order to clarify the mechanism of the specific growth inhibition in MP3/3T3 by 8-Cl-cAMP, we analysed the cell cycle distribution after treatment with 8-Cl-cAMP or 8-Cl-adenosine. More than 90% of cells accumulated into

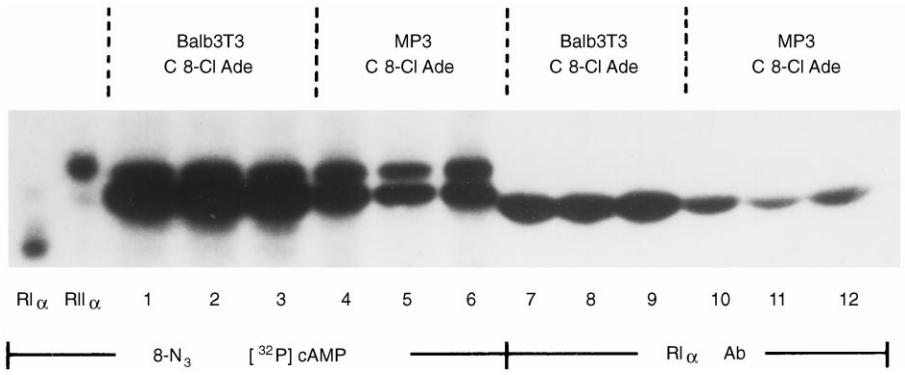

Figure 4. Photoaffinity labelling of cyclic adenosine 3′,5′-monophosphate (cAMP) receptor proteins. The expression of the regulatory subunit of protein kinase A in control (untreated) and treated [8-chloroadenosine 3′,5′-monophosphate (8-Cl-cAMP) 2 μM, 48 h] cells was determined by photoaffinity labelling with 8-N_3-[^{32}P]cAMP as described in [17, 18]. Rabbit skeletal muscle RIα (Sigma) and bovine heart RIIα (Sigma) were used as standards. Immunoprecipitation was performed with anti-RIα antibody [18] following photoaffinity labelling (lanes 7–12), as described in [17, 18]. Data represent one of three separate experiments that gave similar results.

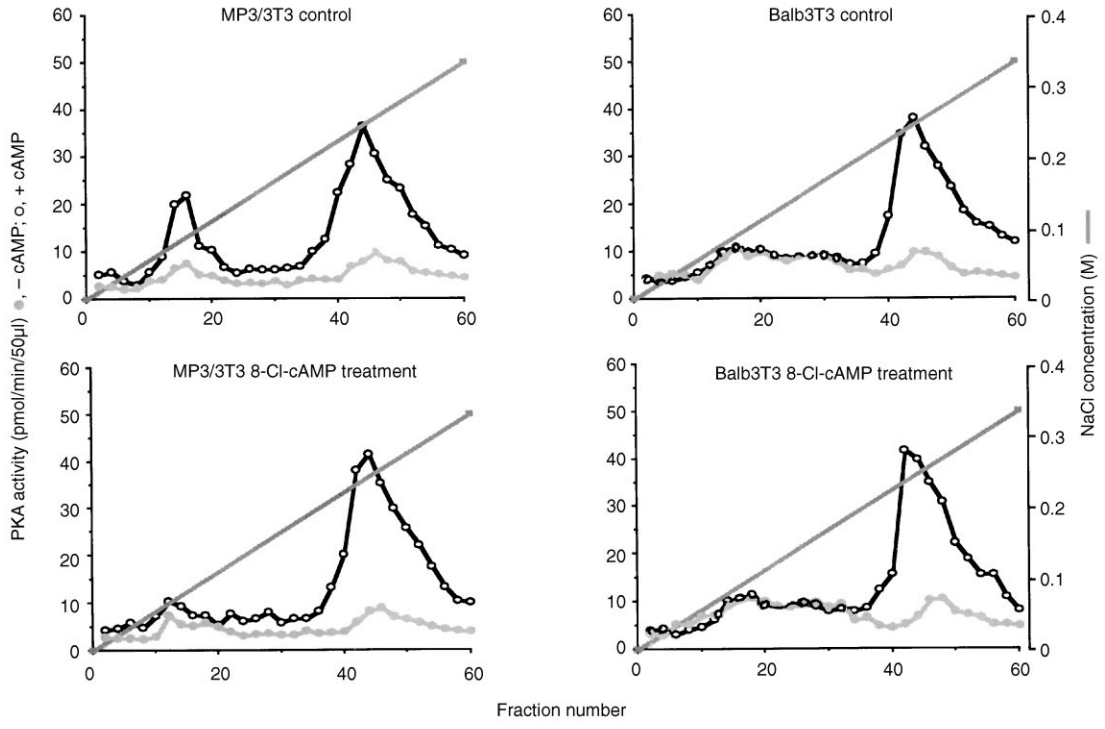

Figure 5. 8-Chloroadenosine 3′,5′-monophosphate (8-Cl-cAMP) downregulates protein kinase A (PKA) type I in *ras*-transformed cells. PKA-I and PKA-II activities in *ras*-transformed and parental cells, before and after treatment with 8-Cl-cAMP (2 μM, 48 h), were measured by DEAE cellulose column chromatography and PKA assays on the eluents. The PKA activity was measured in the absence (●) and presence (○) of 5 μM cyclic adenosine 3′,5′-monophosphate (cAMP) (see Materials and Methods). Each chromatography was repeated three times and yielded similar elution profiles. Data represent one of three separate experiments that gave similar results.

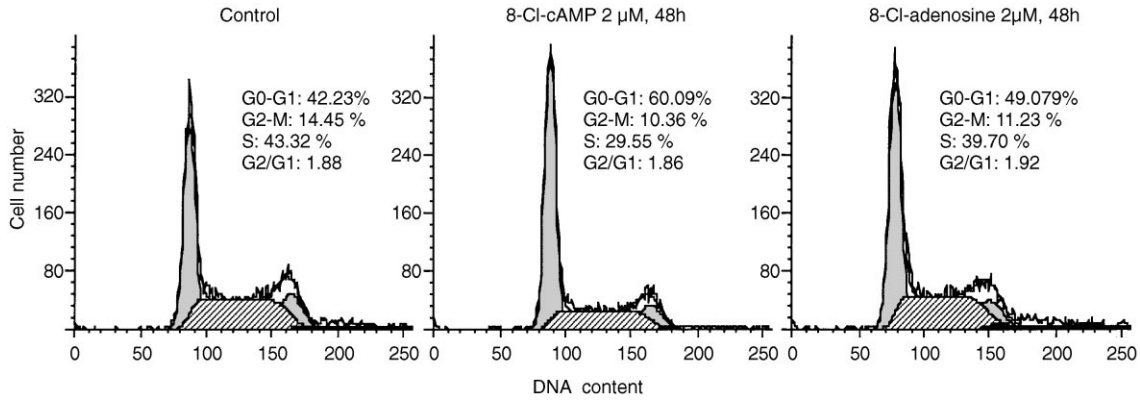

Figure 6. Effect of 8-chloroadenosine 3′,5′-monophosphate (8-Cl-cAMP) on the cell cycle kinetics of *ras*-transformed cells. Cells were synchronised into G_0/G_1 phase by culture for 48 h with 0.5% fetal bovine serum. More than 90% of cells accumulated into G_0/G_1 phase. Cells were harvested and reseeded into a culture dish. After cell attachment to the dish, 8-Cl-cAMP or 8-chloroadenosine (8-Cl-adenosine) was added and cells were harvested at specified times to measure DNA content by propidium iodide (PI) staining (see Materials and Methods). Data represent one of three separate experiments that gave similar results.

G_0/G_1 phase after synchronisation. We measured the cell cycle distribution at 6, 12, 24 and 48 h after reseeding. Cells progressed from G_0/G_1 phase into S phase at 12–24 h after reseeding (data not shown). After 48 h, the S phase population of untreated cells was 43%, whereas that of 8-Cl-cAMP-treated cells was 29% (Figure 6). The reduction of cells in S phase was inversely related to an increase of cells in G_0/G_1 phase (Figure 6). In contrast, 8-Cl-adenosine only minimally affected the cell cycle distribution (Figure 6).

DISCUSSION

We have shown in this study that (i) in the medium containing heat-inactivated serum, which contains a low level of PDE, 8-Cl-cAMP inhibited the growth of *ras*-transformed fibroblasts, but had a minimum inhibitory effect on non-transformed parental cells; (ii) 8-Cl-adenosine produced an equal growth inhibitory effect on both transformed and non-transformed cells; (iii) in the serum-free medium, 8-Cl-cAMP showed a significant growth inhibitory effect only in transformed cells; and (iv) both transformed and non-transformed cells contained PDE IV as the major form of PDE, which exhibits a weak hydrolysing activity towards 8-Cl-cAMP [8, 9]. These results demonstrate that the specific growth inhibitory effect of 8-Cl-cAMP on *ras*-transformed MP3/3T3 cells was not caused by its hydrolysed product, 8-Cl-adenosine.

It has been shown that chemical or viral carcinogenesis elicits a rapid induction of type I PKA isozyme [24, 25]. The *ras*-transformed MP3/3T3 is an example of such a case. Non-transformed 3T3 cells contain only type II PKA, whereas transformed MP3/3T3 exhibit a marked increase in type I PKA (Figure 5). The growth inhibition of MP3/3T3 by 8-Cl-cAMP accompanied almost complete elimination of type I PKA without affecting type II PKA (Figure 5). Thus, 8-Cl-cAMP restored the normal PKA isozyme profile in MP3/3T3 and inhibited proliferation. These results suggest that RIα/PKA-I plays a critical role in cell proliferation in *ras*-transformed MP3/3T3 cells.

It has been believed that cAMP analogues inhibit cell growth through their ability to raise cellular cAMP levels. However, it has also been shown [26, 27] that cellular cAMP levels do not strictly correlate with the transformation or reverse transformation process, suggesting that cellular effectors, such as PKA, other than endogenous cAMP levels, are critically involved in transformation and/or reverse transformation [1]. It has been shown that PKA directly regulates the *ras* signalling pathway by phosphorylating *raf* 1 kinase [13, 28] and that the activated α subunit of the heterotrimeric guanine nucleotide binding protein inhibits proliferative signals from *ras* through cAMP and PKA [14]. We speculate that suppression of RIα/PKA-1 by 8-Cl-cAMP may directly regulate the *ras* signalling pathway without changing cellular cAMP levels. Thus, the negative biochemical signals on the cAMP–*ras* signalling pathway can be introduced by lowering RIα/PKA-I, the growth-stimulatory cAMP receptor protein.

We showed that 8-Cl-cAMP induced cell cycle arrest at the G_0/G_1 phase in the cell cycle-synchronised *ras*-transformed cells. However, 8-Cl-cAMP did not induce any changes in the cell cycle distribution under non-synchronised cell culture conditions, as previously shown [20, 22, 29]. This may be because the cells treated with 8-Cl-cAMP are not yet terminally differentiated, but maybe at various stages of differentiation. We speculate two possible mechanisms of 8-Cl-cAMP effect on cell cycle distribution. One is the effect on the *ras* signalling pathway, which regulates cell cycle progression by the mitogen-activated protein kinase (MAP kinase) pathway [30], namely downregulation of RIα/PKA-I by 8-Cl-cAMP might induce inhibition of the *raf–ras* signalling pathway and lead to cell cycle arrest. The other possibility is that RIα/PKA-I directly regulates the cell cycle progression at the G_0/G_1 to S transition phase and 8-Cl-cAMP inhibits such a role of RIα/PKA-I by downregulating RIα/PKA-I. It has been previously reported that the PKA-I/RIα level sharply increases at the G_1/early S transition phase of the cell cycle [31]. Therefore, RIα may directly regulate the entry of cells from the G_0/G_1 phase to the S phase. It has also been shown that the selective inhibition of various protein kinases, including PKA, in normal fibroblasts produces growth arrest at different check points along the G_1 phase of the cell cycle. Specifically, addition of the PKA inhibitor KT5720 brought about an arrest at a G_1-specific check point approximately 6 h before cell entry into the S phase [32].

The results of the present study demonstrated that 8-Cl-cAMP induces growth inhibition in *ras*-transformed fibroblasts by a mechanism different from that of 8-Cl-adenosine,

namely downregulation of PKA-1. This study also demonstrated that 8-Cl-cAMP is a unique analogue of cAMP. It inhibits the growth of transformed cells while minimally affecting normal cell growth, through selective regulation of PKA isozymes at micromolar concentrations. Such a specific growth inhibitory effect of 8-Cl-cAMP is not mimicked by other analogues of CAMP, such as 8-Br-cAMP, 8-CPT-cAMP, or by the metabolite, 8-Cl-adenosine [33]. It has been shown that both the binding affinity and the activation potency of 8-Cl-cAMP towards PKA are involved in its mechanism of action. 8-Cl-cAMP is an isozyme site-discriminator (ISD) class of cAMP analogue which changes its site-selectivity toward RI and RII [1]. It binds to RII with a high affinity at site B and with low affinity for site A, but binds to RI at site A with a high affinity and at site B with moderately high affinity. At micromolar concentrations, 8-Cl-cAMP binds to RII at site B preferentially which does not lead to RII dissociation from the C subunit, thus keeping PKA-II in holoenzyme, while binding to RI at both site A and site B, facilitating dissociation of RI from the C subunit due to site A and site B co-operactivity, leading to downregulation of PKA-I.

8-Cl-cAMP-induced downregulation of PKA-I can occur with or without upregulation of PKA-II [1, 4] and the PKA-II upregulation by 8-Cl-cAMP occurs with or without upregulation of the RII subunit [1, 4]. In the present study, 8-Cl-cAMP downregulated PKA-I without PKA-II upregulation; the PKA-II holoenzyme level did not change and the RIIα subunit level decreased. We speculate that PKA-II upregulation along with PKA-I downregulation can occur only when the C subunit in the cell is in an excess amount of the total R subunits. Importantly, 8-Cl-cAMP acting as a PKA regulator, requires only micromolar concentrations to inhibit cell growth. However, at unnecessarily high concentrations that exceed cellular cAMP levels it may no longer be protected from the PDE extracellularly/intracellularly and it loses its novel activity due to the formation of metabolites [6, 7]. In the preclinical studies of 8-Cl-cAMP in mice and dogs, the toxic effects were due to the metabolites of 8-Cl-cAMP formed in the plasma [34]. The cAMP PDE is present at high levels in rodents, dogs and bovine plasma, whereas the cAMP hydrolysing enzymes are undetectable in human plasma [6, 34].

Nevertheless, the cAMP PDE levels in human cells and tumours may be variable. In a clinical setting, a continued infusion method without rest will mask the novel growth inhibitory effect of 8-Cl-cAMP. The continued infusion will result in the accumulation of 8-Cl-cAMP to a high concentration that will exceed the cellular clearing concentration leading to production of the cytotoxic metabolites. The phase I clinical study of 8-Cl-cAMP is complete [35]. At doses below the maximum tolerated dose, 8-Cl-cAMP was not toxic but reached plasma concentrations in the potential therapeutic range for growth inhibition [35].

Our present study, together with the successful phase I clinical study [35], strongly support the hypothesis that 8-Cl-cAMP will bring a promising clinical benefit in cancer treatment. Moreover, 8-Cl-cAMP would provide an essential tool to investigate the PKA signalling pathway in transformation and differentiation of various cancer cells.

1. Cho-Chung YS. Role of cyclic AMP receptor proteins in growth, differentiation, and suppression of malignancy: new approaches to therapy [Perspectives in cancer research]. *Cancer Res* 1990, **50**, 7093–7100.
2. Cho-Chung YS, Clair T, Tagliaferri P, *et al.* Site-selective cyclic AMP analogs as new biological tools in growth control, differentiation, and proto-oncogene regulation. *Cancer Invest* 1989, **7**, 161–177.
3. Ally S, Clair T, Katsaros D, *et al.* Inhibition of growth and modulation of gene expression in human lung carcinoma in athymic mice by site-selective 8-Cl-cAMP. *Cancer Res* 1989, **49**, 5650–5655.
4. Rohlff C, Clair T, Cho-Chung YS. 8-Cl-cAMP induces truncation and down-regulation of the RIα subunit and up-regulation of the RIIβ subunit of cAMP-dependent protein kinase leading to type II holoenzyme-dependent growth inhibition and differentiation of HL-60 leukemia cells. *J Biol Chem* 1993, **268**, 5774–5782.
5. Beebe SJ, Corbin JD. Cyclic nucleotide-dependent protein kinases. In Boyer PD, Kerbes EG, eds. The *Enzymes: Control by Phosphorylation*, Part A, Vol. 17. New York, Academic Press, 1986, 43–111.
6. Van Lookeren Campagne MM, Villalba Díaz F, Jastorff B, Kessin RH. 8-Chloroadenosine 3′,5′-monophosphate inhibits the growth of chinese hamster ovary and Molt-4 cells through its adenosine metabolite. *Cancer Res* 1991, **51**, 1600–1605.
7. Lange-Carter CA, Vuillequez JJ, Malkinson AM. 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. *Cancer Res* 1993, **53**, 393–400.
8. Rohlff C, Safa B, Rahman A, Cho-Chung YS, Klecker RW, Glazer RI. Reversal of resistance to adriamycin by 8-Cl-cAMP in adriamycin-resistant HL-60 leukemia cells is associated with reduction of type I cyclic AMP-dependent protein kinase and cyclic AMP response element-binding protein DNA-binding activities. *Mol Pharmacol* 1993, **43**, 372–379.
9. Yokozaki H, Budillon A, Clair T, *et al.* 8-Chloroadenosine 3′,5′-monophosphate as a novel modulator of multidrug resistance. *Int J Oncol* 1993, **3**, 423–430.
10. Han Z, Chatterjee D, Wyche JH. Proliferation of non-transformed cells is inhibited by adenosine metabolite of but not by parental 8-Cl-cyclic AMP. *J Pharmacol Exp Ther* 1993, **265**, 790–794.
11. Anderson PR, Devane SG, Tronick SR. Generation of Balb-MuSV and Ha-MuSV by type C virus transduction of homologous transforming genes from different species. *Cell* 1981, **26**, 129–134.
12. Papageorge A, Lowy D, Scolnick EM. Comparative biochemical properties of p21 *ras* molecules coded for by viral and cellular *ras* genes. *J Virol* 1982, **44**, 509–519.
13. Cook JS, McCormick F. Inhibition by CAMP of Ras-dependent activation of Raf. *Science* 1993, **262**, 1069–1072.
14. Chen J, Lyengar R. Suppression of *ras*-induced transformation of NIH3T3 cells by activated Gαs. *Science* 1994, **263**, 1278–1281.
15. Degerman E, Moos M Jr, Rascon A, *et al.* Single-step affinity purification, partial structure and properties of human platelet cGMP inhibited cAMP phosphodiesterase. *Biochem Biophys Acta* 1994, **1205**, 189–198.
16. Beavo JA, Reifsnyder DH. Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors. *Trends Pharmacol* 1990, **11**, 150–155.
17. Ally S, Tortora G, Clair T, *et al.* Selective modulation of protein kinase isozymes by the site-selective analog 8-chloroadenosine 3′,5′-cyclic monophosphate provides a biological means for control of human colon cancer cell growth. *Proc Natl Acad Sci USA* 1988, **85**, 6319–6322.
18. Nesterova M, Cho-Chung YS. A single-injection protein kinase A-directed antisense treatment to inhibit tumor growth. *Nature Med* 1995, **1**, 528–533.
19. Tortora G, Clair T, Cho-Chung YS. An antisense oligonucleotide targeted against the type IIβ regulatory subunit mRNA of protein kinase inhibits cAMP-induced differentiation in HL-60 leukemia cells without affecting phorbol ester effects. *Proc Natl Acad Sci USA* 1990, **87**, 705–708.
20. Stefano P, Tortora G, Noguchi PD, Marti GE, Washington GC, Cho-Chung YS. Effects of 8-chloroadenosine 3′,5′-monophosphate and N6-benzyl-cyclic adenosine 5′-monophosphate on cell

cycle kinetics of HL-60 leukemia cells. *Cancer Res* 1991, **51**, 6263–6267.
21. Noda M, Selinger Z, Scolnick EM, Bassin RH. Flat revertants isolated from kirsten sarcoma virus-transformed cells are resistant to the action of specific oncogenes. *Proc Natl Acad Sci USA* 1983, **80**, 5602–5606.
22. Tagliaferri P, Katsaros D, Clair T, *et al.* Synergistic inhibition of growth of breast and colon cancer cell lines by site-selective cAMP analogues. *Cancer Res* 1988, **48**, 1642–1650.
23. Steinberg RA, Agard DA. Turnover of regulatory subunit of cyclic AMP-dependent protein kinase in S49 mouse lymphoma cells. *J Biol Chem* 1981, **21**, 10731–10734.
24. Wehner JM, Malkinson AM, Wiser MF, Sheppard JR. Cyclic AMP-dependent protein kinases from Balb3T3 cells and other 3T3 derived lines. *J Cell Physiol* 1981, **108**, 175–184.
25. Cho-Chung YS, Clair T, Shepheard C. Anticarcinogenic effect of N^6,O^2-dibutyryl cyclic adenosine 3′,5′-monophosphate on 7,12-dimethylbenz(a)anthracene mammary tumor induction in the rat and its relationship to cyclic adenosine 3′,5′-monophosphate metabolism and protein kinase. *Cancer Res* 1983, **43**, 2736–2740.
26. Hovi T, Keski-Oja J, Vaheri A. Growth control in chick embryo fibroblasts: no evidence for specific role for cyclic purine nucleotides. *Cell* 1974, **2**, 234–240.
27. Majmudar G, Peterkofsky B. Cyclic AMP-independent processes mediate kirsten sarcoma virus-induced changes in collagen production and other properties of cultured cells. *J Cell Physiol* 1985, **122**, 113–119.
28. Hafner S, Adler SH, Mischak H, *et al.* Mechanism of inhibition of Raf-1 by protein kinase A. *Mol Cell Biol* 1994, **14**, 6696–6703.
29. Tortora G, Tagliaferri P, Clair T, *et al.* Site-selective cAMP analogs at micromolar concentrations induce growth arrest and differentiation of acute promyelocytic, chronic myelocytic, and acute lymphocytic human leukemia cell lines. *Blood* 1988, **71**, 230–233.
30. Joneson T, White MA, Wigler MH, Bar-Sagi D. Stimulation of membrane ruffling and MAP kinase activation by distinct effectors of RAS. *Science* 1996, **271**, 810–812.
31. Tortora G, Pepe S, Bianco C, *et al.* The RIα subunit of protein kinase A controls serum dependency and entry into cell cycle of human mammary epithelial cells. *Oncogene* 1994, **9**, 3233–3240.
32. Gadbois DM, Crissman HA, Tobey RA, Morton Bradbury E. Multiple kinase arrest points in the G_1 phase of nontransformed mammalian cells are absent in transformed cells. *Cell Biol* 1992, **89**, 8626–8630.
33. Budillon A, Clair T, Hartman N, *et al.* Novel growth inhibitory effect of 8-Cl-cAMP is dependent on serum factors that modulate protein kinase A expression but not the hydrolysis of 8-Cl-cAMP. *Int J Oncol* 1996, **9**, 1113–1120.
34. Tomaszewski JE, Sparks RM, Rose LM, Heath JE, Page JG. Plasma drug levels and toxicity produced by intravenous infusions of 8-chloro-cyclic AMP (8-Cl-cAMP, NSC 614491) to Beagle dogs (Abstract 2529). *Proc Am Assoc Cancer Res* 1991, **32**, 425.
35. Tortora G, Ciardiello F, Pepe S, *et al.* Phase I clinical study with 8-chloro-cAMP and evaluation of immunological effects in cancer patients. *Clin Cancer Res* 1995, **1**, 377–384.