Biochemical Pharmacology, Vol. 44, No. 12, pp. 2303–2306, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

8-(4-CHLOROPHENYL)THIO-CYCLIC AMP IS A POTENT INHIBITOR OF THE CYCLIC GMP-SPECIFIC PHOSPHODIESTERASE (PDE V_A)

BRENDAN J. CONNOLLY, P. BARNABY WILLITS, BRIAN H. WARRINGTON and KENNETH J. MURRAY*

SmithKline Beecham Pharmaceuticals, The Frythe, Welwyn, Herts AL6 9AR, U.K.

(*Received* 28 *July* 1992; *accepted* 22 *September* 1992)

Abstract—8-(4-Chlorophenyl)thio-cyclic AMP (8-CPT-cAMP), extensively used as selective activator of cyclic AMP-dependent protein kinase, has been found to be a potent inhibitor of the cyclic GMP-specific phosphodiesterase (PDE V_A). Indeed, 8-CPT-cAMP (IC_{50} = 0.9 μM) inhibited PDE V_A with a potency identical to that of zaprinast. 8-CPT-cAMP was also metabolized by PDE V_A at a rate half that of cyclic GMP. The cyclic GMP-inhibited phosphodiesterase (PDE III) (IC_{50} = 24 μM) and the cyclic AMP-specific phosphodiesterase (PDE IV) (IC_{50} = 25 μM) were also inhibited by 8-CPT-cAMP. In contrast, most of the other cAMP-derivative studies showed little inhibition of any phosphodiesterase isoenzyme. These observations provide further reasons why the mechanism of the physiological effects of 8-CPT-cAMP should be interpreted with caution.

Derivatives of cyclic AMP (cAMP†) (see Fig. 1) are widely used to investigate the role of cAMP in physiological processes [1–3]. It is usually assumed that the effects of cAMP derivatives are due to activation of cAMP-dependent protein kinase (cAMP-PrK) and the interaction of derivatives with purified cAMP-PrK is well documented [1]. However, it has long been known that certain cAMP derivatives also inhibit cyclic nucleotide phosphodiesterases (PDEs) [4] but many of these reports were completed before the current definition of the five isoenzyme families of PDEs (see Table 1 and Ref. 5 for nomenclature). Consequently, many of these early studies were unknowingly conducted on more than one form of PDE and information on the inhibition of specific PDE isoenzymes is largely limited to studies of PDE II (Ref. 6), which is more readily isolated in a pure form. The isoenzyme selectivity of cAMP derivatives as PDE inhibitors is poorly documented and in particular their effects on the cGMP-specific PDE (PDE V_A‡; also known as cGMP-binding PDE [7]) are not known. In this report the effects of a number of cAMP derivatives on five PDE isoenzymes are shown and it is demonstrated that the cAMP derivative 8-CPT-cAMP, commonly used as a selective cAMP-PrK activator, is a potent inhibitor of PDE V_A.

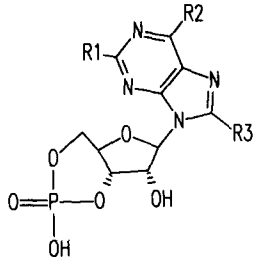

Fig. 1. General structure of cyclic nucleotide derivatives. Substituents R1, R2 and R3 are given in Table 1.

MATERIALS AND METHODS

PDE isoenzymes were partially purified as described previously [8]. No cross contamination was apparent as judged by the fact that all the preparations showed simple Michaelis–Menten kinetics (except PDE II which displayed positive cooperativity [9]) and that the PDEs also responded to physiological and pharmacological modulators in a predictable manner. PDE activity was assayed by the boronate column method as described previously [10]. The IC_{50} values for cAMP derivatives shown in Table 1 were determined by varying the concentration of derivative and using 1 μM [^{3}H]cGMP (37 MBq/mmol) as a substrate for PDE I (in the absence of Ca^{2+} and calmodulin), PDE II and PDE V and with 1 μM [^{3}H]cAMP (37 MBq/mmol) as a substrate for PDE III and PDE IV. Concentration–inhibition curves were fitted to the logistic equation using the program, ALLFIT [11].

The K_m values for cAMP derivatives (K_m^D, shown in Table 1) were determined in a similar manner but using 0.05 μM [^{3}H]cGMP of a high specific activity (596 GBq/mmol) as substrate. This concentration is well below the K_m for cGMP itself (K_m^G) so that

* Corresponding author. Tel. (0438) 782034; FAX (0438) 782580.

† Abbreviations: PDE, cyclic nucleotide phosphodiesterase; CPT, 4-chlorophenylthio; c(G)AMP, cyclic (G)AMP; cAMP-PrK, cAMP-dependent protein kinase; MR, molar refractivity; π, fragmental hydrophobic constant.

‡ The nomenclature is that proposed by Beavo and Reifsnyder [5] in which PDE V refers generally to cGMP-specific family and PDE V_A is the enzyme found in non-retinal tissues.

Table 1. Inhibition of cyclic nucleotide phosphodiesterases by cyclic nucleotide derivatives

No.	Structure R1	R2	R3	PDE inhibition: IC_{50} (bold) or % at 100 μM isoenzyme I	II	III	IV	V	PDE V_A K_m^D (μM)	MR	π	Ref.
I	H	NH_2	S-(*p*-Cl-C_6H_5)	59%	48%	**25**	**24**	**0.9**	0.4	39	3.4	21
II	H	NH_2	SCH_3	33%	25%	**70**	43%	42%	116	13.8	0.6	19
III	H	NH_2	SC_2H_5	54%	31%	**28**	**49**	**11**	5	21.7	1.28	19
IV	H	NH_2	Br	27%	15%	**14**	**93**	26%	128	8.9	0.86	19
V	H	NH_2	N_3	32%	3%	48%	45%	21%	153	10.2	0.46	19
VI	H	NH_2	$N(CH_3)_2$	12%	5%	0%	26%	2%	1995	15.6	0.18	19
VII	H	NH_2	$NHCH_2C_6H_5$	**73**	6%	3%	27%	50%	57	35	1.1	21
VIII	H	NH_2	SCH_2CH_2OH	24%	**67**	**18**	**48**	**57**	20	20	−0.29	21
IX	H	NH_2	*N*-Piperidino	33%	13%	43%	**38**	35%	105	26	0.85	19
X	H	NH_2	$NH(CH_2)_5NH_2$	13%	15%	6%	12%	0%	691	32	−0.43	21
XI	H	$NHCONHC(CH_3)_3$	H	24%	46%	33%	24%	46%	131	1	0	19
XII	H	NHC_6H_5	H	26%	0%	8%	22%	55%	52	1	0	19
XIII	H	$NHCOC_6H_5$	H	14%	13%	5%	17%	13%	—	—	—	—
XIV	H	$NHCOC_3H_7$	H	45%	11%	**27**	82%	10%	222	1	0	19
XV	H	NHC_4H_9	$SCH_2C_6H_5$	**64**	**24**	**48**	**18**	**0.38**	0.2	39.5	2.41	21
XVI	NH_2	OH	S-(*p*-Cl-C_6H_5)	38%	45%	3%	32%	26%	248	—	—	—
XVII	H	NH_2	H	—	—	—	—	—	719	1	0	19
XVIII	NH_2	OH	H	—	—	—	—	—	2	—	—	—

PDE isoenzymes were isolated from various tissues and assayed for activity using 1 μM cyclic AMP or 1 μM cyclic GMP (IC_{50} values) or 0.05 μM cGMP (K_m^D values) as substrate, for details see Materials and Methods. PDE I is the Ca^{2+}/calmodulin-stimulated PDE; PDE II is the cyclic GMP-stimulated PDE; PDE III is the cyclic GMP-inhibited PDE; PDE IV is the cAMP-specific PDE; PDE V is the cGMP-specific PDE; for further details of nomenclature see Ref. 5.

R1, R2 and R3 refer to the general structure shown in Fig. 1.

MR and π are molar refractivity and hydrophobic constants, respectively, as defined and reported in Ref. 19 or calculated in accordance with Ref. 21.

—, Not determined.

under these conditions the IC_{50} value (i.e. the concentration of derivative which reduces the hydrolysis of cGMP by 50%) approximates to the K_m^D (see Eqn 1).

$$IC_{50} = \frac{[cGMP] + K_m^G}{K_m^G} . K_m^D. \quad (1)$$

However, the determination of a K_m^D value does not necessarily imply that the derivative is a substrate for PDE V_A (for full details see Ref. 12).

To study its hydrolysis, 100 μM 8-CPT-cAMP was incubated with PDE V_A for various times and the reaction terminated by boiling and, following centrifugation, the supernatant was analysed by HPLC on a Beckman Ultrasphere IP column using a gradient of methanol in 10 mM KH_2PO_4 (pH 7.0) by measuring the area under the peak of the substrate. The cyclic nucleotides **IX**, **X**, **XII** and **XV** were synthesized as previously described [13–16], **XVI** was obtained from Biolog (Bremem, Germany) and all others from the Sigma Chemical Co. (Poole, U.K.). When necessary, derivatives were purified by chromatography on Sephadex G-25 prior to use [17].

RESULTS AND DISCUSSION

The inhibition of five PDE isoenzymes by a range of cAMP derivatives is shown in Table 1. For PDE isoenzymes I–IV, most of the cAMP derivatives tested were poor inhibitors with IC_{50} values greater than 100 μM. Only derivatives **IV**, **VIII** and **XV** showed significant inhibition of PDE III and IV. This is consistent with previous observations made with preparations that probably contained a mixture of isoenzymes [4].

However, two cAMP derivatives, 8-CPT-cAMP (**I**) and 8-benzylthio-N^6-butyl-cAMP (**XV**), were found to be potent inhibitors of PDE V_A with IC_{50} values less than 1 μM. Paradoxically, an analogous compound 8-CPT-cGMP (**XVI**) was a poor inhibitor of PDE V_A despite the known specificity of this enzyme for cGMP hydrolysis. Francis *et al.* [7] also found 8-CPT-cGMP to be a weak inhibitor of PDE V_A and furthermore showed that other cGMP derivatives were also poor inhibitors with only N^2-hexyl-cGMP showing significant inhibition.

These results made it all the more surprising that 8-CPT-cAMP and related derivatives are potent inhibitors of PDE V_A and so this aspect was studied in more detail. 8-CPT-cAMP is a competitive inhibitor of PDE V_A with respect to cGMP and has a K_i value of 2.5 μM (Fig. 2). This value is very close to that obtained for the commonly used PDE V_A inhibitor zaprinast (M&B 22,948 [5, 18]) which has a K_i of 2.8 μM (data not shown). Hydrolysis of 8-CPT-cAMP by PDE V_A was studied by analysing the reaction products by HPLC (as described in Materials and Methods). The results showed that 8-CPT-cAMP was a good substrate for PDE V_A and the rate of hydrolysis at 100 μM 8-CPT-cAMP was 47% of that obtained with 100 μM cGMP. As 8-

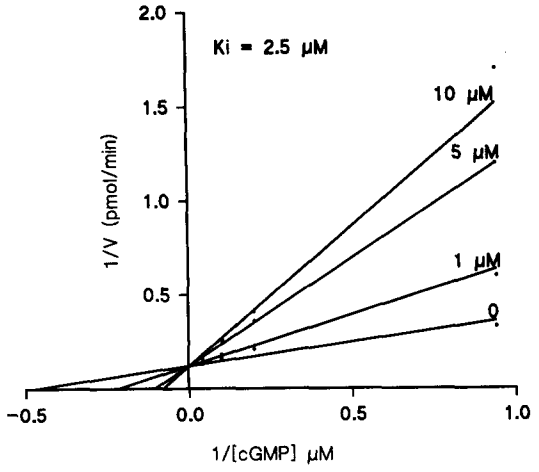

Fig. 2. Competitive inhibition of PDE V_A by 8-CPT-cAMP. Double reciprocal plot showing competitive inhibition of cGMP hydrolysis by 8-CPT-cAMP of PDE V_A. The assay was conducted as described in Materials and Methods using radiolabelled cGMP (1–25 μM) and the indicated concentrations of 8-CPT-cAMP.

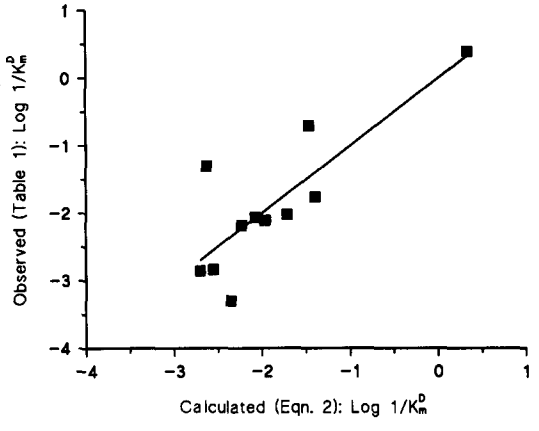

Fig. 3. Plot of observed versus predicted values of log $1/K_m^D$. The observed values of log $1/K_m^D$ are transformed values from Table 1 and the predicted values are those of Eqn 2 given below. Equations 3 and 4 show the results of regression against MR and multiple regression against MR and π, respectively.

Eqn 2
$1/\log K_m = 0.81(\pi) - 2.48$ ($s = 0.65$, $F = 16.84$)
Eqn 3
$1/\log K_m = 0.047(\mathrm{MR}) - 2.84$ ($s = 0.94$, $F = 3.56$)
Eqn 4
$1/\log K_m = 0.73(\pi) + 0.014(\mathrm{MR}) - 2.72$ ($s = 0.67$, $F = 8.65$)

[Table F: Eqn 2 and 3 (1, 9, 0.01) = 10.56; Eqn 4 (2, 8, 0.01) = 8.65.]

CPT-cAMP remained a good substrate for PDE V_A it was considered probable that the critical alignment of the relatively rigid ribose cyclophosphate moiety of 8-CPT-cAMP with respect to the hydrolytic region of the catalytic site of PDE V_A was close to that adopted by cGMP. In addition, it could be concluded that the inhibition was not due to reduced turnover caused by the failure of a high affinity product to leave the hydrolytic site.

To investigate further the PDE inhibitory effect of 8-CPT-cAMP and to allow comparison with cAMP and cGMP, K_m^D values (see Materials and Methods) of the cAMP derivatives for PDE V_A were determined (Table 1). These data suggested that the presence of an N^6-substituent (**XI**, **XII**, **XIV**) or a small, polar 8-substituent (e.g. **IV**, **V**, **VI**) was associated with reduced affinity for PDE V_A, whereas a large or lipophilic 8-substituent enhanced affinity slightly with respect to cGMP (**XVIII**), the effect being most clearly seen with 8-CPT-cAMP (**I**) and the disubstituted cAMP derivative (**XV**) despite the presence of an N^6-substituent in the latter. Regression of these data for the 8-substituted cAMP derivatives (**I–X**) against the descriptors of molar refractivity (MR) and the fragmental hydrophobic constant (π) [19] (covariance = 0.5) of their 8-substituents (Table 1) showed that, within the range of compounds considered, π was a more important determinant of PDE V_A inhibitory activity than MR. Additionally, in a multiple regression using π and MR, MR made no significant contribution to the regression (see legend to Fig. 3) so that the inhibitory potency of 8-substituted cAMP derivatives against PDE V_A appears to be related to the ability of the 8-substituent to make a lipophilic interaction in a region within or close to the guanine-accepting region of the PDE V_A catalytic site. Therefore, it is probably the physicochemical features of an 8-CPT substituent *per se* which enhance binding, rather than a steric effect due to this substituent causing alignment of latent binding functions within the adenine fragment.

8-CPT-cAMP is one of the derivatives of cAMP most commonly used to investigate actions of cAMP-PrK, although recent reports have shown that this particular derivative shows little selectivity for the activation of cAMP-PrK over cGMP-PrK [2, 20]. The observations that 8-CPT-cAMP is an inhibitor and substrate of PDE V_A provide further reasons why the physiological effects of this derivative should be interpreted with caution. As the intracellular concentration of 8-CPT-cAMP in many experiments is unknown, it is not clear if sufficient levels are reached to cause inhibition of PDE V_A and our experiments to address this question were unsuccessful as 8-CPT-cAMP interfered with the cGMP radioimmunoassay. However, 8-CPT-cAMP has the potential to interact with at least three components of the cyclic nucleotide pathway (cAMP-PrK, cGMP-PrK, PDE V_A) and as the end results of these interactions could produce the same physiological response in some tissues and cells (e.g. smooth muscle and platelets), 8-CPT-cAMP is obviously not the derivative of choice for all studies. Indeed, the ability of 8-CPT-cAMP to interact with more than one component of the cyclic nucleotide

pathway may explain the pharmacological potency of this derivative [3, 20].

REFERENCES

1. Beebe SJ and Corbin JD, Cyclic-nucleotide dependent protein kinases. In: *The Enzymes XVII* (Eds. Boyer PD and Krebs EG), pp. 43–111. Academic Press, New York, 1986.
2. Sandberg M, Butt E, Nolte C, Fischer L, Halbrugge M, Beltman J, Jahnsen T, Genieser H-G, Jastorff B and Walter U, Characterization of Sp-5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole-3',5'-monophosphorothioate (Sp-5,6-DCl-cBiMPS) as a potent and specific activator of cyclic-AMP-dependent protein kinase in cell extracts and intact cells. *Biochem J* **279**: 521–527, 1991.
3. Murray KJ, Methods in cycle nucleotide research. In: *Intracellular Messengers. Neuromethods.* (Eds. Boulton AA, Baker GB and Taylor CW), Vol. 20, pp. 313–368. Humana Press, NJ, 1992.
4. Miller JP, Sigman CC, Johnson HL, Novinson T, Springer RH, Senga K, O'Brien DE and Robins RK, Inhibition of cyclic AMP phosphodiesterases by cyclic nucleotide analogs and nitrogen heterocycles. In: *Cyclic Nucleotide Phosphodiesterases. Advances in Cyclic Nucleotide and Protein Phosphorylation Research.* (Eds. Strada SJ and Thompson WJ), Vol. 16, pp. 277–290, Raven Press, New York, 1984.
5. Beavo JA and Reifsnyder DH, Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors. *Trends Pharmacol Sci* **11**: 150–155, 1990.
6. Moit F, Van Haastert PJM and Erneux C, Specificity or cGMP binding to a purified cGMP-stimulated phosphodiesterase from bovine adrenal tissue. *Eur J Biochem* **149**: 59–65, 1985.
7. Francis SH, Thomas MK and Corbin JD, Cyclic GMP-binding cyclic GMP-specific phosphodiesterase from lung. In: *Cyclic Nucleotide Phosphodiesterases: Structure, Regulation and Drug Action* (Eds. Beavo JA and Houslay MD), pp. 117–140. Wiley, Chichester, 1990.
8. Murray KJ, Eden RJ, England PJ, Dolan J, Grimsditch DC, Stutchbury CA, Patel B, Reeves ML, Worby A, Torphy TJ, Wood LM, Warrington BH and Coates WJ, Potential use of selective phosphodiesterase inhibitors in the treatment of asthma. In: *New Drugs for Asthma Therapy* (Eds. Anderson GP, Chapman ID and Morley J), pp. 27–46. Birkhauser, Basel, 1991.
9. Martins TJ, Mumby MC and Beavo JA, Purification and characterization of a cyclic GMP-stimulated cyclic nucleotide phosphodiesterase from bovine tissues. *J Biol Chem* **257**: 1973–1979, 1982.
10. Reeves ML, Leigh BK and England PJ, The identification of a new cyclic nucleotide phosphodiesterase activity in human and guinea-pig cardiac ventricle. *Biochem J* **241**: 535–541, 1987.
11. De Lean A, Munson PJ and Rodbard D, Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay and physiological dose-response curves. *Am J Physiol* **253**: E97–E102, 1978.
12. Van Haastert PJM, Dijkgraaf PAM, Konijn TM, Abbad EG, Petridis G and Jastorff B, Substrate specificity of cyclic nucleotide phosphodiesterase from beef heart and from *Dictyosteliyum discoideum*. *Eur J Biochem* **131**: 659–666, 1983.
13. Michael G, Muhlegger K, Nelboeck M, Thiessen C and Weimann G, Cyclophosphates as substrates and effectors of phosphodiesterase. *Pharm Res Commun* **6**: 203–252, 1974.
14. Dills WL, Beavo JA, Bechtel PJ, Myers KR, Sakai LJ and Krebs EG, Binding of adenosine 3',5'-monophosphate dependent protein kinase regulatory subunit to immobilized cyclic nucleotide derivatives. *Biochemistry* **15**: 3724–3731, 1976.
15. Hong CI, Tritsch GL, Mittelmann A, Hebborn P and Chheda GB, Synthesis and antitumor activity of 5'-phosphates and cyclic 3',5'-phosphates derived from biologically active nucleosides. *J Med Chem* **18**: 465–473, 1975.
16. Miller JP, Yagura TS, Meyer RB, Christensen LF and Robins RK, Synthesis and enzymatic and inotropic activity of some new 8-substituted and 6,8-disubstituted derivatives of adenosine cyclic 3',5'-monophosphate. *J Med Chem* **23**: 242–248, 1980.
17. Corbin JD, Gettys TW, Blackmore PF, Beebe SJ, Francis SH, Glass DB, Redmon JB, Sheorain VS and Landiss LR, Purification and assay of cAMP, cGMP, and cyclic nucleotide analogs in cells treated with cyclic nucleotide analogs. *Methods Enzymol* **159**: 74–82, 1988.
18. Coulson CJ, Ford RE, Marshall S, Walker JL, Woolridge KRH, Bowden K and Coombs TJ, New inhibitor of reagin-mediated anaphylaxis. *Nature* **265**: 545–547, 1977.
19. Hanch C and Leo A, *Substituent Constants for Correlation Analysis in Chemistry and Biology*. J. Wiley & Sons, New York, 1979.
20. Francis SH, Noblett BD, Todd BW, Wells JN and Corbin JD, Relaxation of vascular and tracheal smooth muscle by cyclic nucleotide analogs that preferentially activate purified cGMP-dependent protein kinase. *Mol Pharmacol* **34**: 506–517, 1988.
21. Pomona College Medicinal Chemistry Project, Medicinal chemistry software version 3.42. Daylight Chemical Information Systems, Claremont, CA, 1983.