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EFFECTS OF DIVALENT METALS ON THE SPECIFICITY OF INHIBITORS OF THE CYCLIC NUCLEOTIDE PHOSPHODIESTERASES FROM BOVINE HEART

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Summary

Divalent metals used to support phosphodiesterase (EC 3.1.4.-) activity have been found to influence the substrate and enzyme specificity of many phosphodiesterase inhibitors in studies of the hydrolysis of cyclic AMP and cyclic GMP by the calmodulin-dependent and cyclic AMP-specific phosphodiesterases from bovine heart. Many compounds displayed marked differences in substrate specificity and inhibitory potency in the presence of Mg^{2+}, as compared with Mn^{2+}, when studied with the unactivated form of calmodulin-dependent phosphodiesterase, while few compounds displayed differences in the presence of calmodulin. With a single divalent metal, marked differences in inhibitory potency and substrate specificity were also observed in the absence or presence of calmodulin suggesting that alterations in calmodulin and/or Ca^{2+} levels may greatly affect the response to phosphodiesterase inhibitors. Divalent metals did not alter the effects of inhibitors on the hydrolysis of cyclic AMP by the cyclic AMP-specific phosphodiesterase, however divalent metals would probably indirectly influence the relative cellular level of cyclic AMP hydrolyzed by this enzyme, and therefore the effects of inhibitors, through metal effects on the calmodulin-dependent phosphodiesterase. No correlation was found between the inhibitory activity of the compounds, many of which were cyclic nucleotide analogs, and their ability to activate cyclic AMP-dependent or cyclic GMP-dependent protein kinases or to affect cyclic AMP-dependent protein kinase activity by displacing bound cyclic AMP.

Introduction

We have previously reported [1,2] that the substrate specificity of the calmodulin-dependent cyclic nucleotide phosphodiesterase (EC 3.1.4.-) from bovine heart depends on the divalent metals used to support enzyme activity, both in the absence and presence of calmodulin. The enzyme preferentially hydrolyzed cyclic GMP in the presence of Mg^{2+}, while approximately equal amounts of cyclic GMP and cyclic AMP were hydrolyzed in the presence of Mn^{2+}, Zn^{2+}, Co^{2+} or Ni^{2+}. Marked differences in enzyme activity and substrate specificity were also observed with combinations of Mg^{2+} and other divalent metals [2]. Human heart has been reported by Tipton and Cooke [3] to contain, in total, 7.8 mM Mg^{2+}, 1.2 mM Ca^{2+}, 1 mM Fe^{2+}, 0.5 mM Zn^{2+}, 60 μM Cu^{2+}, 4.6 μM Mn^{2+}, 1 μM Ni^{2+} and 1 μM Co^{2+}. However the amount of each divalent metal that would be available for interaction with the enzyme is unknown. It is therefore apparent that the use of a single divalent metal to monitor the properties of the enzyme in vitro is not entirely adequate to describe the properties of the enzyme in vivo.

The purpose of this study is to determine whether the differing effects of various divalent metals on phosphodiesterase activity also influence the substrate or enzyme specificity of various phosphodiesterase inhibitors. Many compounds have previously been studied for their inhibitory effects on crude and purified cyclic nucleotide phosphodiesterase preparations from various sources in the presence of 1—5 mM Mg^{2+} [4—10]. These studies have not clearly demonstrated agents which specifically inhibit the hydrolysis of a single cyclic nucleotide. In this study we have examined cyclic nucleotide analogs on cyclic nucleotide phosphodiesterases purified from bovine heart. We have arbitrarily studied these compounds in the presence of 5 mM Mg^{2+} or 1 mM Mn^{2+}, as the calmodulin-dependent phosphodiesterase from bovine heart is relatively cyclic GMP-specific with Mg^{2+} but somewhat cyclic AMP-specific with Mn^{2+} [1]. If differences in the inhibitory effects of these compounds are observed in the presence of these divalent metals it would suggest that in vitro assays may not be adequate to indicate the in vivo specificity of these compounds, unless a more physiological mixture of divalent metals is used.

As many of the phosphodiesterase inhibitors examined are cyclic nucleotide analogs they may be pharmacologically less or more active than expected, depending on whether they are hydrolyzed by phosphodiesterase or whether they also inhibit or activate cyclic nucleotide-dependent protein kinases. The cyclic nucleotide analogs examined have previously been studied as substrates of cyclic nucleotide phosphodiesterase with most being resistant to phosphodiesterase activity [6—8]. Their abilities to activate mammalian cyclic AMP-dependent and cyclic GMP-dependent protein kinase and also to displace AMP bound to cyclic AMP-dependent protein kinase have been examined in this study.

Materials and Methods

Cyclic AMP, cyclic GMP and *Crotalus atrox* venom were purchased from Sigma. Cyclic[^{3}H]AMP, cyclic[^{3}H]GMP and [γ-^{32}P]ATP were from New Eng-

land Nuclear. Phosphodiesterase activity was assayed by a procedure adapted from Russell et al. [11]. An appropriate dilution of enzyme was incubated in 40 mM Tris-HCl, pH 7.4 and 5 mM $MgCl_2$ containing $1 \cdot 10^{-8}$ to $3 \cdot 10^{-8}$ M cyclic[^{3}H]AMP or cyclic[^{3}H]GMP (50 000—100000 cpm) in a total volume of 1 ml. When higher concentrations of cyclic nucleotides (usually $1 \cdot 10^{-6}$ M cyclic AMP or cyclic GMP) were required, the indicated amounts of cyclic nucleotides were included. Other additions to or alterations of this assay procedure are as indicated in the legends to the Figures and Tables. After 10 min at 30°C, the reaction was terminated by boiling for 3 min. After cooling in ice for 15 min, 0.1 ml of *C. atrox* venom (1 mg/ml in H_2O) was added to each sample and incubated for 30 min at 30°C. The reaction was terminated by boiling for 3 min. Following cooling in ice, undegraded cyclic nucleotides were removed by centrifugation at $1200 \times g$ for 5 min following the addition of 1.0 ml of Dowex 1 X8 ion-exchange resin (1 : 2 slurry in H_2O). [^{3}H]Adenosine or [^{3}H]-guanosine in the supernatant were detected by liquid scintillation spectrometry. Corrections were made for the binding of approx. 20—30% of the [^{3}H]-adenosine or [^{3}H]guanosine to the ion-exchange resin. The amount of phosphodiesterase used was adjusted so that no more than 15% of the cyclic nucleotide was hydrolyzed during the incubation.

Calmodulin-dependent cyclic nucleotide phosphodiesterase from bovine heart was prepared through the step of rechromatography on DEAE-cellulose as described previously [1]. The active peak was pooled and concentrated using a PM-10 membrane in an Amicon Ultrafiltration chamber. This enzyme preparation was found to be free of calmodulin as described previously [1]. The concentrated enzyme preparation (20 ml) was then dialyzed against 8 l of 1 mM ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetracetic acid (EGTA), pH 7.4/10 mM potassium phosphate buffer, pH 7.4 for 8 h at 4°C and then dialyzed against 8 l of 20 mM potassium phosphate buffer, pH 7.4.

The cyclic AMP-specific phosphodiesterase from bovine heart was obtained as the third peak of phosphodiesterase activity eluting from a column of DEAE-cellulose, as described previously [12]. The peak fractions were pooled and dialyzed extensively against 15 l of 10 mM potassium phosphate buffer, pH 7.4/2 mM 2-mercaptoethanol. The dialysate was rechromatographed by applying it to a column of DEAE-cellulose (4 × 24 cm) which had been equilibrated with the homogenization buffer. The loaded column was eluted with a 1.0 l gradient of 150—400 mM potassium phosphate buffer, pH 7.4/2 mM 2-mercaptoethanol. Fractions of approx. 10 ml were collected and 10-μl aliquots were removed for assay with $1 \cdot 10^{-6}$ M cyclic AMP or cyclic GMP as substrate in the standard procedure. The active fractions were pooled, concentrated using a PM-10 membrane in an Amicon ultrafiltration chamber and dialyzed. This enzyme preparation was free of cyclic GMP phosphodiesterase activity.

Calmodulin was prepared from bovine liver as described previously [12]. Bound Ca^{2+} was removed from this preparation by dialysis against 1 mM EGTA, pH 7.4/20 mM potassium phosphate buffer, pH 7.4, followed by dialysis against 20 mM potassium phosphate buffer, pH 7.4.

Cyclic AMP-dependent protein kinase (type II) was isolated from bovine heart by DEAE-cellulose chromatography as described previously [13]. Cyclic

GMP-dependent protein kinase was isolated from bovine lung as described previously for the enzyme from rat liver [14].

Protein kinase activity was measured by the method of Kuo et al. [15]. The standard assay contained 50 mM potassium phosphate, pH 7.0/5 mM $MgCl_2$/40 μg arginine-rich histone/5 μM [γ-^{32}P]ATP (0.15 μCi), with the appropriate amounts of protein kinase, protein kinase modulator or cyclic nucleotide as indicated, in a final volume of 0.2 ml. The samples were incubated at 30°C for 5 min, the reaction stopped with 20% trichloroacetic acid and the samples processed, as described previously [15], with 20% trichloroacetic acid.

Cyclic AMP binding was assayed by a modification of the method of Gilman [16] as described previously [17]. The binding assay contained, in a final volume of 200 μl, 20 mM potassium phosphate buffer, pH 7.0/0.2 mM Na · EDTA/25 mM 2-mercaptoethanol/20% glycerol/0.1 μM cyclic[^{3}H]AMP. After 1 h at 0°C, the bound cyclic nucleotide was determined by millipore filtration as described previously [17].

Results

The influence of Mg^{2+} (250 μM and 5 mM) and Mn^{2+} (1 mM) on the kinetic parameters of the calmodulin-dependent phosphodiesterase from bovine heart are listed in Table I. In the absence of calmodulin, marked increases in the V value for cyclic AMP hydrolysis were observed with Mn^{2+}, alone or with Mg^{2+}, as compared with Mg^{2+}, while small decreases were noted for cyclic GMP hydrolysis. In the presence of calmodulin, Mn^{2+} alone or with Mg^{2+} produced a marked decrease in the V value for cyclic GMP hydrolysis with little change in the V for cyclic AMP hydrolysis. In the absence of calmodulin, two $K_{m,app}$ values for cyclic GMP were observed in the presence of Mn^{2+}, alone or with Mg^{2+}, as was previously noted with 5 mM Mg^{2+} alone [1]. Mn^{2+} alone or with Mg^{2+} produced little change in the $K_{m,app}$ values for cyclic GMP or cyclic AMP in the absence or presence of calmodulin.

TABLE I

The influence of Mg^{2+} and/or Mn^{2+} on the K_m (μM) and V (pmol/mg per min) values for the hydrolysis of cyclic AMP and cyclic GMP by the calmodulin-dependent phosphodiesterase from bovine heart. The kinetic parameters were determined by Eadie-Scatchard plots [21]. cAMP, cyclic AMP; cGMP, cyclic GMP.

Divalent metal	Minus calmodulin				Plus calmodulin			
	cGMP		cAMP		cGMP		cAMP	
	K_m	V	K_m	V	K_m	V	K_m	V
(A) Mg^{2+}, 5 mM	1.0, 15 *	7200	35	23 200	3.0	12 510	35	32 610
Mn^{2+}, 1 mM	0.6, 15	6860	35	25 470	1.8	7850	35	30 750
Mg^{2+} plus Mn^{2+}	0.6, 15	6751	35	47 450	3.0	7960	35	31 540
(B) Mg^{2+}, 250 μM	2.0, 17	7450	33	18 740	3.0	11 740	31	30 570
Mn^{2+}, 1 mM	0.6, 15	6940	34	24 080	1.3	6120	33	29 300
Mg^{2+} plus Mn^{2+}	1.5, 17	7280	33	31 450	2.0	6890	34	29 780

* Two $K_{m,app}$ values were noted as described previously in the presence of 5 mM Mg^{2+} [1].

TABLE II

The influence of 5 mM Mg^{2+} and 1 mM Mg^{2+} on the inhibitory potency (K_i, determined by Dixon plots [23]) of various commonly used phosphodiesterase inhibitors in the hydrolysis of cyclic AMP and cyclic GMP by calmodulin-dependent phosphodiesterase in the absence or presence of calmodulin (4 μg). cAMP, cyclic AMP; cGMP, cyclic GMP.

Drug	Minus calmodulin						Plus calmodulin					
	5 mM Mg^{2+}			1 mM Mn^{2+}			5 mM Mg^{2+}			1 mM Mn^{2+}		
	cAMP (μM)	cGMP (μM)	$\frac{cAMP}{cGMP}$	cAMP (μM)	cGMP (μM)	$\frac{cAMP}{cGMP}$	cAMP (μM)	cGMP (μM)	$\frac{cAMP}{cGMP}$	cAMP (μM)	cGMP (μM)	$\frac{cAMP}{cGMP}$
3-Isobutyl-1-methylxanthine	3	3	1.0	3	11	0.3	3	6	0.5	4	8	0.5
Theophylline	345	185	1.9	210	180	1.2	120	250	0.5	125	420	0.3
Papaverine	38	33	1.1	18	30	0.6	18	14	1.3	28	28	1.0
Fluphenazine	100	124	0.8	95	120	0.8	18	20	0.9	35	27	1.3

TABLE III

The influence of 5 mM Mg^{2+} and 1 mM Mn^{2+} on the inhibitory potency (I_{50}) of various cyclic nucleotide analogs in the hydrolysis of $1 \cdot 10^{-6}$ M cyclic GMP and cyclic AMP by calmodulin-dependent phosphodiesterase in the absence and presence of calmodulin. The concentrations of the various inhibitory compounds ranged from 10—500 μM with all samples also containing 10 μM $CaCl_2$. All assays were performed in triplicate and the means of three separate experiments (I_{50}, μM) are presented ± S.E. cAMP, cyclic AMP; cGMP, cyclic GMP.

	Minus calmodulin				Plus calmodulin			
	5 mM Mg^{2+}		1 mM Mn^{2+}		5 mM Mg^{2+}		1 mM Mn^{2+}	
	cGMP	cAMP	cGMP	cAMP	cGMP	cAMP	cGMP	cAMP
Cyclic GMP	16 ± 8	<10	<10	15 ± 1	<10	<10	<10	<10
Cyclic IMP	20 ± 3	10 ± 1	<10	<10	18 ± 2	15 ± 3	<10	<10
2′-Deoxy cyclic GMP	35 ± 2	220 ± 10	30 ± 2	80 ± 4	20 ± 3	16 ± 2	<10	<10
8-Bromo cyclic GMP	75 ± 4	100 ± 4	60 ± 3	130 ± 5	30 ± 4	20 ± 2	25 ± 1	25 ± 2
8-Isopropylthio cyclic AMP	80 ± 6	<10	70 ± 3	140 ± 8	30 ± 4	25 ± 3	35 ± 2	25 ± 2
8-Methylthio cyclic AMP	170 ± 5	110 ± 4	75 ± 3	50 ± 2	32 ± 4	20 ± 1	40 ± 3	45 ± 2
8-*p*-Chlorophenylthio cyclic GMP	120 ± 9	320 ± 22	210 ± 8	230 ± 8	45 ± 3	40 ± 2	45 ± 2	55 ± 2
8-Benzylthio cyclic AMP	33 ± 2	50 ± 2	30 ± 3	50 ± 3	50 ± 3	18 ± 1	55 ± 3	25 ± 2
Cyclic AMP	175 ± 6	170 ± 7	45 ± 4	70 ± 5	100 ± 5	40 ± 2	220 ± 9	45 ± 3
8-Methylamino cyclic AMP	>500	>500	250 ± 18	190 ± 7	140 ± 5	200 ± 11	240 ± 11	45 ± 3
8-Bromo cyclic AMP	>500	>500	130 ± 7	160 ± 7	250 ± 9	400 ± 28	280 ± 12	50 ± 2
2′-Deoxy cyclic AMP	160 ± 11	50 ± 2	>500	90 ± 6	270 ± 13	140 ± 8	320 ± 17	>500
Cyclic CMP	>500	>500	>500	>500	>500	>500	180 ± 10	130 ± 5

The K_i values of several commonly used phosphodiesterase inhibitors were determined in the hydrolysis of cyclic GMP and cyclic AMP by the calmodulin-dependent phosphodiesterase, in the presence of 5 mM Mg^{2+} or 1 mM Mn^{2+} (Table II). In the absence of calmodulin, the K_i values of theophylline and papaverine were different with cyclic AMP as substrate in the presence of Mg^{2+} as compared with Mn^{2+}, while with cyclic GMP as substrate the only changes occurred in the K_i values of 3-isobutyl-1-methylxanthine. In the presence of calmodulin, the K_i values of papaverine and fluphenazine were different with cyclic AMP as substrate in the presence of the two metals, while with cyclic GMP as substrate changes occurred with the K_i values of theophylline, papaverine and fluphenazine. The ratio of K_i values obtained with cyclic AMP and cyclic GMP is an indicator of the substrate specificity of the inhibitor. Marked changes in the ratio were observed with 3-isobutyl-1-methylxanthine, theophylline and papaverine in the absence of calmodulin, as the agents were more specific inhibitors of cyclic AMP hydrolysis in the presence of Mn^{2+}. Lesser changes were observed with all compounds in the presence of calmodulin.

The influence of Mg^{2+} and Mn^{2+} on the inhibitory potency (I_{50}) of various cyclic nucleotide analogs was also examined with the calmodulin-dependent phosphodiesterase (Table III). In the absence of calmodulin, the substrate specificity of several compounds varied markedly with the divalent metals. For example, 8-isopropylthio cyclic AMP was more than 8-fold more potent in inhibiting the hydrolysis of cyclic AMP than cyclic GMP in the presence of Mg^{2+}, while it was 2-fold more potent in inhibiting the hydrolysis of cyclic GMP than cyclic AMP with Mn^{2+}. 8-*p*-Chlorophenylthio cyclic GMP was 3-fold more potent in inhibiting the hydrolysis of cyclic GMP than cyclic AMP in the presence of Mg^{2+}, while it was equipotent in inhibiting the hydrolysis of cyclic GMP and cyclic AMP with Mn^{2+}. 2′-Deoxy cyclic GMP, 8-bromo cyclic GMP and 8-benzylthio cyclic AMP were more specific for the inhibition of cyclic GMP hydrolysis than cyclic AMP in the presence of both Mg^{2+} and Mn^{2+}. Cyclic AMP and 8-methylthio cyclic AMP were more effective in inhibiting the hydrolysis of both cyclic AMP and cyclic GMP in the presence of Mn^{2+} than Mg^{2+}.

In the presence of calmodulin, less marked changes in inhibitory substrate specificity were observed with Mg^{2+} and Mn^{2+} (Table III). 8-Methylamino cyclic AMP and 8-bromo cyclic AMP were rather poor inhibitors of cyclic AMP and cyclic GMP hydrolysis (I_{50}, 100 μM) in the presence of Mg^{2+}, while they were more effective on cyclic AMP hydrolysis (I_{50}, 45—50 μM) in the presence of Mn^{2+}. 8-Benzylthio cyclic AMP and cyclic AMP were the only compounds which demonstrated marked substrate specificity in the presence of both Mg^{2+} and Mn^{2+} as they rather selectively inhibited cyclic AMP hydrolysis.

The effects of the inhibitors were also examined with the cyclic AMP-specific phosphodiesterase from bovine heart. This enzyme was purified by rechromatography on DEAE-cellulose (see Materials and Methods) and was found to be free of cyclic GMP hydrolytic activity in the standard assay using $1 \cdot 10^{-6}$ M cyclic[^{3}H]GMP as substrate. As previously shown in rat liver [11], kidney [18], pancreas [19] and human lymphocytes and monocytes [20], the enzyme displays non-linear kinetics in the hydrolysis of cyclic AMP. An Eadie-Scatchard plot [21] of enzyme activity against a wide-range of cyclic AMP concen-

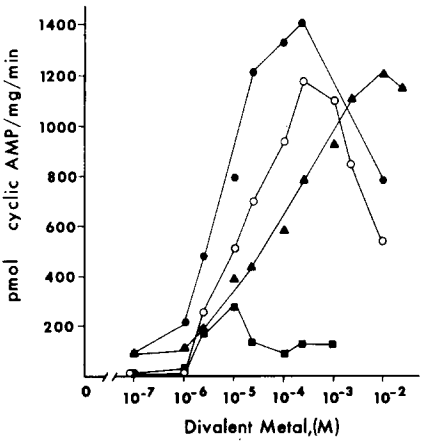

Fig. 1. Effects of varying concentrations of divalent metals on the hydrolysis of $1 \cdot 10^{-6}$ M cyclic AMP by the cyclic AMP-specific phosphodiesterase from bovine heart. The divalent metals Mg^{2+} (▲———▲), Mn^{2+} (●———●), Co^{2+} (○———○) and Zn^{2+} (■———■) were studied.

trations ($1 \cdot 10^{-7}$—$1 \cdot 10^{-4}$ M) indicated two $K_{m,app}$ values (0.9 and 38 μM) for cyclic AMP. The enzyme displayed no activity in the absence of divalent metal while maximal activity was observed in the presence of 0.5 mM Mn^{2+} (Fig. 1). Co^{2+} and Mg^{2+} were somewhat less effective in supporting enzyme activity while comparatively little activity was observed with Zn^{2+}. No differences were observed in the substrate specificity of the enzyme as only cyclic AMP was hydrolyzed in the presence of the various divalent metals. In contrast to the calmodulin-dependent phosphodiesterase, divalent metals did not influence the inhibitory potencies of the various compounds in the inhibition of the cyclic AMP-specific phosphodiesterase (Tables IV, V).

The $K_{a,app}$ value of cyclic GMP-dependent and cyclic AMP-dependent protein kinases was studied with the cyclic nucleotide analogs, as well as the ability of the analogs to displace cyclic AMP bound to cyclic AMP-dependent protein kinase (Table VI). As expected, cyclic GMP and its analogs 8-bromo cyclic GMP and 8-*p*-chlorophenylthio cyclic GMP were the most potent activators of bovine lung cyclic GMP-dependent protein kinase. However 2′-deoxy cyclic GMP was about 400-times less effective than cyclic GMP. Cyclic AMP and its analogs, 8-isopropylthio cyclic AMP, 8-methylthio cyclic AMP, 8-benzylthio cyclic AMP, 8-methylamino cyclic AMP and 8-bromo cyclic AMP were the most

TABLE IV

The influence of 5 mM Mg^{2+} and 1 mM Mn^{2+} on the inhibitory potency (K_i, determined by Dixon plots [23]) of various phosphodiesterase inhibitors in the hydrolysis of cyclic AMP and cyclic GMP by the low K_m cyclic AMP-specific phosphodiesterase from bovine heart.

Compound	Mg^{2+} (μM)	Mn^{2+} (μM)
3-Isobutyl-1-methylxanthine	1.2	1.5
Theophylline	105	98
Papaverine	1	1
Fluphenazine	140	150

TABLE V

The influence of 5 mM Mg^{2+} and 1 mM Mn^{2+} on the inhibitory potency (I_{50}) of various cyclic nucleotide analogs in the hydrolysis of $1 \cdot 10^{-6}$ M cyclic AMP by the low K_m cyclic AMP-specific phosphodiesterase from bovine heart. The concentrations of the various inhibitory compounds ranged from 10—500 μM with all samples also containing 10 μM $CaCl_2$. All assays were performed in triplicate and the means of three separate experiments (I_{50}, μM) are presented ± S.E.

Compound	Mg^{2+}	Mn^{2+}
Cyclic GMP	<10	<10
Cyclic IMP	<10	<10
2′-Deoxy cyclic GMP	<10	<10
8-Bromo cyclic GMP	10 ± 1	12 ± 1
8-Isopropylthio cyclic AMP	25 ± 2	22 ± 1
8-Methylthio cyclic AMP	65 ± 7	71 ± 6
8-*p*-Chlorophenylthio cyclic AMP	215 ± 13	170 ± 9
8-Benzylthio cyclic AMP	35 ± 2	35 ± 2
Cyclic AMP	<10	<10
8-Methylamino cyclic AMP	100 ± 9	140 ± 8
8-Bromo cyclic AMP	25 ± 2	16 ± 1
2′-Deoxy cyclic AMP	<10	16 ± 1
Cyclic CMP	>500	>500

potent activators of bovine heart cyclic AMP-dependent protein kinase. 2′-Deoxy cyclic AMP did not activate the enzyme in the concentration range studied. Cyclic AMP and its analogs most effectively displaced cyclic AMP bound to cyclic AMP-dependent protein kinase. The I_{50} of the compounds in displacing bound cyclic AMP did not correlate with the $K_{a,app}$ value of the cyclic nucleotides with cyclic AMP-dependent protein kinase. For example, the ratio of these parameters was less than 30 with cyclic AMP but over 1500 and 1800 with 8-isopropylthio cyclic AMP and 8-benzylthio cyclic AMP, respec-

TABLE VI

$K_{a,app}$ values of cyclic GMP-dependent protein kinase (cG-PK) from bovine lung and type II cyclic AMP-dependent protein kinase (cA-PK) from bovine heart for various cyclic nucleotides and their analogues, and the concentrations of these nucleotides necessary to displace 50% of cyclic AMP bound to cyclic AMP-dependent protein kinase from bovine heart as described in Materials and Methods. cAMP, cyclic AMP.

Compound	$K_{a,app}$ cG-PK (μM)	$K_{a,app}$ cA-PK (μM)	I_{50} cAMP (μM)
Cyclic GMP	0.012	1.8	200
Cyclic IMP	1.3	0.2	5
2′-Deoxy cyclic GMP	5.0	>10.0	>500
8-Bromo cyclic GMP	0.005	3.0	>500
8-Isopropylthio cyclic AMP	0.500	0.013	20
8-Methylthio cyclic AMP	0.35	0.013	3
8-*p*-Chlorophenylthio cyclic GMP	0.027	0.35	300
8-Benzylthio cyclic AMP	0.320	0.011	20
Cyclic AMP	1.00	0.030	<1
8-Methylamino cyclic AMP	0.37	0.037	6
8-Bromo cyclic AMP	0.8	0.030	4
2′-Deoxy cyclic AMP	>10.0	>10.0	>500
Cyclic CMP	3.0	2.2	50

tively, although all three compounds were similarly potent in activating cyclic AMP-dependent protein kinase (Table VI). Theophylline, papaverine, 3-isobutyl-1-methylxanthine and fluphenazine in a concentration range from 0.1—500 μM did not alter the activity of cyclic AMP-dependent or cyclic GMP-dependent protein kinase nor did they alter the binding of cyclic AMP to cyclic AMP-dependent protein kinase (data not shown).

Discussion

In order to pharmacologically affect a parameter dependent on a single cyclic nucleotide it appears that phosphodiesterase inhibitors must specifically alter the hydrolysis of a single cyclic nucleotide or alter the activity of a single type of phosphodiesterase. Agents could act directly on the phosphodiesterases to compete with the substrates, or act by altering the activity of various factors which regulate phosphodiesterase activity (e.g. calmodulin, calmodulin-binding protein, cyclic AMP-dependent protein kinase, divalent metals, alternate substrates, Ca^{2+} and other possible factors). In addition the subcellular distribution of the enzymes differ as the low K_m cyclic AMP-specific phosphodiesterase is predominantly a membrane-bound enzyme, while the calmodulin-dependent phosphodiesterase is a soluble enzyme [22]. Thus, an inhibitor could localize in one subcellular compartment and thereby cause a specific effect on a single enzyme.

In bovine heart, cyclic GMP is hydrolyzed only by the calmodulin-dependent phosphodiesterase while cyclic AMP is hydrolyzed by both the calmodulin-dependent and the low K_m cyclic AMP-specific phosphodiesterase [13]. DEAE-cellulose chromatography of the soluble and particulate phosphodiesterases in bovine heart indicated that with 1 μM cyclic AMP as substrate, 25% of the cyclic AMP hydrolytic activity is contributed by the calmodulin-dependent phosphodiesterase in the absence of calmodulin, and 75% in the presence of calmodulin [13]. Thus, in the presence of optimal levels of Ca^{2+} and calmodulin, approx. 75% of the cyclic AMP and 100% of cyclic GMP would be hydrolyzed by the calmodulin-dependent phosphodiesterase.

Previously drugs have been routinely examined as possible phosphodiesterase inhibitors in assay systems containing 1—5 mM Mg^{2+} to satisfy the divalent metal requirements of the enzyme. However, the ionic environment of phosphodiesterase in vivo undoubtedly contains Mg^{2+} together with other divalent metals. The in vitro results presented here demonstrate that divalent metals influence the substrate specificity and potency of inhibitors of the calmodulin-dependent phosphodiesterase. More marked changes were observed in the absence of calmodulin than in its presence which suggests that Ca^{2+}, due to its requirement in calmodulin activation of the enzyme, and calmodulin could affect the substrate specificity of the inhibitors. It is, therefore, suggested that the routine study of compounds as inhibitors of calmodulin-dependent phosphodiesterase should include an examination of the inhibitory properties of the compounds in the presence of various divalent metals, as this might more accurately reflect the in vivo activity of the compound.

Many cyclic nucleotide analogs were shown to be potent inhibitors of the hydrolysis of cyclic AMP and cyclic GMP. However, if the intention of the

development of phosphodiesterase inhibitors is to develop compounds which might specifically increase a cyclic AMP-dependent or cyclic GMP-dependent response then their susceptibility to hydrolysis by cyclic nucleotide phosphodiesterases and their effects on cyclic nucleotide-dependent protein kinases must also be considered. Miller et al. [6—8] have examined all these compounds except cyclic CMP as substrates for crude phosphodiesterase preparations from rabbit lung and bovine heart. The 8-substituted analogs were quite resistant to hydrolysis under the conditions used, while the other compounds were hydrolyzed to varying extents. A specific cyclic CMP phosphodiesterase has recently been noted in several mammalian tissues [24]. Miller et al. [6—8] also tested these compounds as inhibitors of the hydrolysis of cyclic AMP and cyclic GMP by the same phosphodiesterase preparations. The results were not comparable to those in the present study due to the differences in enzyme purity and the lack of added Ca^{2+} or calmodulin in the assay incubation mixtures. In order to obtain a cyclic AMP-dependent response the most desirable compounds would be those which specifically inhibit the hydrolysis of cyclic AMP, which are not hydrolyzed by phosphodiesterase, and which specifically increase the activity of cyclic AMP-dependent protein kinase in a single tissue. No such compounds were found in this study. Another choice would be compounds which specifically alter phosphodiesterase activity and have no effect on protein kinase activity. Although several compounds were observed which did not alter protein kinase activity over the range examined, none of the compounds were specific inhibitors of the hydrolysis of a single cyclic nucleotide with the phosphodiesterases isolated from bovine heart. 2′-Deoxy cyclic GMP was one of the most promising compounds as it did not significantly activate cyclic AMP-dependent or cyclic GMP-dependent protein kinase in the levels studied, but in the absence of calmodulin it was 3—6-fold more potent inhibiting cyclic GMP hydrolysis than cyclic AMP hydrolysis. However, in the presence of calmodulin, 2′-deoxy cyclic GMP demonstrated no significant differences in I_{50} values with cyclic GMP and cyclic AMP. The compound was also a potent inhibitor of cyclic AMP hydrolysis by the low K_m cyclic AMP-specific phosphodiesterase. However, Davis and Kuo [9] found that the compound showed more nucleotide and enzyme specificity with the phosphodiesterases purified from guinea pig lung. 2′-Deoxy cyclic GMP inhibited cyclic GMP hydrolysis by the Ca^{2+}-independent cyclic GMP-specific phosphodiesterase present in that tissue with an I_{50} value of 4.5 μM, while it had no detectable effect on the hydrolysis of cyclic AMP by the cyclic AMP-specific phosphodiesterase. Thus, depending on the nature of the phosphodiesterases present in a particular tissue, 2′-deoxy cyclic GMP may be a relatively specific inhibitor of cyclic GMP hydrolysis.

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