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RMI 12330 A, AN INHIBITOR OF ADENYLATE CYCLASE IN RAT LIVER

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Summary

RMI 12330 A, (*N*,-(*cis*-2-phenylcyclopentyl) azacyclotridecan-2-imine hydrochloride), has been reported to inhibit choleratoxin-induced intestinal hypersecretion, presumably via an inhibition of mucosal adenylate cyclase (ATP:pyrophosphate-lyase (cyclizing), EC 4.6.1.1). We report here that the adenylate cyclase activity of a rat liver plasma membrane preparation was inhibited by concentrations of RMI 12330 A ranging from 10 μM to 5 mM. Similar effects were observed when the adenylate cyclase preparation was assayed in the presence of 10 mM NaF, 0.1 μM glucagon or 1 μM (—)-epinephrine plus 10 μM GTP. The effect of RMI 12330 A was not due to the inhibition of the regenerating system present in the incubation medium, since the effect was preserved in its absence. The inhibition brought about by RMI 12330 A was due to a decrease in the maximal velocity of the reaction; the affinity of the enzyme for the substrate remained unmodified. The inhibition was immediate and irreversible, even after several washes of the membranes previously preincubated with the drug. Complete inhibition of cyclase was obtained at a concentration of 370 nmol of RMI 12330 A per mg of membrane protein. The drug acted with a similar dose-response curve upon intact as well as detergent-dispersed cyclase preparations.

Introduction

In spite of extensive studies, little is known of the molecular structure and mechanism of action of the hormone-sensitive adenylate cyclase (ATP:pyrophosphate-lyase (cyclizing), EC 4.6.1.1) system in mammalian cells. The membranous nature of the enzyme system is the main reason for this; an additional reason is that there are almost no drugs which specifically interact with adenylate cyclase [1]. In particular, a specific inhibitor of adenylate cyclase has not as yet been found. Possibilities of drug development based on the cyclic

Abbreviations: Cyclic AMP: Cyclic adenosine 3′5′-monophosphate; RMI 12330 A: *N*-(*cis*-2-phenylcyclopentyl) azacyclotridecan-2-imine hydrochloride.

AMP system have been restricted thus far to inhibitors of cyclic nucleotide phosphodiesterases.

Over the past few years, a new series of organic cycloalkyl molecules has been investigated [2]; the most promising compound, RMI 12330 A (*N*-(*cis*-2-phenylcyclopentyl) azacyclotridecan-2-imine hydrochloride), apparently inhibits choleratoxin-induced intestinal hypersecretion [3] and possessed anti-secretory properties in stomach [4], presumably via an inhibition of adenylate cyclase.

In the present report, the effects of RMI 12330 A on the adenylate cyclase of rat liver have been studied. We demonstrate that RMI 12330 A inhibits cyclase in an irreversible manner, as a result of its binding to hydrophobic component of the enzyme's membrane environment. These studies may lead to new insight into the development of membrane-directed drugs.

Experimental procedure

Materials

RMI 12330 A was the kind gift of Drs. H.R. Rohr and N.L. Wiech, of the Merrell-National laboratories, Cincinnati, Ohio, 45215, U.S.A. Its structure is shown in Fig. 1. This compound was dissolved in ethanol and diluted with 50 mM Tris · HCl buffer, pH 7.6, such that the final ethanol concentration never exceeded 3% (v/v) of the total incubation medium. Porcine crystalline glucagon (Novo laboratories, Bagsvaerd, Denmark), phenylmethyl sulfonyl fluoridine, creatine phosphate and (−)-epinephrine bitartrate (dissolved just prior to use and kept in the dark at 0°C) (Calbiochem, Los Angeles, California, 90024, U.S.A.), dithiothreitol, bovine serum albumin, Lubrol PX and nucleotides (Sigma, St Louis, Missouri, 63110, U.S.A.), creatine kinase (Boehringer, Mannheim, G.F.R), 2-azacyclotridecanone (cyclododecanone isooxime) dissolved in ethanol in the same manner as RMI 12330 A and ϵ-caprolactam (2-oxohexamethylenimine) (Aldrich-Europe, Belgium) were obtained from the commercial sources indicated. [α-^{32}P]ATP (21.5 Ci/mmol) was supplied by New England Nuclear Co., (Boston, Massachusetts, 02111, U.S.A.), and cyclic [8-^{3}H]AMP (13 Ci/mmol) was obtained from the CEA (Saclay, France). Sodium fluoride and all other chemicals were from Merck (Darmstadt, G.F.R.) and of analytical grade.

Methods

Preparation of liver plasma membranes. Female Wistar rats (about 100 g body weight) were used. Liver plasma membranes were prepared according to the procedure devised by Neville [5] up to step 11. The purified membrane preparations were suspended in 1 mM $NaHCO_3$ and stored up to 5 weeks in

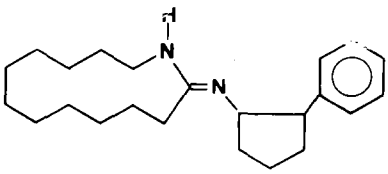

Fig. 1. Structure of RMI 12330 A.

liquid nitrogen without any loss of adenylate cyclase activity. Several batches of liver membranes were used in the experiments reported here; similar results were obtained with all of them. When epinephrine effect was studied, animals were adrenalectomized eight days prior to killing as previously described [6].

Detergent-dispersed adenylate cyclase preparation from rat liver. 3mg of plasma membrane were resuspended in 1.2 ml of the following medium: 0.5 mM ATP, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 5 mM NaF, glycerol 1.66%, 0.66 mM phenylmethyl sulfonyl fluoridine, 5 mM dithiothreitol, Lubrol PX 0.83%, and 50 mM Tris · HCl buffer pH 7.6. This mixture was allowed to stand 30 min at 4°C with occasional stirring; one ml was then centrifuged for 1 h at 4°C and at 165 000 × *g*; 20 µl of the supernatant was used as the source of detergent-dispersed adenylate cyclase.

Adenylate cyclase assay. Adenylate cyclase activity was measured as previously reported [7]. The assay medium contained 0.5 mM [α-^{32}P]ATP 10^6 cpm), 3 mM $MgCl_2$ (except when otherwise indicated), 1 mM EDTA, 1 mM cyclic AMP, 50 mM Tris · HCl pH 7.6, an ATP regenerating system consisting of 25 mM phosphocreatine and 2 mg/ml of creatine phosphokinase, and 20—30 µg of membrane protein in a final volume of 60 µl. When the effect of glucagon was tested, 0.1% bovine serum albumin was added to the assay mixture. Variations from this composition are indicated in the legends to figures. Incubation was initiated by addition of the membranes and was performed for 10 min in a shaking water bath at 33°C. Reactions were terminated by the procedure of Ramachandran [8], or more recently by a modification of the procedure of White [9]: samples were added with 0.2 ml of 0.5 N HCl, boiled for 6 min, buffered with 0.2 ml of 1.5 M imidazole and finally applied to alumina columns. Cyclic AMP was then eluted with 3 ml of 10 mM imidazole pH 7.5. Blank values obtained with this procedure were always lower than 0.005 per cent of the total radioactivity applied. The yield was calculated from previous addition of cyclic [8-^{3}H]AMP. Sample counting was performed after addition of 10 ml Unisolve (Koch-Light laboratory) in an Intertechnique SL 30 liquid scintillation counter. Results are expressed as nmol cyclic AMP formed in 10 min per mg protein at 33°C. The results, obtained from triplicate determinations, agreed within ±5%. The purity of the cyclic AMP formed during the cyclase assay was verified by chromatography on cellulose plates as described previously [7]. Appropriate controls were performed in the presence of 3% ethanol since this compound slightly stimulates the enzyme.

In all experiment, proteins were measured according to Lowry's procedure, using bovine serum albumin as standard.

Results

Effect of RMI 12330 A upon the rat liver adenylate cyclase system

When RMI 12330 A was added at the beginning of the incubation together with the reaction mixture, it strongly inhibited the formation of cyclic AMP. Fig. 2 depicts the effects of increasing amounts of RMI 12330 A upon the basal and the adenylate cyclase activities stimulated by fluoride, (−)-epinephrine plus

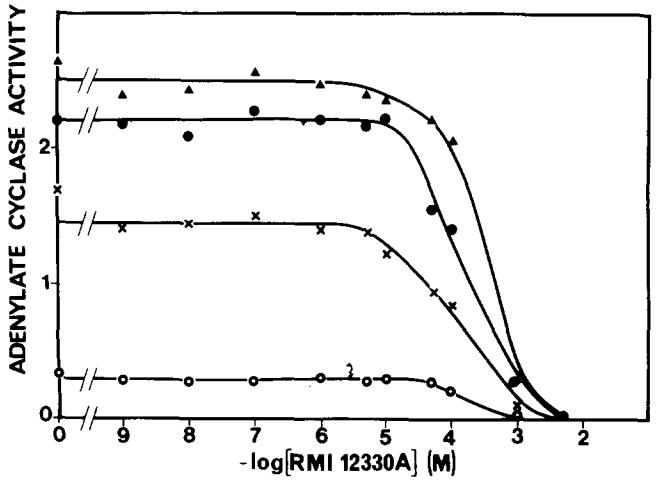

Fig. 2. Dose response curve of the inhibitory action of RMI 12330 A upon rat liver adenylate cyclase activity. Cyclase activity was tested in the presence of 0.5 mM ATP, 3 mM $MgCl_2$, 1 mM EDTA and of increasing concentrations of RMI 12330 A with no other addition (○) or with 10 mM NaF (●), 1 μM epinephrine plus 10 μM GTP (X), 0.1 μM glucagon (▲). RMI 12330 A was dissolved in ethanol, buffered, and diluted to the indicated concentrations so that the final concentration of ethanol never exceeded 3% (v/v). Appropriate controls were performed with ethanol alone. Membranes (25 μg protein) were incubated for 10 min at 33°C and the assays were performed as described in the experimental part. Adenylate cyclase activity is expressed as nmol of cyclic AMP formed in 10 min per mg of membrane protein.

GTP and glucagon respectively. The inhibition of the four activities was equally marked and was related to the concentration of inhibitor. Inhibition was half-maximal at a final RMI 12330 A concentration of 250 μM, and total at 5 mM. These results were obtained with about 25 μg of membrane proteins in the incubation medium.

Mechanism of action of RMI 12330 A

We turned next to the mechanism of the inhibition of adenylate cyclase by RMI 12330 A and determined that it could not have resulted from an effect upon the creatine kinase in the ATP regenerating system used in the cyclase assay, since similar effects were obtained in the absence of a regenerating system.

The effect of RMI 12330 A was further tested as a function of substrate concentration. Experiments were performed under optimal conditions for enzyme activity, namely in the presence of a constant excess (3 mM) of Mg^{2+} over that of ATP, at all ATP concentrations tested. This procedure appeared the most convenient for the maintenance of all the ATP in the form of an equimolar ATP · Mg complex, with a concentration of Mg^{2+} low enough not to be inhibitory. This would not be the case if the ratio ATP/Mg was kept constant, as already described in a previous publication from this laboratory [7]. Under these conditions, and in the presence of 10 mM NaF, RMI 12330 A appeared to lower the maximal velocity of the reaction 3-fold (from 0.5 nmol/2 min/mg protein to 0.16 nmol/2 min/mg protein), while the affinity of the catalytic site for the substrate was not altered (Fig. 3); the apparent K_m for ATP · Mg was

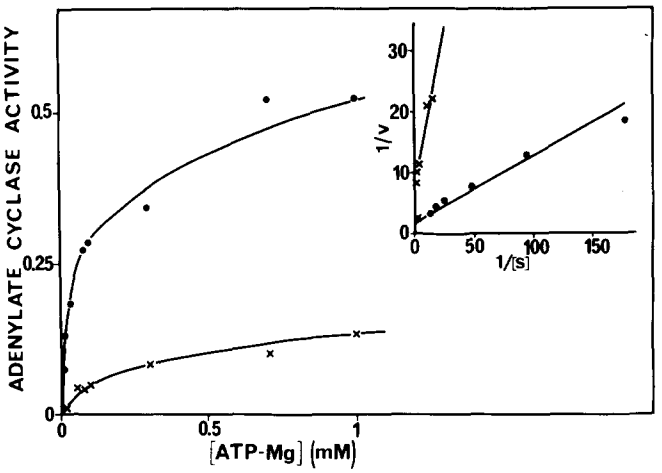

Fig. 3. Effects of increasing amounts of ATP · Mg on the inhibitory action of 1 mM RMI 12330 A upon liver cyclase. The incubation medium contained 17 μg membrane protein, 10 mM NaF, 1 mM EDTA, varying concentrations of ATP and Mg^{2+} so that the Mg^{2+} concentration was always higher than that of ATP by a fixed amount (3 mM). The assay was performed for 2 min in the presence (X) or in the absence (•) of 1 mM RMI 12330 A. For this experiment, the specific activity of the [α-^{32}P]ATP was 2 · 10^6 cpm. A Lineweaver-Burk plot of the data is shown in the insert.

40 μM in the absence as well as in the presence of the inhibitor. Therefore, RMI 12330 A does not seem to exert its inhibitory action by a competition on the catalytic site of the enzyme.

Irreversible binding of RMI 12330 A to plasma membrane

The affinity of RMI 12330 A for the liver plasma membrane and the nature

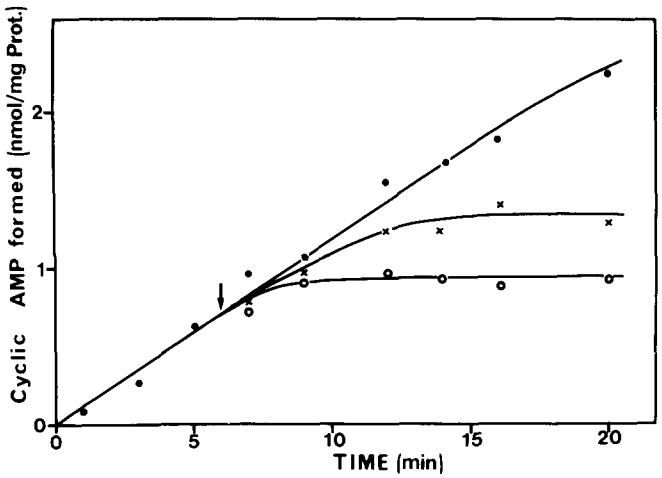

Fig. 4. Time course of the RMI 12330 A effect upon the NaF stimulated adenylate cyclase activity of rat liver plasma membrane. The incubation medium contained 25 μg of membrane protein, 0.5 mM ATP, 3 mM $MgCl_2$, 1 mM EDTA and 10 mM NaF. After 6 min of incubation, RMI 12330 A was added (arrow) at final concentrations of 0 mM (•), 0.5 mM (X) or 1 mM (○), in such a manner as not to modify the concentration of the other components.

TABLE I

EFFECT OF VARYING PREINCUBATION TIME-PERIODS OF RAT-LIVER PLASMA MEMBRANE WITH DIFFERENT CONCENTRATIONS OF RMI 12330 A UPON ADENYLATE CYCLASE ACTIVITY

Membrane proteins (24 μg) were preincubated for different times with varying concentrations of RMI 12330 A at 33°C. Adenylate cyclase was then assayed. The incubation medium contained 10 mM NaF, 1 mM EDTA and 3 mM $MgCl_2$. The effect of RMI 12330 A is expressed as the percent of the activity observed in the absence of drug (1.4 nmol cyclic AMP/10 min per mg protein).

RMI 12330 A (mM)	Adenylate cyclase (% maximal activity) after preincubation time periods				
	0 min	3 min	5 min	10 min	25 min
0.01	99	99	98	99	97.5
0.10	79	88	68	76	77
1.00	18	3	2	0	2
5.00	0	2	0	0	2

of its binding were then studied. Fig. 4 depicts a time course study in which adenylate cyclase was assayed in the presence of 10 mM NaF. The dose-dependent inhibition brought about by RMI 12330 A could be detected 1 min after its addition. Such inhibition was not spontaneously reversible: at 1 mM, RMI 12330 A completely stopped cyclic AMP formation and this effect was sustained for the whole period of incubation (20 min).

Preincubation of the membranes with various concentrations of inhibitor was performed for different time-periods. Table I depicts the results obtained with varying periods of preincubation from 3 to 25 min. No difference was observed between the various periods studied, which indicates that the fixation of RMI 12330 A was immediate.

The possibility that RMI 12330 A might bind to the plasma membranes in

TABLE II

EFFECT OF REPEATED WASHINGS UPON THE ADENYLATE CYCLASE ACTIVITY OF RAT LIVER PLASMA MEMBRANE PREINCUBATED WITH VARYING CONCENTRATIONS OF RMI 12330 A

Membrane proteins (500 μg) were preincubated in 200 μl of 50 mM Tris · HCl, pH 7.6 containing 0, 0.2 or 0.8 mM RMI 12330 A for 10 min at 20°C. The membranes were washed 4 times and, each time, resuspended in the medium described above, without RMI 12330 A. Membranes were then centrifuged at 20 000 × *g* for 15 min between each wash. The last pellet was resuspended in 200 μl of the same buffer. 20 μl of this solution were assayed for cyclase activity. The final incubation medium contained 0.5 mM ATP, 1 mM EDTA, 3 mM $MgCl_2$, and in the presence or absence of 10 mM NaF. The basal activities in the presence of 10 mM NaF were 1.53 and 1.21 nmol cyclic AMP/10 min per mg protein for the intact and washed membranes respectively. The corresponding values in the absence of NaF were 0.307 and 0.217 nmol cyclic AMP/10 min per mg protein. Each value represents the mean of three determinations.

RMI 12330 A (mM)	Adenylate cyclase (percent of maximal activity)			
	NaF 10 mM		None	
	washed	intact	washed	intact
0	100	100	100	100
0.2	75	75	67	62
0.8	37	36	21	23

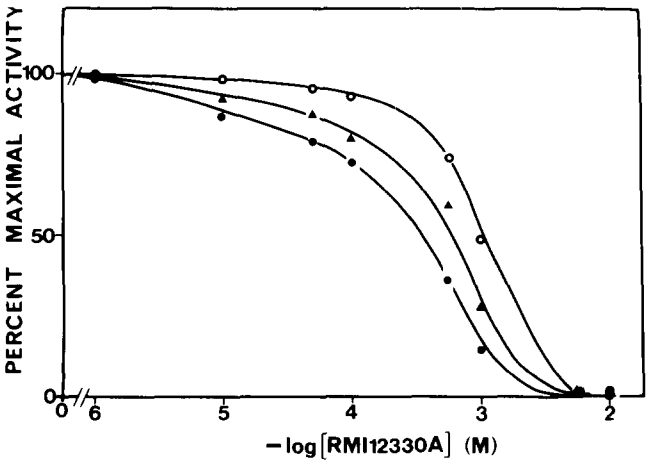

Fig. 5. Inhibitory effect of varying quantities of RMI 12330 A upon adenylate cyclase activity in the presence of different amounts of rat liver plasma membrane in the incubation medium. Different amounts of membrane protein 120 μg (○), 20 μg (▲) and 2 μg (●) were preincubated with varying concentrations of RMI 12330 A in 60 μl of 50 mM Tris · HCl, pH 7.6, 10 mM NaF, 1 mM EDTA and 3 mM $MgCl_2$. Adenylate cyclase activity in the absence of inhibitor was 2.2 nmol/10 min per mg protein.

an irreversible manner was subsequently evaluated. For this experiment, membranes were preincubated with two different concentrations of RMI 12330 A (0.2 and 0.8 mM) at 20°C for 10 min. After 4 cycles of washing by centrifugation and resuspension, the adenylate cyclase was assayed; the results are depicted in Table II. An inhibition of cyclase activity of 25 and 75% respectively was obtained with the two concentrations of RMI 12330 A, whether or not the membranes were washed. This strongly suggests that the action of RMI 12330 A upon adenylate cyclase was due to an irreversible binding with the plasma membranes.

To investigate this hypothesis further, the inhibitory effect of RMI 12330 A was tested as a function of the ratio of this compound to the amount of membrane protein. Fig. 5 represents the effect of the drug at three different concentrations of membrane protein, namely: 2, 20 and 120 μg protein in 60 μl of incubation medium. As expected, a shift in the dose response curve of the inhibitor to the left accompanied the diminution of total membrane protein concentration from 120 to 2 μg. This corresponded to a 20—30% decrease in the apparent affinity of RMI 12330 A. If the binding of RMI 12330 A to membrane is indeed irreverisble, then one has to consider the ratio of RMI 12330 A in μmol over the amount of protein, instead of the usual, final concentration of the inhibitor; regardless of the various experimental conditions (volume of incubation medium, concentration of inhibitor, concentration of membrane protein). Therefore, the amount of RMI 12330 A bound to rat liver plasma membranes and which was necessary to exert inhibitory effects was assessed. Increasing amounts of drug, from 10 μM to 5 mM, were preincubated for 15 min at 33°C with a fixed amount of protein (28.4 μg). Thereafter, the membranes were centrifuged and the supernatant tested as a source of RMI 12330 A on the same batch of protein, and at the same final concentration

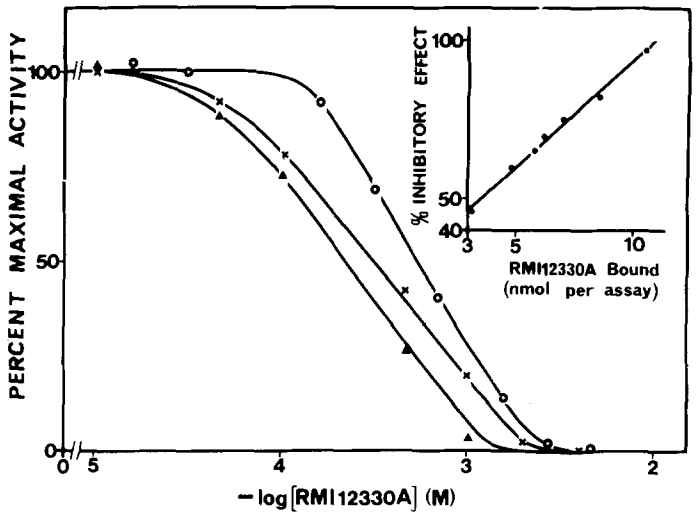

Fig. 6. Diminution of active concentration of RMI 12330 A by preincubation with rat liver plasma membrane. The adenylate cyclase was assayed with 28.4 μg of membrane protein in presence of different amounts of RMI 12330 A (X). The final incubation medium contained 0.5 mM ATP, 10 mM NaF, 3 mM $MgCl_2$ and 1 mM EDTA in Tris · HCl (50 mM) at pH 7.6. The same concentrations of drug were preincubated for 15 min at 33°C in the presence of the same amount of protein (28.4 μg) in 40 μl of Tris · HCl 50 mM pH 7.6. The reaction was terminated by centrifugation 15 min at 2000 × *g* and 4°C. 20 μl of the supernatant were introduced into the incubation medium, containing 28.4 μg of protein and other components at final concentrations indicated above (this corresponds to a 3-fold dilution of the RMI 12330 A). Cyclase was assayed under these conditions (○). The percentage of activity is expressed as a function of the concentration of RMI 12330 A that would have been present in the incubation medium if none had been trapped on the pelleted membranes. The pellet (19.2 μg membrane protein) was resuspended in 20 μl of Tris · HCl (50 mM) at pH 7.6 and assayed for cyclase activity as in the usual procedure (▲). Cyclase activity (○, ▲, X) was expressed as the percentage of the activity with no inhibitor added (2.2 nmol/10 min per mg protein), and plotted as a function of the concentration of RMI 12330 A which was present during the preincubation period (○, ▲) or during the incubation period (X). The relative inhibition of cyclase as a function of the amount of drug bound to membrane protein (28.4 μg/assay) is shown in insert. Total inhibition occurred with 10.5 nmol RMI 12330 A/28.4 μg of membrane protein.

(28.4 μg/assay). Control experiments were performed by direct incubation of the membranes for the cyclase assay with no centrifugation. In these two cases, the results are expressed as a function of the initial concentration of inhibitor (Fig. 6). If no binding of the drug to the membrane had occurred, the two dose response curves should be similar. In fact, the dose vs. response curve of the inhibitor's action was shifted to the right when the supernatants were used as the source of inhibitor. This directly demonstrates that RMI 12330 A was indeed bound to the plasma membranes. For the same percentage of inhibition, the difference between the two dose vs. response curves (before and after preincubation-centrifugation) gives an estimate of the amount of inhibitor bound to the pelleted membranes. From the assay of the cyclase on the various pellets, the relative inhibitory activity of RMI 12330 A could be related to the quantity of drug fixed (Fig. 6). It has been found that for a maximal inhibition of adenylate cyclase, 370 nmol were bound per mg of membrane protein (0.14 mg/mg protein). The sensitivity of the assay did not allow study of the interaction of less than 100 nmol of RMI 12330 A/mg protein.

We also examined the effect of RMI 12330 A upon the adenylate cyclase

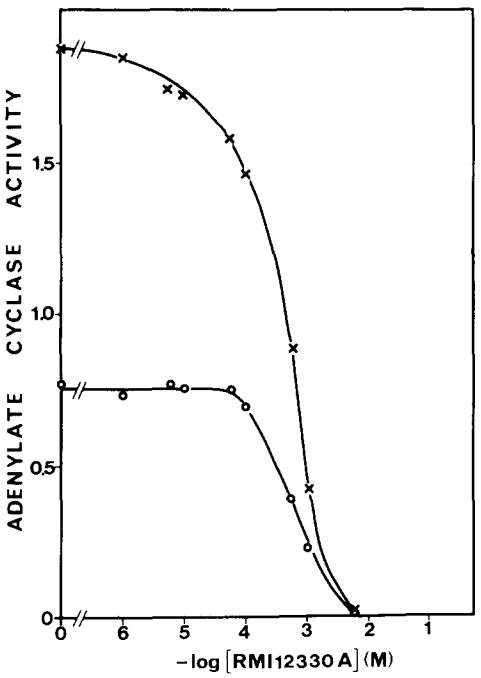

Fig. 7. Action of RMI 12330 A upon detergent-dispersed adenylate cyclase of the rat liver plasma membrane. Detergent-dispersed adenylate cyclase activity (see Experimental Procedure) was assayed (○) under the same conditions as the control (X). The incubation medium contained 0.5 mM ATP, 10 mM NaF, 3 mM $MgCl_2$ and 1 mM EDTA in 50 mM Tris · HCl, pH 7.6. The amounts of protein were identical in both cases (30 μg).

activity of a detergent-dispersed preparation of rat liver plasma membranes. The experiment was carried out with the same amount of protein (30—33 μg/assay) from control and detergent-dispersed membranes and with similar concentrations of inhibitor. The results are depicted in Fig. 7 and show that the "solubilized" enzyme exhibited the same degree of sensitivity to the inhibitor as the control preparation.

Discussion

In the present report, evidence has been obtained for an inhibitory effect of RMI 12330 A upon the hepatic adenylate cyclase. This effect is rapid, bears equally on all cyclase activities tested (Fig. 2) and is due to a non-competitive inhibition of the enzyme (Fig. 3).

We further demonstrate that RMI 12330 A interacts with plasma membranes in an apparently irreversible manner, since its effect persisted in spite of extensive washing of membrane preparations (Table II). As a result of such an irreversible binding, the dose response curve (as a function of the drug concentration) varied greatly depending on the amount of protein present in each enzyme assay. As shown in Fig. 6, 370 nmol of drug per mg of plasma membrane protein were required for maximal inhibition of cyclase. A covalent binding of RMI 12330 A with the membrane appears unlikely; we therefore

assume that hydrophobic bonds play a major role in this interaction *. RMI 12330 A is a very hydrophobic compound and is highly soluble in organic solvents; it reacts very readily with the fluorescent probe 8-anilino-1-naphthalene sulfonic acid increasing the fluorescence of the dye 20-fold: this interaction results in a shift of the emission maximum from 520 to 470 nm (data not shown) similar to that observed when plasma membranes were incubated with the probe.

In this respect, RMI 12330 A appears similar to a serie of drugs which are known to interact with hydrophobic regions of plasma membranes and to produce various changes in enzyme activities [10—15]. These various compounds probably interact differently with discrete components of the membrane. As an example, the polyene antibiotic filipin, which specifically interacts with cholesterol [10], brings about an uncoupling of the epinephrine-sensitive cyclase without [15] or with a less marked effect on the fluoride-stimulated activity [16]. RMI 12330 A appears to interact directly with the catalytic site of cyclase since it inhibits all forms of enzyme activities (i.e. basal, as well as stimulated) (Fig. 2). From the above data, it is likely that the action of RMI 12330 A upon the intestinal hypersecretion induced by choleratoxin [3] is due to an inhibition of cyclase, a system which is classically involved in the pathogenesis of cholera [17]. These results appear particularly interesting since at the present time few drugs are known to inhibit the activity of the adenylate cyclase system.

Acknowledgments

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* The possible role of the lactamimide moiety of RMI 12330 A was investigated. Two lactame components, analogous to the cycloalkyl lactamimide (on the left of structure shown Fig. 1), one with 12 carbons (2-azacyclotridecanone) and another of six carbons (ε-caprolactam) were tested upon liver cyclase activity in the same range of concentration as RMI 12330 A. They were both ineffective.

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