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Simian Virus 40 Small-t Antigen-Induced Theophylline Resistance Is Not Mediated by Cyclic AMP

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Small-t antigen of simian virus 40 renders CV-1 cells resistant to growth arrest induced by theophylline and other methylxanthines. Elevated levels of cyclic AMP are not involved in growth arrest of CV-1 cells by methylxanthines, and small-t antigen does not alter cyclic AMP levels dramatically after infection.

Small-t antigen of simian virus 40 (SV40) has been studied by using deletion mutants that lack small-t antigen but not the viral large-T antigen (21). Small-t antigen enhances the transformation of growth-arrested cells (6, 13, 20, 22) and has been reported to play a role in abortive transformation (7, 16, 24). It has also been shown to prolong cell cycling of growth-arrested primary mouse cells (10). In these systems, small-t antigen appears to function much like a growth factor; this may be a reflection of the ability of the viral protein to disorganize actin cable structures (8) or to interact with two cellular proteins (27).

In permissive monkey kidney (CV-1) cells (18) and in human primary diploid fibroblasts (unpublished data), SV40 small-t antigen allows the growth of cells in the presence of theophylline, a methylxanthine which is an inhibitor of cyclic AMP (cAMP) phosphodiesterase. When uninfected cells are treated with 1 to 2 mM theophylline, they become growth arrested, as detected by [^{3}H]thymidine incorporation into DNA. Arrest occurs in the G_0/G_1 phase of the cell cycle because, after removal of theophylline and addition of serum, cells enter the S phase before cell division (unpublished data). Infection of CV-1 cells by SV40 overcomes the growth arrest. Small-t antigen is responsible for this escape because the mutants that lack small-t antigen show greatly reduced synthesis of both viral and cellular DNAs when theophylline is present throughout the infection. This system allows for biochemical analysis of batch cultures for alterations which may be related to small-t antigen function.

Because of the known effect of theophylline on cAMP phosphodiesterase, it was possible that elevated intracellular cAMP levels were responsible for growth arrest (1, 3) and that small-t antigen might affect the levels of this nucleotide. Consequently, cAMP levels were measured in uninfected and infected cells treated with theophylline, other methylxanthines which vary in the ability to inhibit phosphodiesterase (2–4), or forskolin, a drug which elevates cAMP levels by activating adenylate cyclase (19). Our results show that cAMP levels do not correlate with growth arrest and that infection with wild-type and small-t antigen mutant viruses does not cause major alterations in the levels of intracellular cAMP.

The first indication that cAMP levels were not related to the degree of growth arrest induced by various drugs came from studies of uninfected cells. All of the methylxanthines drastically reduced DNA synthesis (Table 1). This result was surprising because neither theobromine nor 3′-CH_3-xanthine has been reported to be as effective as theophylline in inhibiting phosphodiesterase. In contrast to the methylxanthines, forskolin at low concentrations had no effect on DNA synthesis. Higher concentrations of forskolin did affect cell cycling. Lower concentrations of forskolin were used in these experiments because they caused increases in cAMP levels that were equivalent to the increases induced by theophylline.

Accumulated levels of cAMP were measured in drug-treated cultures by using radioimmunoassay kits (New England Nuclear Corp., Boston, Mass.). Forskolin had a more pronounced effect on cAMP levels than any of the methylxanthines (Table 1). This pattern was also observed after short (45-min) exposures to the various drugs. In CV-1 cells, growth-inhibitory concentrations of theophylline had only a slight effect on cAMP levels. Although the levels of cAMP varied somewhat from experiment to experiment, increases of no more than fourfold were induced by theophylline. The values reported in Table 1 are the sums of intracellular and extracellular cAMP levels. As shown in Fig. 1, nearly all of the increase in cAMP level was found to be extracellular after both theophylline and forskolin treatment of cells. In fact, the intracellular level of cAMP was nearly unaltered in theophylline-treated cells and was only slightly elevated in forskolin-treated cells. This observation also suggested that increased intracellular cAMP levels could not explain growth arrest.

The effects of forskolin and the other methylxanthines on infected cells are shown in Table 2. Cells were infected with wild-type SV40 or with small-t antigen mutant DL-888, and drugs were added immediately after infection. At 48 h postinfection, duplicate plates were used to measure either cAMP levels or the incorporation of [^{3}H]thymidine into cellular DNA. In experiment 1, theophylline-treated cells were compared with forskolin-treated cultures. As with uninfected cells, cAMP levels were increased more by

TABLE 1. Effects of long-term drug treatment on uninfected CV-1 cells

Treatment	Total cAMP concn (pmole/mg of protein)[a]	Thymidine incorporation (cpm/dish, $\times 10^3$)
Control	5.4	41.0
10 μM Forskolin	32.0	61.6
2.0 mM Theophylline	6.4	1.5
2.5 mM Theobromine	3.0	4.4
2.5 mM 3′-CH_3-xanthine	1.0	5.5

[a] cAMP levels were measured after 48 h in the presence of drugs. The data represent accumulated levels of this nucleotide.

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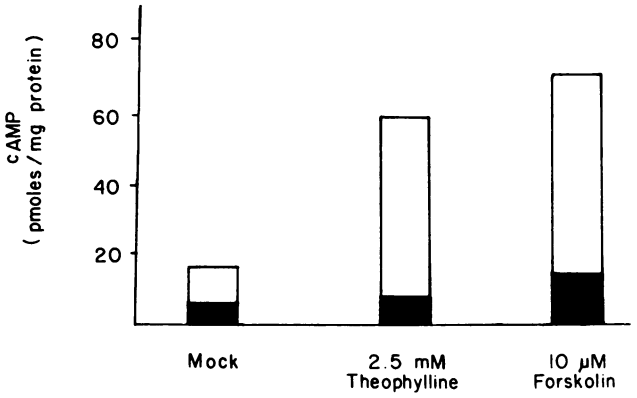

FIG. 1. Effects of drug treatment on intra- and extracellular levels of cAMP. Uninfected cells were treated for 48 h with drugs. After trichloroacetic acid precipitation, the levels of cAMP in media and in cells were determined by using acetylated samples and a radioimmunoassay. The open bars indicate extracellular levels of cAMP; the solid bars indicate intracellular levels of cAMP.

forskolin than by theophylline. However, a significant decrease in thymidine incorporation was observed only in cells infected with mutant DL-888 and maintained in the presence of theophylline. Although forskolin treatment resulted in greater cAMP accumulation, cell cycling was affected only minimally. Interestingly, the levels of cAMP that were present in growth-arrested cells (mutant DL-888 cultures treated with theophylline) did not cause growth arrest of cells infected with wild-type SV40.

In experiment 2 (Table 2), the other methylxanthines were tested in infected cells. No increases in cAMP levels were detected when cells were maintained in the presence of theobromine or 3'-CH_3-xanthine, and, in fact, slight decreases were observed. Nonetheless, the synthesis of cellular DNA was decreased more than 80% in cells infected with mutant DL-888 but was not reduced in cultures infected with wild-type virus.

Because small increases in intracellular cAMP levels could activate the cAMP-dependent (type A) protein kinases, it was important to show that type A kinases were not affected by infection or by the methylxanthines and forskolin. Protein kinase A levels were measured after separation of types I and II by DEAE-cellulose chromatography (Fig. 2). Each fraction was assayed for kinase activity in the presence and absence of cAMP. In addition, because dissociation of the subunits can occur either during extraction of the cells or during the assay itself (9), assays were performed in the presence of the specific inhibitor of the catalytic subunit of type A kinase (26). The profile shown in Fig. 2 is

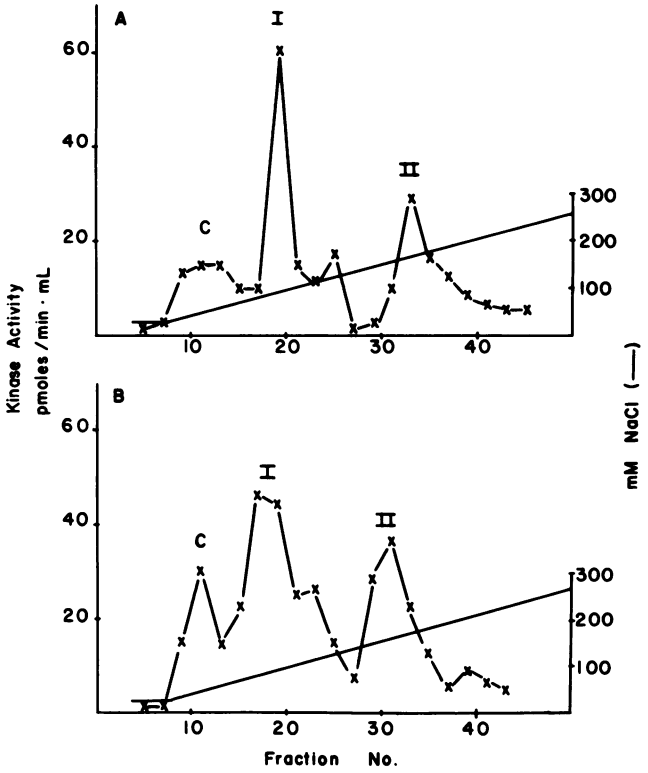

FIG. 2. DEAE-cellulose chromatography of protein kinase A. Cells on plates (5 by 10 cm) were infected with mutant DL-888 (A) or wild-type SV40 (B) and then incubated in the presence of 2 mM theophylline for 48 h. Soluble proteins were applied to DEAE columns (9 by 0.5 cm) and eluted with a linear salt gradient (0 to 400 mM NaCl). Fractions (1.0 ml) were collected and assayed for histone kinase activity in the presence or absence of 10^{-6} M cAMP or in the presence of the inhibitor of the cAMP-dependent catalytic subunit. The figure shows the activity in the presence of cAMP minus the activity not inhibited by the kinase inhibitor. C, Free catalytic subunit; I, type I cAMP-dependent kinase; II, type II cAMP-dependent kinase.

the difference between total kinase activity in the presence of cAMP and residual kinase activity in the presence of the inhibitor.

Extracts were prepared from wild type- or mutant DL-888-infected cells maintained in the presence of theophylline for 48 h and applied to DE-52 columns. Such preparations usually contained a variable amount of the free catalytic subunit, presumably reflecting the dissociation of holoenzyme during extraction. More importantly, similar levels of

TABLE 2. cAMP levels in infected CV-1 cells

Expt	Treatment	Mutant DL-888		Wild type	
		Total cAMP concn (pmole/mg of protein)[a]	Thymidine incorporation (cpm/dish, $\times 10^3$)	Total cAMP concn (pmole/mg of protein)	Thymidine incorporation (cpm/dish, $\times 10^3$)
1	Control	8.3	28.4	6.3	45.1
	10 μM Forskolin	38.5	15.8	41.7	49.1
	2.5 mM Theophylline	25.0	2.2	27.3	41.7
2	Control	2.5	59.4	2.2	90.4
	2.5 mM Theobromine	1.1	10.2	1.0	89.2
	2.5 mM 3'-CH_3-xanthine	2.0	10.2	2.0	73.0

[a] cAMP levels were measured after 48 h in the presence of drugs. The data represent accumulated levels of this nucleotide.

type I and type II cAMP-dependent protein kinase were observed. Thus, the concentrations of theophylline which caused growth inhibition of mutant DL-888-infected cells did not cause substantial reductions in the levels of either holoenzyme, indicating that no activation of the type A kinases had occurred.

Taken together, our observations show that alterations in intracellular cAMP levels are not correlated with the growth arrest of CV-1 cells by methylxanthines or with the ability of small-t antigen to overcome growth arrest. It appears that methylxanthines cause growth arrest in some other fashion. There have been several other effects of methylxanthines reported, some of which could be applicable to the system. For example, dibutyryl cAMP plus theophylline can alter calcium levels in mastocytoma cells (12). The role of calcium ions in cell growth has recently received much attention, especially with regard to the calcium-dependent protein kinase (11) and the mobilization of intracellular calcium in response to serum growth factors (5). In some preliminary experiments, we were not able to detect any major alterations in intracellular calcium distribution in wild type- or mutant DL-888-infected cells, but transient alterations would be difficult to detect.

It has been reported that xanthine derivatives can be incorporated into DNA (25), which might also lead to growth arrest. This seems unlikely in our system because CV-1 cells recover rapidly from theophylline arrest and undergo DNA synthesis within 24 h. In addition, theophylline has no effect on ongoing DNA synthesis, and cells must proceed through the cell cycle before arrest occurs.

In NRK cells theophylline causes a specific decrease in the number of receptors for nerve growth factor (14). The possibility that methylxanthines act at the membrane level is intriguing because of our recent studies on the reversal of small-t antigen effects on CV-1 cells by a series of ionophores (17). Studies of the ionophores have suggested that small-t antigen might affect functions of cellular membrane systems. The key roles played by ion distributions across membranes in growth regulation (15, 23) have made this an interesting possibility. Examination of the activities of various membrane-associated enzymes, possibly in the presence of biologically active small-t antigen, may help to test some of these possibilities.

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