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Cytotoxic Effects of Two Novel 8-Substituted Cyclic Nucleotide Derivatives in Cultured Rat Hepatoma Cells

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SUMMARY

KOONTZ, J. W., AND W. D. WICKS. Cytotoxic effects of two novel 8-substituted cyclic nucleotide derivatives in cultured rat hepatoma cells. *Mol. Pharmacol.* **18:** 65–71 (1980).

The cytotoxic effects of two structurally related cyclic nucleotide analogs have been investigated in a cultured rat hepatoma cell line (H35). Both analogs, 8-H_2NcAMP and 8-OH$(CH_2)_2$HNcAMP, were lethal to growing H35 cells, exhibiting LC_{50}'s of 5–10 and approximately 50–80 μM, respectively. The potency of both analogs was significantly reduced by the concomitant addition of the phosphodiesterase inhibitor, 1-methyl-3-isobutylxanthine. However, a number of differences in the effects of these two derivatives were observed. A variant H35 clone has been selected which is resistant to the lethal effects of 8-H_2NcAMP. This variant retains its sensitivity to 8-OH$(CH_2)_2$HNcAMP, demonstrating quite clearly that the cytotoxic actions of these two analogs are probably exerted at different metabolic loci. Analysis of the effect of either analog on the rapidly turning-over enzyme, tyrosine aminotransferase, showed that after 6 h of exposure, the activity of this enzyme dropped to less than 50% of basal, in contrast to other cyclic nucleotide analogs which induce the enzyme activity severalfold. Neither RNA nor protein synthesis is inhibited by either analog to the degree required to cause a 50% loss in enzyme activity, suggesting that other mechanisms may be operative.

INTRODUCTION

A variety of derivatives of adenosine 3′,5′-monophosphate (cAMP) has been developed over the past few years for the purpose of investigating the involvement of cAMP in biological processes. Early work focused on the ability of these analogs to activate protein kinase *in vitro* and to mimic the action of hormones which were believed to act via cAMP on various biological processes *in vivo* (1–5). One of the goals of such studies was to probe the relationship between the various responses and protein kinase activation in intact cells.

During the course of such studies on the ability of cAMP analogs to stimulate protein kinase and to provoke specific enzyme induction in cultured rat hepatoma (H35) cells, we found that several of these derivatives were cytotoxic (6, 7). The present report deals with a characterization of the effects of two structurally related analogs, 8-aminoadenosine 3′,5′-monophosphate (8-H_2NcAMP) and 8-hydroxyethylaminoadenosine 3′,5′-monophosphate (8-OH$(CH_2)_2$HNcAMP), which proved to be especially toxic to H35 (and other) cells. Previous work showed that both of these derivatives possess little or no ability to induce tyrosine aminotransferase (TAT; EC 2.6.1.5) or to stimulate *in vivo* H_1 histone phosphorylation in H35 cells, although a variety of other cAMP derivatives is quite effective in this regard (1, 3).

The original report describing the synthesis and biochemical screening of these two cyclic nucleotide analogs showed that both are able to activate partially purified bovine brain protein kinase with the following order of effectiveness: 8-H_2NcAMP > cAMP > 8-OH$(CH_2)_2$HNcAMP (8). Of these two analogs, 8-H_2NcAMP was more effectively hydrolyzed by rabbit kidney phosphodiesterase, and this occurred at a rate which was 80% of that observed for cAMP (8). Other studies with these analogs have shown that 8-H_2NcAMP is generally more effective in mimicking cAMP actions in various tissues than is 8-OH$(CH_2)_2$HNcAMP (9, 10).

There is little information in the literature relative to possible metabolic effects of the corresponding nucleosides or free bases of these two analogs. 8-Aminoadenosine has been found to weakly inhibit the growth of *Escherichia coli* (11), to be a competitive inhibitor of ATP-Mg^{2+} in the exchange reaction catalyzed by methionyl-tRNA synthetase (12), and to serve as an effective inhibitor of lactate dehydrogenase when converted to 8-amino AMP (13). Our results suggest that both of the cyclic nucleotide derivatives are likely to exert their lethal effects by virtue of the formation of metabolites

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(5′-nucleotides, etc.) which exert cytotoxic actions by mechanisms yet to be discovered.

MATERIALS AND METHODS

Methods. Procedures for the growth and maintenance of cultured Reuber H35 hepatoma cells (H4-EII-C3) as well as for the determination of cell numbers have been described previously (7, 14). Cells resistant to 8-H_2NcAMP were obtained by growing H35 cells in the presence of increasing concentrations of the analog for several weeks at a time: initially 1, then 10, and finally 100 μM. These cells have now been carried in analog-free medium for at least a year and have retained their resistance to 8-H_2NcAMP.

Measurement of [^{3}H]leucine incorporation into protein was carried out by incubating cells in serum-free medium with 5 μCi/ml [^{3}H]leucine (6.25 μCi/mmol final specific activity). Although this was done in serum-free medium to increase the [^{3}H]leucine specific activity, the cells were maintained in serum-containing medium up to the time of the incubation with labeled precursor. At the appropriate times, the medium was aspirated, and the cells were washed with serum-free medium prior to the addition of fresh serum-free medium containing the appropriate precursor. After 30 min, the medium was aspirated, and the cells were washed twice with cold 0.9% NaCl before the addition of 1 ml 5% TCA to the dish. After standing overnight at 4°C, an aliquot of the TCA-supernatant was removed for counting the acid-soluble material and the remainder of the TCA-soluble material was aspirated. The TCA-precipitable material on the dish was dissolved in 0.3 N NaOH and aliquots were removed for: (a) direct counting of TCA-precipitable material, (b) protein determinations (15), and (c) DNA determinations (16). Control experiments demonstrated that a hot-TCA wash of the precipitated protein did not detectably reduce the observed radioactivity.

Measurement of [^{3}H]uridine incorporation into RNA was carried out essentially as described for [^{3}H]leucine incorporation using a 30-min labeling period with 1 μCi/ml [^{3}H]uridine (4.2 mCi/mmol final specific activity). In control experiments, it was found that greater than 95% of the TCA-precipitable counts were alkali-labile.

[^{3}H]Thymidine incorporation into DNA was analyzed as described for [^{3}H]leucine incorporation.

Preparation of cell extracts and assay of tyrosine aminotransferase activity were carried out as described previously (3, 17).

Each experiment was conducted at least twice with a total of between 8 and 12 separate dishes for each data point. The standard errors of the average values were generally not greater than 10%.

The assay for adenylate deaminase was as described by Coffee (18).

Materials. Tissue culture supplies and media were purchased from Flow Laboratories and Grand Island Biological Co. Inosine, adenosine, and hypoxanthine were purchased from Sigma Chemical Co., St. Louis, Missouri. 1-Methyl-3-isobutyl xanthine was obtained from the G. D. Searle Co., Chicago, Illinois. Nonidet P-40 was a generous gift from Shell International Co., Ltd., London, United Kingdom. Diphenylamine was purchased from J. T. Baker Co. and was used without recrystallization. [4,5-^{3}H]Leucine, [*methyl*-^{3}H]thymidine, and [5-^{3}H]uridine were purchased from New England Nuclear Corp., Boston, Massachusetts.

The cyclic nucleotide analogs were the generous gift of Drs. John Miller, R. Myer, R. K. Robins, and M. Stout of the ICN Nucleic Acid Research Institute, Irvine, California, or were synthesized and purified using a modification of the procedure described by Muneyama *et al.* (8). Purity of the analogs from both sources was checked by thin-layer chromatography, high-pressure liquid chromatography, and comparison of the λ_{max} of the ultraviolet spectrum with published values (8). The analogs from both sources were greater than 95% pure. The contaminants could be accounted for as either unreacted cAMP itself or 8-bromo cAMP. The cAMP is the starting material for the synthesis and 8-bromo cAMP is the intermediate. No further purification was performed because in separate experiments it was verified that neither of these compounds influenced cell growth at the concentrations being used.

RESULTS

Concentration–effect curves. Exposure of H35 cultures to 8-H_2NcAMP and 8-OH$(CH_2)_2$HNcAMP leads to a rounding up of the cells followed by their detachment from the dish. That this phenomenon is due to irreversible cell damage and cytotoxicity is shown by the fact that the sloughed cells will not reattach if replated in analog-free medium after washing and do not exclude trypan blue. Furthermore, simply plating the cells in analog-containing medium does not change the plating efficiency, as measured by the number of cells attached to the dish 18–24 h following subculture (data not shown). References to cell numbers in figures and tables represent the number of cells remaining attached to the dish.

Both 8-H_2NcAMP and 8-OH$(CH_2)_2$HNcAMP have the same effect, i.e., they both produce cell death. However, as can be seen in Fig. 1, there is a marked difference in the LC_{50} of the two analogs. The value found for 8-H_2NcAMP is about 5 μM, whereas that for 8-OH$(CH_2)_2$HNcAMP is 10 times higher, approximately 50 μM. A unique feature of the concentration–effect curve for 8-OH$(CH_2)_2$HNcAMP is the apparent increase in cell number relative to control at concentrations of 1 to 5 μM. This phenomenon has been observed in a number of experiments, but its basis is unknown.

If the two analogs were lethal to H35 cells as the cyclic nucleotides, this order of potencies would have been predicted from their reported effects on protein kinase *in vitro* (8). However, our results have shown that neither of these analogs is capable of activating H35 cell cAMP-dependent protein kinase *in vivo* to a significant extent (1). In an effort to resolve this question, we have made use of 1-methyl-3-isobutylxanthine (MIX), a potent inhibitor of cAMP phosphodiesterase (19). It has been found to convert 8-H_2NcAMP from a weak and transient activator of protein kinase in H35 cells to a strong activator, presumably by inhibiting its hydrolysis by phosphodiesterase (6, 20).

If the cyclic nucleotide form of these derivatives is responsible for the observed toxicity, inhibition of their

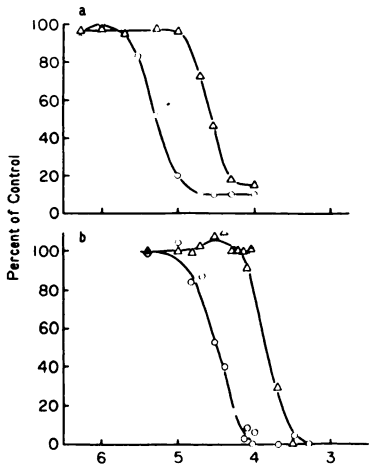

FIG. 1. *Concentration–effect curves for 8-H_2NcAMP and 8-OH$(CH_2)_2$HNcAMP ± MIX*

H35 cells were grown for 3 days in the presence of varying concentrations of the two analogs in the presence or absence of 0.5 mM MIX. The medium was changed on Day 2 and fresh analog added. Control values for the treatment minus MIX were obtained by growing cells in medium free of MIX, whereas control values for the treatment plus MIX was obtained from cells grown in the presence of MIX. ○, Analog alone; △, analog plus MIX. (a) 8-H_2NcAMP. (b) 8-OH$(CH_2)_2$HNcAMP.

hydrolysis should potentiate the action of each derivative. However, exactly the opposite result was obtained. With either analog, the LC_{50} in the presence of MIX was increased significantly (Fig. 1). For 8-H_2NcAMP this increase was approximately 10-fold, and for 8-HO$(CH_2)_2$HNCAMP the increase was the same.

Reversibility. The concentration–effect curves in Fig. 1 were obtained by exposing cultures to the two analogs for 3 days and then determining the number of cells remaining attached to the dish. Using concentrations which gave a maximal response as measured by this procedure, we then determined how long the cells must be exposed to the respective concentration of analog before an irreversible effect is obtained. The effects of short exposure periods and the ability of H35 cells to recover after the removal of analog are seen in Figs. 2a and b.

Recovery of cells from the toxic action of 8-OH$(CH_2)_2$HNcAMP was observed following exposure of up to 16 h (Fig. 2b). Recovery after a 7.5-h exposure was essentially immediate with no apparent lag observed, although the rate of increase in cell number was consistently lower than that found in untreated cells. Recovery after 16 h of exposure to 8-OH$(CH_2)_2$HNcAMP was characterized by a significant lag period of about 48 h before any increase in cell number could be detected. The lag observed in the recovery period could be explained by the possibility that: (a) All of the cells were affected but only gradually recovered; or (b) only a certain proportion of the population was affected, and these cells died off at the same rate that the rest of the population (unaffected) continued to grow. At this point no evidence is available to allow a distinction between these two possibilities. No recovery was observed following either 24 or 32 h of exposure to 8-OH$(CH_2)_2$HNcAMP. Thus, the maximal toxic response to 0.5 mM 8-OH$(CH_2)_2$HNcAMP occurs between 16 and 24 h.

In contrast, the maximal toxic response of H35 cells to 8-H_2NcAMP required considerably less time to develop than was found for 8-OH$(CH_2)_2$HNcAMP. Although significant recovery occurred following 3 h of exposure to 8-H_2NcAMP, irreversible cytotoxicity set in after 7 h or more.

The differences observed between 8-H_2NcAMP and 8-OH$(CH_2)_2$HNcAMP in terms of their kinetics of promoting irreversible cytotoxicity could be a reflection of the ability of cells to hydrolyze sufficient amounts of each cyclic nucleotide analog to the corresponding nucleoside 5′-monophosphate. The effects of phosphodiesterase inhibition support this possibility (Figs. 1a and b). On the other hand, it could also reflect a more fundamental difference in the mechanism of action of these analogs, as will be considered subsequently.

Recovery of quiescent cells following exposure to analogs. The results described in Figs. 2a and b represent the effect of the two analogs on H35 cells which are in log-phase growth and randomly dispersed through the

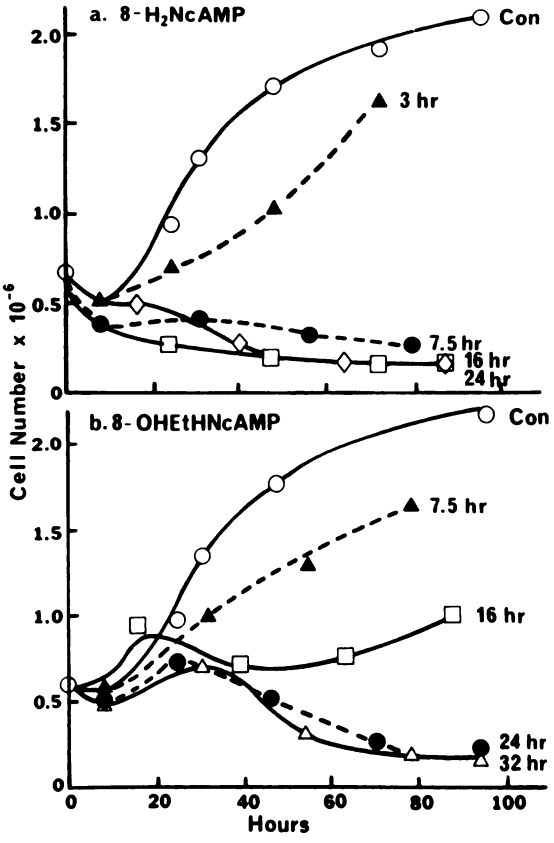

FIG. 2. *Reversibility of the toxic effects of 8-H_2cAMP and 8-OH$(CH_2)_2$HNcAMP*

Randomly growing cells were exposed to the analogs for varying periods. At the appropriate times, a set of dishes was collected and one group was harvested to assay cell number, and the remaining dishes from that set were washed three times with Hanks balanced salt solution and incubated for 5 min at 37°C for the third wash. Fresh serum-containing medium was then added, and the increase in cell number was followed as indicated. Zero time is the time of the addition of analog. (a) 8-H_2NcAMP, 100 μM: ○, control; ▲, 3-h exposure; ●, 7.5-h exposure; ◇, 16-h exposure; □, 24-h exposure. (b) 8-OH$(CH_2)_2$HNcAMP, 500 μM: ○, control; ▲, 7.5-h exposure; □, 16-h exposure; ●, 24-h exposure; △, 32-h exposure.

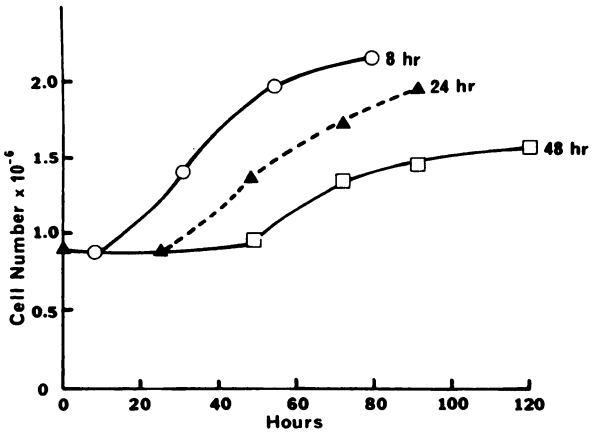

FIG. 3. *Reversibility of the toxic effects of 8-H_2NcAMP in quiescent cells*

The protocol for this experiment is essentially identical to that described for Fig. 1 except that cells were exposed to the analog after having been incubated in serum-free medium for 3 days. Exposure to 100 μM analog was carried out in serum-free medium, but recovery after the removal of analog was in serum-containing medium. Zero time is the time of the addition of analog. ○, 8 h; ▲, 24 h; □, 48 h.

cell cycle. The question arises as to whether or not the same cytotoxic effects would be obtained if the cells had not been engaged in active growth during exposure to the two analogs. In order to address this question, we have used serum starvation to arrest H35 cells in a quiescent state and then exposed them to the analogs for varying lengths of times. Any subsequent recovery and growth of previously treated cells was then followed in serum-containing, analog-free medium. The results shown were obtained with 8-H_2NcAMP but are also representative of the data obtained for 8-OH$(CH_2)_2$HNcAMP (Fig. 3).

Exposure of resting cells to 8-H_2NcAMP or 8-OH$(CH_2)_2$HNcAMP for periods of up to 48 h results in no observable effect on the ability of the cells to be stimulated to grow upon the removal of analog and addition of serum. The decreasing recovery seen after 48 h of exposure is a reflection of the observed decreasing ability of untreated H35 cells to reenter the cell cycle with increasing lengths of time spent in serum-free medium.[1] Thus, untreated cultures show the same rates of growth as those treated with drug for 48 h.

These results are in sharp contrast to those obtained with actively growing cells, where as little as 7 h of exposure to 8-H_2NcAMP results in irreversible toxicity. While there is a variety of fundamental biochemical differences between the resting, quiescent cell and the actively growing cell which could be invoked as the potential site of action of these cytotoxic analogs (21), these results suggest that the cytotoxicity produced by these analogs is not the result of a disruption of cellular maintenance functions, energy production, or a general poisoning of cellular metabolism because any of these would be expected to be lethal to resting as well as growing cells.

Reversal of cytotoxicity by naturally occurring purines. Inhibition of purine biosynthesis or purine nucleotide interconversion has been demonstrated to occur with a number of purine base analogs (22). Indeed, in some cases, the cytotoxicity of such analogs can be significantly reversed by the simultaneous addition of naturally occurring purines (23, 24). As an initial means of exploring possible mechanisms by which these two cyclic nucleotide analogs act, the ability of hypoxanthine or adenosine to influence the cytotoxic response was tested. The results demonstrate that neither of these compounds was able to influence appreciably the toxicity of 8-H_2NcAMP (Fig. 4a). In contrast, hypoxanthine did increase the LC_{50} of 8-OH$(CH_2)_2$HNcAMP approximately 8-fold (Fig. 4b), and adenosine increased the LC_{50} of this analog by more than 10-fold. To determine whether the effects of adenosine and inosine might be to compete (after conversion to nucleotides) with the cAMP analogs for adenylate deaminase, which could potentially activate the drugs, we measured the deamination of the 5′-monophosphate of each of these analogs using commercially available deaminase. The results showed that neither analog was a good substrate for the enzyme, with 8-amino 5′-AMP being deaminated at a rate less than 5% that of AMP and 8-hydroxethylamino 5′-AMP being deaminated at a rate less than 1% that of AMP (data not shown). Thus, the drugs are not likely to be activated by adenylate deaminase, and further, the effects of adenosine and inosine are not attributable to this potential mechanism. The results do suggest that 8-OH$(CH_2)_2$HNcAMP does exert part of its cytotoxic effects by interference with purine metabolism. The inability of these naturally occurring purine nucleosides to reverse 8-H_2NcAMP toxicity suggests that this analog does not interfere with purine metabolism and, consequently, that the two analogs may well have different mechanisms of toxicity.

Selection of cells resistant to cytotoxic effects. We have succeeded in selecting cells which appear to be essentially fully resistant to the toxic action of 8-

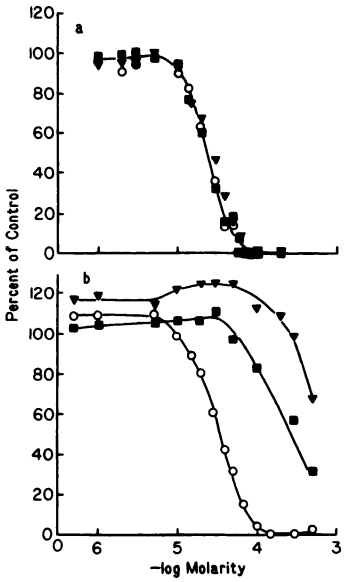

FIG. 4. *Concentration–effect curves for 8-H_2NcAMP and 8-OH$(CH_2)_2$HNcAMP in the presence of adenosine or hypoxanthine*

Details for this experiment are essentially as those described for Fig. 1. Analog alone, ○; analog plus 100 μM adenosine, ▼; analog plus 100 μM hypoxanthine, ■. (a) 8-H_2NcAMP. (b) 8-OH$(CH_2)_2$HNcAMP.

[1] Koontz, J. W., unpublished observations.

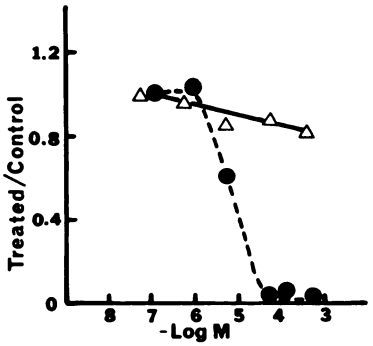

FIG. 5. *Concentration–effect curves for 8-H_2NcAMP in cells selected for resistance to 8-H_2NcAMP*

Details of this experiment are essentially as those described for Fig. 1. ●, Sensitive cells; △, resistant cells.

H_2NcAMP. This phenotype has remained stable for at least a year and over many generations. Figure 5 illustrates a comparison of the concentration–effect curves for the toxicity of 8-H_2NcAMP in the "wild-type" and resistant H35 cells. The resistant population is virtually unaffected by a concentration of 8-H_2NcAMP which is at the LC_{50} for the sensitive cells and is only mildly affected at concentrations 100 times this level.

Figure 6 compares the action of 0.5 mM 8-H_2NcAMP with that of 0.5 mM 8-OH$(CH_2)_2$HNcAMP in the resistant cells. Despite being resistant to the toxic effects of 8-H_2NcAMP, these cells proved to be still fully sensitive to 8-OH$(CH_2)_2$HNcAMP. MIX was found to be capable of completely preventing the toxic actions of 8-OH$(CH_2)_2$HNcAMP in these cells as well as in the wild-type cells (Fig. 6). The ability of MIX to reverse the toxicity of 8-OH$(CH_2)_2$HNcAMP in cells resistant to 8-H_2NcAMP suggests that a loss of phosphodiesterase activity could not be the biochemical basis for the resistance of these cells to 8-H_2NcAMP. Otherwise, an inhibitor of phosphodiesterase activity would be expected to have no effect on cyclic nucleotide analog toxicity.

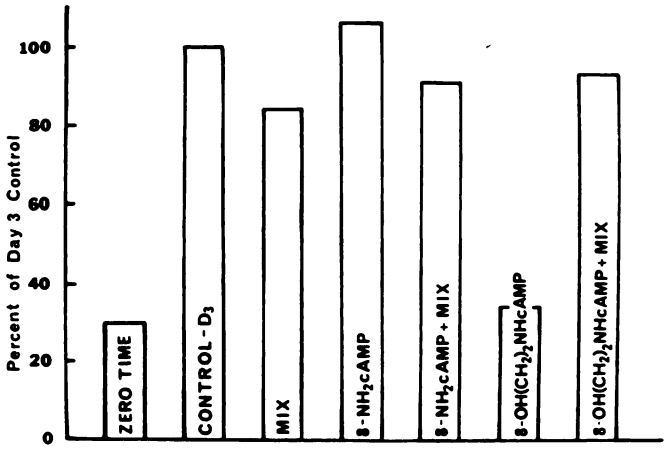

FIG. 6. *Effect of MIX on 8-OH$(CH_2)_2$HNcAMP toxicity in 8-H_2NcAMP-resistant cells*

Cells were grown as described under Methods. Additions were made as indicated at time zero. Cells were harvested following 3 days of exposure to drugs. Control—D_3 refers to control cultures on Day 3. Concentrations of drugs were as follows: 8-H_2NcAMP, 0.1 mM; 8-OH$(CH_2)_2$HNcAMP, 0.5 mM; MIX, 0.5 mM.

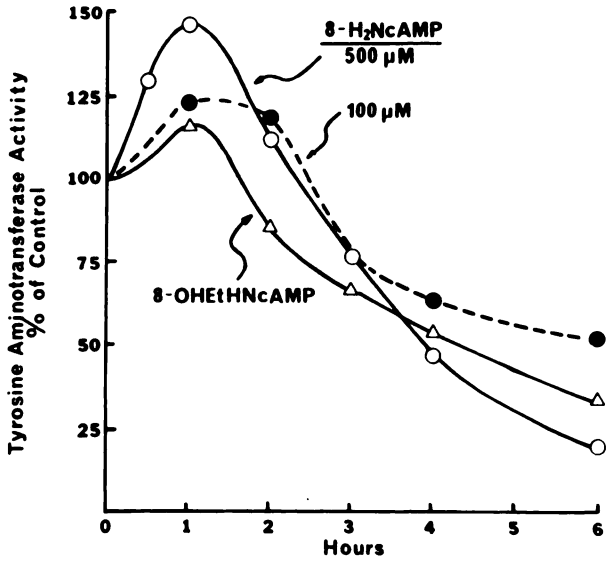

FIG. 7. *Tyrosine aminotransferase activity in cells exposed to 8-OH$(CH_2)_2$HNcAMP and 8-H_2NcAMP*

Cells were incubated in the presence of (△) 0.5 mM 8-OH$(CH_2)_2$HNcAMP, (●) 100 μM, or (○) 500 μM 8-H_2NcAMP, harvested at the indicated times, and assayed for enzyme and protein as described under Materials and Methods.

Effect on tyrosine aminotransferase activity. Previous work in this laboratory has demonstrated that a variety of 6- and 8-substituted cyclic AMP analogs can mimic N^6,$O^{2'}$-dibutyryl cAMP in inducing tyrosine aminotransferase in H35 cells (3). It was also found that neither 8-H_2NcAMP nor 8-OH$(CH_2)_2$HNcAMP is able to significantly induce this enzyme at 3 to 4 h unless MIX is also present (6). Because of its short half-life (25, 26) and essential noninducibility by these two analogs, we have monitored changes in the activity of this enzyme as a possible index of functional inhibition of protein synthesis by the two derivatives. Both of these analogs were, in fact, found to provoke a dramatic loss of enzyme activity after a lag of 2–3 h (Fig. 7). Although the extent of the loss is variable, the loss of at least 50% of the aminotransferase activity within 6 h has been observed in three separate experiments, as is also true of the lag time.

The concentration of 8-H_2NcAMP which produced the greatest loss of tyrosine aminotransferase activity (0.5 mM) was used because that concentration is optimal for induction with other cyclic nucleotide analogs such as N^6,$O^{2'}$-dibutyryl cAMP, 8-BrcAMP, and 8-OHcAMP (3). As seen in Fig. 7, this concentration of 8-H_2NcAMP did cause a modest rise in aminotransferase activity at 1 h. This temporary induction was found consistently with 8-H_2cAMP and is paralleled by a comparable change in the activation of protein kinase (1). The transient nature of the induction response is likely to be due to the susceptibility of this analog to hydrolysis by phosphodiesterase. This is substantiated by independent studies which demonstrated that TAT induction by 8-H_2NcAMP is both enhanced and prolonged by MIX (6, 27). A fivefold lower concentration of 8-H_2NcAMP produced less of an increase in tyrosine aminotransferase activity at 1 h, and the subsequent loss of enzyme activity was also less dramatic.

To determine whether these analogs were causing a

TABLE 1

Effect of analogs on DNA, RNA, and protein synthesis in H35 cells

H35 cells at about one-third confluency were exposed to 0.5 mM 8-$OH(CH_2)_2HNcAMP$ and the indicated concentrations of 8-H_2NcAMP for the periods of time indicated. The procedures used for measuring the incorporation of the appropriate labeled precursors into acid-precipitable material are described in Materials and Methods. The values indicated represent the percentage of incorporation relative to that in untreated companion cultures.

Synthetic process	Period of exposure	Analogs		
		8-$OH(CH_2)_2H$-NcAMP (500 μM)	8-H_2NcAMP	
			100 μM	500 μM
	h			
Protein	3	103	118	134
	6	90	122	80
RNA	3	90	95	70
	6	82	106	59
DNA	3	106	90	90
	6	109	100	100

loss of tyrosine aminotransferase activity by inhibiting protein or RNA synthesis, we measured the incorporation of [^{3}H]leucine or [^{3}H]uridine into acid-precipitable material in the presence or absence of the analogs. The results (Table 1) revealed that the only significant inhibition at 3 h was observed with 500 μM 8-H_2NcAMP on RNA synthesis. However, by 6 h both 500 μM 8-$OH(CH_2)_2HNcAMP$ and 500 μM 8-H_2NcAMP produced a mild inhibition of protein synthesis. This may well result from the observed inhibition of RNA synthesis. Although the inhibition of RNA and protein synthesis is not striking, it appears to exhibit a concentration dependence similar to that found with the loss of tyrosine aminotransferase activity. However, the degree of inhibition found does not appear to be sufficient to account for the observed loss of enzyme activity, especially since 0.1 mM 8-H_2NcAMP does not affect macromolecular synthesis but leads to a greater than 50% loss of aminotransferase activity in 6 h (Fig. 7). The possibility is being investigated that these analogs may exert a specific effect on the synthesis or degradation of this enzyme or cause a general enhancement of protein degradation.

Essentially no inhibition of [^{3}H]TdR incorporation into DNA was observed over the time period examined. Because of the effects of these analogs on cell growth, it was expected that inhibition of DNA synthesis would eventually be found. It is likely that this would have been the case had a longer exposure period been employed. On the other hand, the lack of an effect on DNA synthesis during periods of exposure of up to 6 h emphasizes once again the fact that the effects of the analogs are not due to a rapid and generalized metabolic toxic action which causes an immediate cessation of growth. Rather, the observed response appears to be a more gradual effect requiring some incremental accumulation of a specific toxic agent.

DISCUSSION

The initial direction of these studies was to determine whether or not the cytotoxic effects of the two 8-amino substituted cAMP analogs were manifestations of cyclic nucleotide-mediated phenomena. The results of the experiments with MIX strongly suggest that this is not the case, and it appears that hydrolysis of the analogs to the corresponding 5′-nucleoside monophosphate is a prerequisite for cytotoxicity. However, even in the presence of MIX, 8-H_2NcAMP and probably 8-$OH(CH_2)_2HNcAMP$ are toxic when added at sufficiently high concentrations. Although this could be due to an inherent cytotoxic action of the cyclic nucleotide analogs themselves, it is more likely to be due simply to incomplete inhibition of the phosphodiesterase by MIX. MIX is a competitive inhibitor of phosphodiesterase, and thus the inhibition can be overcome with sufficient concentrations of substrate (20). Previous results have shown that neither analog is capable of completely mimicking cAMP in provoking tyrosine aminotransferase induction or specific H_1 histone phosphorylation (protein kinase activation) in the absence of MIX (1, 3, 6). Increases in the cAMP content of intact H35 cells with 0.2 mM MIX average 20- to 30-fold, consistent with at least a 90% inhibition of phosphodiesterase in the absence of cytotoxic derivatives (28).

An additional question addressed was whether these two analogs exert their cytotoxic action by similar mechanisms. Several lines of evidence presented in the current report suggest that the mechanisms of toxicity exerted by these two analogs are different. 8-H_2NcAMP requires much less than a generation time of exposure before it is irreversibly cytotoxic, whereas 8-$OH(CH_2)_2HNcAMP$ requires a full generation time or more before its effect is irreversible. Furthermore, the potency of 8-$OH(CH_2)_2HNcAMP$ can be decreased by the addition of naturally occurring purine nucleosides, whereas the toxicity of 8-H_2NcAMP is totally unaffected by these compounds. Thus, if 8-H_2NcAMP does affect purine nucleotide metabolism, it is not likely to be by way of *de novo* purine metabolism. Finally, a variant H35 cell population selected for resistance to 8-H_2NcAMP retains full sensitivity to the cytotoxic action of 8-$OH(CH_2)_2HNcAMP$.

Examination of chemical models of these two analogs suggests no obvious major structural differences other than the increased bulk of the ethanolamine group at the 8-position of 8-$OH(CH_2)_2HNcAMP$ relative to the amino group at the 8-position of 8-H_2NcAMP. However, the differences in the ability of these analogs to activate cAMP-dependent protein kinase (1, 8) and their differential sensitivity to phosphodiesterase (8) indicate that they possess structural variations of sufficient magnitude to alter their biochemical activities.

An interesting feature of the data is that tyrosine aminotransferase activity falls rapidly in cells treated with both analogs, 8-H_2NcAMP producing a more dramatic effect than 8-$OH(CH_2)_2HNcAMP$. This enzyme has a relatively short half-life (about 90 min), and thus the measurement of its specific activity should be a sensitive indicator of changes in the rates of protein synthesis or degradation. Although both protein and RNA syntheses are inhibited to a slight degree by these analogs, the observed inhibition appears to be insufficient to account for the loss of enzyme activity. Other possibilities can be suggested: For example, the rate of deg-

radation of this enzyme (and proteins in general) might be enhanced without any major change in protein synthesis which would result in the loss of activity. In preliminary experiments, however, no change in the rate of general protein degradation was seen, whether 8-H_2NcAMP was present during the labeling period or during the chase period (data not shown).

Another possible mode of action for these analogs is that they are incorporated into nucleotide pools and eventually into RNA. If this were so, RNA and protein synthesis could appear to be nearly normal with respect to incorporation of a labeled precursor, and yet the products could be functionally defective (e.g., loss of tyrosine aminotransferase catalytic activity, etc.) due to incorporation of the nucleotide analog into mRNA possibly causing insertion of the wrong amino acid into proteins. Incorporation of base analogs into nucleic acids has been proposed as being required for the cytotoxic action of such compounds as 5-azacytidine (29) and 8-azaguanine (30) and is also a possibility in the present case.

Although the incorporation of the nucleotide analog into RNA could eventually lead to the inhibition of precursor incorporation in RNA and protein, it might take longer than the 6-h period during which we conducted labeling experiments. We are currently investigating the metabolism of these analogs with respect to their incorporation into nucleotide pools as well as their possible incorporation into nucleic acids.

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