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ACTIVATION OF CYCLIC AMP-DEPENDENT PROTEIN KINASES I AND II BY CYCLIC 3',5'-PHOSPHATES OF 9-β-D-RIBOFURANOSYLPURINE AND 1-β-D-RIBOFURANOSYLBENZIMIDAZOLE

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SUMMARY

Analogs of cyclic AMP lacking the 6-amino group--9-β-D-ribofuranosylpurine cyclic 3',5'-phosphate (I)--or the 1- and 3-nitrogens as well as the 6-amino group--1-β-D-ribofuranosylbenzimidazole cyclic 3',5'-phosphate (II)--were effective activators of both type I (cAKI) and type II (cAKII) isozymes of cAMP-dependent protein kinase. An analog with a pyrimidine ring fused to the benzimidazole ring system of II--3-β-D-ribofuranosyl-8-aminoimidazo[4,5-*g*]-quinazoline cyclic 3',5'-phosphate (III)--was equipotent to I or II as an activator of cAKII but only 1/10 as potent as I or II as an activator of cAKII. The results show that neither cAKI nor cAKII requires the 6-amino group and that they may have different sensitivities to the effects of alterations in the electron distribution in the pyrimidine ring.

INTRODUCTION

The two isozymic forms of cAK,[2] cAKI and cAKII, differ significantly in the portions of their respective cAMP binding locales that are adjacent to the 1-, 2-, 3-, and 6-positions of cAMP (1-3). The lone pair electrons of the nitrogen in the 3-position of the adenine ring appear to be involved in cAMP binding to R_{II} (4) but apparently are not involved in cAMP binding to R_I (1,5). An analog of cAMP that lacks a nitrogen in the position analogous to the 3-position in cAMP--*lin*-benzo-cAMP (see Figure 1 for structures)--was nearly as active as cAMP as an activator of cAKII (6).

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2. Abbreviations: cAMP, adenosine cyclic 3',5'-phosphate; cIMP, inosine cyclic 3',5'-phosphate; cNMP, 9-β-ribofuranosylpurine cyclic 3',5'-phosphate; cRBMP, 1-β-D-ribofuranosylbenzimidazole cyclic 3',5'-phosphate; *lin*-benzo-cAMP, 3-β-D-ribofuranosyl-8-aminoimidazo[4,5-*g*]-quinazoline cyclic 3',5'-phosphate; cAK, cAMP-dependent protein kinase; cAKI and cAKII, the type I iszoyme and type II isozyme, respectively, of cAK; R_I and R_{II}, the regulatory subunits of cAKI and cAKII, respectively.

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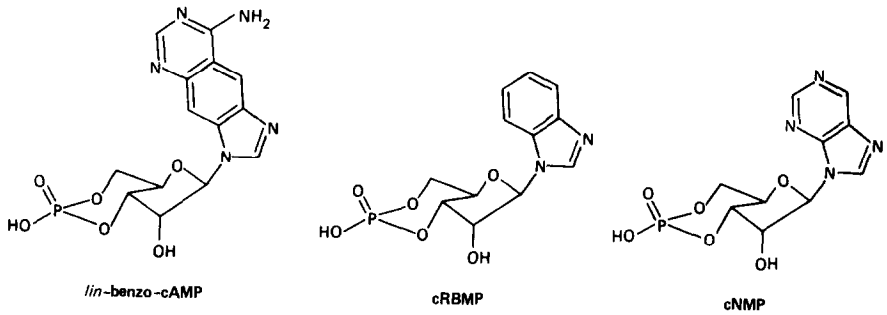

Figure 1

In an effort to understand why *lin*-benzo-cAMP is capable of activating cAKII and to further our knowledge of the similarities and differences in the cAMP binding sites on cAKI and cAKII, we have synthesized a cAMP analog--cRBMP (see Figure 1)--that contains only the benzimidazole portion of the imidazo[4,5-*g*]quinazoline ring system of *lin*-benzo-cAMP. Since this analog lacks an amino group in the position analogous to the 6-position of cAMP, we have also synthesized a derivative--cNMP (see Figure 1)--that lacks the 6-amino group but that contains the 1- and 3-nitrogens of cAMP.

The new cAMP analogs were compared with *lin*-benzo-cAMP for their relative abilities to activate cAKI from rabbit and porcine skeletal muscle and cAKII from bovine brain and cardiac muscle.

MATERIALS AND METHODS

The *lin*-benzo-cAMP was prepared from 3-β-D-ribofuranosyl-8-amino-imidazo[4,5-*g*]quinazoline (6,7) by the procedure of Schmidt et al. (6). A modification of the method of Yoshikawa et al. (8) provided the 5'-phosphates of nebularine and 1-β-D-ribofuranosylbenzimidazole in 70% and 55% yields, respectively, which were converted to their cyclic 3',5'-phosphates as described below. The 5'- and cyclic 3',5'-phosphates were homogeneous, as judged by chromatography on Merck cellulose F_{254} plates in four different solvent systems: A, isopropanol:H_2O:HCOOH (7:2:1, v/v); B, isopropanol:H_2O: conc. NH_4OH (7:2:1, v/v); C, H_2O saturated n-butanol; and D, n-butanol, H_2O, CH_3COOH (2:1:1, v/v).

Nebularine-3',5'-cyclic phosphate (cNMP). The 5'-phosphate of nebularine (0.8 mmole) was dried by evaporation from anhydrous pyridine and dissolved in 80 ml of pyridine. This solution was added, dropwise over a period of 30 min, to a refluxing solution of 600 mg (3 mmoles) of dicyclohexylcarbodiimide in 100 ml of anhydrous pyridine. The mixture was refluxed an additional 30 min and then left overnight at room temperature. Water (10 ml) was added and the mixture was brought to dryness under reduced pressure. This procedure was repeated several times to remove traces of pyridine. The residue was taken

up in 200 ml of water, brought to pH 7 with NH_4OH, filtered, and loaded unto a 200/400 mesh 25 × 1.5 cm column of Dowex 1X2(HCO_3^-), which was eluted with a linear gradient of 0-1 M NH_4HCO_3. The desired product was found in the fractions eluting between 0.3 and 0.4 M NH_4HCO_3, which were pooled and brought to dryness. The residue was dried by evaporation from aqueous methanol. The product was dissolved in the minimal volume of water, precipitated with ethanolic ether as the ammonium salt, and stored *in vacuo* over P_2O_5. UV spectra: pH 7, λ_{max} 263 nm (ε_{max} = 6.9 × 10^3); pH 1, λ_{max} 262 nm (ε_{max} = 5.9 × 10^3). Chromatography of cNMP in solvent systems A, B, C, and D gave R_f values of 0.43, 0.57, 0.14, and 0.41, respectively (nebularine 5'-phosphate gave R_f values of 0.31, 0.17, 0.05, and 0.36, respectively).

1-β-D-Ribofuranosylbenzimidazole-3',5'-cyclic phosphate (cRBMP). The 5'-phosphate (1.0 mmole) of 1-β-D-ribofuranosylbenzimidazole was converted to the cyclic phosphate and isolated as described for cNMP except that the column was eluted with 0.05 M HCOOH. The fractions containing the product (between 300 and 400 ml) were pooled and dried under reduced pressure (at <40°). The product crystallized as the free acid from 50% aqueous ethanol in the form of rough prisms. UV spectra: pH 7, λ_{max} 248 nm (ε_{max} = 6.4 × 10^3), λ_{max} 273 nm (ε_{max} = 3.6 × 10^3), λ_{max} 281 nm (ε_{max} = 3.0 × 10^3); pH 1, λ_{max} 269 nm (ε_{max} = 6.4 × 10^3), λ_{max} 276 nm (ε_{max} = 5.4 × 10^3). Chromatography of cRBMP in solvent systems A, B, C, and D gave R_f values of 0.60, 0.81, 0.25, 0.61, respectively (1-β-D-ribofuranosylbenzimidazole 5'-phosphate gave R_f values of 0.31, 0.17, 0.05, and 0.36, respectively).

RESULTS AND DISCUSSION

Each of the analogs was examined for its ability to activate cAKI from rabbit and porcine muscle and cAKII from bovine brain and heart. The results are shown in Table 1. Since K_a values are a function of enzyme concentration (15,16), all K_a values for cAMP and the analogs were determined at the same concentration (43 nM) of cAKI or cAKII. Under the assay conditions, the K_a values for cAMP with all four protein kinases were comparable, ranging from 47 to 72 nM. Therefore, the K_a values were compared directly without further normalization of the data.

Analogs with ring systems made up of an imidazole ring annellated to either a pyrimidine ring or a benzene ring are analogs of adenine that lack either the 6-amino group (cNMP) or the 1- and 3-nitrogens as well as the 6-amino group (cRBMP). Both cNMP and cRBMP were approximately 1/4 as potent as cAMP with the two cAKI isozymes and approximately 1/2 as potent as cAMP with the two cAKII isozymes. Clearly, neither isozyme requires the 6-amino group to be activated by cAMP. This conclusion is consistent with our previous reports that 6-substituted derivatives of cNMP and N^6-substituted derivatives

Table 1

Activation of cAMP-Dependent Protein Kinases by *lin*-benzo-cAMP, cRBMP, and cNMP[a]

Cyclic Nucleotide	Protein Kinase Activation,[b] K_a' cAKI Rabbit Muscle	cAKI Porcine Muscle	cAKII Bovine Brain	cAKII Bovine Heart	Protein Kinase Specificity,[c] $\frac{K_a'\ (cAKI)}{K_a'\ (cAKII)}$
cAMP	1.0	1.0	1.0	1.0	1.0
lin-benzo-cAMP	0.011	0.020	0.42	0.59	0.031
cRBMP	0.25	0.24	0.57	0.70	0.39
cNMP	0.22	0.27	0.40	0.63	0.48

[a] Previously described methods of others were used to purify rabbit skeletal muscle cAKI (9), porcine skeletal muscle cAKI (10), bovine brain cAKII (11), and bovine heart cAKII (12). The protein kinase assays were performed using the paper disk method previously described (13). The assay for the kinase contained, in 0.1 ml: 5 µmol of sodium acetate (pH 6.0), 1 µmol of $MgCl_2$, 100 µg of calf thymus histone (Worthington, grade HLY), 0.5 nmol of γ-[^{32}P]-ATP (150,000 cpm), 4.3 pmol of protein kinase holoenzyme, and various concentrations of the cyclic nucleotide being tested as an activator (10^{-9}-10^{-3} M). The concentration of the holoenzyme was based on the cAMP- or cGMP-binding capacity of each enzyme preparation (14). The catalytic activity of the kinase was measured in the presence of at least seven different concentrations of the cyclic nucleotide being tested as an activator, varied over at least a 100-fold concentration range. The amount of product formed was determined at three or more time points (5-20 min) to ensure that linear rates were being measured. The K_a for each analog was determined from the x-intercept (calculated from linear regression analysis, correlation coefficients ≥0.990) of a line described by a double reciprocal plot of the above data (picomoles of phosphate transferred to histone)$^{-1}$ vs [cyclic nucleotide analogs]$^{-1}$. With each protein kinase, at least three separate K_a determinations were made that yielded values within 15% of the reported value.

[b] The potency of each cyclic nucleotide as an activator of cAK was expressed as a K_a' value, where K_a' = [(apparent K_a for cAMP)/(apparent K_a for the analog)], and where the K_a values for cAMP are 47 nM, 72 nM, 60 nM, and 57 nM for the rabbit muscle cAKI, porcine muscle cAKI, bovine brain cAKII, and bovine heart cAKII, respectively.

[c] The protein kinase specificity is expressed as [K_a'(cAKI)]/[K_a'(cAKII)] = [(K_a' for rabbit muscle cAKI) + (K_a' for porcine muscle cAKI)]/[(K_a' for bovine heart cAKII) + (K_a' for bovine brain cAKII)].

of cAMP are approximately equipotent to cAMP as activators of bovine brain cAKII (17-19).

The results with cRBMP also indicate that neither isozyme requires the 1- and 3-nitrogens to be activated by cAMP. We have shown previously that 1-deaza-cAMP was equal in activity to cAMP as an activator of cAKII from either bovine brain or cardiac muscle, but that 3-deaza-cAMP was only approximately 1/20 as

potent as an activator of these cAKII isozymes (4). Based on these earlier data, we had suggested that the lone pair electrons of the nitrogen in the 3-position of cAMP are involved in the interaction of cAMP with cAKII isozymes (4), but the results with cRBMP show that the weak activity of 3-deaza-cAMP as a cAKII activator is due to factors other than or in addition to the absence of the 3-nitrogen from the purine ring.

The *lin*-benzo-cAMP contains a pyrimidine ring fused to the benzimidazole ring system of cRBMP. Although *lin*-benzo-cAMP was equipotent to both cRBMP and cNMP as an activator of cAKII, it was only 1/10 as potent as either cRBMP or cNMP as an activator of cAKI. If *lin*-benzo-cAMP is viewed as a 5,6-disubstituted derivative of cRBMP, then the N-5 of *lin*-benzo-cAMP is analogous to the α-atom of a 2-substituent on cAMP. We have shown previously that cAKI has stricter steric requirements than does cAKII for the binding locale on the protein kinases adjacent to the 2-position of cAMP. This is exemplified by 2-substituted derivatives such as 2-$H_3C(H_2C)_3$-cAMP and 2-$H_5C_6HC{=}HC$-cAMP, which are 10- to 20-fold more potent activators of cAKII than of cAKI (1). Therefore, the 30-fold greater potency of *lin*-benzo-cAMP as an activator of cAKII than of cAKI may be due to its being analogous to a 1,2-disubstituted cAMP. The pyrimidine ring of *lin*-benzo-cAMP apparently is a steric hindrance to the binding of this analog to R_I, but does not significantly affect binding to R_{II}.

Jastorff et al. (20) has suggested that the binding of the adenine moiety of cAMP to cAKI may involve dipole-induced dipole interactions. This is consistent with our finding that an intact pyrimidine ring is vital for activation of both isozymes (3,21,22). Furthermore, the electron distribution in the pyrimidine ring as well as its aromaticity appear to be important for the binding of cAMP to R_I and R_{II} (5), since cyclic AMP derivatives with electron-withdrawing 2-substituents are more potent than cAMP as activators of cAKI and less potent than cAMP as activators of cAKII (1). Such substituents would produce a reduction in the electron density and an alteration in the

electron distribution in the pyrimidine ring. Therefore, certain electron distributions may be much more favorable to the binding of an analog to R_I, whereas other electron distributions may be much more favorable to the binding of an analog to R_{II}. The pyridine ring of 3-deaza-cAMP, the benzene ring of cRBMP, and the pyrimidine ring of cAMP all have different electron distributions (4,23). The poor ability of 3-deaza-cAMP to activate cAKII may be due to an electron distribution in its pyridine ring that is unfavorable for its interaction with the cAMP binding site on R_{II}. Likewise, the electron distribution in the benzene ring of cRBMP may be acceptable for binding to R_{II}.

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