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MAPPING CYCLIC AMP BINDING SITES ON TYPE I AND TYPE II CYCLIC AMP-DEPENDENT PROTEIN KINASES USING 2-SUBSTITUTED DERIVATIVES OF CYCLIC AMP

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SUMMARY

Twenty-one derivatives of cyclic AMP with substituents or modification in the 2-position were examined for their ability to activate rabbit skeletal muscle type I cyclic AMP-dependent protein kinase (PK I) and bovine heart type II cyclic AMP- dependent protein kinase (PK II). PK I had stricter steric requirements than did PK II for the binding locale on the protein kinases adjacent to the 2 position of cyclic AMP. Derivatives with substituents that caused electron withdrawal from the purine ring were better than cyclic AMP as activators of PK I, but were less active than cyclic AMP as activators of PK II.

INTRODUCTION

Many derivatives of cAMP[1] can efficiently activate various cAMP-dependent protein kinases (1-3). Most of the published studies on the activation of protein kinases by cAMP derivatives were completed before it became generally recognized that there are two isozymic forms of cAMP-dependent protein kinase, referred to as type I and type II (4,5). There have been no systematic studies comparing the relative abilities of various cAMP derivatives to activate well-characterized type I and type II cAMP-dependent protein kinases, but a few reports on a small number of cAMP derivatives have appeared (6,7).

The differences between PK I and PK II appear to be caused principally by differences in their regulatory subunits (5,8). If these differences

1. Abbreviations: cAMP and 2-aza-cAMP refer to adenosine cyclic 3',5'-phosphate and 7-amino-4-β-D-ribofuranosylimidazo[4,5-d]-1,2,3-triazine cyclic 3',5'-phosphate, respectively. The cAMP derivatives with substituents in the 2-position are designated by the chemical formula of the substituent group in the 2-position (e.g., 2-H_3C-cAMP refers to 2-methyladenosine cyclic 3',5'-phosphate). PK I and PK II refer to the type I isozyme and the type II isozyme, respectively, of cAMP-dependent protein kinase.

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between the regulatory subunits of the two isozymes extend to the respective cAMP binding sites on these proteins, then certain derivatives of cAMP may be more potent activators of PK I than of PK II, and vice versa. Since we previously demonstrated a quantitative relationship between the relative potency of a number of 2-substituted cAMP derivatives as activators of bovine brain PK II and parameters describing the hydrophobic, steric, and electronic character of the substituents on these compounds (9), we decided to compare the abilities of a number of 2-substituted derivatives of cAMP to activate the well-characterized (5) rabbit muscle PK I and bovine heart PK II.

MATERIALS AND METHODS

Preparation and assay of protein kinases. The rabbit skeletal muscle PK I and the bovine heart PK II were purified as described by Hoffmann et al. (5). Protein kinase assays were performed using the paper disk method (10,11). The assay mixture (0.1 ml) contained 5 µmol of sodium acetate, pH 6.0; 1 µmol of $MgCl_2$; 15 µg of histone (Worthington HLY); 0.5 nmol of γ-[^{32}P]-ATP (150,000 cpm); 3.7 pmol of protein kinase holoenzyme, and one of various concentrations of the cAMP analog being tested. The concentration of holoenzyme used was based on the cAMP binding capacity of each enzyme preparation (10).

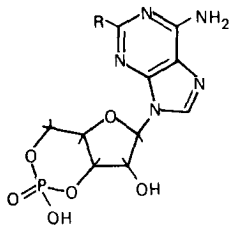
The catalytic activity of the kinase was measured in the presence of at least seven different concentrations of the cAMP analog being tested varied over at least a 100-fold range. The incubation time (3 to 12 min) was determined from pilot experiments to ensure that the data were kinetically valid. The K_a for each analog was determined from the x-intercept (calculated from linear regression analysis) of a line described by a double reciprocal plot of (pmol of phosphate transferred to histone)$^{-1}$ versus (concentration of cyclic nucleotide analog)$^{-1}$ (11). At least three separate K_a determinations were made for each analog with each protein kinase, and the K_a values were considered adequately precise when the results of at least three experiments yielded values within 10% of each other. The K_a' value--the ratio of the apparent K_a for cAMP to the apparent K_a for the analog--was calculated for each analog using cAMP K_a values of 47 nM for rabbit skeletal muscle PK I and 57 nM for bovine heart PK II.

Cyclic AMP derivatives. The 2-substituted cAMP derivatives and 2-aza-cAMP were synthesized as previously described (9,12,13). The structures are shown in Table 1.

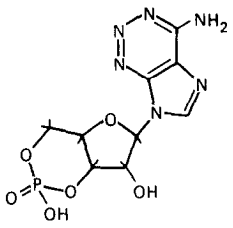
Computations. Multiple regression analysis was accomplished with a stepwise regression program adapted for the NIH PROPHET computer system [a specialized computer resource developed by the Chemical/Biological Information-Handling Program, Division of Research Resources, National Institutes of Health, described in detail elsewhere (14)]. The constants for π, the Hansch hydrophobic parameter; E_s, the Taft steric parameter; $\mathcal{F}$, the electron inductive parameter; and $\mathcal{R}$, the electron resonance parameter were taken from our previous publication (9). The constants for molar refractivity, MR, were taken from Hansch et al. (15).

TABLE 1

Activation of PK I and PK II by 2-Substituted Derivatives of cAMP[a]



2-Substituted-cAMP



2-Aza-cAMP

Compound Number	R	K_a' Rabbit Muscle PK I	K_a' Bovine Heart PK II	K_a' (PK I) / K_a' (PK II)
1	CH_3	0.20	0.15	1.3
2	C_2H_5	0.080	0.21	0.38
3	nC_3H_7	0.047	0.32	0.15
4	nC_4H_9	0.019	0.43	0.044
5	nC_6H_{13}	0.43	0.85	0.51
6	nC_8H_{17}	4.7	1.4	3.4
7	$nC_{10}H_{21}$	0.51	0.23	2.2
8	iC_4H_9	0.034	0.047	0.72
9	CF_3	4.8	0.29	17.
10	$CH_2CH_2C_6H_5$	0.39	0.47	0.83
11	$CH{=}CHC_6H_5$	0.14	1.9	0.074
12	2-thienyl	0.075	0.19	0.39
13	C_6H_5	0.017	0.020	0.85
14	$S{-}CH_3$	0.020	0.20	0.10
15	$S{-}C_2H_5$	0.030	0.20	0.15
16	$S{-}nC_3H_7$	0.034	0.30	0.11
17	$S{-}CH_2C_6H_5$	0.78	0.58	1.3
18	Cl	3.7	0.33	11.
19	NH_2	0.31	0.18	1.7
20	$N(CH_3)_2$	0.018	0.060	0.30
21	"2-aza"[b]	0.47	0.062	7.6

[a] The protein kinase assays were performed and the K_a' values determined as described in MATERIALS AND METHODS.

[b] "2-Aza" refers to 2-aza-cAMP.

RESULTS AND DISCUSSION

The ability of 2-substituted cAMP derivative to activate rabbit skeletal muscle PK I and bovine heart PK II was examined. Because K_a values are a function of enzyme concentration (16,17), all K_a values for cAMP and the

derivatives were determined at the same concentration of type I and type II holoenzyme (37 n*M*). The results are given in Table 1.

From multiple regression analysis, we previously demonstrated a striking relationship between the relative potency of a number of 2-substituted cAMP derivatives as activators of bovine brain PK II (18) and parameters describing the hydrophobic, steric, and electronic character of the substituents on these compounds (9). This analysis yielded EQ. 1:

$$\text{Log } (K_a') = -0.72(\pm 0.21) + 0.39(\pm 0.11)\pi + 0.63(\pm 0.13)E_s + 0.65(\pm 0.60)\mathfrak{F} + 2.6(\pm 1.1)\mathfrak{R} \quad (1)$$

$$n = 14,\ r = 0.97,\ s = 0.15$$

where n is the number of data points, r is the coefficient of multiple correlation, and s is the standard deviation. The numbers in parentheses are the 95% confidence intervals. Multiple regression analysis of the data presented here on bovine heart PK II on the 14 compounds used for EQ. 1 (cAMP, *(1-6, 8, 9, 13-16, 18)* yielded EQ. 2:

$$\text{Log } (K_a') = -0.74(\pm 0.15) + 0.36(\pm 0.053)\pi + 0.62(\pm 0.06)E_s + 0.53(\pm 0.29)\mathfrak{F} + 2.2(\pm 0.49)\mathfrak{R}$$

$$n = 14,\ r = 0.96,\ s = 0.16$$

EQ. 1 and 2 are comparable, and, within the limits of the set of compounds examined, bovine brain PK II and bovine heart PK II are quite similar in the portion of the cAMP binding site near the 2-position of cAMP. The primary limitations of these equations are the limited variation among the substituents relative to constants for E_s and $\mathfrak{R}$, and the presence of constants for only lipophilic substituents in the data set.

The other cAMP derivatives were not included in the regression analysis,[2] but their K_a' values reported here for bovine heart PK II are quite similar to those reported previously for bovine brain PK II (9,13,18).

2. Compounds *10-12, 17, 19,* and *20* were not included because their substituent constants were unknown or difficult to estimate, and 2-$n$$H_{21}C_{10}$-cAMP [*7*] was not included because its anomalously low K_a' value probably is caused by coiling of the sidechain or aggregation in aqueous solution (9). Inclusion of 2-aza-cAMP [*21*] was not appropriate in an analysis of substituents.

We do not yet know if all PK IIs have similar cAMP binding sites, but data from our laboratory (M. Scholten, A. Beck, and J. P. Miller, unpublished results) on the activation of type II isozymes from porcine skeletal muscle and rat liver by 2-substituted cAMP derivatives suggest that all PK IIs are similar in the portion of the cAMP binding site near the 2-position of cAMP. In addition, the type II isozymes from bovine brain, bovine heart, and rat liver are remarkably similar in their requirement for the lone-pair electrons of the nitrogen in the 3-position (18).

Multiple regression analysis of the data in Table 1 for rabbit skeletal muscle PK I, with the values for π, E_S, $\mathfrak{F}$, and $\mathfrak{R}$ used above, revealed that the type I activity cannot be described by the parameters used to describe the type II activity with this set of 2-substituted cAMP derivatives. The relationship between K_a' and either E_S or π is not linear for PK I, as it is for PK II. This difference between the two isozymes is illustrated best by examining the relationship between the K_a' values and molar refractivity for the straight-chain 2-alkyl- and 2-alkylthio-cAMP derivatives (Figure 1). PK II exhibits an initial loss in binding from the introduction of a 2-CH_3 or a 2-C_2H_5 on cAMP that was overcome gradually as additional hydrophobic binding became available with longer chain substitutents. PK I demonstrates stricter steric requirements; increasing substituent size from 2-CH_3 to 2-$\underline{n}C_4H_9$, or from 2-SCH_3 to 2-S-$\underline{n}C_3H_7$, resulted in a progressive loss in binding, but longer-chain substituents, 2-$\underline{n}C_6H_{13}$ and 2-$\underline{n}C_8H_{17}$, overcame the steric hindrance to binding, presumably by hydrophobic interaction with the kinase. It appears as if there is significantly more steric interference to binding for PK I than there is for PK II, but that hydrophobic binding by the longer-chain substituents overcame the negative steric effects for both isozymes.

Rabbit muscle PK I, like bovine heart PK II and bovine brain PK II (9), was activated only poorly by derivatives with branching in the first or second atom of the side chain (e.g., compounds *8*, *12*, *13*, and *20*). The potency of

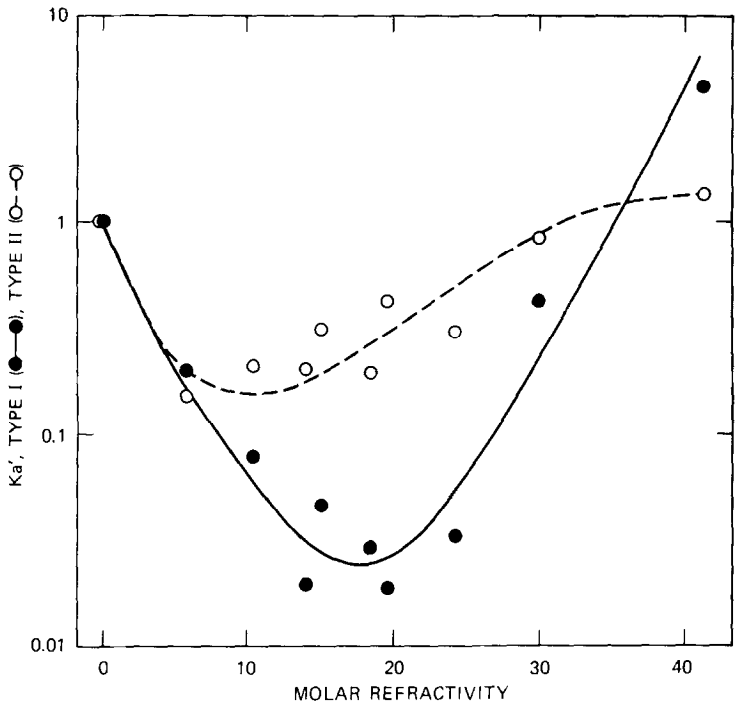


FIGURE 1. Relationship between K_a' values and molar refractivity for type I and type II cAMP-dependent protein kinases. The K_a' values were taken from Table 1. The 2-alkyl- and 2-alkylthio-cAMP derivatives used in this analysis and their respective MR values are as follows: cAMP, 0; *1*, 5.65; *2*, 10.3; *14*, 13.8; *3*, 15.0; *15*, 18.4; *4*, 19.6; *16*, 24.2; *5*, 29.9; and *6*, 41.2.

derivatives with branching at the third atom of the side chain depended on the nature of the first two atoms of the side chain: 2-$H_5C_6H_2CH_2C$-cAMP [*10*] and 2-$H_5C_6H_2CS$-cAMP [*17*] had comparable K_a' values with the two kinases, but 2-$H_5C_6HC{=}HC$-cAMP [*11*], in which the first two atoms of the side chain are more sterically restricted than in *10* or *17*, is 10 times more potent as an activator of PK II than it is of PK I. This observation is consistent with the greater steric restrictions to binding to PK I than to PK II illustrated in Figure 1. The more potent activation of PK II than of PK I by *11* could also result from an interaction between the π electrons of the -C=C-moiety of the 2-$CH{=}CHC_6H_5$ substituent and the PK II.

Another major difference between the two isozymes is the relationship of the K_a' values to electronic effects exerted by substituents in the 2-position. 2-Cl-cAMP [*18*] and 2-F_3C-cAMP [*9*], both of which exert relatively

strong electron-withdrawing effects on the purine ring, were more potent activators of PK I than of PK II. The data suggest that electron withdrawal from the purine ring improved binding to PK I but was detrimental to binding to PK II. Although only two of the derivatives exhibited strong electron-withdrawing effects, the results are consistent with our previous observation that the lone-pair electrons of N-3 are involved in the binding of cAMP to type II protein kinases (18) and with the considerably greater ability of 2-aza-cAMP [*21*] to activate the type I than the type II kinase. The potent activation of PK I by *9* and *18* could also result from an electrostatic interaction between the 2-Cl and 2-CF_3 substituents and the PK I.

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