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INHIBITORY EFFECTS OF CORDYCEPIN ON CYCLIC NUCLEOTIDE-DEPENDENT AND CYCLIC NUCLEOTIDE-INDEPENDENT PROTEIN KINASES*

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Abstract—The *in vitro* effect of cordydepin was tested using various protein kinase preparations. These included cyclic AMP-dependent protein kinase (A-PK) from bovine heart, cyclic GMP-dependent protein kinase (G-PK) from fetal guinea pig lung, and two cyclic nucleotide-independent nuclear protein kinases (PK-I and PK-II) prepared from rat hepatoma 3924A and rat liver. The 50 per cent inhibitory concentrations (ID_{50}) of cordycepin for A-PK and G-PK ranged from $1.5–5.0 \times 10^{-4}$ M and $2.5–8.0 \times 10^{-4}$ M, respectively, depending on the presence or absence of cyclic AMP and cyclic GMP in the assay. The ID_{50} of cordycepin with either hepatoma 3924A or rat liver PK-I and PK-II was 4.5×10^{-5} M and 1.0×10^{-3} M, respectively. The inhibitory effect of cordycepin was competitive with respect to ATP in all cases. The K_m for ATP was increased 3-fold and 5-fold by 5×10^{-4} M cordycepin for G-PK and A-PK, respectively, while the K_m for ATP was increased 10-fold and 4-fold by 1×10^{-3} M cordycepin for PK-I and PK-II, respectively.

Cordycepin (3′-deoxyadenosine) has been found to be an efficacious inhibitor of RNA synthesis [1–4] and cell growth [5, 6]. Although this antibiotic has been shown to be an inhibitor of polyadenylic acid (poly(A))† synthesis [7, 8] and hence, poly(A)mRNA, cordycepin has also been documented to have an inhibitory effect on all species of nuclear [1, 2, 4, 9, 10] and cytoplasmic RNA [3, 11–13]. However, a selective and dose-dependent effect by cordycepin on nuclear RNA does appear to exist in some tumor cell systems. This has been shown by the greater sensitivities of nuclear ribosomal RNA and poly(A) synthesis than heterogeneous nuclear RNA synthesis to cordycepin [2, 3, 7, 9].

It has been hypothesized that the *in vivo* effect of cordycepin is mediated through its conversion to 3′-dATP which in turn acts as a competitive inhibitor of RNA polymerases [1, 14] or poly(A) polymerase [14] or both. This pathway for the metabolic activation of cordycepin has been based largely on the data of Klenow [15] who showed that approximately 10 percent of cordycepin is converted to 3′-dATP by Ehrlich ascites tumor cells. However, *in vitro* studies with 3′-dATP have shown: (1) no order of specificity to the inhibitory effect of 3′-dATP on various RNA polymerases and poly(A) polymerases [1, 14, 16] and (2) an inhibitory potency no greater than cordycepin itself in homologous systems [1–4, 16–18].

In view of the unique role that protein kinases may play in the regulation of cell growth and transcription [19, 20], it was felt that an examination of the *in vitro* effects of cordycepin on cyclic nucleotide-dependent protein kinases as well as on cyclic nucleotide-independent nuclear protein kinases from normal and neoplastic liver might provide an alternate explanation for the inhibitory effect of this nucleoside antibiotic on transcription.

MATERIALS AND METHODS

Materials. Cyclic AMP, cyclic GMP, casein (vitamin-free), Tris buffer, ATP and cordycepin were obtained from Sigma Chemical Co. [γ-^{32}P]ATP (16 Ci/m-mole) was purchased from New England Nuclear Corp. Arginine-rich histone (HA) was obtained from Worthington Biochemicals. All other chemicals were of reagent grade and filtered, deionized water was used.

Animals. Male Sprague–Dawley rats (ARS/Sprague–Dawley) weighing 250 g were used for preparation of protein kinases from liver nuclei. ACI/N rats (Laboratory Supply Co.) bearing intramuscularly transplanted hepatoma 3924A were obtained from Dr. Harold P. Morris, Howard University.

Preparation of protein kinases. Nuclear protein kinases were prepared from purified rat liver or hepatoma nuclei [21]. Nuclei were suspended in deionized water and stirred for 30 min at 4°. Saturated $(NH_4)_2SO_4$ (neutralized to pH 7.9 with NH_4OH) was added to a final concentration of 0.25 M and stirring was continued for 1 hr. The suspension of disrupted nuclei was sonicated for two 10-sec bursts 1 min apart at 4° using a microtip probe at full power (Heat Systems-Ultrasonics, Inc.). The sonicate was dialyzed overnight against 1 l of buffer containing: 0.025 M $(NH_4)_2SO_4$-0.05 M Tris–HCl (pH 7.9)-25% glycerol-5 mM $MgCl_2$-0.1 mM EDTA-2 mM 2-mercaptoethanol. The dialyzed preparation was centrifuged at 210,000 *g* for 30 min and the supernatant fluid chromatographed on QAE Sephadex A-25 using a linear gradient of 0.1–0.5 M $(NH_4)_2SO_4$ in the aforementioned buffer as previously described [22]. This procedure resolved

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† Abbreviations used are: G-PK, cyclic GMP-dependent protein kinase; A-PK, cyclic AMP-dependent protein kinase; PK-I, nuclear protein kinase I; PK-II, nuclear protein kinase II; poly (A), polyadenylic acid.

two main protein kinase activities, one eluting in the wash fraction (PK-I) and the other (PK-II) eluting at 0.018 M $(NH_4)_2SO_4$. The specific activities (pmoles ^{32}P transferred/5 min/mg protein) were 14,000 and 5,000 for PK-I from rat liver and hepatoma, respectively, and 135,000 and 83,000 for PK-II from rat liver and hepatoma, respectively. No phosphoprotein phosphatase was present in any of the preparations. Details of this procedure will be reported elsewhere (R. I. Glazer, manuscript in preparation).

Cyclic GMP-dependent protein kinase was purified from guinea pig fetal lungs through the step of Sephadex G-200 gel filtration [23], and cyclic AMP-dependent protein kinase was purified from bovine hearts through the step of DEAE-cellulose chromatography [24]. The stimulatory modulator of cyclic GMP-dependent protein kinase, which is free from the inhibitory modulator of the cyclic AMP-dependent enzyme, was prepared through the step of DEAE-cellulose chromatography [25].

Protein kinase assays. Nuclear PK-I and PK-II from rat liver or hepatoma 3924A were assayed at 37° for 5 min in a mixture (0.2 ml) containing: 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 20 mM magnesium acetate, 0.5 μCi [γ-^{32}P]ATP (0.25 Ci/m-mole), and 300 μg of casein. Acid insoluble radioactivity was measured on glass fiber filtter discs as previously described [22]. One unit of nuclear protein kinase activity is defined as the amount of enzyme that transferred 1 pmole of ^{32}P from [γ-^{32}P]ATP to recovered casein in 5 min at 37° under the assay conditions.

Cyclic GMP-dependent protein kinase was assayed in the presence of 40 μg stimulatory modulator as described recently [25]. The cyclic AMP-dependent enzyme was assayed under the condition reported earlier [24]. Arginine-rich histone was the substrate in both cases. One unit of the cyclic nucleotide-dependent protein kinase activity is defined as that amount of enzyme that transferred 1 pmole of ^{32}P from [γ-^{32}P]ATP to recovered histone in 10 min at 30° under the assay conditions.

RESULTS

The effect of varying concentrations of cordycepin on A-PK and G-PK are presented in Table 1. A dose-dependent decrease occurred in either basal or cyclic nucleotide-dependent activity for both classes of protein kinases. There was a significant reduction in the inhibitory concentration of cordycepin when A-PK and G-PK were assayed in the presence of both cyclic AMP and cyclic GMP.

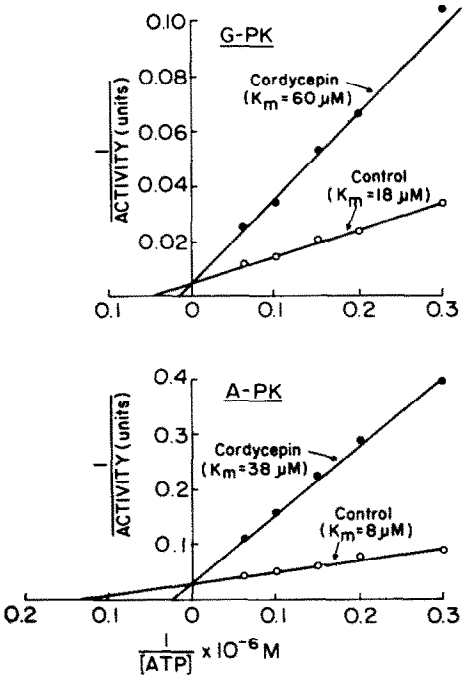

Fig. 1. Kinetics of inhibition by cordycepin of cyclic GMP-dependent and cyclic AMP-dependent protein kinases. The cyclic GMP enzyme (4.1 μg) was assayed in the presence of 4×10^{-7} M cGMP, whereas the cyclic AMP enzyme (3.5 μg) was assayed in the presence of 4×10^{-7} M cAMP. The concentration of cordycepin was 5×10^{-4} M.

The inhibitory effect of cordycepin on A-PK and G-PK in the presence of cyclic AMP and cyclic GMP, respectively, was competitive with respect to ATP (Fig. 1). The K_m for ATP was increased 3- to 5-fold in the presence of 5×10^{-4} M cordycepin.

Two predominant protein kinase activities were resolved on QAE Sephadex from nuclear extracts of rat liver or hepatoma 3924A. Assays of their activities as a function of the concentration of cordycepin is shown in Fig. 2. PK-I, from either rat liver or hepatoma 3924A was inhibited 50 percent by approxi-

Table 1. Dose-dependent inhibition by cordycepin of cyclic GMP-dependent and cyclic AMP-dependent protein kinases*

	Protein kinase activity (10^2 units/mg protein)					
	Cyclic GMP-dependent			Cyclic AMP-dependent		
Cordycepin (M)	Basal	Cyclic GMP	Cyclic AMP	Basal	Cyclic GMP	Cyclic AMP
None (control)	17.1(100)	125.6(100)	24.9(100)	8.6(100)	16.0(100)	57.7(100)
1×10^{-6}	17.3(101)	119.8 (95)	23.9 (96)	8.6(100)	15.4 (96)	56.0 (97)
1×10^{-5}	17.1(100)	106.8 (85)	21.0 (84)	8.3 (97)	10.9 (68)	51.7 (90)
1×10^{-4}	16.6 (97)	83.9 (67)	19.5 (78)	8.0 (93)	9.1 (57)	40.0 (69)
1×10^{-3}	7.8 (46)	28.3 (23)	8.8 (35)	2.9 (34)	3.4 (21)	5.4 (9)
2×10^{-3}	5.4 (32)	17.3 (14)	6.8 (27)	2.3 (27)	2.9 (18)	3.4 (6)

* The cyclic GMP enzyme (4.1 μg) and the cyclic AMP enzyme (3.5 μg) were incubated in the presence and absence of 0.4×10^{-6} M of either cyclic nucleotide. The values shown in parentheses indicate the percent of respective controls.

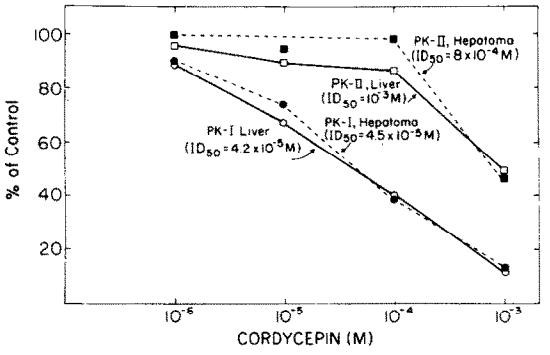

Fig. 2. Dose–response of inhibition by cordycepin two nuclear protein kinases from hepatoma 3924A and rat liver. The concentration of PK-I from rat liver or hepatoma 3924A was 5 μg and 20 μg. respectively. The concentration of PK-II from rat liver of hepatoma 3924A was 5 μg and 0.4 μg. respectively.

mately 4×10^{-5} M cordycepin. In contrast. PK-II from both tissues was inhibited 50 per cent by a 20-fold higher concentration of cordycepin.

The kinetics of cordycepin inhibition of rat liver and hepatoma PK-I and PK-II showed competitive inhibition (Fig. 3). The K_m for ATP was increased 10-fold for PK-I and 4-fold for PK-II.

DISCUSSION

The present study has shown that cordycepin is a competitive inhibitor of all protein kinases tested, and in particular of one form of protein kinase found in the nucleus of normal and neoplastic liver. The concentration of cordycepin required to suppress phosphorylation of mixed histone by A-PK and G-PK or of casein by PK-I and PK-II is within the range found to be cytotoxic to normal and tumor cells in culture [5, 6]. In the study by Plunkett and Cohen [5]. 1×10^{-4} M cordycepin inhibited the growth of L cells by 50 per cent, and using L1210 cells Johns and Adamson [6] found that 4×10^{-5} M inhibited cell growth by about 50 per cent. Similarly, the synthesis of total RNA, 45S ribosomal precursor RNA and tRNA in cultures of Novikoff hepatoma cells were inhibited 50 per cent by 1×10^{-4} M cordycepin [1, 26]. The synthesis of nuclear and ribosomal RNA was reduced by 25–50 per cent and 80–100 per cent, respectively at 1×10^{-5} and 1×10^{-4} M cordycepin [27]. A recent study by Puvion *et al.* [28] has also shown approximately 60 per cent inhibition of nucleolar and nuclear RNA in isolated liver cells by 1×10^{-4} M cordycepin.

Collectively. these data suggest that cordycepin itself may be an effective inhibitor of processes associated with transcription. Since the concentrations of 3′-dATP required to inhibit RNA polymerases or poly(A)polymerase are equivalent to the amount of cordycepin required to inhibit RNA synthesis, it is unlikely that 3′-dATP is the actual intracellular inhibitor *in vivo*. In support of this contention is the report by Klenow [15] showing that in Ehrlich ascites tumor cells. less than 10 per cent of the cordycepin is converted to 3′-dATP in 3 hr. Moreover. it is known that cordycepin is an effective inhibitor within a few min [4. 13] a time period wherein little 3′-dATP would be expected to form. Since it has recently been demonstrated that nuclear protein kinases may stimulate transcription by RNA polymerases [29, 30] and that phosphorylation of nonhistone chromosomal proteins may increase the gene transcription of histone mRNA [31], it remains a possibility that cordycepin may function *in vivo* by altering the pattern of phosphorylation of chromatin. Since cordycepin also inhibits both A-PK and G-PK purified from tissue extracts. it is conceivable that the antibiotic may affect cytoplasmic constituents as well.

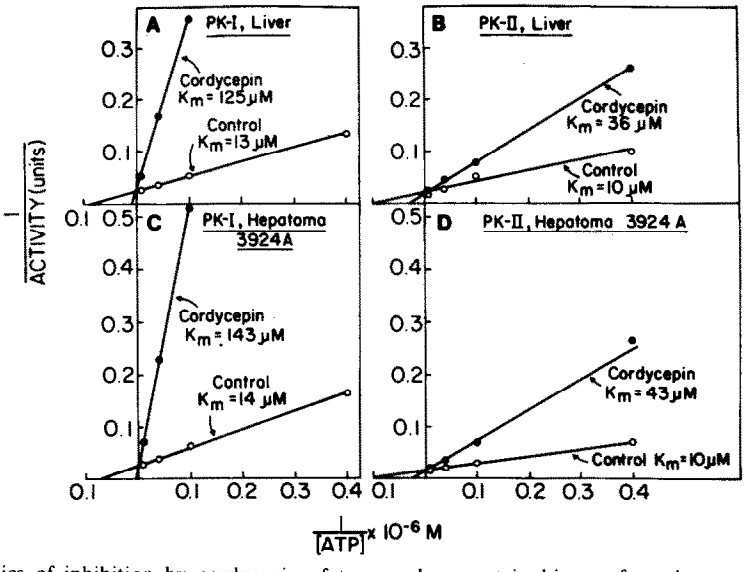

Fig. 3. Kinetics of inhibition by cordycepin of two nuclear protein kinases from hepatoma 3924A and rat liver. The assay conditions were as described in Fig. 2. The concentration of cordycepin was 1×10^{-3} M.

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