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EFFECTS OF BIKAVERIN ON PURINE NUCLEOTIDE SYNTHESIS AND CATABOLISM IN EHRLICH ASCITES TUMOR CELLS *IN VITRO**

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Abstract—Bikaverin, a growth-inhibitory fungal metabolite, inhibits the incorporation of radioactive purine bases into nucleic acids and also into acid-soluble nucleotides. It also inhibits energy metabolism and induces the breakdown of intracellular ATP. Evidence is presented that these effects may be due to inhibition of ATP synthesis by bikaverin, probably at the level of the tricarboxylic acid cycle or oxidative phosphorylation.

Bikaverin (Fig. 1) is a fungal metabolite that has been isolated from *Fusarium oxysporum* and related species of *Fusarium, Gibberella fujikuroi*, and *Mycogone jaapii*. In early studies it was also known as lycopersin, passiflorin, mycogonin and "fungal vacuolation factor" [1–9]. Bikaverin was reported to have specific antiprotozoal activity against *Leishmania brasiliensis*, but was not effective against other protozoa, or against certain fungi and bacteria [4]. Fuska *et al.* have found that it inhibits the growth of Ehrlich ascites carcinoma, lymphoma L5178Y, sarcoma 37, lymphoadenoma NK/LY, and HeLa cells in culture [10, 11], and initial biochemical studies [10–12] showed that 25 μl/ml of bikaverin inhibited incorporation of [^{14}C]adenine and [^{14}C]valine into acid-insoluble material by *ca.* 50 per cent in cultured Ehrlich ascites tumor cells. We have investigated the effects of bikaverin on purine ribonucleotide synthesis and metabolism, and the results of these studies are presented here. It appears that the main effect of bikaverin may be as an inhibitor of ATP synthesis.

MATERIALS AND METHODS

Sources of most materials [13], methods of preparation and incubation of Ehrlich ascites tumor cells [14, 15], and procedures for the separation and measurement of radioactivity in purine bases, ribonucleosides and ribonucleotides [14, 16] have been reported previously. Bikaverin is not readily soluble in water, but is soluble in dilute solutions of ethanol or dimethylsulfoxide; however, in the latter cases the solvents themselves produced appreciable inhibition of purine nucleotide synthesis. Hence a fine suspension of bikaverin in distilled water, prepared with the aid of sonication, was used in these experiments.

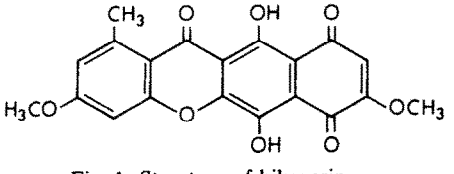

Fig. 1. Structure of bikaverin.

Briefly, to measure the effects of bikaverin on purine ribonucleotide synthesis and interconversion, Ehrlich ascites tumor cells were incubated as 2.5 per cent suspensions in 100 μl of calcium-free Krebs–Ringer medium containing 25 mM phosphate and 5.5 mM glucose for 20 min, with and without bikaverin; 100 μM [^{14}C]adenine, [^{14}C]guanine or [^{14}C]hypoxanthine (the specific activity of each was *ca.* 50 mCi/m-mole) was then added, and incubation was continued for an additional 30 min. Radioactivity in perchloric acid-soluble purine ribonucleotides (ATP, ADP, adenylate, GTP, GDP, guanylate, inosinate and xanthylate), purine ribonucleosides (adenosine, inosine, xanthosine and guanosine), and purine bases (adenine, hypoxanthine, xanthine, guanine and uric acid) was measured.

Perchloric acid-insoluble fractions from such cells were washed three times with cold 0.4 M perchloric acid, dissolved in 0.1 N NaOH, and portions were taken for measurement of radioactivity.

Studies of the effect of bikaverin on energy metabolism and purine nucleotide catabolism used cells containing radioactive ATP. Briefly, Ehrlich ascites tumor cells were first incubated as described above with [^{14}C]adenine. After 30 min, unused [^{14}C]adenine was removed by centrifugation and resuspension in fresh medium. Cells were then incubated under various conditions, with and without bikaverin, for an additional 30 min, and concentrations of radioactive metabolites were measured. Details are given elsewhere [17] and in the legend to Table 2.

Concentrations of phosphoribosyl pyrophosphate (P-ribose-PP) in cells incubated aerobically in Krebs–Ringer medium containing 25 mM phosphate and

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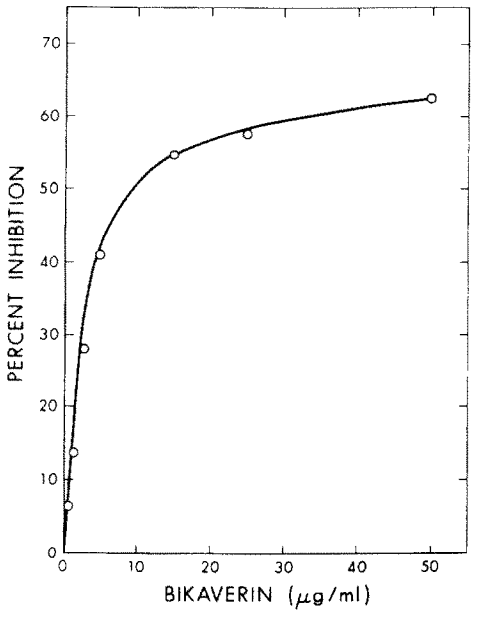

Fig. 2. Effects of bikaverin on incorporation of [^{14}C]adenine into nucleic acids. Ehrlich ascites tumor cells (2 per cent suspensions) were incubated in 100 μl of calcium-free Krebs–Ringer medium containing 25 mM phosphate and 5.5 mM glucose, with and without bikaverin, at 37° with shaking and in an air atmosphere. After 20 min, [^{14}C]adenine was added to a final concentration of 100 μM, and incubation was continued for 30 min. Radioactivity in perchloric acid-insoluble material was determined. Values reported are averages of duplicate measurements and are representative of results obtained in three experiments. Within each experiment, average deviation of individual analyses from the mean was less than 10 per cent.

5.5 mM glucose were determined as described previously [18, 19].

RESULTS

The aim of this study was to determine the mechanism by which bikaverin inhibited the incorporation of [^{14}C]adenine into nucleic acids. In order to be able to relate the present work with that previously reported [10–12], the relation between bikaverin concentration and inhibition of [^{14}C]adenine incorporation into acid-insoluble material was determined first. As shown in Fig. 2, bikaverin was a potent inhibitor of this process under the conditions used here. In other experiments (data not shown), similar dose–response curves were obtained when the incorporation of [^{14}C]guanine and [^{14}C]hypoxanthine into acid-insoluble material was measured in the presence of a range of bikaverin concentrations. These data suggested either that bikaverin affected nucleic acid synthesis itself or that it inhibited the synthesis of purine nucleoside triphosphates from radioactive purine bases.

Experiments were next conducted to determine if bikaverin inhibited the conversion of purine bases to nucleotides. Figure 3 shows that bikaverin inhibited total nucleotide synthesis from [^{14}C]adenine, [^{14}C]guanine and [^{14}C]hypoxanthine, and that the relations between degree of inhibition and bikaverin concentration were similar to that observed in Fig. 2. Nucleotide synthesis from adenine and guanine was inhibited to about the same extent, whereas nucleotide synthesis from hypoxanthine was less effectively inhibited.

The experiments of Fig. 3 also involved the measurement of radioactivity in individual purine ribonucleotides, as well as in adenosine, inosine and hypoxanthine (other purine bases and nucleosides had negligible radioactivity). Examination of the incorporation of radioactivity into individual metabolites, particularly when [^{14}C]adenine was used as precursor, indicated that bikaverin was interfering with the energy metabolism (i.e. ATP synthesis) of the cells in some way. Thus, Table 1 shows that the ratio of radioactivity in ATP to that in ADP was reduced in cells treated with bikaverin, that this ratio was inversely related to bikaverin concentration, and that the magnitude of this effect followed a dose–response relationship similar to those found in Figs. 2 and 3. An additional criterion of energy metabolism is the percentage of total nucleotide radioactivity found in adenylate plus inosinate. Normally this is *ca.* 1.5 per cent, but as shown in Table 1, it was considerably increased in cells treated with bikaverin. A further consequence of poor energy metabolism is accelerated dephosphorylation of purine nucleotides to form in-

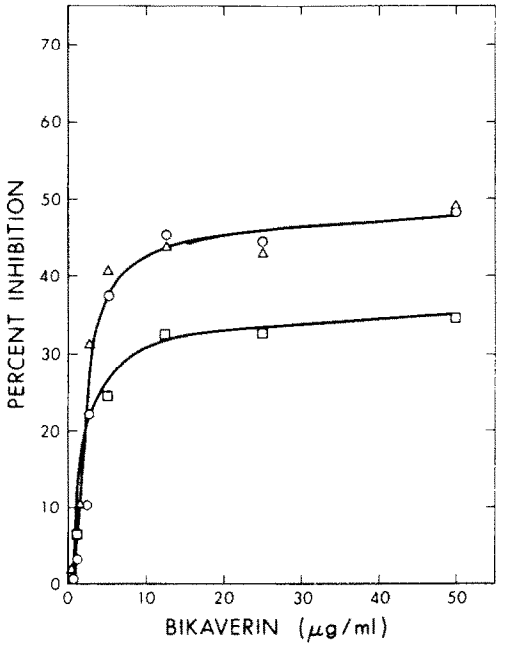

Fig. 3. Effects of bikaverin on incorporation of radioactive purines into acid-insoluble nucleotides. Ehrlich ascites tumor cells were incubated as described in Fig. 2, with and without bikaverin. After 20 min, [^{14}C]adenine (○), [^{14}C]guanine (△) or [^{14}C]hypoxanthine (□) was added to a final concentration of 100 μM, and incubation was continued for 30 min. Perchloric acid-soluble protein ribonucleotides were separated by thin-layer chromatography, the radioactivity in each was measured, and the sum of radioactivity in all acid-soluble purine nucleotides was calculated. 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 per cent.

Table 1. Effects of bikaverin on adenine nucleotide metabolism*

Bikaverin (μg/ml)	ATP / ADP	AMP + IMP (Per cent of total nucleotides)	Hypoxanthine + inosine (Per cent of total nucleotides formed)
0	9.09	1.55	1.18
0.5	9.01	1.45	2.04
1.0	8.46	1.39	2.17
2.5	7.53	5.56	5.89
5	4.99	5.83	7.84
12.5	3.22	6.52	8.10
25	2.76	6.80	9.32
50	2.29	8.84	9.74

* Ehrlich ascites tumor cells were incubated as described in Fig. 2, with and without bikaverin. After 20 min, [^{14}C]adenine was added to a final concentration of 100 μM, and incubation was continued for 30 min. Radioactivity in individual acid-soluble purine nucleotides and in adenosine, inosine and hypoxanthine was determined. 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 per cent.

osinate and hypoxanthine (adenosine is rapidly deaminated and did not accumulate in any of these experiments.) Table 1 shows that bikaverin treatment led to up to almost a 10-fold increase in purine nucleotide dephosphorylation.

Because both nucleotide synthesis and the catabolism of nucleotides that accompanies disturbances in energy metabolism [17] were proceeding simultaneously in the preceding experiments, further experiments were carried out to study the effects of bikaverin on energy metabolism more directly. Thus tumor cells were first incubated with [^{14}C]adenine and glucose in order to label the intracellular pool of ATP, and unused adenine was then removed to prevent any further synthesis of radioactive nucleotides. The cells were then incubated with and without 50 μg/ml of bikaverin, and the radioactivity in individual purine nucleotides was determined over the course of incubation for 60 min; data for both 30- and 60-min periods will be presented (except for cells incubated anaerobically without glucose, in which case ATP concentrations were almost completely depleted in 30 min). In order to assess the effects of bikaverin on different aspects of energy metabolism, cells were incubated under four conditions: (a) aerobically plus glucose, (b) aerobically without glucose, (c) anaerobically plus glucose and (d) anaerobically without glucose. It should be pointed out that, whereas in the experiments described previously cells were exposed to bikaverin for 20 min prior to addition of the radioactive precursor, in the following experiments bikaverin was added at the beginning of the period of measurement. Thus in terms of time of exposure of cells to drug, 30 min in the earlier experiments corresponds to 50 min in the experiments about to be described.

The results of these experiments are shown in Table 2. In control cells incubated aerobically plus glucose for 30 to 60 min, there was a 20 per cent decrease in radioactivity in total adenine nucleotides and in total purine nucleotides; this is believed to represent, at least in part, incorporation into nucleic acid. (It should be noted that in this particular case, data for 30 and 60 min are from separate experiments; hence separate sets of zero time values are given. In the other experiments both sets of zero time values apply to both.) These decreases were also observed in cells incubated anaerobically with glucose or aerobically without glucose, although their magnitude was slightly less in these cases; the changes were also greater at 60 min than at 30 min. Under these three sets of conditions there were increases in the concentrations of purine nucleoside monophosphates (adenylate, inosinate and xanthylate) in the control cells, but these three nucleotides did not account for a large percentage of the total radioactive purine nucleotides. Radioactivity in guanine nucleotides was relatively small and did not change appreciably in the course of these experiments.

In tumor cells incubated aerobically plus glucose, bikaverin induced further reductions in ATP (19 per cent at 30 min and 49 per cent at 60 min), total adenine nucleotides (16 per cent at 30 min and 38 per cent at 60 min), and total radioactive purine nucleotides (14 per cent at 30 min and 35 per cent at 60 min); the concentrations of adenylate, inosinate and xanthylate were increased. The loss of radioactive purine nucleotides could be almost completely accounted for by increases in radioactive inosine and hypoxanthine. Similar changes were produced by bikaverin in cells incubated anaerobically with glucose. In this case, however, concentrations of radioactive purine nucleoside monophosphates increased appreciably only after a 60-min incubation with bikaverin. In both of these situations, of course, glycolysis was the main mechanism of energy generation in these cells.

Effects of bikaverin on energy metabolism were much more apparent in cells incubated aerobically without glucose, in which case oxidative phosphorylation was the main source of ATP generation; fatty acids and other endogenous metabolites provide substrates for the tricarboxylic acid cycle. The data of Table 2 show that under these conditions, bikaverin produced large decreases in concentrations of radioactive ATP (85 per cent at 30 min and 97 per cent at 60 min), total adenine nucleotides (55 per cent at 30 min and 70 per cent at 60 min), and total radioactive purine nucleotides (34 per cent at 30 min and 44 per cent at 60 min). At the same time the concentrations of radioactive adenylate, inosinate and xanthylate increased very markedly (369, 1218 and 1483 per cent at 30 min respectively). However, the concentrations of radioactive adenylate and inosinate decreased somewhat by 60 min, whereas that of xanthylate continued to increase. Again, radioactivity in in-

Table 2. Effects of bikaverin on energy metabolism*

Incubation conditions	Bikaverin	Incubation time (min)	Radioactive metabolites (nmoles/g cells) ATP	ADP	AMP	Total adenine nucleotides	IMP	XMP	Total guanine nucleotides	Total nucleotides
Aerobic + glucose	−	0	2199	111	15	2325	3	5	31	2364
	−	30	1627	215	30	1872	8	7	33	1920
	+	30	1330	216	43	1589	28	12	25	1654
	−	0	2930	320	41	3291	6	12	34	3343
	−	60	2337	215	24	2576	9	7	30	2622
	+	60	1195	280	86	1561	73	31	35	1700
Anaerobic + glucose	−	0	2127	152	28	2307	4	4	35	2350
	−	30	1916	212	56	2184	19	7	35	2245
	+	30	1612	265	57	1934	19	10	32	1995
	−	60	1799	160	39	1998	10	8	46	2060
	+	60	1294	241	119	1654	74	22	30	1780
Aerobic − glucose	−	0	2735	248	52	3035	4	5	35	3079
	−	30	2179	333	134	2646	32	12	39	2729
	+	30	335	366	495	1196	407	178	41	1822
	−	60	1789	318	97	2204	69	16	50	2339
	+	60	54	140	465	659	261	335	63	1318
Anaerobic − glucose	−	0	2426	115	18	2559	3	6	36	2604
	−	30	447	266	311	1024	201	141	30	1396
	+	30	150	212	382	744	335	179	34	1292

* Two ml of 2% (v/v) Ehrlich ascites tumor cell suspensions 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 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. Cells were then incubated for 0, 30 and 60 min with and without 50 μg/ml of bikaverin in an atmosphere either of air or of 100% N_2, and with and without 5.5 mM glucose. For the experiments done aerobically plus glucose, data for 30 and 60 min are from separate experiments; hence separate zero time values are given. In the other experiments, both sets of data are from the same experiment and the single set of zero time values applies to both times. Values reported are averages of duplicate measurements and are representative of results obtained in at least two experiments. Within each experiment, average deviation of individual analyses from the mean was less than 7 per cent.

osine and hypoxanthine increased as a result of dephosphorylation of purine nucleoside monophosphates.

Finally, experiments were also conducted using cells incubated anaerobically in the absence of glucose. Under these conditions, neither glycolysis nor oxidative phosphorylation can support energy generation, and ATP concentrations declined considerably even in control cells as a result of its utilization for energy-requiring processes. As in all of the cases studied here and previously [17], concentrations of radioactive ADP did not increase proportionally to the extent of ATP loss; instead, radioactive purine nucleoside monophosphates were formed, and these in turn could be dephosphorylated. Thus in control cells incubated under these conditions there was an 82 per cent decrease in ATP, a 60 per cent decrease in total adenine nucleotides, and a 46 per cent decrease in total radioactive purine nucleotides. Incubation with bikaverin led to a further 44 per cent loss in ATP and 28 per cent decrease in total adenine nucleotides, but to only an 8 per cent decrease in total radioactive purine nucleotides; concentrations of radioactive inosinate and xanthylate increased considerably.

In light of all of the effects of bikaverin described above, it seemed appropriate to measure its effect on the accumulation of PP-ribose-P in tumor cells incubated with glucose. Accumulation of PP-ribose-P was the same in control cells incubated aerobically for 30 or 60 min as in cells incubated with 50 μg/ml of bikaverin.

DISCUSSION

These studies have shown that bikaverin inhibits ATP synthesis and hence energy metabolism in Ehrlich ascites tumor cells incubated *in vitro*. Although the primary studies of the effects of bikaverin on energy metabolism used a relatively high concentration of this drug, the data of Table 1 show that it affected ATP synthesis at lower concentrations as well; in fact the dose–response relationship for these effects was similar to that regarding nucleotide synthesis and incorporation into nucleic acids.

The fact that ATP synthesis was affected to a much greater extent in cells incubated aerobically without glucose than in those incubated with glucose, whether aerobically or anaerobically, suggests that bikaverin primarily inhibits mitochondiral metabolism rather than glycolysis or related reactions. In this respect bikaverin resembles 2,4-dinitrophenol more than it does 2-deoxyglucose. Bikaverin also resembles 2,4-dinitrophenol in that purine nucleoside monophosphates tend to accumulate as major products of ATP catabolism, whereas the monophosphates are mainly dephosphorylated in cells treated with 2-deoxyglucose [17]. Even though inhibition of mitochondrial energy metabolism might not be expected to cause ATP breakdown in cells incubated anaerobically with glucose, both bikaverin (see Table 2) and 2,4-dinitrophenol (J. F. Henderson and M. L. Battell, unpublished results) produce a certain amount of ATP catabolism under these conditions also.

Chemically, bikaverin is a quinone, and inhibitory effects of quinones on electron transport, oxidative phosphorylation, respiration and glycolysis are well known [20]. In each system, there are several potential sites of action, and individual quinones vary considerably in their relative potencies on the different biochemical reactions. Obviously, further studies, using isolated mitochondrial and perhaps also reconstituted glycolytic systems, are required to elucidate the exact mechanism or mechanisms by which bikaverin inhibits ATP synthesis and energy metabolism.

Although it might be expected that inhibition of energy metabolism could lead to inhibition of incorporation of purine bases into acid-soluble nucleotides and into nucleic acids, this work has not definitely established that there is a cause-and-effect relationship betwen these different effects of bikaverin. Although bikaverin did not inhibit PP-ribose-P accumulation in cells incubated aerobically with glucose, inhibition of purine nucleotide formation under similar conditions might be due to increased intracellular concentrations of purine ribonucleoside monophosphates; these compounds are potent inhibitors of the purine phosphoribosyltransferases [21, 22].

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