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EFFECTS OF RMI 12330A, A NEW INHIBITOR OF ADENYLATE CYCLASE ON MYOCARDIAL FUNCTION AND SUBCELLULAR ACTIVITY

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1 RMI 12330A, a lactam-imine, at concentrations of 10^{-4} M and higher, inhibited basal as well as isoprenaline and NaF-stimulated adenylate cyclase activity of guinea-pig heart homogenates. However, RMI 12330A was a more potent inhibitor of histamine-stimulated adenylate cyclase (IC_{50} of 1.5×10^{-5} M).

2 In the isolated work-performing heart of the guinea-pig, RMI 12330A (IC_{50} of 1.1×10^{-6} M) depressed all cardiac functions: pressures developed, dP/dt, contractile force, dF/dt, work performance and stroke work. Left atrial pressure rose and the positive inotropic response to increasing heart rate (staircase) became negative. Histamine, isoprenaline and ouabain no longer caused positive inotropic effects.

3 Increasing the perfusate calcium concentration from 2.5 mM to 4.5 and 6.5 mM completely restored cardiac function after its depression by RMI 12330A.

4 RMI 12330A uncoupled mitochondrial oxidative phosphorylation; the classical uncoupler, dinitrophenol, had the same effects on cardiac dynamics as RMI 12330A.

5 RMI in high doses inhibited hydrolytic activity of Na^+, K^+-ATPase of crude and purified heart preparations (IC_{50} of 1.7×10^{-4} M) and inhibited ouabain binding to the same enzymes (IC_{50} of 1.5×10^{-4} M).

6 A lactam-imine analogue of RMI 12330A that had no effect on adenylate cyclase, was also without effect on any of the systems examined.

Introduction

It has been suggested that cyclic adenosine 3',5'-monophosphate (cyclic AMP)-dependent and independent protein kinases and calcium-dependent and possibly calcium-independent phosphorylase kinases modulate cardiac function by phosphorylating a wide spectrum of substrates, including contractile proteins, sarcoplasmic reticulum and sarcolemma (Krebs, Stull, England, Huang, Bröstrom & Vandendeede, 1973; Schwartz, Entman, Kaniike, Lane, Van Winkle & Bornet, 1976). It is possible that cyclic nucleotides (under certain circumstances) and/or calcium mediate the inotropic responses to some agonists, as well as modulate contractility secondary to normal stimulation, and may exert influences on important electrical events that in turn regulate contraction (Reuter, 1974; Coraboeuf, 1978). The possibility that oscillations of adenylate cyclase and guanylate cyclase may regulate contractile responses to perturbations in heart is supported by experiments on isolated cardiac cells and intact preparations (Brooker, 1975; Wollenberger, Babskii, Krause, Genz, Blohm & Bogdanova, 1973). It has been hypothesized that cyclic AMP-dependent, protein kinase-catalyzed phosphorylation of cardiac muscle sarcolemma might cause changes in calcium influx or efflux from the cell and hence regulate contraction or relaxation (Wollenberger, Will & Krause, 1975; Gervais, Lane, Anner, Lindenmayer & Schwartz, 1976).

From results of experiments with an endogenous inhibitor of adenylate cyclase, we suggested that cyclic nucleotides may mediate the calcium-induced cardiac contractile changes, particularly in response to ouabain-Na^+, K^+-ATPase interaction (Schwartz, Entman, Ezrailson, Lehotay & Levey, 1977) corroborating the suggestion of Himms-Hagen (1970) of a reciprocal relationship between Na^+, K^+-ATPase and adenylate cyclase in adipocytes. More recently, Lelievre, Paraf, Charlemagne & Sheppard (1977) using wild type and ouabain-resistant plasmocytoma cell lines, suggested that adenylate cyclase and Na^+,

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K^+-ATPase may be closely coordinated in control of membrane activity and that a protein of M_r 37,000 may control the action of ouabain on the Na^+, K^+-ATPase with respect to digitalis resistance (Lelievre, Zachowski, Laget, Charlemagne & Paraf, 1979).

Merrell Research Center has prepared a new compound *cis-N*-(2-phenylcyclopentyl) azacyclotridec-1-en-2-amine monohydrochloride coded RMI 12330A, which has been reported to inhibit hormone-stimulated and basal adenylate cyclase of rat liver plasma membrane preparations (Siegel & Wiech, 1977; Guellaen, Mahu, Mavier, Berthelot & Hanoune, 1977). In both studies RMI 12330A inhibited basal cyclase activity as well as activity stimulated by NaF, adrenaline, and glucagon. When tested on rat plasma membrane preparations, RMI 12330A, inhibited Mg^{2+}-ATPase activity but had little or no effect on Na^+, K^+-ATPase activity (Wiech, Siegel & Hogan, 1977).

This paper deals with the effects of RMI 12330A on cardiac adenylate cyclase as well as on several other relevant enzymatic systems, including Na^+, K^+-ATPase, digitalis binding, sarcoplasmic reticulum calcium transport, mitochondrial energetics and calcium transport and on the work performance of the isolated working guinea-pig heart particularly with respect to its effects on the inotropic action of digitalis glycosides, histamine, catecholamines and calcium.

Methods

Isolated work-performing heart preparation

Guinea-pigs of either sex, weighing 550 to 650 g, were anaesthetized with 30 mg/kg sodium pentobarbitone and protected with 20 mg heparin sodium intraperitoneally. Artificial respiration was maintained during thoracotomy. The heart was removed and perfused in a working heart perfusion system described by Neely, Liebermeister, Battersby & Morgan (1967), modified by Wiester, Bonventre & Grupp (1973). In this system perfusion fluid (venous return) enters through the left atrium into the ventricle and is pumped by the ventricle through the aortic resistance and the coronary system. A strain gauge arch was sewn to the right ventricle to record isometric wall tension from the ventricle. Other parameters recorded were: aortic pressure and flow, atrial pressure, venous return, left intraventricular pressure and its derivative dP/dt, right ventricular contractile force and its derivative dF/dt, heart rate, ECG, coronary flow and temperature of the perfusion fluid. Venous return was monitored with a Biotronex flow meter, dP/dt and dF/dt with Grass 7P20C differentiators and the pressures and ECG with a Grass P7 polygraph. In the three pacing experiments, the heart was stimulated with a Grass S8 stimulator at 5 Hz at supra-threshold voltage for 0.5 ms duration. The following functions could be calculated from the recorded parameters: cardiac output, stroke volume, cardiac work, stroke work, coronary resistance, total peripheral resistance, and the rate of pressure development during volume or during pressure load. The perfusion fluid used in this study was a Krebs-Henseleit solution saturated with 95% O_2 and 5% CO_2 (to adjust pH to 7.4) and kept at a constant temperature of 37.0°C ± 0.2°C. The composition of the perfusion fluid was (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, CaEDTA 0.5, $NaHCO_3$ 25 and glucose 5.5. The fluid was not recirculated.

In an isolated working heart preparation, changes in the concentrations of agonists and antagonists in the perfusion fluid frequently slightly change ion concentration, temperature, or flow. Any of these changes would cause changes in baseline observations. To avoid this, all agents were infused with Harvard infusion pumps at rates of 0.04 to 0.91 ml/min (added to about 26 to 34 ml/min venous return). This method makes it possible to deliver exact concentrations of the agents, to begin and end infusions exactly, and to add additional agents at any time.

Adenylate cyclase assay

The ventricles from guinea-pigs were minced with scissors and then homogenized with a Polytron for 3 × 3 s at maximum speed. The homogenization medium contained 0.25 M sucrose, 5 mM Tris.Cl and 1 mM EGTA (pH 7.4). The homogenate was filtered through four layers of cheesecloth and centrifuged for 20 min at 1000 *g*. The pellet was washed twice by resuspension and recentrifugation at the same speed. The final pellet was resuspended in the same medium at a protein concentration of 7 to 10 mg/ml and quick frozen in small aliquots.

The assay medium consisted of the following: 100 mM Tris.Cl (pH 7.2); 1 mM ATP; 2 mM Mg^{2+}; 1 mM cyclic AMP (and about 6000 ct/min of [^{3}H]-cyclic AMP); 10 mM phosphocreatine; 10 units/ml creatine-phosphokinase; 0.1% BSA; 10^{-5} M guanosine triphosphate (GTP); 12.5 mM sucrose and 50 μM EGTA (both added with the membrane preparation); and 80 to 150 μg membrane protein, in a final volume of 250 μl. All reagents were added to the assay tubes on ice. The tubes were then transferred to a 30°C shaking incubator and preincubated for 5 min to allow enzymatic activity to reach a steady state. [α-^{32}P]-adenosine triphosphate ([α-^{32}P]-ATP) (1 to 2 μCi) was then added and the reaction was allowed to proceed for 10 to 20 min. The reaction was stopped by addition of 100 μl 1% SDS. After addition of 650 μl H_2O to each tube, the labelled cyclic AMP was iso-

lated by the dual Dowex-alumina column method of Salomon, Londos & Rodbell (1974). Production of [^{32}P]-cyclic AMP was linear with time and protein concentration under the conditions stated above.

Na^+, K^+-ATPase preparation and assay

Previously described methods were used to prepare Na^+, K^+-ATPase from the outer medulla of lamb kidney (Lane, Potter & Collins, 1979) and beef heart (Pitts & Schwartz, 1975). Na^+, K^+-ATPase activity, unless otherwise noted, was assayed in a medium containing in 2.5 ml final volume, 5 mM $MgCl_2$, 5 mM Na_2ATP, 100 mM NaCl, 10 mM KCl, 25 mM histidine chloride (pH 7.4), 2 mM phosphoenolpyruvate, 0.5 mM NADH and 0.02 ml pyruvate kinase/lactate dehydrogenase suspension (Sigma). The reaction was started with the addition of enzyme. The oxidation of NADH was continuously monitored at 340 nm at a temperature of 37°C. Mg^{2+}-ATPase activity is defined as ATPase activity in the presence of 10^{-3} M ouabain, which for the kidney and beef heart enzyme was negligible. For the homogenate from guinea-pig heart, Na^+, K^+-ATPase activity is defined as ouabain-sensitive activity, i.e., total ATPase activity minus Mg^{2+}-ATPase activity.

[^3H]-ouabain binding assay

[^{3}H]-ouabain binding was studied as previously described (Wallick, Anner, Ray & Schwartz, 1978) using four binding media: (a) Mg; (b) Mg + inorganic phosphate; (c) Mg plus ATP plus Na; and (d) Mg plus ATP + Na + K. Each binding medium contained in a final volume of 1.0 ml, 2.5×10^{-6} M [^{3}H]-ouabain (100 mCi/mmol), 42 mM Tris.Cl (pH 7.4). In addition, when used, Mg^{2+} was 5 mM, Na_2ATP was 5 mM, NaCl was 100 mM, KCl was 1 mM and inorganic phosphate was 5 mM. The reaction (30°C) was initiated by the addition of an appropriate amount of enzyme and stopped by the addition of 0.1 ml of 10^{-2} M unlabelled ouabain. The protein was collected on a 0.2 μm Millipore filter and rinsed three times with 5 ml of cold water. The filter containing the protein was dissolved in a scintillation mixture containing Biosolv and Fluoralloy TLA (Beckman) and radioactivity was assayed in a liquid scintillation counter.

Isolation of mitochondria

Mitochondria were isolated from rabbit (male, New Zealand, white, 3 to 4 kg) hearts by Nagarse treatment. The rabbits were killed by a blow on the neck, the hearts were removed and immediately placed in a ice-cold solution containing 180 mM KCl, 10 mM EGTA, 0.5% bovine serum albumin (BSA, Fraction V) and 10 mM HEPES buffer, pH 7.4. All other steps from then on were carried out at 0 to 4°C. The fat pads and atria were removed and the ventricles (3 to 4 g) were finely minced with a pair of scissors. The minced muscle was homogenized in a glass homogenizer with a teflon pestle (Thomas, size C) with 10 ml of the above solution and 5 mg Nagarse/g tissue. The homogenate was immediately diluted to 10 vol (1 g tissue: 10 ml solution) and centrifuged immediately at 7000 *g* for 5 min. The supernatant was discarded and the pellet was resuspended in the above solution (1 g/10 ml) with gentle homogenization and centrifuged at 500 *g* for 10 min. The pellet was discarded and the supernatant was centrifuged at 6500 *g* for 10 min. The resulting crude mitochondrial pellet was washed twice by resuspension and centrifugation before final suspension in a small volume to give 20 to 30 mg protein/ml of a solution containing 180 mM KCl, 0.1 mM EGTA, 0.5% BSA and 10 mM HEPES buffer, pH 7.4. Mitochondrial protein was measured by the method described by Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as standard.

Measurement of respiration

Respiration was measured with a Clark oxygen electrode fitted to a Gilson polarograph. The assays were carried out 30°C in a 1.5 ml solution containing 250 mM sucrose, 2.55 mM Pi, 10 mM HEPES, pH 6.8 and 1 mg mitochondrial protein. The substrates used were 6.6 mM glutamate + 6.6 mM malate or 6.6 mM succinate (+rotenone, 3.3 μM, added in 10 μl ethanol) or 50 μM palmitylcarnitine + 6.6 mM malate. State 3 rate was initiated with 376 nmol ADP. The definitions of state 3, state 4, and the calculations of Respiratory Control (RC) and ADP/O ratios were carried out following the procedure of Estabrook (1967).

Measurement of calcium uptake

Calcium uptake by isolated mitochondria was carried out at room temperature (22°C) by the murexide method (Harigaya & Schwartz, 1969). The assay solution (3 ml) contained 250 mM sucrose, 4 mM Pi, 0.2 mM murexide, 10 mM HEPES (pH 6.8) and 1.5 mg mitochondrial protein. The substrate was 5 mM succinate (+3.3 μM rotenone, added in 20 μl ethanol). The reaction was started with 66 μM $CaCl_2$. The decrease in optical density at 472 to 508 nm was recorded in an Aminco Dual Beam Spectrophotometer Model DW2a.

Measurement of mitochondrial ATPase

ATPase activity was measured in intact and sonicated mitochondria according to Lardy & Wellman (1953). The medium contained 100 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$ and 50 μg sonicated mitochondria in a

final volume of 1 ml. When intact mitochondria were used, the medium was supplemented with 250 mM sucrose. After preincubation at 30°C for 5 min in the presence of RMI 12330A, the reaction was started with 10 mM ATP (pH adjusted to 7.5). The reaction was allowed to continue for 10 min and terminated with 1 ml of ice-cold 10% trichloracetic acid solution. The precipitate was removed by centrifugation at 3000 *g* for 15 min and the liberated Pi in the supernatant was measured according to Bonting, Simon & Hawkins (1961).

Isolation of sarcoplasmic reticulum

SR vesicles from dog heart were prepared as previously described (Sumida, Wang, Mandel, Froehlich & Schwartz, 1978) with an additional centrifugation at 143,000 *g* in the final buffer (10 mM Tris/maleate, pH 6.8, and 100 mM KCl or 30 mM Tris/maleate, pH 6.8, without KCl). The modification of the procedure improved the stability of the SR preparatons with less than 10% loss in yield.

Sarcoplasmic reticulum Ca^{2+}-ATPase

SR Ca^{2+}-ATPase activity was measured at 37°C in a histidine buffer (25 mM, pH 7.2) containing 0 to 100 μM RMI 12330A, 5 mM $MgCl_2$, 5 mM NaN_3, 100 mM KCl, 5 mM ATP, and 14 μM free Ca^{2+} (100 μM added $CaCl_2$ and 100 μM added EGTA). Basal Mg^{2+}-ATPase activity was measured in the buffer solution without added $CaCl_2$. In the procedure SR vesicles (50 μg/ml) were incubated with all the ligands except ATP, and after 6 to 8 min of preincubation, ATP was added to initiate the reaction. The reaction was quenched after 6 min with 10% cold trichloro-acetic acid. The precipitate was removed by centrifugation and the Pi in the supernatant was assayed using ammonium molybdate and ferrous sulphate as described for mitochondrial ATPase. The specific Ca^{2+}-ATPase was the total ATPase less basal Mg^{2+}-ATPase.

Calcium binding and uptake

Experiments were carried out with an Aminco DW2a dual wavelength spectrophotometer, using the calcium sensitive dye, Arsenazo III, at 570 and 600 nm. In the Ca^{2+} binding experiments the reaction medium contained 40 mM Tris/maleate (pH 6.8), 100 mM KCl, 10 mM $MgCl_2$, 30 μM (added) $CaCl_2$, 333 μM ATP, 33 μM arsenazo III, and 0.4 mg/ml SR. The experiments were carried out at 30°C with 8 min preincubation of the SR before addition of 10 μl of 100 mM ATP to initiate calcium binding. Ca^{2+} uptake experiments were carried out in the presence of 5 mM oxalate under the same conditions.

Drugs used

RMI 12330A and RMI 12119A (3,4-dihydro-*N*-(tricyclo[3.3.1.1^{3,7}]dec-1-yl)-2 H-pyrrol-5-amine monohydrochloride were provided by Merrell National Laboratories, Cincinnati, Ohio and were dissolved in 5% ethanol. Nagarse was obtained from Enzyme Development Corporation, New York, N.Y. All other chemicals were of highest purity available commercially. Ouabain (Lilly), histamine (Sigma), isoprenaline (Winthrop) and 2,4-dinitrophenol (Sigma) were used.

Cumulative dose-response curves were obtained and the effective dose level (ED_{50}) determined by probit analysis (Goldstein, 1964). Effectiveness of antagonists were analyzed with the methods of Arunlakshana & Schild (1959) and Van Rossum (1963). All results are expressed as mean ± standard error of the mean. The significance of the difference between means was determined by Student's *t* test. *P* values <0.05 were considered significant.

Results

The isolated work-performing heart of the guinea-pig responded to the adenylate cyclase inhibitor, RMI 12330A, with negative inotropic effects. Figure 1

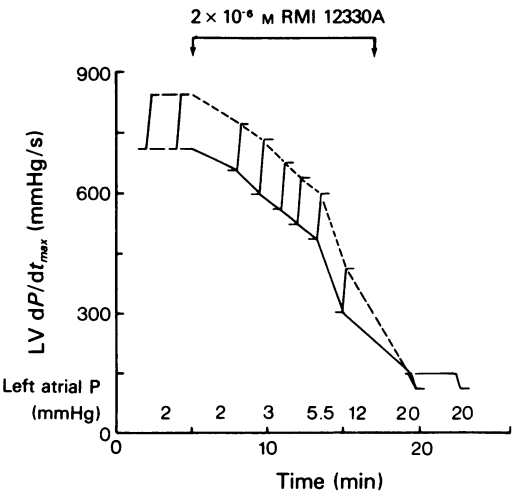

Figure 1 Effects of an infusion of RMI 12330A 2×10^{-6} M on left ventricular (LV) d*P*/d*t* *max* during contraction in the isolated work-performing heart preparation of the guinea-pig. Solid line denotes basal contractility, broken line connects points of higher contractility achieved with increasing heart rate by electrical pacing from 200 to 245 beats per min (positive staircase response). Note at 20 min the reversal of the positive staircase by RMI 12330A. Left atrial pressure (mmHg) is also indicated.

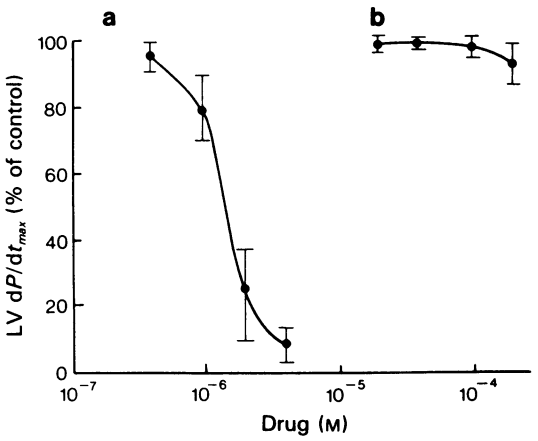

Figure 2 Cumulative dose-response curves of 2 lactam-amines: (a) RMI 12330A, an effective adenylate cyclase inhibitor (n = 8), and (b) RMI 12119A, an ineffective one (n = 3). Vertical bars indicate ± s.e. mean. ID_{50} (50% inhibition of contractility) is 1.1×10^{-6} M for RMI 12330A.

shows an example of a response to 2×10^{-6} M: the basal dP/dt *max* during contraction fell over 15 min from 720 to 150 mmHg/s (solid line). During control, brief 5 s tests of the staircase response by electrical stimulation of the heart to raise heart rate from 200 to 245 beats/min produced a positive staircase from 720 to 860 mmHg/s (broken line in Figure 1); after 15 min, the positive staircase was converted to a negative one. At the same time left atrial pressure rose from 2 to 20 mmHg, indicating the development of cardiac failure. The dose-response curve to RMI 12330A was very steep (Figure 2a). The dose inhibiting dP/dt by 50% (ID_{50}) was determined to be 1.1×10^{-6} M RMI 12330A. A lactam-imine analogue of RMI 12330A, designated RMI 12119A, had no effect on adenylate cyclase activity, and also did not influence cardiac contraction (Figure 2b). In Table 1 all the cardiac variables we could measure or calculate are listed. Only left atrial pressure increased, all other variables decreased and the positive staircase was converted to a negative one. Figure 3 compares a 10 fold higher dose of the ineffective analogue, RMI 12119A, with

Table 1 Effects of RMI 12330A 2×10^{-6} M and dinitrophenol 7×10^{-6} M on the isolated work-performing heart of the guinea-pig

Cardiac function	*Control -ATPase**	*RMI 12330A*	*Dinitrophenol*
Heart rate (beats/min)	215 ± 20	165 ± 21*	174 ± 15*
Aortic pressure systol/diastol (mmHg) mean	61/31 44 ± 5	35/20 25 ± 1.5*	42/26 31 ± 0.7*
LV pressure systol/diastol† (mmHg)	62/1	36/20	45/15
LV dP/dt contraction (mmHg/s) relaxation	+1092 ± 39 −1142 ± 37	+270 ± 45** −195 ± 21**	+475 ± 39** −690 ± 47**
RV contract. force (g)	5.4 ± 0.1	1.7 ± 0.2**	2.8 ± 0.3**
RV dF/dt contraction (g/s) relaxation	+96 ± 2 −119 ± 3	+30 ± 1** −36 ± 1.7**	+50 ± 2* −61 ± 3
LA pressure (mmHg)	1.6 ± 0.4	19.4 ± 4**	16.3 ± 2**
Coronary flow (ml/min)	9.3 ± 0.4	10.5 ± 0.5	10.3 ± 0.6
Venous return (ml/min)	33	33	33
Cardiac work (mmHg × ml/min)	1462 ± 86	828 ± 54*	1033 ± 61%
Stroke work (work/heart rate)	6.8 ± 0.2	5.02 ± 0.5**	5.94 ± 0.3*
Total periph. res. (mmHg ml^{-1} min^{-1})	1.35 ± 0.1	0.75 ± 0.25*	0.95 ± 0.1*
Staircase (mmHg s^{-1}100^{-1} beats)	+155 ± 24	−38 ± 15**	−14 ± 8**

* $P < 0.05$; ** $P < 0.01$; † LV end-diastolic pressure was not measured.
Results are shown as means ± s.e. mean. RMI 12330A experiments, n = 21; dinitrophenol n = 3.
LV = left ventricle; RV = right ventricle; LA = left atrium.

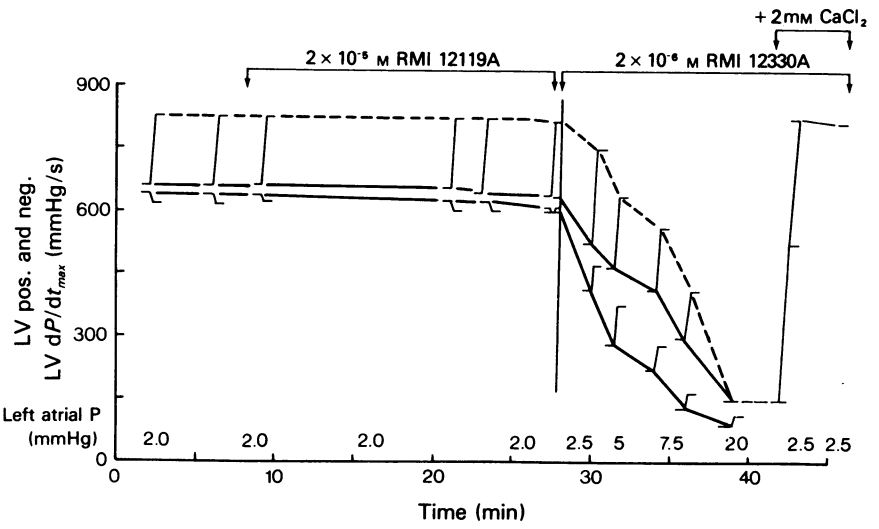

Figure 3 Effects of RMI 12119A and RMI 12330A (see legend of Figure 2) on d*P*/d*t* *max* during contraction (upper solid line), and relaxation (lower solid line) of the guinea-pig isolated heart. The broken line connects the maxima of a staircase response to electrical pacing of the heart from 180 to 260 beats/min. Left atrial pressure (mmHg) is indicated in numbers. Addition of 2 mM $CaCl_2$ to the 2.5 mM present in the perfusate reversed all effects produced by RMI 12330A.

the effective, RMI 12330A. In this figure d*P*/d*t* *max* during relaxation is also shown. Twenty minutes exposure to RMI 12119A did not alter cardiac dynamics. The positive staircase in contraction and relaxation remained unchanged, as did left atrial pressure. The addition of 2×10^{-6} M RMI 12330A, in contrast, immediately decreased contractility; d*P*/d*t* during relaxation fell faster than d*P*/d*t* during contraction, left atrial pressure rose to 20 mmHg and the staircase response disappeared. Infusion of 2 mM $CaCl_2$ in addition to the 2.5 mM present in the perfusate restored within 2 min 80% of cardiac function and the positive staircase response and brought atrial pressure back towards normal.

Since RMI 12330A uncoupled mitochondrial oxidative phosphorylation (see below), we exposed 3 working heart preparations to 7×10^{-6} M dinitrophenol (DNP), an uncoupling agent. This concentration of DNP had effects on cardiac dynamics of the guinea-pig isolated heart similar to that described for RMI 12330A, a decrease in contractility, increase in left atrial pressure and conversion of the positive staircase to a negative one. This inhibition by DNP was also reversed by increasing the Ca^{2+} concentration of the perfusate.

Schwartz *et al.* (1977) found that the effects of cardiac glycosides and catecholamines were attenuated or blocked by a putative adenylate cyclase inhibitor. We, therefore, used ouabain and isoprenaline before and after exposure to RMI 12330A. In addition, histamine was added because RMI 12330A interferes primarily with histamine-stimulated adenylate cyclase (see below). Table 2 lists the results of 8 experiments. For control, the effects of the positive inotropic agonists, histamine, isoprenaline and ouabain in concentrations close to the ED_{50} (see Table 2) on d*P*/d*t* *max*, contractile force and d*F*/d*t* were recorded at normal Ca^{2+} concentrations. Then 2×10^{-6} M RMI 12330A was infused and the effects of the agonists were tested in normal (i.e. 2.5 mM), 4.5 mM and 6.5 mM Ca^{2+} concentration. The first block of Table 2 shows basal contraction, the negative inotropic effects of RMI 12330A, and the beneficial effects of increasing concentrations of calcium. The positive inotropic effects of histamine, isoprenaline and ouabain were greatly reduced in the presence of RMI 12330A; however, increased concentrations of calcium also restored the positive inotropic effects of the agonists.

Adenylate cyclase activity

RMI 12330A, at concentrations up to 10^{-4} M, did not influence basal adenylate cyclase activity (Figure 4a) or activity stimulated by isoprenaline or NaF (data not shown). However, as shown in Figure 4(a), RMI 12330A was an effective inhibitor of cyclase activity stimulated by histamine with an IC_{50} of 1.5×10^{-5} M (mean of 5 experiments) when tested against 10^{-5} M histamine. RMI 12119A, the ineffective analogue of RMI 12330A, did not inhibit basal cyclase activity in concentrations up to 10^{-4} M; the IC_{50} against histamine was greater than 10^{-4} M.

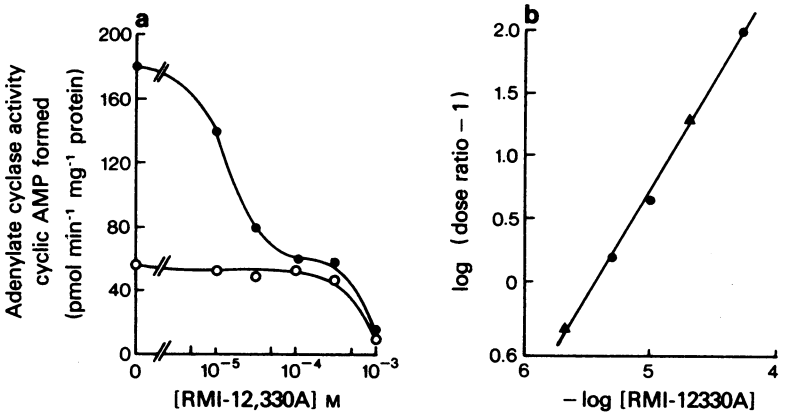

Figure 4 (a) Effect of RMI 12330A on basal (O) and histamine (10^{-5} M) stimulated (●) guinea-pig ventricle adenylate cyclase. The assay followed the procedure of Salomon *et al.* (1974). A typical experiment is shown which was repeated on five different guinea-pig ventricle preparations. (b) Schild plot of the inhibition of histamine-stimulated guinea-pig ventricle adenylate cyclase by RMI 12330A: dose-response curves to histamine (10^{-7} to 10^{-3} M) were obtained in the absence and presence of various concentrations of RMI 12330A. Data from 2 different ventricle preparations were analyzed according to Arunlakshana & Schild (1961).

Time course studies indicated that the inhibition of histamine-stimulated adenylate cyclase by RMI 12330A was rapidly reversed within less than 15 s upon addition of a higher concentration of histamine. Addition of RMI 12330A to a cyclase assay already containing histamine resulted in a rapid inhibition of activity, a new steady state being obtained in less than 15 s. Thus, the inhibitory effect of RMI 12330A represents a readily reversible reaction.

Saturating concentrations of histamine caused a five to seven fold stimulation of adenylate cyclase activity in the cardiac preparations. The dose-response curve to histamine was progressively shifted to the right in a parallel fashion by increasing concentrations of RMI 12330A, suggestive of competitive antagonism. A Schild plot (Figure 4b) of the data from two membrane preparations was linear. However, the slope of the plot, 1.72, was significantly greater than

Table 2 Effects of histamine, isoprenaline and ouabain on the function of the isolated heart of the guinea-pig with and without RM 12330A

	Ca^{2+} (mM)	*dP/dt max during contraction* (mmHg/s)	*Contractile force* (g)	*dF/dt max during contraction* (g/s)
Basal contraction	2.5	+1092 ± 40	5.4 ± 0.1	+97 ± 2
+2.0 × 10^{-6} M	2.5	+270 ± 45	1.7 ± 0.25	+30 ± 1
RMI 12330A	4.5	+613 ± 21	3.1 ± 0.2	+52 ± 3
	6.5	+1170 ± 35	5.6 ± 0.2	+94 ± 5
4.4 × 10^{-7} M histamine	2.5	+1580 ± 36**	7.1 ± 0.3*	+148 ± 5**
+2.0 × 10^{-6} M	2.5	+410 ± 44*	2.2 ± 0.1	+31 ± 3
RMI 12330A	4.5	+910 ± 51*	4.6 ± 0.3*	+91 ± 6**
	6.5	+1715 ± 28**	6.8 ± 0.2*	+155 ± 5**
7.2 × 10^{-9} M isoprenaline	2.5	+1690 ± 25**	7.6 ± 0.6*	+150 ± 5**
+2.0 × 10^{-6} M	2.5	+460 ± 46*	2.6 ± 0.3*	+38 ± 2*
RMI 12330A	4.5	+820 ± 59*	3.9 ± 0.5	+60 ± 5
	6.5	+1695 ± 31**	7.4 ± 0.4*	+148 ± 4**
5.6 × 10^{-7} M ouabain	2.5	+1405 ± 28**	6.5 ± 0.7*	+132 ± 6**
+2.0 × 10^{-6} M	2.5	+246 ± 13	2.5 ± 0.6	+33 ± 3
RMI 12330A	4.5	+1310 ± 50**	5.4 ± 0.8*	+133 ± 5**
	6.5	+1800 ± 62**	6.5 ± 0.8*	+124 ± 4*

*$P < 0.05$; **$P < 0.01$.
Results are shown as means ± s.e. mean ($n = 8$).

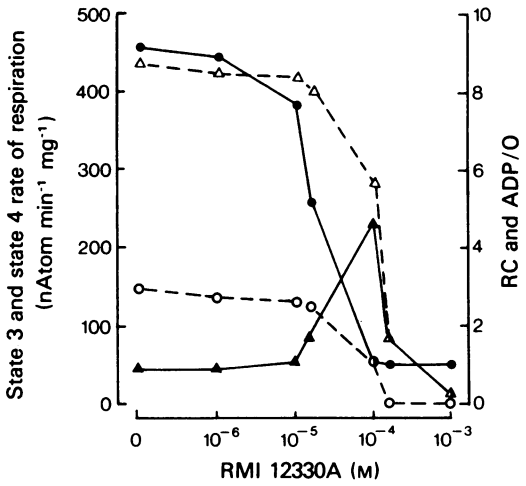

Figure 5 Effects of RMI 12330A on respiratory activities of isolated heart mitochondria of rabbit. (△) = state 3; (▲) = state 4; (●) = RC; (○) = ADP/O. The substrates were 6.6 mM glutamate + 6.6 mM malate. RMI 12330A was added before substrates and ADP.

the theoretical value of one expected for simple competition.

Effects of RMI 12330A on respiratory activities of isolated mitochondria

RMI 12330A up to a concentration of 10^{-5} M had little effect upon state 3 and state 4 respiratory rates or upon RC and ADP/O ratios. Concentrations above 10^{-5} M and up to 10^{-4} M decreased state 3 rates, but increased state 4 rates with a concomitant reduction in RC and ADP/O ratios (Figure 5). RMI 12330A above 10^{-4} M inhibited state 3 rates and decreased state 4 rates. This inhibition of RMI 12330A at higher concentrations was observed with NAD-linked substrates, succinate and palmitylcarnitine but not with ascorbate + TMPD (Figure 6) indicating that the site of inhibition is between coenzyme Q and cytochrome c. The classical uncoupler failed to release this inhibition of respiration at high concentrations of RMI 12330A. Concentrations of RMI 12330A between 10^{-5} and 10^{-4} M released the oligomycin-inhibited state 3 respiration in a manner similar to DNP (Figure 6).

Effects of RMI 12330A on Ca^{2+} uptake by isolated mitochondria

Energy-linked Ca^{2+} uptake of isolated mitochondria was also inhibited by RMI 12330A in a concentration-dependent manner (Figure 7). When mitochondria were completely uncoupled at 10^{-4} M, a small uptake (20%) of Ca^{2+} was still observed. Ca^{2+} uptake was completely abolished at a RMI 12330A concentration of 10^{-3} M.

Effects of RMI 12330A on mitochondrial ATPase

The ATPase activity of sonicated mitochondria was 90 to 95% inhibited by oligomycin. Oligomycin-sensitive ATPase activity of sonicated mitochondria

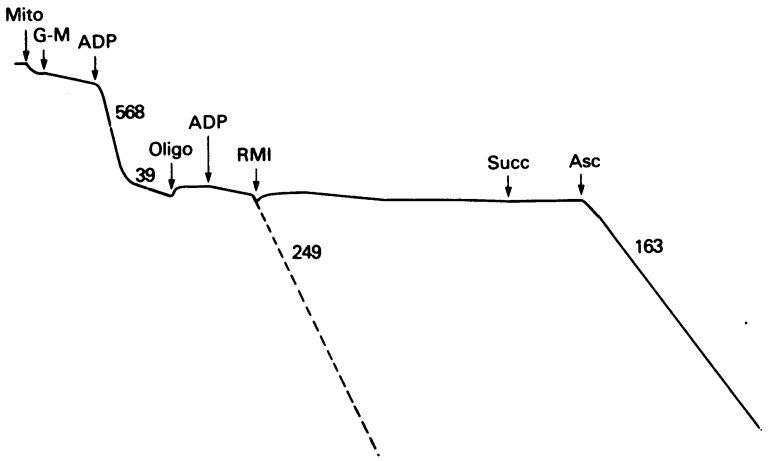

Figure 6 Oxygraph trace showing the effects of RMI 12330A on oligomycin (Oligo) inhibited respiration of isolated mitochondria (Mito). The assay contained 1 mg mitochondrial protein, 6.6 mM each of glutamate and malate (G-M), 376 nmol ADP, 10 μg oligomycin. The other conditions were as described under Methods. Solid line 10^{-3} M RMI; broken line 6.6×10^{-5} M RMI. Succinate (Succ) 6.6 mM and ascorbate (Asc) 5 mM + N,N,N′,N′-tetramethyl-*p*-phenylenediamine 100 μM was added.

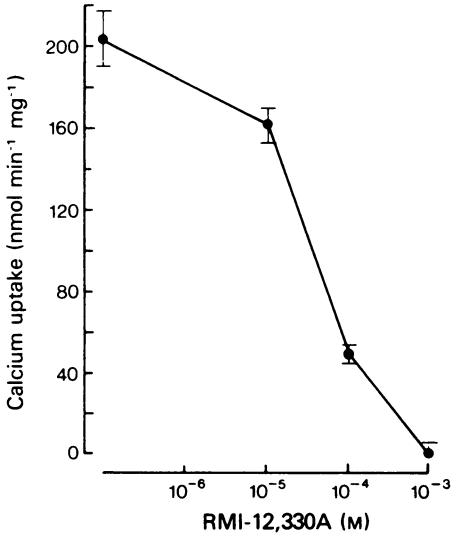

Figure 7 Effect of RMI 12330A on calcium uptake of isolated mitochondria, (n = 4); vertical lines show s.e. mean. RMI was added before addition of succinate (+rotenone) and calcium.

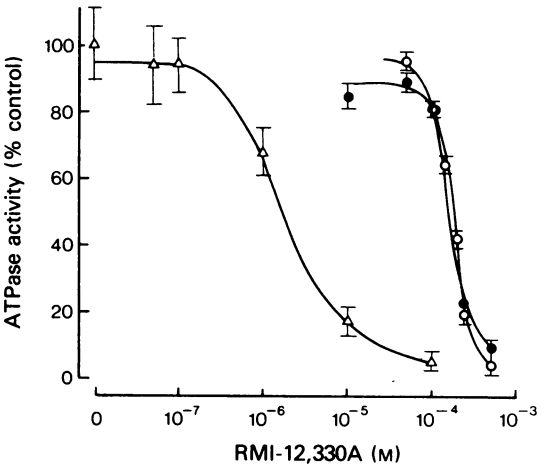

Figure 8 Inhibition of Na^+, K^+-ATPase and mitochondrial ATPase by RMI 12330A. Enzyme activity was assayed as described in Methods. Na^+, K^+-ATPase from sheep kidney (●), beef heart (○), and sonicated mitochondria prepared from rabbit heart (△).

was also inhibited by RMI 12330A (Figure 8). A small stimulation of the enzyme activity was observed at 10^{-7} M in some experiments although it was found not to be statistically significant. Since the mitochondrial protein concentration used to assay ATPase activity was small, it is possible that the inhibition appears to have occurred at lower concentrations than those used to inhibit respiration and Ca^{2+} uptake. The ATPase activity of intact mitochondria was very small (less than 10% of the sonicated mitochondria) and RMI 12330A had little effect upon it up to a concentration of 10^{-4} M.

Na^+, K^+-ATPase activity and ouabain binding

The effect of RMI 12330A on the Na^+, K^+-ATPase activity of a crude homogenate from guinea-pig heart was tested. In the absence of RMI 12330A, the total ATPase activity was 67 μmol mg^{-1} h^{-1} and the ouabain-insensitive activity (Mg^{2+}-ATPase) was 40 μmol mg^{-1} h^{-1}. Thus the Na^+, K^+-ATPase activity was 18 μmol mg^{-1} h^{-1}. The response of the total ATPase activity and the Mg^{2+}-ATPase to RMI 12330A was similar. The Na^+, K^+-ATPase activity (the difference between the total activity and the ouabain insensitive activity) did not appear to be affected. This conclusion is consistent with previous reports (Wiech *et al.*, 1978). However, the dose-response curve of this crude homogenate was quite complex and contained multiple inflection points. Because of the difficulty in measuring Na^+, K^+-ATPase in crude homogenates, the effect of RMI 12330A on preparations of Na^+, K^+-ATPase devoid of Mg^{2+}-ATPase activities was studied. RMI 12330A inhibited the hydrolytic activity of Na^+, K^+-ATPase prepared from sheep kidney and beef heart (Figure 8) with an IC_{50} of 1.6×10^{-4} and 1.8×10^{-4} respectively. RMI 12330A also inhibited ouabain binding to the Na^+, K^+-ATPase purified from sheep kidney. The degree of inhibition of ouabain binding depended upon the binding conditions employed. In the presence of MgPi and of MgATPNa, ouabain binding was inhibited 75% by 5×10^{-4} M RMI 12330A. In the presence of Mg alone or MgATPNaK (conditions under which ouabain binding is slower) ouabain binding was inhibited 95% and 85% respectively. Since the drug inhibited ouabain binding both in the presence and absence of ATP, it seemed unlikely that the drug was interfering with binding of ATP to the enzyme. On the other hand, since RMI 12330A also inhibited mitochondrial ATPase and Ca^{2+}-ATPase activity of sarcoplasmic reticulum, a kinetic study was carried out.

For concentrations of RMI 12330A less than 2×10^{-4} M the double-reciprocal plot was parallel (Figure 9). At higher concentrations the plots became non-linear. The reciprocal plot pattern is typical of uncompetitive inhibition, with a reversible binding of the inhibitor (I) to the enzyme-substrate (ES) complex yielding an inactive ESI complex. The inhibitor does not bind to free enzyme. For this simple case replots

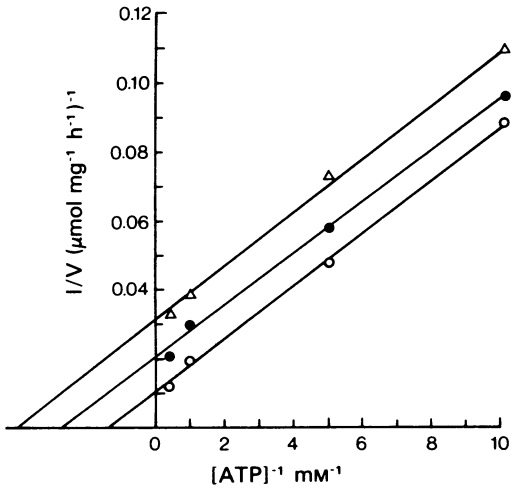

Figure 9 Double reciprocal plot: effect of ATP on specific activity of enzyme (Na^+, K^+-ATPase) isolated from beef heart in the presence of zero (○), 0.15 mM (●), and 0.25 mM (△) RMI 12330A.

$1/V max$ and $1/Km$ versus I will be linear. Replots for RMI 12330A show curves which are concave upward suggesting multiple inhibitory sites for RMI 12330A. A Hill plot yielded an n value of 3.2.

The compound RMI 12119A, an analogue of RMI 12330A, in the range 10^{-8} to 10^{-3} M had no effect on hydrolytic activity or ouabain binding of Na^+, K^+-ATPase isolated from lamb kidney.

Effects of RMI 12330A on Ca^{2+}-ATPase of sarcoplasmic reticulum

The specific activity of Ca^{2+}-ATPase of sarcoplasmic reticulum used in this study ranged from 50 to 70 μmol Pi mg^{-1} h^{-1}. The percentage inhibition of the enzyme by RMI 12330A is shown in Table 3. The dose-dependent inhibition curve shows that RMI 12330A at 50 μM inhibited approx. 50% of the Ca^{2+} ATPase activity, but the drug became less potent at high concentration, with 100 μM of the drug inhibiting only 60% of the activity. In the medium containing 5 mM oxalate (a Ca^{2+} precipitating reagent used in the Ca^{2+} uptake experiments) RMI 12330A was totally ineffective in inhibiting the Ca^{2+}-ATPase. Under the same conditions, with or without 5 mM oxalate, RMI 12330A at 40 μM inhibited 20% of the total basal Mg^{2+}-ATPase activity (data not shown).

Effects of RMI 12330A on sarcoplasmic reticulum calcium binding and uptake

The apparent total calcium binding of the sarcoplasmic reticulum preparations ranged from 60 to 76 nmol/mg, and the total uptake averaged 230 nmol/mg in the presence of 5 mM oxalate. RMI 12330A had inhibitory effects on both the calcium binding and the rate of calcium uptake as shown in Table 3. The inhibition of the apparent total binding was significant at drug concentrations greater than 30 μM. The drug at 100 μM inhibited 60% of the calcium binding. The inhibition by the drug of the rate of calcium uptake was linear. At 50 μM, RMI 12330A decreased the rate of uptake by 85%. The total calcium uptake was not affected by the drug.

Discussion

Cyclic nucleotides play an important part in the contractile process of the heart. Therefore, the effects of agents decreasing or increasing their availability are of great interest. Levey, Lehotay, Canterbury, Bricker & Meltz (1975) reported the isolation of an endogenous substance that inhibited adenylate and guanylate cyclase activity, blocking glycogenolysis and calcium uptake in cardiac sarcoplasmic reticulum. Schwartz *et*

Table 3 Percentage inhibition induced by RMI 12330A of dog cardiac sarcoplasmic reticulum, Ca^{2+}-ATPase, Ca^{2+}-binding and Ca^{2+}-uptake

[*RMI 12330A*] (μM)	*Ca^{2+}-ATPase**		*Ca^{2+}-binding*	*Ca^{2+}-uptake*
	−oxalate	*+oxalate*		
0	0	0	0	0
5	7	0	—	—
10	12	0	1	11
20	24	0	—	—
30	36	0	—	52
40	42	0	—	—
50	47	—	25**	84
100	58	—	57	—

*Average of triplicate; **average of duplicate.

al. (1977) studied the effects of this substance on myocardial contractility and found blockade of cardiotonic and cardiodepressive agents and conversion of the positive staircase to a negative one. Unfortunately the chemical nature of this substance is not known, making the study of mechanism of action difficult.

Recently a chemically well-defined compound, RMI 12330A, became available, which was reported to inhibit adenylate cyclase but not Na^+, K^+-ATPase (Siegel & Wiech, 1976; Cheng, Munro, Reavis & Woodward, 1976; Guellaen *et al.*, 1977). This made possible a systematic study of the effects of an adenylate cyclase inhibitor on myocardial function and on subcellular organelles.

RMI 12330A in concentration above 10^{-4} M inhibited in our studies adenylate cyclase activity of broken cell membrane preparations and this was true whether activity was measured in the absence or presence of activators of adenylate cyclase, such as histamine, isoprenaline, or NaF. We have previously shown (Johnson & Mizoguchi, 1977; Johnson, Weinstein & Green, 1979a and b) that histamine-activated adenylate cyclase in guinea-pig heart has the characteristics of an H_2-receptor system as defined by Black, Duncan, Durant, Ganellin & Parsons (1972). RMI 12330A appears to be a more potent inhibitor of the histamine-activated cyclase than of basal cyclase activity or activity stimulated by isoprenaline or NaF. Although the effects of RMI 12330A on histamine-stimulated cyclase revealed competitive kinetics with histamine, on the basis of parallel dose-response curves, the Schild plot had a slope considerably greater than one, perhaps indicative of positive cooperativity. However, another and perhaps more likely explanation is that there is substantial nonspecific binding of this compound to the cardiac membranes. This might result, particularly at low doses, in free concentrations of inhibitor considerably below the total amounts added to the assay. As a consequence of this, the slope of the Schild plot would be erroneously high. Guellaen *et al.* (1977) in fact presented evidence for substantial binding of RMI 12330A to rat liver membranes. These results substantiate earlier reports describing the effects of RMI 12330A on adenylate cyclase activity in liver plasma membranes (Siegel & Wiech, 1976). The studies reported that RMI 12330A inhibited basal cyclase activity as well as activity stimulated by NaF, adrenaline, VIP, GTP and GMP-P (NH)P and glucagon. RMI 12330A also decreased cholera-induced intestinal secretion (Siegel & Wiech, 1976) and antagonized acid secretion in the Shay and Gosh-Schild rat models of peptic ulcer (Cheng *et al.*, 1976). Cyclic AMP is believed to be involved in both the cholera toxin effects and in acid secretion induced by histamine or pentagastrin. It was suggested that RMI 12330A had anti-secretory properties similar to the H_2-receptor antagonist, metiamide (Cheng *et al.*, 1976). Our observations that RMI 12330A was an effective inhibitor of histamine-stimulated adenylate cyclase may explain the inhibitory action of this compound on acid secretion.

RMI 12330A has some interesting effects on cardiac dynamics in the isolated work-performing heart preparation. These effects must be understood on the basis of the peculiarities of the work performing heart preparation. Since venous return is kept constant in this preparation, negative effects which shift Starling function curves to the right, must lead to an increase in left atrial pressure and left ventricular and end-diastolic pressure. The functional limit of the preparation is reached when left atrial pressure is about 20 mmHg. RMI 12330A reduced all the variables of cardiac dynamics greatly. Particularly, it increased left atrial pressure quickly, leading to signs of 'frank' cardiac failure. This explains the steepness of the dose-response curve (Figure 2). The negative effects involved all the cardiac variables that we measured and calculated. Of special significance was that dP/dt during relaxation decreased faster than the one during contraction and that the positive staircase response was converted to a negative staircase response. In contrast to this, in cardiac failure, which had developed spontaneously in the absence of RMI 12330A, the positive staircase response was reduced but always present and did not convert to a negative one. Cardiac glycosides, histamine or catecholamines given during the RMI 12330A exposure were greatly reduced in their positive inotropic effects. They did not restore a positive staircase response. However, increasing the calcium concentration of the perfusate above the normal 2.5 mM restored not only all inhibited variables of cardiac dynamics, including left atrial pressure and positive staircase response, but also the responses to the three positive inotropic agents. Most of these observations are compatible with the report of the effects of another adenylate cyclase inhibitor (Schwartz *et al.*, 1977). Basal contraction was not decreased in their experiments whereas a substantial reduction occurred in ours. The functional significance of the left atrium in our system versus the free response of a myocardial strip used in the other study may explain the difference (see above). Both studies revealed negative staircase, a reduced response to ouabain and catecholamines and a positive response to calcium during adenylate cyclase inhibition. We did not use acetylcholine but added histamine in our study since RMI 12330A inhibited histamine-stimulated adenylate cyclase in concentrations that were similar to the ones we used in the isolated hearts. Also, histamine stimulates ventricular contraction of the guinea-pig by H_2-receptor stimulation (Verma & McNeill, 1977) and we showed that

RMI 12330A inhibited histamine-stimulated adenylate cyclase, apparently by interactions with the histamine H_2-receptor (although the inhibition may not be strictly competitive). This action, therefore, could be a 'true' effect of adenylate cyclase inhibition on cardiac dynamics. However, the effects of catecholamines were also reduced, but RMI 12330A had effects on *in vitro* catecholamine-stimulated adenylate cyclase only in concentrations 10 or more times higher than the ones used in the isolated hearts. Furthermore, cardiac glycoside effects were reduced similarly, and the positive staircase became negative, effects which are not commonly linked to cyclic AMP activity. Further support for the possibility that the adenylate cyclase inhibitory action of RMI 12330A is indeed the cause of the cardiac effects we observed, is the fact that dP/dt during relaxation was influenced earlier and decreased at a faster rate than the one during contraction. Since cyclic AMP is probably involved in relaxation (Kirchberger, Tada, Repke & Katz, 1972), decreased levels produced by inhibition of adenylate cyclase should be revealed in the relaxation phase first.

It is clear from this study that RMI 12330A inhibits several systems and is not specific for adenylate cyclase. A possible clue as to the RMI 12330A mechanism of action can be found in our studies with isolated mitochondria. RMI 12330A appeared to be acting like a classical uncoupler of oxidative phosphorylation of mitochondria at low concentrations, in a manner similar to DNP. At higher concentrations the action of RMI 12330A on isolated mitochondria was complex. The inhibition of state 3 and state 4 rates of respiration may be due to inhibition of electron transport chain between coenzyme Q and cytochrome c and probably mitochondrial ATPase activity. Although inhibition of ATPase activity of sonicated mitochondria was observed, the fact that DNP did not release the inhibition of respiration by high concentrations of RMI 12330A indicated that the inhibition of respiration was probably not due to the inhibition of ATPase activity *in vivo*. The concentrations at which RMI 12330A affected the myocardial function of isolated working heart preparations were similar to those which uncoupled oxidative phosphorylation of isolated mitochondria. It is, therefore, quite likely that the negative inotropic effect of RMI 12330A may be due to the uncoupling effect of this drug. Indeed, exposure of the isolated heart to DNP 7×10^{-6} M revealed identical effects to the ones seen with RMI 12330A on those cardiac dynamics we observed (Table 1). The conversion of the staircase and the improvement with increasing calcium concentrations were also seen.

In our membrane preparations of crude homogenates of the guinea-pig heart and in purified sheep kidney and partially purified beef heart, RMI 12330A in high doses inhibited the hydrolytic activity of Na^+, K^+-ATPase. Ouabain binding to the same enzymes was also inhibited. The results of the studies on the guinea-pig homogenates were more variable than those on the other preparations. Multiple binding sites on the enzymes for RMI 12330A are suggested. It is tempting to speculate that the differences between the results on the 'crude homogenate' (guinea-pig homogenates) and the more highly purified preparations (beef heart and sheep kidney) are due to the presence of adenylate cyclase in the 'crude homogenate'.

It is clear that several of the actions of RMI 12330A can be understood on the basis of adenylate cyclase inhibition, albeit a rather specific inhibition, namely that of the histamine-stimulated adenylate cyclase. It also seems clear that RMI 12330A interferes with calcium-related mechanisms, which include histamine, catecholamine and cardiac glycoside-induced effects on contractility. Increasing calcium concentration restored function to all the depressed cardiac tissues. Finally, RMI 12330A uncoupled mitochondrial oxidative phosphorylation in a manner similar to DNP. Since RMI 12330A affects different enzymatic and dynamic systems of the heart, it is possible that each of the observed effects are caused by different mechanisms. On the other hand, it is possible that all of these factors, adenylate cyclase activity, calcium delivery and oxidative phosphorylation are processes that involve membrane activity and are in fact membrane-associated-enzymatic systems. RMI 12330A may inhibit these membrane processes probably by altering membrane organization.

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