NOTES

Reversal of Simian Virus 40 Small-t-Antigen-Induced Theophylline Resistance

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Simian virus 40 small-t antigen allows the growth of CV-1 cells in concentrations of theophylline that normally prevent cell cycling. This effect is completely overcome by two ionophores that uncouple mitochondria and by the anti-mitochondrial drug methylglyoxal bis-(guanyldiazide).

Although the ability to make a small-t antigen has been maintained in papovaviruses isolated from mice, monkeys, and humans, the function of this protein and its value to the life cycles of the viruses is almost totally unknown. The simian virus 40 (SV40) small-t antigen is involved in the efficient transformation of some growth-arrested cell lines (2, 9, 14) but appears to play no role once the cell is transformed. It is also dispensable for viral productive growth in monkey cells. Among the other functions related to the expression of the SV40 small-t antigen are the disorganization of actin cables (6), the association with two cellular proteins (17), and the increased resistance to theophylline or cyclic AMP (cAMP) derivatives of monkey kidney CV-1 cells, clone TC7 (13). When CV-1 cells are treated with 1 to 2 mM theophylline, DNA synthesis is reduced by over 90% within 24 to 48 h. When cells are infected with wild-type (WT) virus, both cellular and viral DNA syntheses occur at the same levels in theophylline-treated and -untreated cultures. However, cells infected with SV40 deletion mutants that lack small-t antigen are nearly as sensitive to theophylline as are uninfected cells. Theophylline does not appear to directly inhibit DNA synthesis because addition of theophylline to infected cells 1 to 2 h before addition of [³H]thymidine does not result in decreased thymidine incorporation by cells infected with small-t mutants (K. Rundell, unpublished data). In addition, after removal of theophylline from cultures, a period of time is required before DNA synthesis can be detected (13), suggesting that cells must progress through the cell cycle and that they have not been blocked in the S phase itself. A direct role for small-t antigen in the stimulation of cellular DNA synthesis is highly unlikely based on studies of mutants with deletions that affect large-T, but not small-t, antigen (16).

We have been studying the theophylline resistance patterns of infected CV-1 cells in an effort to determine the site of action of small-t antigen and to obtain clues as to its specific effects on the cell. First, we attempted to increase the theophylline resistance of uninfected cells or cells infected with small-t-antigen mutants. Because small-t antigen is involved in the disorganization of actin microfilaments, we used drugs such as cytochalasins B and D to determine whether the disorganization of actin cables could directly increase the theophylline resistance of CV-1 cells. However, concentrations of these drugs that did alter actin cable

structure did not alter the theophylline sensitivity patterns of these cells. Similarly, we were unable to identify other chemicals or drugs that increased the resistance of CV-1 cells.

In an alternate approach, we have identified agents that overcome the effect of small-t antigen on CV-1 cells and increase the sensitivity of WT-infected cells to theophylline. The first of these agents found was methylglyoxal bis-(guanyldiazide) (MGBG). MGBG greatly increased the sensitivity of WT-infected CV-1 cells to theophylline, at concentrations where MGBG alone was not toxic to the cells (Table 1). Confluent cultures of CV-1 cells were infected with WT SV40 (5 to 10 PFU per cell) and then incubated in serum-free medium containing various concentrations of theophylline and MGBG for 36 h at 37°C. [³H]thymidine was added for 6 h, cellular and viral DNAs were fractionated by the procedure of Hirt (7), and then radioactivity in cellular DNA was measured. As expected, 1.5 mM theophylline had little effect on the synthesis of cellular DNA in WT-infected cells but reduced DNA synthesis in cells infected with the small-t-antigen deletion mutant, *d888*. At the concentration used in this experiment, MGBG did not reduce cellular DNA synthesis in the absence of theophylline but eliminated the theophylline resistance of the WT-infected cells.

MGBG is best known as an inhibitor of S-adenosylmethionine decarboxylase, an enzyme involved in the synthesis of polyamines (5). It also affects mitochondria, causing pronounced mitochondrial swelling *in vivo* (12) and reducing oxygen consumption by isolated mitochondria (4, 11). In a variety of experiments with either radioactive polyamine precursors (putrescine or ornithine) to measure rates of synthesis of the polyamines or dansylation of total polyamine pools in cells, we were unable to obtain any evidence for alteration in polyamine metabolism in infected cells. This suggested that the mitochondrial effects of MGBG might be responsible for increasing the theophylline sensitivity of CV-1 cells.

This possibility was supported by experiments which demonstrate synergistic effects between MGBG and a K⁺ ionophore, valinomycin. By using isolated mitochondria (4), it has been shown that MGBG can decrease oxygen consumption and that mitochondria preincubated with valinomycin become even more sensitive to inhibitory effects of MGBG. In these studies, it was suggested that MGBG may compete with K⁺ binding sites on the outer surface of the inner mitochondrial membrane and that valinomycin further

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TABLE 1. Effect of MGBG on theophylline resistance of WT-infected CV-1 cells

Theophylline (mM) ^a	Thymidine incorporation ^b (cpm × 10 ⁻⁴ per dish)		
	d1888	WT	WT + 10 μM MGBG ^a
0	4.3	8.1	6.6
1.5	0.7	7.6	0.5
2.0	0.4	6.2	0.2

^a Theophylline and MGBG were added in serum-free medium immediately after infection of confluent cultures in 35-mm dishes.

^b At 36 to 42 h postinfection, 1 μCi of [³H]thymidine was added, and then radioactivity in cellular DNA was measured.

reduced extramitochondrial K⁺ levels. The inhibitory effects of MGBG on theophylline-treated, WT-infected CV-1 cells were also potentiated by valinomycin (Fig. 1). In the absence of theophylline, neither MGBG nor valinomycin blocked progression of the infected cells into DNA synthesis (data not shown).

Further indication for mitochondrial involvement in theophylline resistance was obtained using two ionophores, nonactin (a K⁺ ionophore) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, an H⁺ ionophore). Either ionophore was able to increase the theophylline sensitivity of WT-infected cells at concentrations where the ionophores alone had little or no effect on DNA synthesis (Table 2). Increased sensitivity to theophylline was observed over a relatively narrow range of concentrations of the ionophores, and the effective concentrations were just below concentrations that were cytotoxic.

FCCP and nonactin can affect ion gradients across the

FIG. 1. Effect of valinomycin on MGBG-induced theophylline sensitivity. Confluent cultures of CV-1 cells in 35-mm dishes were infected with WT SV40 and then incubated in serum-free medium containing 1.5 mM theophylline and various concentrations of MGBG. One set of plates contained valinomycin at 10 nM (triangles), the other set did not contain valinomycin (circles). After 36 h at 37°C, incorporation of [³H]thymidine into cellular DNA was measured. The values represent the total incorporated thymidine per dish of cells.

plasma membrane, as well as across intracellular membranes such as the mitochondrial membrane, but experiments with Ehrlich ascites cells (3) have suggested that low concentrations of FCCP affect mitochondria preferentially. In addition, the drug MGBG causes morphological and functional alterations of mitochondria, but effects on plasma membrane functions have not been reported. In some preliminary experiments, we tried to assess the effects of MGBG on plasma membrane transport by using transport of 2-aminoisobutyric acid (AIB), a non-metabolizable amino acid analog whose transport is sensitive to changes in the membrane potential (8). In the experiment shown in Table 3, a combination of theophylline and MGBG reduced cellular DNA synthesis over 90% in WT-infected CV-1 cells, but these concentrations of drugs did not depress the transport of AIB. Although not shown here, the transport of both AIB and 2-deoxyglucose was reduced approximately twofold by theophylline alone, even in WT-infected cells in which theophylline had no effect on cellular DNA synthesis. However, rates of transport were not further reduced by MGBG and did not parallel the ability of cells to incorporate thymidine into DNA.

The enhancement of theophylline sensitivity of cells by MGBG or the two ionophores was particularly pronounced in the case of WT-infected cells. At appropriate concentrations of these drugs, the difference in thymidine incorporation between cells in the presence and absence of theophylline was over 10-fold. Although not shown here, subtoxic concentrations of these drugs also increased the theophylline sensitivity of uninfected cells two- to threefold. However, pronounced effects such as those observed in WT-infected cells have not been observed.

The mechanisms by which theophylline causes growth arrest in cell culture are not well understood. Because theophylline is an inhibitor of cAMP phosphodiesterase, elevated levels of cAMP have been considered as one possible mechanism. However, this interpretation has been questioned by studies in which cholera toxin, which elevates intracellular cAMP levels, was shown to enhance cell cycle progression of 3T3 cells in response to growth factors (15). Similarly, we have been unable to detect major differences in cAMP concentrations in cells infected with WT SV40 or with the small-t-antigen deletion mutants in the presence of theophylline (unpublished data). It is certainly possible that theophylline has a variety of effects on cells in culture and that some effect other than the inhibition of cAMP phosphodiesterase may be responsible for growth inhibition.

TABLE 2. Enhancement by ionophores of theophylline sensitivity of WT-infected CV-1 cells

FCCP (μM) ^a	Nonactin (μM) ^a	Thymidine incorporation ^b (cpm × 10 ⁻⁴ per dish)	
		Untreated	2 mM theophylline ^a
0	0	10.7	9.4
4	0	9.6	8.1
6	0	10.8	5.4
8	0	10.2	1.2
0	0.2	18.6	8.6
0	0.4	16.1	2.3
0	0.6	9.4	1.1

^a Theophylline, FCCP, or nonactin was added in serum-free medium immediately after infection.

^b At 36 to 42 h postinfection, 1 μCi of [³H]thymidine was added, and then radioactivity in cellular DNA was measured.

TABLE 3. Transport of AIB in CV-1 cells infected in the presence and absence of theophylline and MGBG

Theophylline (mM) ^a	MGBG (μM) ^a	Thymidine incorporation ^b (cpm × 10 ⁻⁴ per dish)	AIB transport ^c (nmol/20 min)
1.5	0	12.3	4.11
1.5	10	2.1	4.91
1.5	12	1.3	4.62
2.0	0	8.8	4.29
2.0	10	0.9	4.38

^a Theophylline or MGBG was added in serum-free medium immediately after infection.

^b [³H]thymidine was added from 36 to 42 h postinfection, and then radioactivity in cellular DNA was measured.

^c A parallel set of plates was incubated for 40 h. Cells were washed twice with warm phosphate-buffered saline and then incubated with 0.2 mM AIB (2.78 mCi/mmol) for 20 min at 37°C. Cells were washed rapidly with phosphate-buffered saline three times and then solubilized in phosphate-buffered saline containing 0.5% Nonidet P-40. Background radioactivity was determined using a zero time point and was subtracted for each measurement.

Our experiments suggest that theophylline sensitivity, particularly in the case of WT-infected CV-1 cells, is enhanced by MGBG and ionophores, all of which have pronounced effects on mitochondrial function. Whether this phenomenon is related to cAMP is unknown, but these results suggest that one effect of theophylline may be to alter ion distributions across mitochondrial membranes or, possibly, across other cellular membranes. Because the SV40 small-t antigen is responsible for the theophylline resistance of CV-1 cells, we speculate that small-t antigen may enhance cellular respiration in one of a variety of ways. For example, small-t antigen might stimulate mitochondrial oxidative phosphorylation directly or indirectly via increased intracellular NADH or other metabolites. Increased NADH levels would result if small-t antigen were to increase the glycolytic activity of cells, possibly by interaction with one of the key glycolytic enzymes. It is of particular interest here that small-t antigen interacts with two cellular proteins, 56K and 32K, which are found as soluble, cytoplasmic proteins in a variety of cell lines (17). Alternatively, direct effects of small-t antigen on plasma membrane functions cannot be ruled out. Although no apparent alteration in AIB transport was observed in our experiments, a variety of substrates for transport should be tested, and direct measurements of ion fluxes or plasma membrane potential itself should be performed.

Finally, a known function of small-t antigen is the disorganization of actin cable structures. It is conceivable that cytoskeletal rearrangements could alter ion fluxes across membranes or directly affect mitochondrial activity. Mitochondrial associations with microfilaments have not been reported, but descriptions of mitochondrial associations with microtubules (1) and with intermediate filaments (10) underline the potential relationship of cytoskeleton structure to organelle function. It has been disappointing in this regard that we have been unable to detect small-t antigen or the associated cellular proteins either in cytoskeleton preparations or in a variety of subcellular fractions from standard fractionation protocols. Possibly, the development of cell lines that express small-t antigen as the sole viral product or

the availability of monoclonal antibodies that recognize protein sequences unique to small-t antigen will allow detection of a discrete subcellular localization for this protein.

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