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SELECTIVE INHIBITION OF MACROMOLECULAR SYNTHESIS IN TRANSFORMED MOUSE CELLS BY MEANS OF ATP-TREATMENT[1]

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SUMMARY: External ATP causes a rapid and reversible increase in passive permeability in transformed cells but not in untransformed 3T3 cells. By this technique certain impermeant inhibitors of protein or DNA synthesis were sealed into transformed cells. With 1-β-D-arabinofuranosylcytosine 5'-triphosphate the inhibition of DNA synthesis was maintained for at least 20 h. The present results suggest that treatment with ATP makes it possible to achieve selective toxicity for transformed cells *in vitro* and possibly *in vivo*.

Recently it has been found that treatment of spontaneously or virus transformed 3T3 cells (3T6, SV3T3 and PY3T3[2]) with external ATP causes a rapid and striking increase in membrane permeability, allowing the entry and efflux of nucleotides and phosphate esters which normally do not cross the plasma membrane (1-5). The permeability change induced by ATP is rapidly reversible and the treated cells grow at a normal rate (2). None of these effects are obtained with untransformed 3T3 cells.

The present results show that selective inhibition of both protein and DNA synthesis can be achieved in transformed cells treated with external ATP and certain inhibitors which are ordinarily impermeant. The inhibitors were selectively sealed into the transformed cells by this reversible permeabilization procedure, and in this way the inhibited state was maintained.

1. A preliminary result of this work was presented at the XIth International Congress of Biochemistry, Toronto, Canada, 1979, in abstract: p. 482.

2. Abbreviations: 3T6, spontaneously transformed Swiss 3T3 cells; SV3T3, 3T3 cells transformed by SV40; PY3T3, 3T3 cells transformed by polyoma virus; DME, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GDPCP, gyanylyl β,γ-methylene diphosphonate, GDPNP, guanylyl imidophosphate; araCTP, 1-β-D-arabinofuranosylcytosine 5'-triphosphate.

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Materials and Methods

Cells. Stock cultures of Swiss 3T3, 3T6 and SV3T3 cells were grown in Dulbecco's modified Eagle's medium (DME) containing 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin as described (1). The cells were subcultured into 33 mm Falcon dishes in DME plus 10% FCS and were used as described (5).

Protein synthesis in ATP-treated cells and in cell extract. Transformed 3T3 cells were permeabilized in buffer A by external ATP and protein synthesis in the cells were determined in the presence of external additions in buffer B as described previously (5). Buffer A: 0.1 M Tris/HCl, pH 8.2, 50 mM NaCl, 50 µM $CaCl_2$ and 5 mg/ml Dextran 500; Buffer B: 50 mM Tris/HCl, pH 8.0, 95 mM potassium acetate, 5 mM sodium acetate, 5 mM magnesium acetate and 5 mg/ml Dextran 500. Cell extracts were prepared as described by Weber et al. (6). The reaction mixture for protein synthesis in cell extracts contained in a 60 µl volume: 35 µl cell lysate (120-150 µg protein), 1.7 mM ATP, 0.25 mM GTP, 8.3 mM creatine phosphate, 3 units creatine phosphokinase, 0.7 mM dithiothreitol, 18.3 mM Hepes/KOH (pH 7.4), 83 mM potassium acetate, 3 mM magnesium acetate, 165 µM of each of 19 amino acids and [^{14}C]leucine at 1 µCi/ml. The mixture was incubated at 37°C for 30 min and TCA-precipitable counts were measured.

Sealing normally impermeant drugs in ATP-treated 3T6 cells. ATP-treated 3T6 cells in buffer A were further incubated with the indicated drugs in buffer B for 15 min at 37°C. The medium were replaced by 2 ml DME containing 10% FCS and the cultures were incubated at 37°C in the CO_2-incubator to seal the membranes (2,5). When the cells were treated with ara-CTP, the cultures were washed once with DME before the sealing procedure. After the incubation of the indicated period, the cells were labeled with [^{3}H]thymidine (0.02 µM, 0.33 µCi/ml) or [^{3}H]uridine (1 µM, 0.5 µCi/ml) for 30 min at 37°C and radioactivity in TCA-insoluble material was measured. For the assay of protein synthesis, the cultures were washed twice with buffer B and incorporation of [^{14}C]leucine into protein was determined as described (5). Protein was determined by the method of Lowry et al. (7).

Materials

[^{14}C]leucine (289 to 294 mCi/mmole), [methyl-^{3}H]thymidine (20 Ci/mmole) and [6-^{3}H]uridine (22.4 Ci/mmole) were obtained from New England Nuclear. Cycloheximide, puromycin, chloramphenicol, aurin tricarboxylic acid and 1-β-D-arabinofuranosylcytosine 5'-triphosphate (araCTP) were purchased from Sigma. Guanylyl β,γ-methylene diphosphonate (GDPCP) and guanylyl imidophosphate (GDPNP) obtained from Boehringer Mannheim Biochemicals. Sparsomycin was a generous gift from Dr. Sidney Peska, Roche Institute of Molecular Biology, Nutley, New Jersey.

Results

Protein synthesis in ATP-treated normal and transformed 3T3 cells

The nonhydrolyzable GTP analogue, GDPCP, and the antibiotic sparsomycin inhibited protein synthesis in ATP-treated 3T6 cells and cell extracts but not in untreated 3T6 cells (Fig. 1). The dose response of protein synthesis to GDPCP in ATP-treated cells was very similar to that observed for cell extracts. Another GTP analogue, GDPNP, showed a similar inhibition of protein synthesis in ATP-treated 3T6 cells and cell extracts. Aurin tricarboxylic acid was effective only for the cell-free system, while puro-

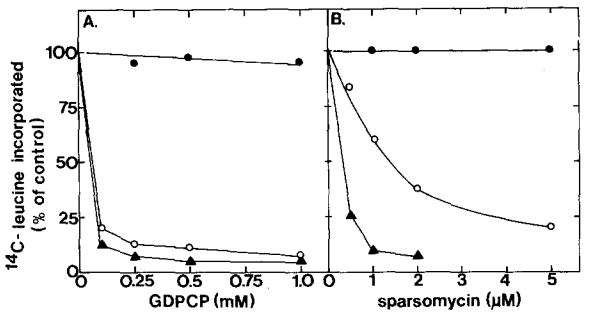

Figure 1. Effect of GDPCP and sparsomycin on protein synthesis in ATP-treated 3T6 cells and in cell extracts. Protein synthesis in 3T6 cells (●), in ATP-treated 3T6 cells (○) and in cell extracts from 3T6 cells (▲) was determined in the presence of various concentrations of GDPCP (A) or sparsomycin (B). The cells or cell extracts were incubated with [^{14}C]leucine and the incorporation of the isotope into TCA-insoluble material was measured.

mycin and cycloheximide were powerful inhibitors of protein synthesis in ATP-treated and untreated cells (data not shown). The inhibition by GDPCP or sparsomycin was observed when glycolytic factors (5) provided energy (Fig. 1) and also when ATP plus phosphoerol pyruvate or succinate plus factors for oxidative phosphorylation (5) were used (data not shown). In no case was significant inhibition noted for chloramphenicol, which is an inhibitor of mitochondrial protein synthesis (data not shown).

Table 1 shows that protein synthesis in another transformed cell line, SV3T3 was also inhibited by GDPCP or GDPNP, but only when the cells were permeabilized by ATP. Sparsomycin, however, did not inhibit protein synthesis in ATP-treated SV3T3 cells. As expected, protein synthesis in untransformed 3T3 cells was not affected by any of these drugs under the same experimental conditions.

The effect of ATP on permeability can be reversed by incubation of the permeabilized cells with DME or neutral buffer (1-3). Protein synthesis in ATP-treated 3T3 cells has also been restored by incubation of the cells with DME (5). This reversible permeability change was used to seal normally impermeant inhibitors into transformed cells. The cells permeabilized by ATP were treated with various concentrations of GDPCP for 15 min at an alkaline pH, followed by incubation for 60 min with DME

Table 1

Differential inhibition of protein synthesis by several drugs in normal and transformed 3T3 cells

Cells	Inhibitors	SV3T3	3T3
		[^{14}C]leucine incorporated (pmoles/mg protein)	
ATP-treated	None	31.1 (100%)	16.6 (100%)
"	GDPCP 1 mM	3.95 (13%)	15.0 (90%)
"	GDPNP 1 mM	11.2 (36%)	16.3 (98%)
"	Sparsomycin 5 μM	29.2 (94%)	16.6 (100%)
Untreated	None	28.6 (100%)	16.9 (100%)
"	GDPCP 1 mM	28.6 (100%)	16.6 (98%)
"	GDPNP 1 mM	30.8 (108%)	16.9 (100%)
"	Sparsomycin 5 μM	27.2 (95%)	16.9 (100%)

Protein synthesis in SV3T3 and 3T3 cells, which were preincubated with or without ATP in buffer A (pH 8.2) for 6 - 8 min, was determined in the presence of the indicated inhibitors in buffer B.

in the presence of serum. Table 2 shows that protein synthesis was inhibited by more than 90%, when compared with ATP-treated 3T6 cells which had not been given the inhibitor (lane 3), in which protein synthesis had been restored by incubation with DME. Protein synthesis in other control cells that received GDPCP but not ATP was also not affected (lane 8). The inhibition by GDPCP was specific for protein synthesis: uridine uptake, an indicator for restoration of the permeability barrier (2), RNA synthesis and DNA synthesis were not inhibited by GDPCP under the conditions described in Table 2 (data not shown). The inhibition of protein synthesis by GDPCP in ATP-treated and resealed 3T6 cells continued for at least 3 hr after the drug was sealed in (Table 2). However, partial recovery of protein synthesis was noted after 9 hr and by 20 hr recovery was complete.

DNA synthesis in ATP-treated transformed and untransformed 3T3 cells

Ara-C is a synthetic nucleoside shown to be active against a variety of animal tumors and certain human neoplasms (8,9). In order for ara-C to

Table 2

Effect of GDPCP on protein synthesis in ATP-treated and resealed 3T6 cells

Experimental Conditions			Protein Synthesis
Stage I	Stage II	Stage III	
			pmoles/mg protein
-ATP	--	--	30.2
+ATP	--	--	1.52
"	GDPCP 0 mM	DME + serum	53.9
"	" 0.1mM	"	18.2
"	" 0.5mM	"	7.43
"	" 1.0mM	"	3.13[a]
-ATP	" 0 mM	"	55.0
"	" 1.0mM	"	60.6

GDPCP was sealed into 3T6 cells by means of ATP treatment as described in Methods. After 3T6 cells were treated with ATP in buffer A, pH 8.2, for 8 min (Stage I), the cells were incubated with various concentrations of GDPCP for 15 min in buffer B (Stage II). The medium was replaced by DME plus 10% FCS and the cells were incubated for another 60 min (Stage III), following which protein synthesis in the cultures was determined in buffer B as described in Materials and Methods.

[a]After 3 h in DME + serum this value rose to 7.8; it reached 22.6 in 9 h and 41.1 in 20 h.

exert its effect, it must be converted to the triphosphate, araCTP, which is an inhibitor of DNA polymerase (10) and which cannot cross the cell membrane. When ATP-treated 3T6 cells were incubated with araCTP for 15 min and subsequently with DME, the drug was sealed into the cells and inhibited DNA synthesis even after 20 hr of incubation (Fig. 2A). By contrast, DNA synthesis in untransformed 3T3 cells was unaffected by the same treatment with araCTP (Fig. 2B). The viability of ATP-treated 3T6 cells as measured by colony formation was also reduced by araCTP, but considerable experimental variation was encountered (data not shown).

Discussion

In the present study the GTP analogues, GDPCP and GDPNP, and sparsomycin inhibited protein synthesis in 3T6 cells permeabilized by ATP. The sensi-

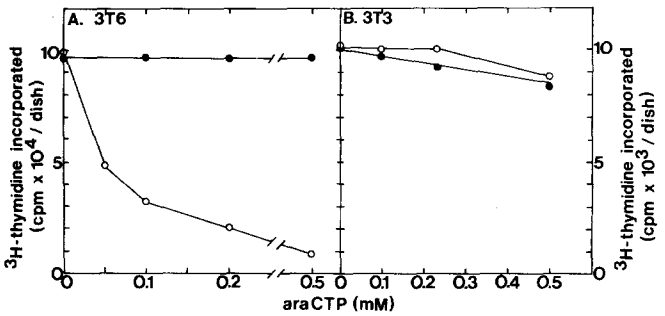

Figure 2. Selective inhibition of DNA synthesis in transformed 3T3 cells by means of ATP-treatment. (A) ATP-treated (O) or untreated 3T6 cells (●) were incubated with various concentrations of araCTP in buffer B for 15 min as described in Table 2. Cells were washed once with DME and were incubated for 20 hr at 37° with DME + 10% FCS. At the end of incubation, uptake of [^{3}H]thymidine into TCA-insoluble material was measured in each culture. (B) The same experiment was done for both ATP-treated (O) and untreated 3T3 cells (●).

tivity of these cells to the inhibitors was close to that of equivalent cell extracts. Similar results were obtained with another transformed line, SV3T3 except that sparsomycin was inactive. Perhaps the increase in permeability obtained with these SV3T3 cells was not sufficient to allow entry of sparsomycin. Protein synthesis in untransformed 3T3 cells was unaffected by any of these inhibitors even when the cells were treated with ATP, confirming eariler studies on the differential effect of ATP on normal and transformed 3T3 cells (1-5). The reversible permeabilization has been used to seal an inhibitor in transformed cells. The nucleotide analogues, GDPCP or araCTP, were sealed in ATP treated 3T6 cells and inhibited protein synthesis or DNA synthesis, respectively. It is interesting that the effect of GDPCP on protein synthesis in whole cells was unstable presumably due to slow metabolism or leakage of the drug from the cells, so that recovery of protein synthesis was complete after 20 hr. The effect of araCTP on DNA synthesis was prolonged in 3T6 cells for 20 hr, but it was not sufficient to kill a large fraction of the cells consistently. By adjustment of dosage or by repeating the procedure it may be possible to improve the killing of transformed cells. In any case, with a suitable impermeant inhibitor it should be possible to achieve selective toxicity for transformed cells *in vitro*, and it is conceivable that the technique could be used *in vivo*.

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