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PRELIMINARY COMMUNICATION

ADENOSINE TRIPHOSPHATE CATABOLISM IN EHRLICH ASCITES TUMOR CELLS TREATED WITH DACTYLARIN

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Dactylarin is a tricyclic, quinone-containing antibiotic of the geodin type (1), which Miko et al. (2) have recently shown to be an inhibitor of energy generating processes in Ehrlich ascites tumor cells in vitro. At relatively low dactylarin concentrations oxidative phosphorylation was uncoupled, whereas at higher concentrations aerobic glycolysis was inhibited also. Related compounds (substances $66A_2$ and $66A_3$) had similar metabolic effects.

Studies of other compounds that inhibit energy generating processes have shown that (a) they induce the catabolism of ATP, (b) the conditions under which catabolism is maximal differ from one inhibitor to another, and (c) the relative rates of alternative pathways of ATP catabolism also vary, depending on the inhibitor and the conditions used (3-6).

In this study the extent and pathways of ATP catabolism in dactylarin-treated Ehrlich ascites tumor cells have been investigated, and it has been found that dactylarin has unique effects on this process.

Sources of most materials, methods of tumor cell preparation and incubation, and procedures for the separation and measurement of radioactivity in purine bases, ribonucleosides and ribonucleotides have been reported previously (7,8). ATP and its metabolites were separated by thin-layer chromatography, and their radioactivity was measured. The initial total concentration and specific activity of ATP were determined by these methods plus high-performance liquid chromatography; it has previously been established (3) that there is no compartmentation of radioactive and non-radioactive ATP with respect to catabolism. Changes in concentrations of metabolites were calculated from the initial specific activity of ATP. Dactylarin and the related compound $66A_3$ were prepared in 0.154 M sodium chloride; as the molecular weight of $66A_3$ is not known, drug concentrations are expressed in μg/ml. In stock solutions of 250 μg/ml, $66A_3$ was completely dissolved, but dactylarin was only partially dissolved; the latter was completely dissolved at the final concentrations used, however.

To study ATP catabolism, tumor cells were first incubated with [^{14}C]-adenine to produce radioactive ATP. Unused [^{14}C]adenine was removed by centrifugation and resuspension in fresh medium. Concentrations of radioactive metabolites were measured in cells incubated for an additional 30 min under various conditions, with and without dactylarin or $66A_3$. Details are given in the legend of Table 1.

The effects of dactylarin and $66A_3$ on ATP catabolism were studied first under conditions in which the cells relied solely on oxidative phosphorylation for energy generation, i.e. aerobic incubation in the absence of glucose. Table 1 shows that dactylarin treatment induced measurable ATP catabolism at 0.15 μg/ml, and 90 percent of cellular ATP was catabolized at a concentration of 3.12 μg/ml; 0.78 μg/ml (ca. 2.5 μM) produced ca. 50 percent catabolism in the time period (30 min) studied. $66A_3$ had approximately the same dose-response relationship, though low concentrations were not quite as toxic as dactylarin.

Because $66A_3$ was more water soluble than dactylarin, further experiments were carried out using the former compound. The effects of $66A_3$, therefore, were next studied in cells that depended upon both oxidative phosphorylation and glycolysis for energy generation (i.e. aerobic incubation in the presence of glucose); in these cells glycolysis is the predominant pathway under these conditions. Table 1 shows that 0.78 µg/ml $66A_3$ produced about the same degree of ATP catabolism under these conditions as in the absence of glycolysis, but higher drug concentrations were much less effective. Thus, glycolytic activity seemed partially to protect ATP from catabolism.

Table 1. ATP catabolism induced by dactylarin and $66A_3$*

Drug	Conditions	ATP concentration (percent of control)						
		Drug concentration (µg/ml)						
		0.15	0.31	0.78	1.56	3.12	6.25	12.5
Dactylarin	O_2-glucose	83.1	61.9	42.5	17.6	9.07	6.37	4.59
$66A_3$	O_2-glucose	98.6	86.0	53.8	11.7	6.72	4.65	1.63
$66A_3$	O_2+glucose			53.2	48.4	43.6	24.1	16.8
$66A_3$	N_2+glucose			100	100	100	100	100

*Two ml of 2 percent (v/v) Ehrlich ascites tumor cell suspension in calcium-free Krebs-Ringer medium containing 25 mM phosphate and 5.5 mM glucose was incubated in 10-ml Erlenmeyer flasks at 37° with shaking, with an air atmosphere. After 20 min, [^{14}C]adenine (ca. 50 mCi/mmole) was added to a final concentration of 100 µM, and incubation was continued for 30 min to synthesize [^{14}C]ATP. Unutilized [^{14}C]adenine was then removed by centrifugation and resuspension of the cells twice in fresh, warmed medium containing glucose. Portions (100 µl total volume) were then incubated with and without drug, under various conditions. Values reported are averages of duplicate measurements and are representative of results obtained in two experiments. Within each experiment, average deviation of individual analyses from the mean was less than 7 percent. Control ATP, 2285 nmoles/g cells.

Finally, the effect of $66A_3$ was studied in cells that depended solely on glycolysis (i.e. anaerobic incubation with glucose). Table 1 shows that under these conditions even 12.5 µg/ml (as well as 25 µg/ml; data not shown) had no effect on ATP concentrations.

These studies, then, confirm the findings of Miko et al. (2) that the most sensitive target of dactylarin and $66A_3$ action was oxidative energy generating processes of the cell, and are compatible with their findings that these compounds uncouple oxidative phosphorylation. In agreement with Horakcva et al. (9), low drug concentrations had no effect on glycolysis; in contrast to the results of Miko et al. (2), however, concentrations up to 25 µg/ml also did not appear to affect glycolysis in the present study.

These results may be compared with similar studies carried out using ethidium (4), isometamidium (4) and bikaverin (5). Isometamidium (250 µM) produced very substantial ATP catabolism under both aerobic and anaerobic conditions in the presence of glucose, and also aerobically in the absence of glucose. Ethidium (250 µM) produced 50 percent ATP catabolism when cells were incubated aerobically without glucose, and only 7-14 percent catabolism in cells glycolyzing either aerobically or anaerobically. Bikaverin (50 µg/ml) resembled ethidium in these respects, though it was more potent. Dactylarin and $66A_3$ thus are different from these other compounds in both their potency and their relationship between incubation conditions and extent of ATP catabolism (i.e. different effects under conditions of oxidative phosphorylation, aerobic glycolysis, and anaerobic glycolysis).

The second phase of this study was to investigate the relative rates of alternative pathways of ATP catabolism in cells treated with dactylarin and $66A_3$; only results with the latter compound are reported. Following catabolism to adenylate, two alternative pathways potentially may be followed; one is deamination to inosinate, while the second is dephosphorylation to adenosine (which in these cells normally is deaminated rapidly to inosine; the latter is converted partially to hypoxanthine). Any inosinate formed may also be metabolized via two alternative routes; one is conversion to xanthylate, while the second is dephosphorylation to inosine. Because the incubation medium used does not contain glutamine, xanthylate is not further metabolized to guanine nucleotides.

Initial studies indicated that hypoxanthine and inosine were formed as a result of $66A_3$-induced ATP catabolism, and experiments were conducted to ascertain the relative extent to which these compounds were formed via dephosphorylation of adenylate and deamination of adenosine, and via the alternative pathway of deamination of adenylate and dephosphorylation of inosinate. To do this, cells were treated with deoxycoformycin to inhibit adenosine deaminase (10). This approach has been used previously to determine the relative rates of these pathways in Ehrlich ascites tumor cells (11) and in human erythrocytes (6). Inhibition of adenosine deaminase by deoxycoformycin did not alter the extent of ATP catabolism or the amounts of hypoxanthine and inosine that were formed; virtually no adenosine accumulated in either the presence or the absence of deoxycoformycin. The conclusion can therefore be drawn that adenylate catabolism proceeded totally via deamination to inosinate, and that none was dephosphorylated to adenosine.

Table 2. Metabolites of ATP in cells treated with $66A_3$

Drug concn (μg/ml)	Metabolite concentration (percent of control)* Adenine nucleotides	Adenylate	Inosinate	Xanthylate	Hypoxanthine + inosine
0.78	67.2	164	224	275	184
1.56	34.6	213	436	377	309

*Control values (nmoles/g cells): adenine nucleotides, 2622; adenylate, 33, inosinate, 4; xanthylate, 6; and hypoxanthine + inosine, 87.

Table 2 shows the relative changes in concentrations of major intermediates and end products of ATP catabolism in cells treated with moderate doses of $66A_3$. It is seen first that the decrease in concentration of total adenine nucleotides was not as great as that of ATP (shown in Table 1); this is accounted for chiefly by some accumulation of adenylate. Much of the adenylate formed during ATP catabolism was deaminated to inosinate, however, and of this inosinate, some accumulated, some was converted to xanthylate, and some was dephosphorylated to inosine; part of the latter was then phosphorolyzed to hypoxanthine. The pattern of ATP catabolism in dactylarin-treated cells was the same as when $66A_3$ was used.

As a way of comparing the relative rates of alternative pathways in these experiments to those found in previous studies for ethidium, isometamidium and bikaverin, the relative extents of accumulation of adenylate, inosinate and xanthylate were calculated. Inosinate accumulation by cells treated with ethidium and isometamidium was ca. 60 percent greater than adenylate accumulation, whereas in bikaverin-treated cells this value was 345 percent; in $66A_3$-treated cells inosinate accumulation exceeded that of adenylate by ca. 40 percent. Xanthylate accumulation was considerably greater than that of adenylate in cells treated with ethidium (395 percent), isometamidium (232 percent) and bikaverin (402 percent); in contrast, this value was ca. 70 percent in cells treated with $66A_3$. Though these experiments cannot be compared precisely, such calculations do indicate that $66A_3$ induces a pattern of ATP catabolism that differs somewhat from those produced by other inhibitors.

The present results, together with other studies (3-6), show that the relative rates of alternative pathways of purine metabolism in cells treated with a variety of inhibitors of energy generating processes vary considerably depending on the inhibitor and conditions used. The enzymatic bases of these differences remain to be precisely defined.

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