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EFFECT OF MODIFICATION OF THE 1-, 2-, AND 6-POSITIONS OF 9-β-D-RIBOFURANOSYLPURINE CYCLIC 3′,5′-PHOSPHATE ON THE CYCLIC NUCLEOTIDE SPECIFICITY OF ADENOSINE CYCLIC 3′,5′-PHOSPHATE- AND GUANOSINE CYCLIC 3′,5′-PHOSPHATE-DEPENDENT PROTEIN KINASES*

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Abstract—A group of analogs of adenosine cyclic 3′,5′-phosphate (cAMP) and guanosine cyclic 3′,5′-phosphate (cGMP) with modifications in the 1-, 2- (or N^2), and 6 (or N^6)-positions of the purine ring were compared as activators of a cAMP-dependent protein kinase [PK(cAMP)] from bovine brain and of a cGMP-dependent protein kinase [PK(cGMP)] from lobster tail muscle. The results suggest that the 6-amino group of cAMP is not required for the activation of PK(cAMP) by cAMP and that the 6-oxygen and 2-amino moieties of cGMP are required for the activation of PK(cGMP) by cGMP. In the case of PK(cGMP) activation by cGMP, the 6-oxygen apparently accepts a proton from the enzyme and the 2-amino group apparently donates a proton to the enzyme.

The original proposal of Kuo and Greengard [1] that the diverse actions of cAMP†† on eukaryotic physiology are all mediated through cAMP-dependent protein kinases remains the principal working hypothesis for the mechanism of action of cAMP [2, 3]. After the discovery of cGMP-dependent protein kinases [4], it was proposed that the regulation of cellular functions by cGMP is mediated by these enzymes.

Our results [5–8] and those of other investigators [9, 10] on the abilities of certain 2-, 6-, and 8-substituted derivatives of 9-β-D-ribofuranosylpurine cyclic 3′,5′-phosphate to activate PK(cAMP) and PK(cGMP) suggested that PK(cGMP) is specific for an amino group in the 2-position of the purine ring and that PK(cAMP) is specific for an amino group in the 6-position. Furthermore, modification or substitution of the 6-amino group of cAMP (e.g. N^6-ethyl-cAMP [5]) or the 2-amino group of cGMP (e.g. cXMP [7]) often results in analogs that demonstrate significantly reduced specificity for their respective protein kinase relative to that of the parent cyclic nucleotide from which they are derived. The acute specificities of PK(cAMP) and PK(cGMP) for their respective unmodified cyclic nucleotide activators appear to lie in the regulatory subunits of the two protein kinases [10]. As part of our continuing investigations of the similarities and differences between the cyclic nucleotide binding sites on the PK(cAMP) and PK(cGMP), we report here some new cyclic nucleotide derivatives that provide additional insight into the nature of the cyclic nucleotide specificity of these protein kinases.

MATERIALS AND METHODS

Cyclic nucleotide analogs. The structures of the cyclic nucleotide analogs are shown in Table 1. Previously reported methods were used to synthesize compounds **1**, **9**, **12** and **15** [11], **2**, **5**, **6** and **7** [12], **4** [7], **11** and **13** [13], **14** [5], **19** [6], and **20** [14]. Compounds **3**, **8**, **10**, **16–18**, **21–23** and **24** were synthesized as described below. All the new compounds yielded C, H, N analyses that were ± 0.4% of theoretical values. In addition, the u.v. and ^{1}H n.m.r. spectra of each of the new compounds were consistent with each structure.

2-Azaadenosine-1-oxide cyclic 3′,5′-phosphate (**3**). 5-Amino-1-β-D-ribofuranosylimidazole-4-carboxamidoxime 3′,5′-cyclic phosphate (5 g, 15 moles) [11] was dissolved in 50 ml of 6 N hydrochloric acid (at −30°); 1.1 g of sodium nitrite (in 5 ml of water) was added dropwise with stirring over a 10-min period. The solution was stirred for an additional 30 min at −30°, then 20 ml of ethanol was added and the solution was adjusted to pH 7 with conc. ammonium hydroxide. The solution was desalted with Dowex

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†† Abbreviations: cAMP, adenosine cyclic 3′,5′-phosphate; cGMP, guanosine cyclic 3′,5′-phosphate; cIMP, inosine cyclic 3′,5′-phosphate; cXMP, xanthosine cyclic 3′,5′-phosphate; PK(cAMP), cAMP-dependent protein kinase; and PK(cGMP), cGMP-dependent protein kinase.

50 (H^+, 100–200 mesh), evaporated with ethanol, and filtered to yield 2.0 g (58%) of **3**. *Anal.* ($C_9H_{11}N_6O_7P \cdot H_2O$) C, H, N.

2-Methyladenosine-1-oxide cyclic 3',5'-phosphate (**8**). 5-Amino-1-β-D-ribofuranosylimidazole-4-carboxamidoxime 3',5'-cyclic phosphate (1 g, 3 mmoles) [11] was dissolved in 1.5 ml of 2 N sodium hydroxide and 10 ml of water. Under cooling in an ice-bath, 1 ml of acetaldehyde dissolved in 10 ml of methanol was added to the solution. The mixture was stirred for 3 hr at room temperature. Chloranil (0.3 g), dissolved in 10 ml of methanol, was added to the mixture, which was then stirred at room temperature overnight. The solvent was evaporated and the residue was dissolved in 50 ml of water. The aqueous solution was extracted with ethyl acetate (3 × 50 ml) and passed onto a column of Dowex 1 × 3 (formate, 3 × 9 cm). The column was washed with 200 ml of water, and the nucleotide was eluted with a gradient of 400 ml of 50% ethanol in the mixing chamber and 400 ml of 2 N formic acid in 50% ethanol in the reservoir. Evaporation of fractions containing the product gave a residue that crystallized on addition of ethanol to give 300 mg (28%) of **8**. *Anal.* ($C_{11}H_{14}N_5O_7P \cdot H_2O$) C, H, N.

1-Benzyladenosine cyclic 3',5'-phosphate (**10**). To a solution of 10 g (30.4 mmoles) of cAMP and 4.62 g (30 mmoles) of 1,5-diazabicyclo[5.4.0]undec-5-ene in 50 ml of dimethylsulfoxide was added 4 ml of benzyl bromide. After overnight stirring, the solution was diluted with 100 ml of water and 100 ml of ethanol. After the solution stood for 3 days, some cAMP had precipitated; this was removed by filtration and concentrated *in vacuo* to a small volume. The concentrated solution was diluted with 100 ml of water and passed onto a column of Dowex 1 × 8 (formate, 3 × 15 cm), which was then eluted with 50% aqueous ethanol. The product appeared in the first 300 ml of eluant. Evaporation of the fractions and recrystallization of the residue from aqueous ethanol gave 2.10 g (16%) of **10**. *Anal.* ($C_{17}H_{18}N_5O_6P \cdot H_2O$) C, H, N.

1-Methylinosine cyclic 3',5'-phosphate (**16**). To a solution of 300 mg (0.9 mmole) of cIMP and 150 mg of 1,5-diazabicyclo[5.4.0]undec-5-ene in 3 ml of dimethylsulfoxide was added 1 ml of methyl iodide. The reaction solution was stirred for 4 hr at room temperature and then diluted with 20 ml of ethanol. The product (87 mg, 26%) was collected by filtration and recystallized twice from ethanol–water to yield 43 mg (13%) of **16**.

1-Benzylinosine cyclic 3',5'-phosphate (**17**). To a solution of 2.1 g (6.3 mmoles) of cIMP in 24 ml of water were added ethanolic sodium carbonate (12 g in 120 ml) and benzyl bromide (4 ml). After overnight stirring at room temperature, the solid material was removed by filtration and washed with 100 ml of 80% ethanol. The filtrate and washings were combined and evaporated to dryness. The residue was dissolved in 50 ml of water and passed onto a column of Dowex 50 × 8 (H^+, 3 × 15 cm), which was then eluted with water. Appropriate fractions were collected and evaporated. The residue was dissolved in a small volume of ethanol, and diethyl ether was added to the solution. The resulting precipitate was collected, washed with diethyl ether, and dried to give 1.37 g (50%) of **17**. *Anal.* $C_{17}H_{17}N_4O_7P \cdot 2H_2O$) C, H, N.

2-Azainosine cyclic 3',5'-phosphate (**18**). 5-Amino-1-β-D-ribofuranosylimidazole-4-carboxamide 3',5'-cyclic phosphate (338 mg, 1 mmole) [11] was dissolved in 20 ml of 6 N hydrochloric acid (at −25°), and sodium nitrite (80 mg, 1.15 mmole, in 3 ml of water) was added dropwise with stirring over a 10-min period. The solution was stirred at −30° for an additional 30 min, and ethanol (20 ml) was added. The solution was adjusted to pH 7 with conc. ammonium hydroxide and then applied to a Dowex 50 (H^+, 3 × 20 cm) column. Elution of the column with water and evaporation of the appropriate fractions gave a semisolid, which crystallized from ethanol, yielding 160 mg (47%) of **18**. *Anal.* ($C_9H_{10}N_5O_7P \cdot \frac{1}{2}H_2O$) C, H, N.

1-Methylguanosine cyclic 3',5'-phosphate (**21**). To a suspension of cGMP · $4H_2O$ sodium salt (2.0 g, 10 mmoles) in 0.67 N sodium hydroxide (15 ml) was added methyl iodide (3 ml). After vigorous stirring at room temperature in a closed container for 24 hr, the solution was applied to a column of Dowex 1 × 2 (formate, 3 × 15 cm) that was eluted with a gradient of 600 ml of water in the mixing chamber and 600 ml of 4 N formic acid in the reservoir. The fractions containing the product, which eluted at the end of the gradient, were evaporated to dryness, and the residue was washed with ethanol and dried to give 1.45 g (44%) of **21**. *Anal.* ($C_{11}H_{14}N_4O_7P$) C, H, N.

1-Aminoguanosine cyclic 3',5'-phosphate (**22**). The procedure for preparing 1-aminoguanosine [15] was used to aminate cGMP · $4H_2O$ sodium salt (1.0 g, 5 mmoles) using hydroxylamine-*O*-sulfonic acid except that 1 N ammonium hydroxide was used instead of 1 N sodium hydroxide. After evaporation of the solvent, the crude product was dissolved in 10 mM triethylamine (500 ml), and the solution, after being brought to pH 9.7 with carbon dioxide, was applied to a column of DEAE cellulose (Whatman DE-52) previously equilibrated with 10 mM triethylamine (pH 9.7). The column was eluted with a gradient of 1 liter of 10 mM triethylamine (pH 9.7) in the mixing chamber and 1 liter of 0.1 M triethylamine (pH 9.7) in the reservoir. The fractions containing product were lyophilized, the residue was dissolved in water (50 ml), and the solution was applied to a column of Dowex 1 × 2 (formate, 1 × 15 cm, previously equilibrated with water that was brought to pH 10 with ammonium hydroxide). The column was eluted with a gradient of 400 ml of water (brought to pH with ammonium hydroxide) in the mixing chamber and 400 ml of 0.8 M ammonium formate in the reservoir. The first band eluting from the column was evaporated to dryness and crystallized from methanol/ethanol to give 0.42 g (23%) of **22** as the ammonium salt. *Anal.* ($C_{10}H_{16}N_7O_7 \cdot H_2O$) C, H, N.

6-Phenyl-3-(β-D-ribofuranosyl cyclic 3',5'-phosphate)-imidazo[1,2-a]purin-8-one (**23**). To a suspension of cGMP · $4H_2O$, sodium salt (3.0 g, 15 mmoles) in dimethylsulfoxide (45 ml) containing 3.0 g of 1,5-diazobicyclo[5.4.0]undec-5-ene was added 3.0 g of α-bromoacetophenone. The mixture was stirred at room temperature for 20 hr. It was then diluted with 350 ml of water, adjusted to pH 7.5 with 1 N hydro-

chloric acid, and filtered. The filtrate was adjusted to pH 1.5 with conc. hydrochloric acid, stirred for 10 min, filtered, washed with water and acetone, and dried *in vacuo* at room temperature to yield 1.53 g (22%) of **23**. *Anal.* ($C_{18}H_{16}N_5O_7P \cdot H_2O$) C, H, N.

6-*(p-Methoxyphenyl)-3-(β-D-ribofuranosyl cyclic 3',5'-phosphate)-imidazo*[1,2-a]*purin-8-one* (**24**). To a suspension of cGMP·$4H_2O$, sodium salt (3.0 g, 15 mmoles) in dimethylsulfoxide (45 ml) containing 3.0 g of 1,5-diazobicyclo[5.4.0]undec-5-ene was added 3.4 g of α-bromo-*p*-methoxyacetophenone. The mixture was stirred at room temperature for 65 hr. It was then diluted with 350 ml of water, adjusted to pH 5.5 with 1 N hydrochloric acid, and filtered. The filtrate was adjusted to pH 1.5 with conc. hydrochloric acid, filtered, and dried to give 837 mg. This was dissolved in 50 ml of water at pH 7.5 and adjusted to pH 1.5. The resulting gel was suspended in acetone, filtered, and dried *in vacuo* at 70° to give 628 mg (13%) of **24**. *Anal.* ($C_{19}H_{18}N_5O_8P$) C, H, N.

Enzyme preparations and assays. The bovine brain PK(cAMP) and the lobster tail muscle PK(cGMP) were purified as described by Kuo and Greengard [4, 9]. The protein kinase assays were performed using the paper disk method described previously [5, 6]. The assay for the kinase contained, in 0.1 ml: 5 μmoles of sodium acetate (pH 6.0), 1 μmole of $MgCl_2$, histone [Worthington HLY, 100 μg for PK(cAMP) and 25 μg for PK(cGMP)], 0.5 nmole of γ-[^{32}P]ATP (150,000 cpm), 5.1 pmoles of protein kinase holoenzyme, and various concentrations of the cAMP analog being tested as an activator (1 nM to 0.5 mM). The concentration of the holoenzyme was based on the cAMP-binding capacity of each enzyme preparation [16]. The catalytic activity of the kinase was measured in the presence of a number of concentrations (at least seven) of the cyclic AMP analog 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 reaction rates were measured. 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 the above data (pmoles of phosphate transferred to histone)$^{-1}$ vs [cyclic nucleotide]$^{-1}$ [6]. Only those apparent K_a values that resulted from lines with correlation coefficients ≥ 0.990 were considered acceptable. All data are the results of triplicate determinations which were reproducible within 15 per cent of the value reported.

The K_a'(cAMP) is equal to (apparent K_a for cAMP)/(apparent K_a for the analog), and the K_a'(cGMP) is equal to (apparent K_a for cGMP)/(apparent K_a for the analog). The K_a value for cAMP was 68 nM with the bovine brain PK(cAMP), and the K_a value for cGMP was 90 nM with the lobster tail muscle PK(cGMP). The K_a' values for the activation of bovine brain PK(cAMP) by compounds **1**, **2**, **4**, **5**, **6**, **7**, **9**, **12**, **14**, **15**, **19** and **20** have been reported previously [6, 7, 11, 14]. The K_a' values for the activation of lobster muscle PK(cGMP) by compounds **4**, **5**, **6**, **14** and **19** have also been reported previously [6, 7]. The K_a'(cAMP)/k_a'(cGMP) ratio is calculated as a measure of the relative specificity of each analog as either a PK(cAMP) activator or a PK(cGMP) activator.

RESULTS

Each cyclic nucleotide analog was examined for its ability to activate PK(cAMP) from bovine brain and PK(cGMP) from lobster tail muscle. The potency of each analog relative to either cAMP (for the bovine brain enzyme) or cGMP (for the lobster tail muscle enzyme) as an activator of each kinase was quantitated by determining K_a' values. In addition, the relative specificity of each analog for either PK(cAMP) or PK(cGMP) was estimated by examining the ratio of the K_a' for PK(cAMP) to the K_a' for PK(cGMP). The K_a' values and the K_a'[PK(cAMP)]/K_a'[PK(cGMP)] ratios are shown in Table 1.

The bovine brain PK(cAMP) exhibited a basal activity of 1.4 units/pmole enzyme in the absence of cAMP, where 1 unit is that amount of activity which transfers 1 pmole of $H_2{}^{32}PO_4^-$ to histone in 20 min under the assay conditions described above. At 5 μM cAMP, the bovine brain PK(cAMP) exhibited its maximal activity of 13 units/pmole enzyme. All of the analogs were able to maximally activate this enzyme to the same degree as that seen after activation by 5 μM cAMP. Likewise, the lobster muscle PK(cGMP) exhibited a basal activity of 2.1 units/pmole enzyme and a maximally stimulated activity at 5 μM cGMP of 11 units/pmole enzyme; all the analogs were able to maximally activate this enzyme to the same degree as that seen after activation by 5 mM cGMP. In the cases of analogs that were very poor activators, this conclusion is based on extrapolation of the double reciprocal plot to yield an estimated V_{max} which in every case was comparable to that for either cAMP (18 units/pmole enzyme) or GMP (15 units/pmole enzyme).

Activation of PK(cAMP) and PK(cGMP) by cyclic nucleotide analogs containing a 6-amino functionality. A group of 1-substituted, 2-substituted, and 1,2-disubstituted cAMP analogs were examined as PK(cAMP) and PK(cGMP) activators. The cAMP-N^1-oxide (**1**), like cAMP, was highly specific for PK(cAMP). The 2-aza analog of **1**, 2-aza-cAMP-N^1-oxide (**3**), although considerably less active than cAMP as an activator of PK(cAMP), was still highly specific for PK(cAMP). The 2-methyl-cAMP-N^1-oxide (**8**), which—like **3**—is modified in the 1- and 2-positions, demonstrated approximately the same K_a' values and the same degree of specificity for PK(cAMP) as did **3**. The compound, 2-aza-cAMP (**2**), which contains only the modification of the 2-position, was specific for PK(cAMP) but demonstrated significantly less specificity for PK(cAMP) then did cAMP. Derivatives containing certain types of substituents in the 2-position were found to be less specific for PK(cAMP) than **2** was. The compounds 2-amino-cAMP (**4**) and 2-hydroxy-cAMP (**5**) were only slightly more potent as PK(cAMP) activators than as PK(cGMP) activators, and 2-mercapto-cAMP (**6**) was found to be slightly more potent as a PK(cGMP) activator than as a PK(cAMP) activator. In contrast, 2-methyl-cAMP (**7**) demon-

Table 1. Activation of cAMP-dependent and cGMP-dependent protein kinases by 1-, 2-, and 6-substituted derivatives of cNMP*

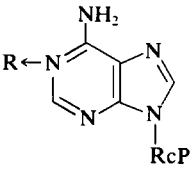
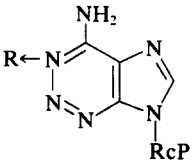
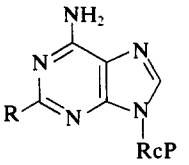
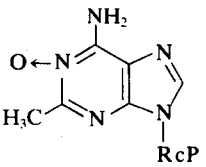
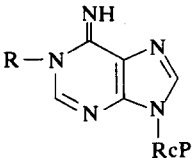
Compound	R	Protein kinase activation		
		K_a'(cAMP) [bovine brain PK(cAMP)]	K_a'(cGMP) [lobster muscle PK(cGMP)]	K_a'(cAMP)/ K_a'(cGMP)
				
cAMP		1.0	0.015	67
1	O	0.45	0.0078	58
				
2		0.076	0.0040	19
3	O	0.080	0.0012	67
				
4	NH_2	0.12	0.045	2.6
5	OH	0.032	0.0050	6.4
6	SH	0.012	0.023	0.52
7	CH_3	0.16	0.0017	94
				
8		0.040	0.00046	87
				
9	CH_3	0.060	0.011	5.5
10	$CH_2C_6H_5$	0.80	0.22	3.5

Table 1. *Contd.*

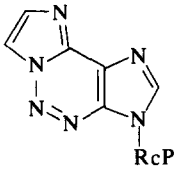
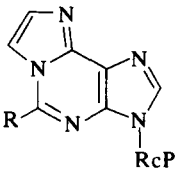
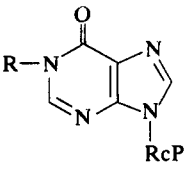
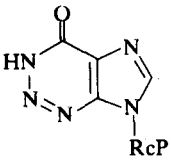
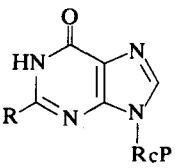
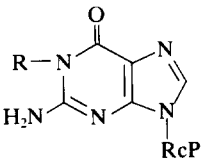
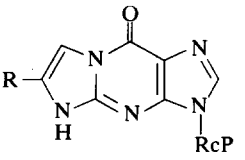
Compound	R	Protein kinase activation: K_a'(cAMP) [bovine brain PK(cAMP)]	K_a'(cGMP) [lobster muscle PK(cGMP)]	K_a'(cAMP)/ K_a'(cGMP)
				
11		0.69	0.11	6.3
				
12	H	0.65	0.11	5.9
13	CH_3	0.16	0.0016	100
				
14	H	0.59	0.085	6.9
15	OH	0.023	0.0020	12
16	CH_3	0.38	0.045	8.4
17	$CH_2C_6H_5$	0.18	0.073	2.4
				
18		0.11	0.0088	13
				
cGMP	NH_2	0.023	1.0	0.023
19	OH	0.016	0.010	1.6
20	CH_3	0.38	0.0045	84

Table 1. *Contd.*

Compound	R	Protein kinase activation: K_a'(cAMP) [bovine brain PK(cAMP)]	K_a'(cGMP) [lobster muscle PK(cGMP)]	K_a'(cAMP)/ K_a'(cGMP)
				
21	CH_3	0.020	0.034	0.59
22	NH_2	0.050	0.15	0.33
				
23	C_6H_5	0.070	0.12	0.58
24	C_6H_4-*p*-OCH_3	0.22	0.35	0.62

* RcP = β-D-ribofuranosyl cyclic 3,5-phosphate.

strated a specificity for PK(cAMP) similar to that of cAMP.

Activation of PK(cAMP) and PK(cGMP) by cyclic nucleotide analogs containing a 6-imino functionality. The two examples of this type of derivative were 1-methyl-cAMP (**9**) and 1-benzyl-cAMP (**10**). Compound **9** was somewhat less active than cAMP as a PK(cAMP) activator, but was approximately equal in activity to cAMP as a PK(cGMP) activator. Similarly, **10** was almost as potent as cAMP as a PK(cAMP) activator, but was fifteen times more potent than cAMP as a PK(cGMP) activator.

Activation of PK(cAMP) and PK(cGMP) by cyclic nucleotide analogs containing a 1,N^6*-etheno functionality.* These analogs include 1,N^6-etheno-cAMP (**12**) and its 2-aza (**11**) and 2-methyl (**13**) derivatives. Both **11** and **12** were potent activators of both PK(cAMP) and PK(cGMP) and, like **9** and **10**, were comparable to cAMP as activators of PK(cAMP) and considerably more active than cAMP as activators of PK(cGMP). In contrast, **13**—like **7** and **8**—exhibited a specificity for PK(cAMP) similar to that of cAMP.

Activation of PK(cAMP) and PK(cGMP) by cyclic nucleotide analogs containing a 6-oxo functionality. A group of 1-substituted- and 2-substituted-cIMP analogs, 1-substituted-cGMP analogs, and 1,N^2-etheno-cGMP analogs were examined as PK(cAMP) and PK(cGMP) activators. The 1-methyl- (**16**), and 1-benzyl-cIMP (**17**) demonstrated comparable K_a' values to those for cIMP (**14**) with PK(cAMP) and PK(cGMP). The 1-hydroxy-cIMP (**15**), although less active than **14** with either PK(cAMP) or PK(cGMP), demonstrated approximately the same specificity for PK(cAMP) as that of **14**, **16**, and **17**. The specificity of 2-aza-cIMP (**18**) for PK(cAMP) was not significantly different from that of cIMP (**14**). The 2-methyl-cIMP (**20**), like 2-methyl-cAMP (**7**), was highly specific for PK(cAMP). In contrast, cXMP (**19**), although considerably less active than cAMP and cGMP as activators of PK(cAMP) and PK(cGMP), respectively, was equally active with both protein kinases. The 1-substituted-cGMP derivatives (**21** and **22**) were found to be equal in potency to cGMP as PK(cAMP) activators but somewhat less active than cGMP as PK(cGMP) activators. In comparison, the 1,N^2-etheno-cGMP analogs (**23** and **24**), like **21** and **22**, were less active than cGMP as PK(cGMP) activators; however, unlike **21** and **22**, they were more active than cGMP as PK(cAMP) activators.

DISCUSSION

The structural differences between cAMP and cGMP reside in the 1-, 2-, and 6-positions of the purine ring. Cyclic AMP contains an amino group in the 6-position and a proton in the 2-position, whereas cGMP contains an amino group in the 2-position, a proton in the 1-position, and an oxygen in the 6-position. The cyclic nucleotide derivatives studied here were chosen because they contained modifications in either one, two, or three of these apparently critical positions.

The specificity of PK(cAMP) for a 6-amino group was retained by all the analogs that contained the 6-amino functionality and either a 1-oxide (compounds **1**, **3** and **8**), a 2-aza (compounds **2** and **3**) and/or a 2-methyl (compounds **7** and **8**) modification. In contrast, the specificity of PK(cAMP) for the 6-

amino group was either eliminated or substantially reduced by all the analogs that contained the 6-amino functionality and either a 2-amino (compound **4**), 2-hydroxy (compound **5**), or 2-mercapto (compound **6**) modification. Therefore, substituents in the 2-position that are capable of forming a hydrogen bond appear to yield derivatives with significantly less specificity for PK(cAMP) than that exhibited by cAMP.

When the 6-amino group was changed to a 6-imino group by substitution at the 1-position (compounds **9** and **10**), the resulting analogs exhibited an increased specificity for PK(cGMP) compared to cAMP. The analogous 1-substituted cIMP derivatives (compounds **16** and **17**) demonstrated K_a' values and cyclic nucleotide specificities similar to those for **9** and **10**. These results suggest that the activation of PK(cGMP) by cGMP involves the donation of a proton to the 6-oxygen on cGMP and that a 6-imino group can serve this proton-accepting function. Furthermore, an analog containing a 6-amino group did not activate PK(cGMP) possibly because of the inability of the 6-amino to accept a proton at physiological pH. Since some of the compounds containing either a 6-oxygen (compounds **14**, **16**, **17**, **18**, **20**, **24**) or a 6-imino (compound **10**) group were able to efficiently activate PK(cAMP), the activation of PK(cAMP) by cAMP may not require the 6-amino group. Consistent with this conclusion, nebularine cyclic 3′,5′-phosphate (6-deamino-cAMP) demonstrates a K_a' of 0.40 with PK(cAMP),* showing that PK(cAMP) does not require the 6-amino functionality for activation by cAMP.

The 1,N^6-etheno modification yielded some analogs (compounds **11** and **12**) that demonstrated a significant increase in specificity for PK(cGMP) compared to cAMP. As in the case of the analogs with 6-oxygen and 6-imino groups, the 1,N^6-etheno functionality yielded a 6-nitrogen that can serve as a proton acceptor during activation of PK(cGMP) by the analogs.

The considerable increase in potency as PK(cGMP) activators of 1-benzyl-cAMP (**10**), 1-benzyl-cIMP (**17**) and 1,N^6-etheno-cAMP may also be a result of hydrophobic interactions between the benzyl or etheno moieties and PK(cGMP). Such an interaction would rationalize the considerably larger K_a' value with PK(cGMP) of 1-benzyl-cAMP (**10**) than of 1-methyl-cAMP (**9**).

The apparent requirement, mentioned above, of PK(cGMP) for a hydrogen bonding moiety in the 2-position is further demonstrated by the vast differences in protein kinase specificity between cGMP, cXMP (**19**), and 2-methyl-cIMP (**20**). The weak activity of **19** may be attributed to the diketo form in which cXMP exists [17], suggesting that the 2-amino may serve as a proton donator. This conclusion is consistent with the ability of the 1,N^2-etheno-cGMP derivatives (compounds **23** and **24**) to efficiently activate PK(cGMP). When the 2-amino or substituted 2-amino functionality is replaced by a methyl group (compound **20**), the resulting compound exhibits a drastically reduced ability to activate PK(cGMP).

* T. S. Yagura, Z. Kazimierczuk, D. Shugar and J. P. Miller, *Biochem. biophys. Res. Commun* **97**, 737 (1980).

The results presented here lead to the following generalizations. First, the 6-amino group is probably not required for the activation of PK(cAMP) by cAMP. Second, both the 6-oxygen and the 2-amino moieties are probably required for the activation of PK(cGMP) by cGMP. The 6-oxygen apparently accepts a proton from the enzyme and the 2-amino apparently donates a proton to the enzyme. These conclusions must be limited to a comparison of bovine brain PK(cAMP) with lobster tail muscle PK(cGMP). Two isozymic forms (referred to as type I and type II) of PK(cAMP) have been described [18]. The bovine brain PK(cAMP) use here is a type II isozyme [19]. The type I and type II isozymes appear to differ in at least those portions of their respective cAMP-binding sites that are adjacent to the 2-position of cAMP [20]. Therefore, the differences reported here between a type II PK(cAMP) and the lobster tail muscle PK(cGMP) may not extend to a type I PK(cAMP).

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