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ACTIVATION AND DISSOCIATION OF ADENOSINE 3′,5′-MONOPHOSPHATE-DEPENDENT AND GUANOSINE 3′,5′-MONOPHOSPHATE-DEPENDENT PROTEIN KINASES BY VARIOUS CYCLIC NUCLEOTIDE ANALOGS*

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Abstract—The effects of various analogs of adenosine 3′,5′-monophosphate (cyclic AMP), guanosine 3′,5′-monophosphate (cyclic GMP), and inosine 3′,5′-monophosphate (cyclic IMP) in stimulating and dissociating cyclic AMP-dependent and cyclic GMP-dependent classes of protein kinases were examined. With the possible exceptions of the dibutyryl derivative of cyclic GMP and the cyclothiophosphate analog of cyclic AMP, all compounds tested were capable of maximally stimulating both classes of protein kinases, and some analogs were more reactive than the parent compounds. The effectiveness of these compounds in activating these protein kinases paralleled their ability to cause the dissociation of the enzymes into subunits. Some compounds, notably the 8-thio and 8-methylthio analogs of cyclic AMP, which were at least as reactive as cyclic AMP in dissociating and stimulating the cyclic AMP-dependent enzyme, did not compete, but unexpectedly cooperatively enhanced the binding of the radioactive cyclic AMP to this enzyme, suggesting that these sulfur-containing analogs may interact with specific sites on the enzyme different from cyclic AMP. Many of the cyclic AMP analogs, like cyclic GMP, supported the stimulatory action of the protein kinase modulator on arginine-rich histone phosphorylation catalyzed by cyclic GMP-dependent protein kinase. The modulator, however, inhibited the cyclic AMP-dependent class of protein kinase activated by any of the cyclic nucleotides and analogs under the same assay conditions.

AN OVERWHELMING body of evidence supporting the role of adenosine 3′,5′-monophosphate (cyclic AMP) as a universal mediator for the actions of a wide variety of biological stimuli[1] has been accumulated. It has also become clear that guanosine 3′,5′-monophosphate (cyclic GMP) may play a separate and an independent role for regulating cellular metabolism and function.[2–6] We have proposed earlier that the actions of cyclic AMP and cyclic GMP may be mediated through regulation of cyclic AMP-dependent and cyclic GMP-dependent protein kinases respectively.[5,7]

Tubercidin 3′,5′-monophosphate, a structural analog of cyclic AMP, has been shown to be more effective than cyclic AMP in activating phosphorylase b kinase

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in a skeletal muscle preparation.[8] We[9] reported that it is as effective as cyclic AMP in activating cyclic AMP-dependent protein kinases from various sources and is more active than cyclic AMP in activating the cyclic GMP-dependent class of enzyme. Muneyama *et al.*[10] have synthesized a series of 8-substituted cyclic AMP analogs and demonstrated that many of them are more effective than cyclic AMP in activating bovine brain cyclic AMP-dependent protein kinase while resisting hydrolysis by pig brain phosphodiesterase or even inhibiting the activity of that enzyme. Posternak and Cehovic[11] reported that many 2- and 8-substituted cyclic AMP analogs are also more effective than cyclic AMP in stimulating the release of growth hormone from rat pituitary gland. They reported that 8-thio and 8-bromo analogs of cyclic AMP are at least as potent as N^6, 2′-*O*-dibutyryl cyclic AMP on pituitary slices, suggesting that these analogs may be also quite permeable to cell membrane. The structures of some of the cyclic nucleotides are shown in Fig. 1.

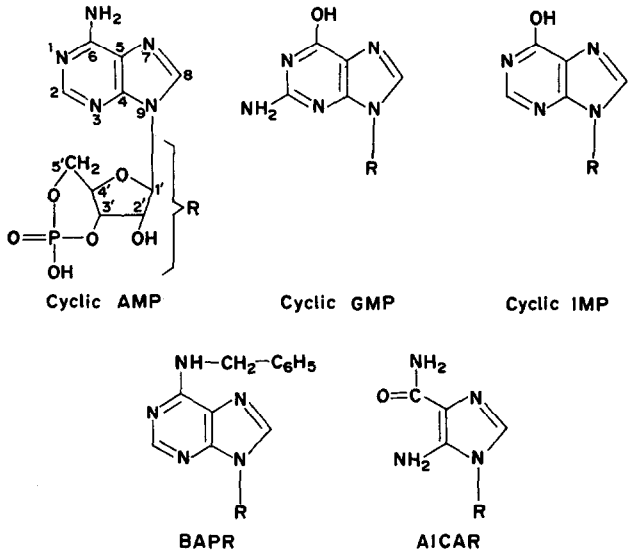

FIG. 1. Structures of some cyclic nucleotides and analogs.

In view of the recent availability of many derivatives of cyclic nucleotides and their potential usefulness as pharmacological agents, we investigated in some depth the effects of the analogs of cyclic AMP, cyclic GMP and cyclic IMP on the activation and dissociation of both cyclic AMP-dependent and cyclic GMP-dependent classes of protein kinases. We also studied the binding of these analogs to cyclic AMP-dependent enzyme and their influences on the action of protein kinase modulator in regulating enzyme activity. The modulator has been shown to either stimulate or inhibit these two classes of protein kinases under certain assay conditions.[12,13]

EXPERIMENTAL PROCEDURE

Materials. Cyclic AMP-^{3}H (24·1 Ci/m-mole) was purchased from Schwarz/Mann; cyclic AMP, cyclic GMP, inosine 3′,5′-monophosphate (cyclic IMP) and glyceralde-

hyde-3-phosphate dehydrogenase were purchased from Boehringer-Mannheim. The following cyclic nucleotide analogs were kindly provided by Boeringer-Mannheim: N^2,2′-*O*-dibutyryl cyclic GMP, 6-benzylamino-purine-riboside 3′,5′-monophosphate (BARP, EG-3), 8-bromo cyclic AMP (PA-1), 8-benzylamino cyclic AMP (PA-2), 8-bromo cyclic IMP (PA-17), 8-benzylamino cyclic IMP (PA-18), 8-bromo cyclic GMP (PA-22), 6-benzyl cyclic AMP (MK-63), amino-imidazolcarboxamideriboside 3′,5′-monophosphate (AICAR, MK-82) and 8-benzylamino cyclic GMP (MK-206). The following cyclic AMP analogs were the gifts of Nucleic Acid Research Institute, ICN: 8-thio (YH-4511), 8-oxo (YG-81C), 8-amino (YH-471), 8-methylthio (YH-85111), 8-methylamino (YH-131), 8-β-hydroxyethylamino (YH-161) and 8-β-hydroxyethylthio (YH-121). Many of the above analogs were also purchased from Plenum Scientific Research and were found to be similar to the samples provided by Boehringer-Mannheim and ICN in their reactivity. Catalase and horse liver alcohol dehydrogenase were obtained from Sigma.

Preparation and assay of protein kinase and protein kinase modulator. Cyclic GMP-dependent protein kinase from lobster tail muscle[14] and cyclic AMP-dependent protein kinase from bovine heart[7] were both purified to the step of DEAE-cellulose chromatography according to the procedure described elsewhere. Cyclic AMP-dependent protein kinase from bovine brain was purified to the step of hydroxylapatite chromatography;[15] the 7·7 S enzyme from the second protein peak was used in the present studies. The protein kinase modulator was purified to an apparent homogeneity from lobster tail muscle.[13] Protein kinase activity was assayed under the standard incubation system which contained, in a final volume of 0·2 ml: sodium acetate buffer, pH 6·0, 10 μmoles; substrate protein, 20–40 μg; [γ-^{32}P]-ATP, 1·0 nmole, containing about $1{\cdot}8 \times 10^6$ cpm; magnesium acetate, 2 μmoles; cyclic nucleotide or analog, up to 2 μmoles; and, if present, lobster muscle protein kinase modulator, 5 μg. The substrate protein used was either 20 μg of arginine-rich histone for the experiments involving the lobster muscle and bovine heart protein kinases or 40 μg of histone mixture for those involving the bovine brain enzyme respectively. The reaction was allowed to proceed for 5 min at 30°. The ^{32}P-histone was recovered from the incubation mixture by the addition of trichloroacetic acid–tungstate precipitating solution, as described elsewhere.[16] One unit of protein kinase is defined as that amount which transfers 1 pmole (10^{-12} mole) of ^{32}P from [γ-^{32}P]-ATP to histone in 5 min at 30° under the assay conditions.

Dissociation of protein kinases and sucrose density gradient ultracentrifugation. The incubation conditions for dissociation of the protein kinases and the method for determination of molecular size of the enzyme subunits by means of sucrose density gradient ultracentrifugation were essentially the same as described earlier.[15] In brief: an aliquot of bovine brain cyclic AMP-dependent protein kinase (containing 0·16 mg protein) or lobster muscle cyclic GMP-dependent protein kinase (containing 1·4 mg protein), in a final volume of 0·22 ml, was preincubated with or without added cyclic nucleotides or analogs for 5 min at 30° in the presence of 50 mM sodium acetate buffer (pH 6·0), 0·3 mM EGTA and 2·5 mM 2-mercaptoethanol. The enzyme solutions were then quantitatively layered onto individual centrifuge tubes containing 4·8 ml of a 5–20% (w/v) sucrose density gradient. The sucrose solution also contained the same other ingredients as in the preincubation solution. Separation of the dissociated enzyme subunits was carried out at 3° by ultracentrifugation in a Beckman

SW 39 L rotor at 37,500 rev/min for 16 hr. The values of the sedimentation coefficients (S) for each protein component were estimated by the method of Martin and Ames,[17] using catalase (11·6 S), glyceraldehyde-3-phosphate dehydrogenase (7·7 S) and horse liver alcohol dehydrogenase (5·4 S) as the standard marker proteins.

Assay for cyclic AMP binding. The binding of cyclic AMP by protein kinase was assayed in an incubation system which contained, in a final volume of 0·2 ml: 50 mM sodium acetate buffer (pH 4·0) and 1 pmole cyclic AMP-^{3}H, containing about 6300 cpm, in the presence and absence of nonradioactive cyclic nucleotides or analogs. The incubation, initiated by the addition of 110 μg of bovine brain cyclic AMP-dependent enzyme, was carried out for 60 min at 0°. The incubation mixture was then diluted to 1 ml with cold 20 mM potassium phosphate buffer, pH 6·0, and the amount of the bound radioactive cyclic AMP measured according to the method of Gilman.[18]

RESULTS AND DISCUSSION

The dose-dependent activation of bovine heart cyclic AMP-dependent protein kinase by cyclic AMP, cyclic GMP and cyclic IMP, and their analogs, in order of decreasing effectiveness, as judged by their activities observed at 10^{-7} M, is presented in Table 1. All compounds tested, with the possible exception of N^2,2′-*O*-dibutyryl cyclic GMP, were capable of maximally activating the enzyme. Many analogs were

TABLE 1. ACTIVATION OF BOVINE HEART CYCLIC AMP-DEPENDENT PROTEIN KINASE BY VARIOUS CYCLIC NUCLEOTIDES AND ANALOGS

Experiment No.	Compound	Cyclic AMP-dependent enzyme activity (units)* at 10^{-8} M	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M	10^{-3} M
I	8-Bromo cyclic AMP	63	149	173	172	132	71
	BAPR	35	143	176	172	172	169
	Cyclic AMP	36	137	169	174	151	99
	6-Benzol cyclic AMP	41	99	177	181	177	148
	8-Benzylamino cyclic AMP	33	70	156	164	148	126
	8-Bromo cyclic IMP	31	51	144	168	152	141
	Cyclic IMP	30	51	143	172	148	135
	AICAR	27	39	104	178	173	163
	8-Bromo cyclic GMP	19	25	43	156	180	160
	Cyclic GMP	21	21	46	139	164	182
	8-Benzylamino cyclic GMP	22	20	30	64	142	170
	N^2-2′-*O*-dibutyryl cyclic GMP	13	14	12	15	19	86 (139)†
II	8-Methylthio cyclic AMP	71	141	163	170	140	68
	8-Oxo cyclic AMP	68	148	155	165	167	127
	8-(β-Hydroxyethylthio) cyclic AMP	67	133	163	143	92	73
	8-Bromo cyclic AMP	63	145	148	126	80	62
	8-Amino cyclic AMP	63	145	147	164	154	113
	8-Thio cyclic AMP	63	134	146	155	53	15
	Cyclic AMP	45	134	147	151	124	67
	8-Methylamino cyclic AMP	55	105	161	166	158	120
	8-(β-Hydroxyethylamino) cyclic AMP	36	85	150	168	161	125
	Cyclic GMP	24	24	56	144	156	159
	Adenosine 3′,5′-cyclothio-phosphate	27	28	26	70	132	98

* The basal activities were 20·5 and 23·5 for experiments I and II respectively.

† The value in parentheses was obtained at 10^{-2} M.

TABLE 2. ACTIVATION OF LOBSTER TAIL MUSCLE CYCLIC GMP-DEPENDENT PROTEIN KINASE BY VARIOUS CYCLIC NUCLEOTIDES AND ANALOGS

Experiment No.	Compound	Cyclic GMP-dependent enzyme activity (units)* at 10^{-8} M	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M	10^{-3} M
I	8-Bromo cyclic GMP	9·7	17·9	19·7	19·7	18·9	18·7
	8-Benzylamino cyclic AMP	5·3	12·2	16·7	19·7	19·1	15·5
	Cyclic GMP	5·8	9·4	18·8	22·0	18·7	18·0
	6-Benzoyl cyclic AMP	4·7	6·9	12·2	18·3	18·5	19·4
	8-Benzylamino cyclic GMP	4·4	6·3	12·1	18·1	18·4	18·6
	Cylic AMP	5·0	6·0	10·9	20·5	22·0	18·2
	8-Bromo cyclic IMP	4·9	5·8	10·6	16·9	18·7	18·6
	BAPR	4·2	4·1	10·2	15·8	21·9	18·5
	AICAR	4·5	5·3	8·8	16·2	19·4	16·2
	8-Benzylamino cyclic IMP	4·8	5·6	7·6	15·2	21·7	17·6
	Cyclic IMP	5·6	5·7	6·5	13·2	21·5	18·2
	N^2-2′-*O*-dibutyryl cyclic GMP	3·9	4·1	3·9	3·9	7·6	17·6 (20·5)†
II	Cyclic GMP	8·6	15·4	20·5	22·1	22·6	20·3
	8-Bromo cyclic AMP	8·2	10·9	17·6	20·7	17·4	4·5
	8-Thio cyclic AMP	7·8	10·2	17·8	20·4	18·5	5·2
	8-Methylthio cyclic AMP	6·6	9·4	15·7	20·9	16·1	4·9
	8-(β-Hydroxyethylthio) cyclic AMP	6·3	8·7	14·5	19·6	17·1	4·1
	8-Oxo cyclic AMP	7·0	10·3	12·5	21·0	22·2	18·8
	8-Amino cyclic AMP	6·9	7·4	12·2	18·9	22·6	19·3
	8-Methylamino cyclic AMP	7·7	8·9	10·9	21·0	19·8	16·7
	Cyclic AMP	6·4	8·9	11·9	18·6	20·4	9·7
	8-(β-Hydroxyethylamino) cyclic AMP	6·2	6·2	9·7	14·1	18·8	14·6
	Adenosine 3′,5′-cyclothiophosphate	5·5	5·6	5·9	5·6	7·2	5·3

* The basal activities were 4·9 and 6·3 for experiments I and II respectively.
† Value in parentheses was obtained at 1×10^{-2} M.

more active than cyclic AMP. Some representatives of the compounds listed in Table 1 were also tested for activating bovine brain cyclic AMP-dependent enzyme, and results similar to those observed with the heart enzyme were obtained. The relative potency of some 8-substituted analogs of cyclic AMP to activate the bovine brain enzyme has been reported earlier by Muneyama *et al.*[10] Our results obtained with both bovine heart and brain enzymes were in agreement with their findings. The activation of the bovine heart cyclic AMP-dependent enzyme by 10^{-2} M dibutyryl cyclic GMP may be due to a possible contamination of free cyclic GMP in this preparation, since 10^{-5} M cyclic GMP, representing a possible 0·1 per cent contamination, would have stimulated the enzyme to the same level. We have shown earlier[19] that N^6, 2′-*O*-dibutyryl cyclic AMP at 5×10^{-4} M is able to maximally activate brain bovine cyclic AMP-dependent protein kinase; the reactivity of this preparation may also be due to a possible contamination of free cyclic AMP.

It should be mentioned that maximal or near maximal activation of the enzyme by 8-oxo and 8-amino cyclic AMP was noted over a 10,000-fold range of their concentrations, whereas only over a 100-fold range of concentrations was noted for activation by 8-bromo and 8-thio cyclic AMP. At a supramaximal concentration of 10^{-4} M, the inhibitory activity of the latter two analogs became evident. For most of the remaining compounds listed in Table 1, including cyclic AMP, the range of

concentration at which a near maximal enzyme activity was observed was about 1000-fold.

The same compounds listed in Table 1 were also examined for their effects on lobster muscle cyclic GMP-dependent protein kinase; the results, in order of decreasing potency in activating the enzyme, are shown in Table 2. With the exception of adenosine 3′,5′-cyclothiophosphate, all compounds tested were found to be active and, at different concentrations were capable of maximally activating the lobster enzyme. Dibutyryl cyclic GMP activated the lobster muscle enzyme when it was present at concentrations higher than 10^{-3} M; its effect, however, may be due to a possible contamination of free cyclic GMP in the preparation. It is interesting that 8-bromo cyclic GMP was more reactive than cyclic GMP in activating the cyclic GMP-dependent enzyme. A sharp inhibition of the cyclic GMP-dependent class of protein kinase by a supramaximal concentration (10^{-3} M) of 8-bromo and 8-thio analogs of cyclic AMP was also noted, similar to that seen for bovine heart cyclic AMP-dependent protein kinase (Table 1). Cyclic AMP itself also became inhibitory at 10^{-3} M. The enzyme activity, however, was maintained at a near maximal level in the presence of the same concentration of 8-oxo and 8-amino [and also 8-methylamino and 8-(β-hydroxyethylamino)] analogs of cyclic AMP.

The apparent K_a values (concentrations that cause a half-maximal stimulation of the enzymes) for various cyclic nucleotides and their analogs of cyclic AMP-depen-

TABLE 3. SUMMARY OF APPARENT K_a VALUES OF CYCLIC NUCLEOTIDES AND ANALOGS FOR BOVINE HEART AND BOVINE BRAIN CYCLIC AMP-DEPENDENT PROTEIN KINASES AND LOBSTER MUSCLE CYCLIC GMP-DEPENDENT PROTEIN KINASE

Compound	Apparent K_a (M)		
	Cyclic AMP-dependent enzyme (bovine heart)	Cyclic AMP-dependent enzyme (bovine brain)	Cyclic GMP-dependent enzyme (lobster muscle)
8-Bromo cyclic AMP	1×10^{-8}	ND*	3×10^{-7}
8-Thio cyclic AMP	2×10^{-8}	5×10^{-8}	3×10^{-7}
8-Methylthio cyclic AMP	2×10^{-8}	6×10^{-8}	5×10^{-7}
8-Oxo cyclic AMP	2×10^{-8}	1×10^{-8}	2×10^{-6}
8-Amino cyclic AMP	2×10^{-8}	4×10^{-8}	2×10^{-6}
8-(β-Hydroxyethylthio) cyclic AMP	3×10^{-8}	ND	7×10^{-7}
Cyclic AMP	3×10^{-8}	1×10^{-7}	2×10^{-6}
BAPR	4×10^{-8}	ND	3×10^{-6}
8-Methylamino cyclic AMP	8×10^{-8}	2×10^{-7}	1×10^{-6}
8-Benzylamino cyclic AMP	1×10^{-7}	4×10^{-7}	2×10^{-7}
8-(β-Hydroxyethylamino) cyclic AMP	1×10^{-7}	ND	6×10^{-6}
Cyclic IMP	2×10^{-7}	2×10^{-6}	8×10^{-6}
8-Bromo cyclic IMP	2×10^{-7}	ND	2×10^{-6}
6-Benzoyl cyclic AMP	1×10^{-6}	ND	2×10^{-6}
AICAR	1×10^{-6}	ND	3×10^{-6}
8-Benzylamino cyclic IMP	1×10^{-6}	ND	3×10^{-6}
Cyclic GMP	2×10^{-6}	2×10^{-5}	5×10^{-8}
8-Bromo cyclic GMP	3×10^{-6}	ND	2×10^{-8}
8-Benzylamino cyclic GMP	2×10^{-5}	ND	2×10^{-6}
Adenosine 3′,5′-cyclothiophosphate	2×10^{-5}	ND	Not active†
N^2-2′-O-dibutyryl cyclic GMP	3×10^{-3}	ND	3×10^{-4}

* ND, not determined.

† Highest concentration tested: 1×10^{-3} M.

TABLE 4. DISSOCIATION OF BOVINE BRAIN CYCLIC AMP-DEPENDENT PROTEIN KINASE BY VARIOUS CYCLIC NUCLEOTIDES AND ANALOGS

Cyclic nucleotides and analogs (5×10^{-6} M)	Relative extent of dissociation	
	Without histone	With histone (200 μg/ml)
None	None*	Partial
Cyclic AMP	Partial†	Complete‡
8-Thio cyclic AMP	Patial	Complete
8-Oxo cyclic AMP	Partial	ND§
8-Benzyl cyclic AMP	Partial	ND
8-Amino cyclic AMP	Partial	ND
BAPR	Partial	ND
8-Methylthio cyclic AMP	Partial	ND
8-Methylamino cyclic AMP	Slight ¦	Complete
8-Benzylamino cyclic AMP	Slight	Complete
Cyclic IMP	None	ND
Cyclic GMP	None	ND
8-Bromo cyclic GMP	None	ND

* The enzyme remained and sedimented as 7·7 S holoenzyme.

† The 7·7 S component completely disappeared, accompanied by the appearance of components of intermediate molecular sizes for the free catalytic subunit of 3·6 S (see reference 15).

‡ The 7·7 S holoenzyme was completely transformed into the 3·6 S free catalytic subunit.

§ Not determined.

¦ The 7·7 S component did not completely disappear, but was accompanied by the appearance of components of intermediate molecular sizes without 3·6 S free catalytic subunit.

dent protein kinases from bovine heart and bovine brain, and of cyclic GMP- dependent protein kinase from lobster tail muscle, based upon the data shown in Tables 1 and 2 and other more detailed experiments, using some intermediate concentrations of the nucleotides, are summarized in Table 3. The K_a values for the 8-bromo, 8-thio, 8-methylthio, 8-oxo, 8-amino, 8-(β-hydroxyethylthio) and 8-methylamino analogs of cyclic AMP and BAPR were found to be comparable to those of cyclic AMP toward the bovine heart cyclic AMP-dependent enzyme. Similar results were obtained with several of the analogs tested with the cyclic AMP-dependent enzyme from bovine brain. 8-Bromo cyclic GMP was the only analog which exhibited an affinity comparable to that of cyclic GMP toward lobster muscle cyclic GMP-dependent protein kinase. It should be mentioned that 8-benzylamino cyclic AMP was more reactive than 8-benzylamino cyclic GMP in activating both classes of protein kinases (Tables 1, 2 and 3).

It has been recently shown[15,20-25] that cyclic AMP-dependent protein kinases from various tissue consisted of a regulatory subunit (which binds cyclic AMP) and a catalytic subunit, and that the mechanism by which cyclic AMP activates protein kinases involves removal of the regulatory subunit, thus yielding the active, cyclic AMP-independent form of the enzyme. It was shown earlier[15,25] that a bovine brain cyclic AMP-dependent protein kinase was dissociated into catalytically active components of different molecular sizes by histone. The effectiveness of some analogs, in the presence and absence of histone, in causing dissociation of the bovine brain cyclic AMP-dependent protein kinase was compared with cyclic AMP, and the results, in decreasing order, are presented in Table 4. As can be seen, their relative

TABLE 5. DISSOCIATION OF LOBSTER TAIL MUSCLE CYCLIC GMP-DEPENDENT PROTEIN KINASE BY CYCLIC GMP AND ANALOGS

Compound (5×10^{-6} M)	7·7 S Holoenzyme (%)	3·6 S Catalytic subunit (%)
None (control)	91	9
8-Bromo cyclic GMP	54	46
Cyclic GMP	62	38
8-Benzylamino cyclic AMP	76	24
8-Benzylamino cyclic GMP	80	20

effectiveness agreed quite well with the apparent K_a values (Table 3) for those compounds, indicating that the dissociation of the holoenzyme is a prerequisite to activation of the enzyme.

We have also shown earlier[15] that the cyclic GMP-dependent class of protein kinase, purified from lobster tail muscle, analogous to the cyclic AMP-dependent class of protein kinases from various mammalian tissues, consists of a regulatory (cyclic GMP-binding) and a catalytic subunit, and that cyclic GMP activates the enzyme by promoting dissociation of the holoenzyme into the free catalytic and regulatory subunits. Some analogs were compared with cyclic GMP for their effectiveness in causing dissociation of the lobster enzyme; the results are shown in Table 5. In the absence of the added compound, the cyclic GMP-dependent enzyme preparation used was found to consist of 91 per cent holoenzyme (7·7 S) and 9 per cent catalytic subunit (3·6 S). Cyclic GMP and its analogs, at 5×10^{-6} M, partially dissociated the enzyme. Their relative effectiveness, in decreasing order, was 8-bromo cyclic GMP > cyclic GMP > 8-benzylamino cyclic AMP > 8-benzylamino cyclic GMP, in good agreement with the data obtained for their activation of the lobster enzyme (Tables 2 and 3).

Interaction of cyclic AMP with cyclic AMP-dependent protein kinase presumably precedes its action on dissociation and concomitant activation of the enzyme; a study concerning binding of the cyclic nucleotides and their analogs to the enzyme may provide some insight into the nature of the initial step(s) involved in the dissociation and activation process. A direct measurement of the binding of the cyclic nucleotide analogs to the enzyme was not possible due to the inavailability of the radioactive preparations. A study of the analogs competing with radioactive cyclic AMP for binding to the bovine brain enzyme was thus conducted, and the results, in order of decreasing effectiveness, are shown in Table 6. It was surprising that the relative ability of the analogs to compete with cyclic AMP for binding did not parallel their ability to activate and dissociate the enzyme. Thus, the 8-amino and 8-oxo analogs of cyclic AMP, in spite of their apparent K_a values being lower than that of cyclic AMP toward the bovine brain enzyme (Table 3), were less effective than other analogs in competing with the radioactive cyclic AMP for binding to the enzyme. The 8-thio and 8-methylthio analogs of cyclic AMP, which were also more active than cyclic AMP, conversely enhanced the binding of cyclic AMP. The 8-benzylamino and 8-methylamino analogs of cyclic AMP, which were more active than cyclic IMP in stimulating and dissociating the enzyme, were found to be less effective than cyclic IMP in competing with cyclic AMP for binding. It is interesting that BAPR, which presumably has a K_a value quite similar to that for cyclic AMP, as

TABLE 6. COMPETITION BY SOME CYCLIC NUCLEOTIDES AND ANALOGS OF THE BINDING OF RADIOACTIVE CYCLIC AMP TO BOVINE BRAIN CYCLIC AMP-DEPENDENT PROTEIN KINASE

Compound	Bound ^{3}H-cyclic AMP in presence of* (% remaining)	
	2 pmoles	10 pmoles
Cyclic AMP	68	41
BAPR	74	37
Cyclic IMP	79	58
8-Amino cyclic AMP	87	80
8-Benzlamino cyclic AMP	93	84
8-Oxo cylic AMP	100	74
Cyclic GMP	99	93
8-Methylamino cyclic AMP	99	95
8-Bromo cyclic GMP	104	105
8-Benzylamino cyclic GMP	106	107
8-Thio cyclic AMP	123	117
8-Methylthio cyclic AMP	123	115

* The amount of the radioactive cyclic AMP bound to the enzyme in the absence of added nonradioactive cyclic nucleotides or analogs was 2800 cpm; this value was taken as 100 per cent binding. The values presented are means of duplicate samples, the difference between each sample being less than 3 per cent.

judged indirectly from their relative affinity toward the bovine heart enzyme (Table 3), was approximately as effective as cyclic AMP itself in competing with the binding of radioactive cyclic AMP. The 8-bromo and 8-benzylamino analogs of cyclic GMP, at the concentrations tested, like cyclic GMP itself, were not effective.

The lack of correlation between their effectiveness to compete with cyclic AMP for binding and to activate (and dissociate) the enzyme suggests a possible existence of multiple binding sites for the compounds on the enzyme. One type of binding site common to cyclic AMP may be assigned to those compounds that compete with cyclic AMP for binding, whereas another type of binding site distinguishable from that of cyclic AMP may be assigned to those that do not. The positive cooperativity observed with the 8-thio and 8-methylthio analogs on cyclic AMP binding seems to support this line of thinking. Since some compounds tested were capable of maximally activating (Tables 1 and 3) and dissociating (Table 4) the bovine enzyme, it is conceivable that interaction of the compounds with either type of binding site (which may be categorized as the cyclic AMP type and the 8-thio cyclic AMP type respectively) on the regulatory subunit could bring about full enzyme dissociation and activation. It is also possible that the compounds at higher concentrations would interact with both types of binding sites. This possibility is strengthened, at least in part, by the observation that the positive cooperativity of the 8-thio and 8-methylthio analogs for the cyclic AMP binding was reduced as the concentration of the analogs increased from 2 to 10 pmoles (Table 6).

Alternatively, all nucleotides and their analogs may share a common binding site. The discrepancy observed between the competitive binding and the enzyme dissociation (and activation) may therefore be attributable to the possibilities that the efficacy for inducing dissociation does not parallel the affinity for binding and that the rates of binding of those compounds are different.

We have reported earlier[12,13] that a heat-stable protein kinase modulator, shown to inhibit cyclic AMP-dependent protein kinase activity,[26] is also able to augment the activity of the cyclic GMP-dependent class of enzymes in the presence of an optimal concentration of cyclic GMP (but not cyclic AMP) when a specific protein, such as arginine-rich histone, was used as substrate. The analogs were tested in this system to examine the extent to which they mimicked cyclic GMP. The results are summarized in Table 7. Minimum concentrations of the individual compounds shown to give maximal or near maximal stimulation of the enzyme were used for the study. As can be seen, cyclic GMP and its analogs were all capable of supporting, in different degrees, the effect of the modulator in augmenting the activity of the cyclic GMP-dependent enzyme. Cyclic IMP and 8-bromo cyclic IMP, but not the 8-benzylamino analog, were also effective. Cyclic AMP and its analogs either could or could not support the augmenting action of the modulator. It seems that no general rules can be derived from these data allowing one to predict the properties of these analogs with respect to their effects on modulator action.

TABLE 7. COMPARATIVE EFFECT OF CYCLIC NUCLEOTIDE ANALOGS ON THE ACTIVITY OF LOBSTER TAIL MUSCLE CYCLIC GMP-DEPENDENT PROTEIN KINASE IN THE ABSENCE AND PRESENCE OF PROTEIN KINASE MODULATOR

Compound	Concn (M)	Cyclic GMP-dependent enzyme activity (units)	
		Without modulator	With modulator
None (basal)	0	6·0	5·7
Cyclic GMP	1×10^{-5}	22·0	38·0
Cyclic AMP	1×10^{-3}	20·8	17·7
Cyclic IMP	5×10^{-3}	21·5	36·2
8-Bromo cyclic GMP	1×10^{-6}	18·8	32·5
8-Benzylamino cyclic AMP	1×10^{-5}	18·5	26·1
8-Bromo cyclic AMP	1×10^{-5}	19·9	17·4
8-Thio cyclic AMP	1×10^{-5}	19·2	25·4
8-Methylthio cyclic AMP	1×10^{-5}	18·9	14·7
8-(β-Hydroxyethylthio) cyclic AMP	1×10^{-5}	21·9	15·7
8-Methylamino cyclic AMP	1×10^{-5}	19·6	19·3
8-Oxo cyclic AMP	1×10^{-4}	22·3	20·1
8-Amino cyclic AMP	1×10^{-4}	23·3	17·0
6-Benzoyl cyclic AMP	1×10^{-4}	19·3	26·5
8-Benzylamino cyclic GMP	1×10^{-4}	19·3	26·3
8-Bromo cyclic IMP	1×10^{-4}	19·1	27·8
BAPR	1×10^{-4}	19·4	23·2
AICAR	1×10^{-4}	20·6	22·5
8-Benzylamino cyclic IMP	1×10^{-4}	19·5	15·2
8-(β-Hydroxyethylamino) cyclic AMP	1×10^{-4}	21·5	16·7
N^2,2′-*O*-dibutyryl cyclic GMP	1×10^{-2}	18·5	23·2

All the compounds studied in Table 7 were also examined for their effect on the action of modulator on cyclic AMP-dependent protein kinases prepared from either bovine heart or bovine brain using the same protein (arginine-rich histone) as substrate. In all cases the modulator inhibited the activity of cyclic AMP-dependent enzymes stimulated by any of the compounds.

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