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RMI 12330A, AN INHIBITOR OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASES AND ADENYLATE CYCLASE IN KIDNEY PREPARATIONS

NICHOLAS H. HUNT and TONY EVANS

Department of Experimental Pathology, John Curtin School of Medical Research, The Australian National University, Canberra, A.C.T. (Australia)

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Summary

N-(*cis*-2-phenylcyclopentyl)azacyclotridecan-2-imine hydrochloride (RMI 12330A) inhibited cyclic AMP and cyclic GMP phosphodiesterase activities in kidney preparations from rat and mouse. The drug was effective in the concentration range 0.1—1 mM. The agent was much less effective in inhibiting chick kidney cyclic nucleotide phosphodiesterases. The onset of inhibition of rat particulate cyclic AMP phosphodiesterase activities was rapid (less than 30 s) and irreversible. The inhibition of the low K_m forms of cyclic AMP phosphodiesterase in mouse kidney homogenates was of the non-competitive type. RMI 12330A inhibited cyclic AMP phosphodiesterase activities in intact rat renal tubules. Adenylate cyclase activity, both basal and stimulated, was inhibited in all three species by the drug. Since RMI 12330A affects cyclic GMP metabolism as well as cyclic AMP metabolism, caution must be exercised in interpreting its effects upon cellular processes in terms of its actions upon the adenylate cyclase-cyclic AMP pathway alone.

Introduction

Cyclic AMP is the second messenger for a number of peptide hormones, catecholamines and prostaglandins. Agents which specifically affect the activities of adenylate cyclase and cyclic AMP phosphodiesterase, the enzymes of cyclic AMP metabolism, are useful tools for determining the importance of

Abbreviation: RMI 12330A, *N*-(*cis*-2-phenylcyclopentyl)azocyclotridecan-2-imine hydrochloride.

cyclic AMP in the regulation of cellular processes. For example, there is controversy over the role of cyclic AMP as a possible regulator of normal and neoplastic cellular growth [1]. This could be clarified by the use of a specific inhibitor of adenylate cyclase, but no such agents have thus far been discovered [2].

Recently a cycloalkyl compound, RMI 12330A, has been reported to inhibit adenylate cyclase activity in rat liver [3,4] and rabbit mucosal [4] particulate preparations. The potential usefulness of this agent has been assessed further here by studying the specificity of its action upon adenylate cyclase.

Materials

RMI 12330A was the gift of Dr. N.L. Wiech of Merrell-National Laboratories, Cincinnati, OH 45215, U.S.A. [α-^{32}P]ATP (approx. 20 Ci/mmol), cyclic [2,8-^{3}H]AMP (approx. 40 Ci/mmol) and cyclic [8-^{3}H]GMP (approx. 25 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, U.K. Cyclic AMP, cyclic GMP, ATP, Dowex X8 200—400 mesh (Cl^- form), collagenase (Cat. C2139) and 5′-nucleotidase (grade IV, Cat. N5880) were from Sigma Chemical Co, St. Louis, MO 63178, U.S.A. Phospho*enol*pyruvate and pyruvate kinase (EC 2.7.1.40, spec. act. approx. 200 i.u./mg) were from Boehringer Mannheim, Australia. Bovine serum albumin (fraction V) was from Armour Pharmaceuticals, Eastbourne, U.K. Bovine parathyroid hormone (code 69TP, 1000 units/mg) was prepared as described previously [5]. All other reagents were of AR grade.

Assay of phosphodiesterase activity. Rats (male Lewis strain, weight approx. 200 g), mice (male B10A strain, weight approx. 25 g) or chicks (male, aged 7—14 days) were killed by cervical dislocation. Kidneys were removed and washed in a buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$ and 250 μM EGTA ('phosphodiesterase assay buffer') at 4°C. For rat and mouse the renal cortex was removed into phosphodiesterase assay buffer. These tissues and intact chick kidney were homogenized by 12 strokes of a tight-fitting pestle in a Dounce glass-glass homogenizer at 4°C. The homogenates were either assayed directly or were centrifuged for 30 min at 100 000 $\times g$ (4°C). The particulate fraction was resuspended and rehomogenized in phosphodiesterase assay buffer. Cyclic AMP and cyclic GMP phosphodiesterase activities were determined by the method of Broudreau and Drummond [6]. Preliminary studies confirmed the observation [7] that RMI 12330A did not inhibit 5′-nucleotidase activity. Thus the second stage of the phosphodiesterase assay was not inhibited by the drug. Time of incubation and dilution of the sample were adjusted to keep substrate conversion below 25%. Average cyclic nucleotide substrate concentration during the incubation was calculated [8] and used in the plots of $[S]/v$ vs. [S] [9]. The presence of two forms of the enzyme in each species was suggested by the shapes of the plots. Least-squares regression was used to calculate K_m values after assignment of the break-off points from the plots of $[S]/v$ vs. [S]. The kinetics of competition were examined by plotting $1/v$ vs. [i] [10] for four different values of [i].

Assay of adenylate cyclase activity. Rat, mouse or chick kidneys were

removed and washed at 4°C in 50 mM Tris-HCl, pH 7.8, containing 0.013% (w/v) bovine serum albumin, 30 mM KCl and 4.5 mM $MgCl_2$ ('adenylate cyclase assay buffer'). Cortices were removed from rat and mouse kidney into adenylate cyclase assay buffer. These tissues, and chick kidney, were homogenized in the same buffer by six strokes of a Dounce glass-glass homogenizer. The homogenates were assayed for adenylate cyclase activity by the method of Salomon et al. [11], slightly modified [12]. Protein determinations were by the method of Lowry et al. [13]; standards and blanks were modified to eliminate errors due to interference by Tris buffers [14].

Isolated renal tubules. Rat renal tubule suspensions were prepared by the method of Larkins et al. [15]. More than 95% of freshly prepared cells excluded trypan blue; this value decreased to 85—90% after 2 h incubation in the experiments described in Table II.

Results and Discussion

Kinetic data from the hydrolysis of cyclic AMP by mouse, chick and rat kidney phosphodiesterases show non-linear forms when plotted as $[S]/v$ vs. $[S]$. Similar data have been obtained for this enzyme from many sources and are considered to indicate the presence of high-affinity and low-affinity forms [16]. Table 1 gives estimates of K_m and V for kidney or kidney cortex homogenates from three species. In subsequent experiments the effects of RMI 12330A upon cyclic AMP phosphodiesterase activity were studied at substrate concentrations of 1 and 100 μM.

The hydrolysis of cyclic AMP by phosphodiesterases in homogenates of mouse and rat renal cortex was inhibited by RMI 12330A at both low and high substrate concentrations (Fig. 1). The effect was significant ($P < 0.01$, Student's paired t-test) in the concentration range 10^{-4}—10^{-3} M of drug. Similar results were obtained when the effect of the drug on separated fractions was studied (Fig. 2). High and low K_m phosphodiesterase activities associated with particles or with the 100 000 $\times g$ supernatant were inhibited by concentrations of RMI 12330A from 10^{-4} to 10^{-3} M.

TABLE I

K_m AND V OF CYCLIC AMP PHOSPHODIESTERASES FROM CHICK KIDNEY AND FROM RAT AND MOUSE RENAL CORTEX PREPARATIONS

Assays were performed at substrate concentrations between 0.5 and 500 μM. Units: for K_m =M; for V pmol/min per μg protein. Mean ± S.E. from three to five experiments (duplicate determinations). First of each pair of values corresponds to the high-affinity form of the enzyme.

Species	K_m			V		
	Homogenate	Soluble	Particulate	Homogenate	Soluble	Particulate
Mouse	—	6.83 ± 1.52	3.19 ± 0.10	—	0.60 ± 0.09	0.14 ± 0.02
		106 ± 9	194 ± 8		3.83 ± 0.26	1.86 ± 0.37
Rat	2.36 ± 0.01	3.16 ± 0.55	2.21 ± 0.46	0.60 ± 0.07	0.76 ± 0.12	0.14 ± 0.05
	178 ± 22	111 ± 14	283 ± 24	6.7 ± 1.1	4.8 ± 0.7	4.0 ± 1.1
Chick	3.54 ± 0.42	3.53 ± 0.60	2.24 ± 0.20	0.20 ± 0.02	0.25 ± 0.06	0.11 ± 0.01
	300 ± 71	179 ± 39	518 ± 90	2.9 ± 0.7	3.1 ± 0.5	2.8 ± 0.8

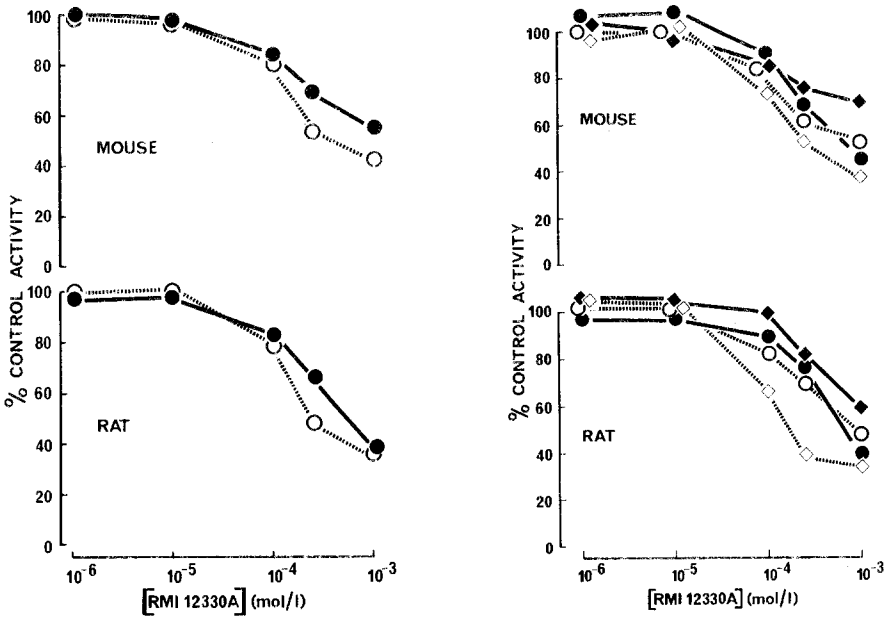

Fig. 1. Effect of RMI 12330A on hydrolysis of cyclic AMP by kidney homogenates. •, 1 μM cyclic AMP; ○, 100 μM cyclic AMP. Values are means of five determinations (mouse) and three determinations (rat). S.E. were less than or equal to 6.1% of mean values.

Fig. 2. Effect of RMI 12330A on hydrolysis of cyclic AMP by kidney preparations. •, 100 000 × *g* supernatant, 1 μM cyclic AMP; ○, 100 000 × *g* supernatant, 100 μM cyclic AMP; ■, 100 000 × *g* particles, 1 μM cyclic AMP; □, 100 000 × *g* particles, 100 μM cyclic AMP. Values are means of two experiments.

Analysis of the kinetics of inhibition of cyclic AMP phosphodiesterase in mouse renal cortex homogenates by the method of Dixon [10] demonstrated that the effect upon the low K_m enzyme was of the non-competitive type. The K_i was 2.1 mM. The same analysis of the effects of RMI 12330A on the high K_m form yielded non-linear plots, the significance of which was not clear. A non-competitive mode of action has also been shown for the inhibition of rat kidney adenylate cyclase activity by the drug [3].

The hydrolysis of cyclic AMP by rat kidney phosphodiesterases was inhibited when whole cells were incubated with RMI 12330A for 30 or 120 min, followed by washing (Table II).

The onset of the effect of RMI 12330A was rapid; inhibition of the hydrolysis of cyclic AMP by rat kidney particulate phosphodiesterase was seen after only 30 s exposure to the drug (Table III). Similar degrees of inhibition of the hydrolysis of cyclic AMP were produced by: (a) incubating particulate preparations for 10 min or 120 min with RMI 12330A, washing, and assaying for 10 min (1 μM cyclic AMP) or 120 min (100 μM cyclic AMP), or (b) adding the drug to aliquots of the same original preparations and assaying phosphodiesterase activity in its presence for 10 or 120 min. Thus, washing did not reduce the degree of inhibition of either the low or high K_m forms of the enzyme and the action of RMI 12330A probably is irreversible.

The hydrolysis of cyclic AMP by chick kidney preparations was affected

TABLE II

EFFECT OF RMI 12330A ON CYCLIC AMP PHOSPHODIESTERASE ACTIVITIES IN INTACT RAT RENAL TUBULES

Renal tubules were preincubated for 30 or 120 min with 10^{-3} M RMI 12330A or buffer alone. Cells were than washed twice, homogenized and assayed as described in the text. The experiment was performed twice, with duplicate determinations. Values are means of cyclic AMP conversion rate (pmol/min per μg protein).

	Cyclic AMP (μM)	Incubation time (min)		
		0	30	120
Control	1	0.159	0.137	0.119
RMI 12330A	1	—	0.126	0.071
Control	100	2.488	2.514	2.244
RMI 12330A	100	—	1.621	0.872

only slightly by RMI 12330A (Fig. 3, upper panel), in contrast to the effects seen with the mammalian tissues (Figs. 1 and 2). Cyclic GMP phosphodiesterase activity was examined at two substrate concentrations, 2 and 200 μM, which corresponded to the high and low-affinity forms which were present in all species. The hydrolysis of cyclic GMP by chick kidney was inhibited by RMI 12330A (Fig. 3, lower panel). However, the effects of the agent on cyclic GMP hydrolysis by mouse and rat renal cortex were greater (Fig. 4). An exception to this generalisation was that very little effect upon the soluble form of the enzyme from the rat was seen at low substrate concentration.

Although chick kidney cyclic nucleotide phosphodiesterases were inhibited only slightly by RMI 12330A, adenylate cyclase activity was markedly affected (Fig. 5). Basal, parathyroid hormone-stimulated and NaF-stimulated activities were inhibited to a similar degree in mouse, rat and chick kidney particulate preparations. The effective concentrations of RMI 12330A were $5 \cdot 10^{-5}$—

TABLE III

CYCLIC AMP PHOSPHODIESTERASE ACTIVITY IN RAT KIDNEY PARTICLES AFTER EXPOSURE TO RMI 12330A FOR VARIOUS TIMES

Particulate preparations were preincubated for 0.5, 10 or 120 min with 1 mM RMI 12330A or in buffer alone. They were then diluted 20-fold in phosphodiesterase assay buffer and centrifuged at 2000 × g for 2 min. After one additional wash, membranes were resuspended in buffer and assayed (1 or 100 μM cyclic AMP substrate). Values are means (from two experiments) of cyclic AMP conversion (pmol/min per μg protein).

Preincubation time (min)	Preincubation treatment	Substrate concn. cAMP (μM)	
		1	100
0.5	Buffer alone	0.035	0.913
	RMI 12330A	0.025	0.581
10	Buffer alone	0.032	0.818
	RMI 12330A	0.019	0.247
120	Buffer alone	0.029	0.679
	RMI 12330A	0.011	0.087

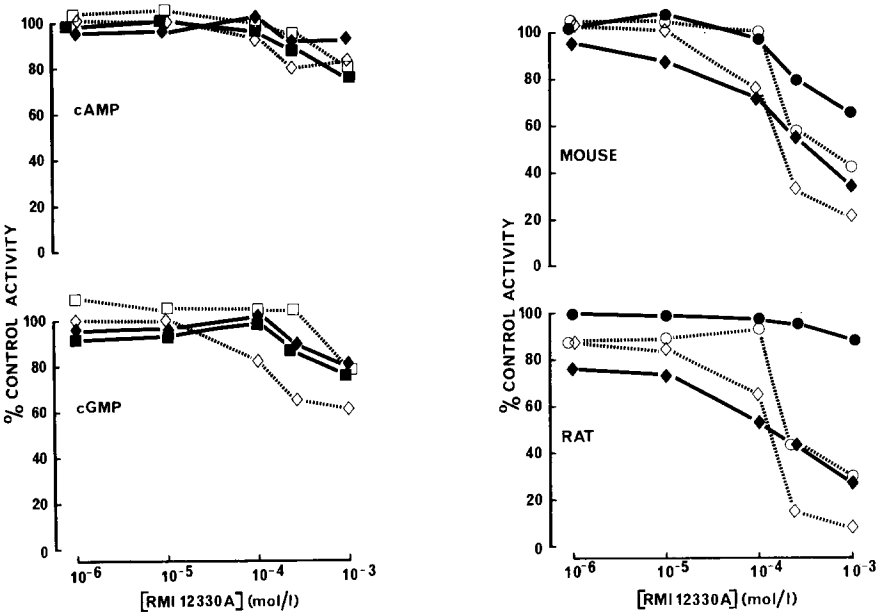

Fig. 3. Effect of RMI 12330A on hydrolysis of cyclic AMP and cyclic GMP by chick kidney preparations. ■, 100 000 × *g* supernatant, low substrate concentration (1 μM cyclic AMP, 2 μM cyclic GMP); □, 100 000 × *g* supernatant, high substrate concentration (100 μM cyclic AMP, 200 μM cyclic GMP); ◆, 10 000 × *g* particles, low substrate concentration; ◇, 100 000 × *g* particles, high substrate concentration. Values are means of four experiments. S.E. were less than or equal to 10.2% of mean values.

Fig. 4. Effect of RMI 12330A on hydrolysis of cyclic GMP by kidney preparations. ●, 100 000 × *g* supernatant, 2 μM cyclic GMP; ○, 100 000 × *g* supernatant, 200 μM cyclic GMP; ◆, 100 000 × *g* particles, 2 μM cyclic GMP; ◇, 100 000 × *g* particles, 200 μM cyclic GMP. Values are means of two experiments.

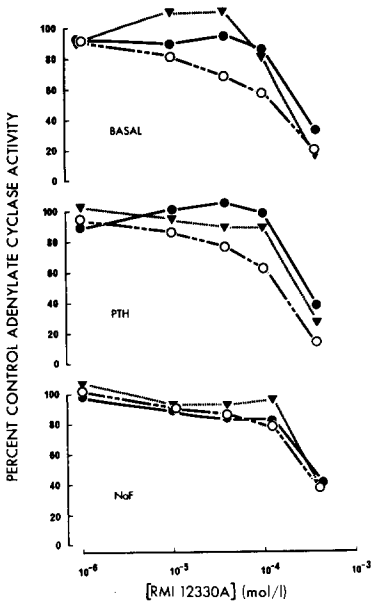

Fig. 5. Effect of RMI 12330A on adenylate cyclase activity in kidney homogenates. ●, rat; ▼, mouse; ○, chick. PTH, parathyroid hormone. Values are means from two experiments.

$5 \cdot 10^{-4}$ M, i.e. similar to those which inhibited cyclic nucleotide phosphodiesterases.

The effects of RMI 12330A upon cellular processes could be due to its inhibition of one or more of the three enzymes which have been studied. Since cyclic GMP metabolism is affected, the drug cannot be used to discriminate between cyclic AMP-dependent and cyclic AMP-independent actions of agonists. The effects of RMI 12330A on guanylate cyclase activity have not been studied to determine whether that enzyme is inhibited also.

It has been demonstrated that RMI 12330A inhibits the activities of adenylate cyclase (Refs. 3, 4 and 7, and Fig. 5), phosphodiesterase (Figs. 1—4) and Mg-ATPase [7] in various tissues but not those of ($Na^+ + K^+$)-ATPase [7] or 5′-nucleotidase (Ref. 7; Hunt, N.H. and Evans, T., unpublished results). The enzymes which are inhibited are Mg^{2+} dependent whereas those which are not affected have no Mg^{2+} dependence. However, increasing the Mg^{2+} concentration in the phosphodiesterase assay from 1 to 20 mM did not overcome the RMI 12330A inhibition (Hunt, N.H., unpublished results). Furthermore, chick adenylate cyclase was strongly inhibited by RMI 12330A whereas chick phosphodiesterases were fairly resistant to inhibition. It may be concluded that the drug probably does not act by interfering with the interaction of Mg^{2+} with substrate or enzyme.

The susceptibilities of the various forms of cyclic AMP and cyclic GMP phosphodiesterases to inhibition by RMI 12330A were varied. In particular, the chick phosphodiesterases were very resistant to inhibition although the adenylate cyclase of the same species was not. It has been suggested [17] that the complexity of the phosphodiesterases make them the most suitable target for pharmacological intervention and this is exemplified by their different responses to RMI 12330A.

The nature of the inhibition of phosphodiesterases and adenylate cyclase by RMI 12330A has not been elucidated. Hydrophobic bonding has been suggested as a mechanism for its effect upon liver adenylate cyclase activity [3]. Whatever the mechanism, it is clear that considerable caution must be exercised in interpreting pharmacological effects of this drug in terms of its effects upon cyclic nucleotide metabolism.

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