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EFFECTS OF SEVERAL NEWER CARDIOTONIC DRUGS ON CARDIAC CYCLIC AMP METABOLISM

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Abstract—The purpose of this study was to investigate the possible roles of selective inhibition of cyclic nucleotide phosphodiesterase (PDE) isozymes, adenylate cyclase activation, and tissue cyclic 3′,5′-adenosine monophosphate (cyclic AMP) elevation in the positive inotropic action of five new cardiotonic drugs. Three PDE isozymes (PDE I, II and III), homogenates, and slices of guinea pig ventricles were used. The inotropics amrinone, milrinone, AR-L 115BS, MDL 17,043, and RMI 82,249 all inhibited cyclic AMP hydrolysis by PDE III in a concentration-dependent manner, as did the PDE inhibitors aminophylline and 1-methyl-3-isobutylxanthine (MIX). All drugs except for AR-L 115BS inhibited PDE III at concentrations lower than those producing a standard inotropic response. A significant correlation ($r = 0.80$, $P < 0.05$) was observed between PDE III inhibition and inotropic activity for six of the drugs. Only aminophylline and MIX, but none of the cardiotonic drugs, inhibited cyclic AMP hydrolysis by PDE I and II and cyclic 3′,5′-guanosine monophosphate (cyclic GMP) hydrolysis (amrinone not tested) by PDE I. Further, none of the cardiotonic drugs inhibited the calmodulin-stimulated cyclic AMP hydrolysis by PDE I, indicating their lack of calmodulin antagonist activity. These drugs also did not stimulate adenylate cyclase activity but all increased net cyclic AMP formation from ATP in guinea pig ventricular homogenates through inhibition of cyclic AMP breakdown. Amrinone, milrinone, MDL 17,043 and RMI 82,249, but not AR-L 115BS, raised cyclic AMP levels significantly ($P < 0.05$) in guinea pig ventricular slices. Also, amrinone, MDL 17,043 and RMI 82,249, but not AR-L 115BS, potentiated forskolin-induced cyclic AMP increase. These data taken together suggest that the specific inhibition of cyclic AMP PDE III isozyme and the consequent elevation of tissue cyclic AMP levels in cardiac tissue are an important mechanism of action of amrinone, milrinone, MDL 17,043 and RMI 82,249. Because AR-L 115BS did not increase cyclic AMP levels, it is likely that another mechanism may participate in the inotropic response to AR-L 115BS.

Conventional cardiotonic drugs for the treatment of heart failure have many limitations, including induction of arrhythmias (cardiac glycosides), a narrow therapeutic index (cardiac glycosides), and a lack of oral activity (dopamine and related compounds) [1, 2]. Recently, the search for new types of cardiotonic agents resulted in the discovery of amrinone and many other agents which are not structurally related to catecholamines or cardiac glycosides. The new agents include amrinone, its more potent analog milrinone (WIN 47,203), MDL 17,043 (fenoximone), RMI 82,249 and AR-L 115BS (Vardax) (Fig. 1) [3–7]. They are either pyridine or imidazole derivatives and exhibit similar cardiovascular profiles, including oral inotropic and vasodilator activities and smaller chronotropic effects than catecholamines. The inotropic effects of these agents do not appear to be mediated by direct interaction with beta adrenoceptors or Na^+,K^+-ATPase [5, 7–11]. On the other hand, cardiac glycosides and catecholamines are known to produce their inotropic effects by inhibiting Na^+,K^+-ATPase and stimulating beta adrenoceptors respectively [2].

Earlier studies failed to detect any significant effect of amrinone on cardiac PDE and cyclic AMP levels [8, 12], suggesting a unique mechanism of cardiotonic action. However, recent studies demonstrate the ability of amrinone to inhibit a crude cyclic AMP PDE or the PDE fraction III (PDE III isozyme) from cardiac tissues and to elevate cyclic AMP levels in cardiac muscle preparations [13–15]. Milrinone also was found to inhibit crude cardiac PDE and to increase cyclic AMP levels [3, 15]. Like amrinone, MDL 17,043 selectively inhibits dog heart cyclic AMP PDE III in its inotropic concentration range [9]. AR-L 115BS produces a weak inhibition of crude PDE and fraction III cardiac PDE [15–17]. As for RMI 82,249, conflicting findings were reported [6, 15]. Although milrinone, AR-L115BS and MDL 17,043 increase cardiac cyclic AMP levels [3, 9, 13, 14], their potential direct effects on adenylate cyclase have not been reported.

In this study we examined effects of five newer cardiotonic agents and two standard PDE inhibitors on three PDE isozymes and correlated their PDE inhibitory activities with cardiotonic activities. This study also represents a systematic evaluation of effects of these agents on cyclic AMP metabolism using isolated PDE isozymes, homogenates, and relatively intact slices of guinea pig ventricles.

MATERIALS AND METHODS

Assay of cyclic 3′,5′-nucleotide phosphodiesterase. Cyclic 3′,5′-nucleotide phosphodiesterase (PDE) activity was determined by the radiometric method

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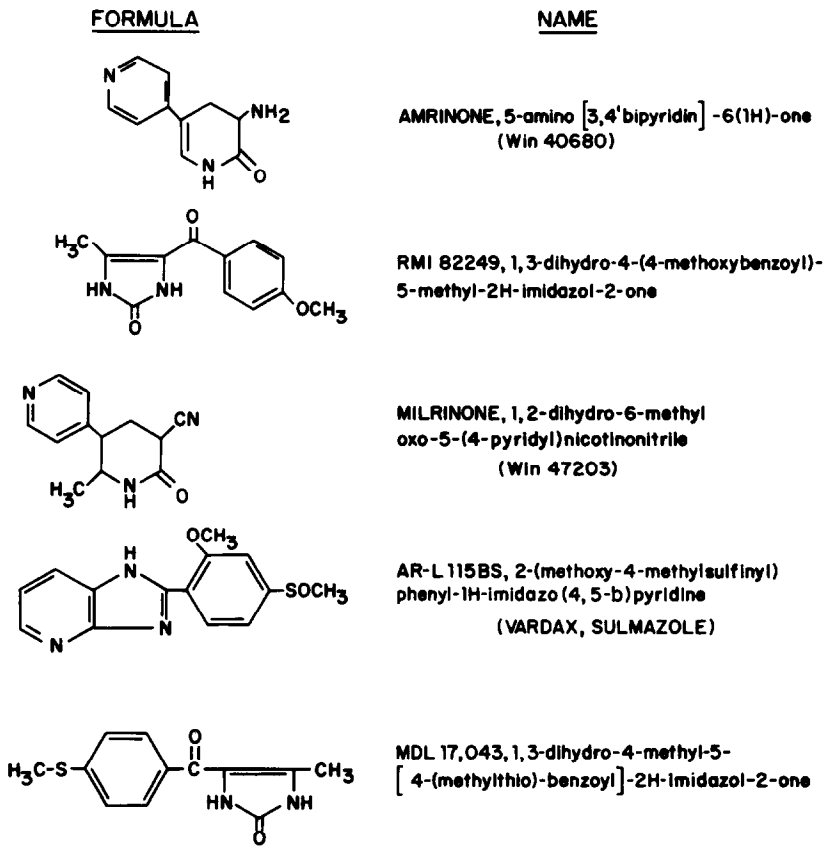

Fig. 1. Chemical structures of newer cardiotonic agents.

of Brooker *et al.* [18] using PDE isozymes I, II and III isolated from guinea pig ventricle. The substrate used for drug inhibition studies was either 1 μM cyclic AMP or 1 μM cyclic GMP. In kinetic studies the concentration of cyclic AMP varied between 0.1 and 10 μM or 1 and 100 μM. The reaction mixture was incubated for 10 min at 30°.

Isolation of cyclic nucleotide PDE isozymes. Ventricles from male Hartley guinea pigs (400–500 g) were homogenized in 10 vol. of reagent grade water using a Brinkmann Polytron PT-10 (30 sec, setting 5) and then sonicated for 4 min in an ice-bath at the half-maximum power output using a Branson sonifier cell disrupter 200. The sonicated homogenates were centrifuged at 30,000 *g* for 20 min at 4°. Three major forms of guinea pig ventricular cyclic nucleotide PDE were isolated by DEAE-cellulose anion exchange liquid chromatography using the stepwise elution procedure described by Thompson *et al.* [19]. DEAE-cellulose chromatography was performed in a column (1.5 × 40 cm) with a bed volume of 32 ml. The 30,000 *g* supernatant fraction (approx. 7 ml) was applied to the column equilibrated with 70 mM sodium acetate buffer, pH 6.5. The loaded column was washed with two column volumes of buffer (70 mM sodium acetate buffer, pH 6.5, containing 5 mM mercaptoethanol) followed by successive elutions with one column volume of each of 220, 350, and 700 mM sodium acetate buffers, pH 6.5, containing 5 mM mercaptoethanol at a flow rate of 0.6 ml/min. Ventricles from Hartley guinea pigs (400–500 g) were also used in preparing homogenates or slices for studies on cyclic AMP formation.

Accumulation of cyclic AMP generated from ATP in guinea pig ventricular homogenates. Cyclic AMP formation from ATP in the absence of a PDE inhibitor was assayed by the method of Tse *et al.* [20]. Briefly, ventricles were homogenized for 30 sec in 3 vol. buffer (50 mM Tris–HCl buffer, pH 7.5, containing 10 mM $MgCl_2$) using a Brinkmann Polytron PT-10 at a speed setting of 5. The homogenates were filtered through a thin layer of glass wool and diluted 5-fold with the same buffer. Samples (50 μl) of the above homogenates were incubated for 10 min at 30° with the reaction mixture (150 μl) containing 50 mM Tris–HCl (pH 7.5), 120 mM $MgCl_2$ and 1 mM ATP in the presence and absence of drug. The reaction was terminated by placing the tubes in a boiling water bath for 3 min. Aliquots of the 1000 *g* supernatant fraction were assayed for cyclic AMP by a protein binding assay (Diagnostic Products Co.).

Assay of adenylate cyclase in ventricular homogenates. Cyclic AMP formation from ATP in the presence of a PDE inhibitor represents adenylate cyclase activity and was measured by the method of Kanoff and Greengard [21] with the following changes. A 1 mM concentration of MIX was used, and ventricular homogenates were incubated at 30° for 10 min. The reaction mixture contained 100 mM Tris maleate buffer (pH 7.8), 0.6 mM ethylene-

glycolbis(amino-ethylether)tetra-acetate (EGTA), 1 mM MIX, 2 mM $MgCl_2$, 0.1 mM GTP and 1 mM ATP. Cyclic AMP was determined as described above.

Effect of agents on cyclic AMP levels in guinea pig ventricular slices. The procedure for slice preparation and incubation was similar to that previously reported [22] with the following modifications. Calcium chloride in the final incubation medium was replaced with 0.6 mM EGTA, unless otherwise indicated. The Dowex 1-X8 column step for cyclic AMP purification was omitted since it did not make any difference in cyclic AMP measurement. Protein was precipitated in trichloroacetic acid and redissolved in 1 N NaOH for protein determination. The final incubation of slices was carried out for 10 min at 37° in the presence and absence of an agent.

Determination of positive inotropic activity of agents in isolated guinea pig left atria. We chose guinea pig atria over ventricular preparations for determination of inotropic activity because the atria was found to be more stable (less irregular beats) and to respond to inotropic agents with a greater magnitude. Guinea pig left atria were allowed to equilibrate for at least 1 hr in the organ baths containing Krebs–Henseleit bicarbonate buffer aerated with 5% CO_2 and 95% O_2 at 32°. Resting tension was set at 1 g. Atria were stimulated electrically (voltages 10% above threshold) with rectangular pulses of 1 msec duration at a frequency of 1 Hz by impulses generated from a Grass S-44 stimulator and distributed by a Buxco multichannel stimulus-distribution unit. Isometric force developed by the atria was measured with Grass FT 03 force displacement transducers connected to Buxco preamplifiers. A concentration–response relationship for each drug was determined using a cumulative dosing regimen which involved addition to atrial preparations of increasing concentrations of each drug at 5-min intervals. This duration was sufficient for equilibration prior to administration of the next dose. Since the cumulative dosing resulted in an attenuated response to MDL 17,043, its concentration response was determined by measuring responses of individual atrial preparations exposed to only one concentration (100, 300 or 1000 μM) of the drug. Signals were recorded on Hewlett–Packard or Brush oscillographs. The concentration causing an increase in atrial force of 750 mg (i.e. EC_{750}) was determined by log-linear regression analysis. The EC_{750} represents the concentration producing a large (i.e. approximately 75%) increase in contractile force.

Other procedures. Protein concentration was determined by the method of Lowry *et al.* [23] with bovine serum albumin as a standard. Data presented in this report are mean ± standard errors of the means (S.E.M.) and were analyzed statistically using analysis of variance, Duncan's Multiple range test, and Student's *t*-test. The IC_{50} (concentration producing 50% inhibition) values and correlation coefficients were obtained by a least squares analysis [24] of experimental data. Stock solutions (10 mM) of MIX, MDL 17,043, RMI 82,249 and AR-L 115BS were prepared by dissolving them in 100% dimethyl sulfoxide (DMSO). Subsequent dilution was made with reagent grade water. Other drugs were dissolved in water. Amrinone and milrinone were synthesized by Drs. R. Doll and E. Smith (Medicinal Chemistry, Schering Corp.). RMI 82,249 and MDL 17,043 were supplied by Dr. R. C. Dage (Merrell-Dow) and AR-L 115BS from Karl Thomae GmbH (West Germany). [^{3}H]Cyclic AMP (sp. act. 42.5 Ci/mmole) and [^{3}H]cyclic GMP (sp. act. 34.5 Ci/mmole) were obtained from the New England Nuclear Corp. and calmodulin and other drugs from the Sigma Chemical Co.

RESULTS

Isolation and kinetic properties of PDE fractions I, II and III. An extract (30,000 *g* supernatant) of guinea pig ventricular homogenates was subjected to DEAE-cellulose chromatography to separate the various PDE isozymes. These were identified by monitoring cyclic AMP degrading activity. Three peaks were typically obtained and labeled as PDE I, II and III according to the order of elution from the column (Fig. 2). Highest cyclic AMP PDE activity was obtained in peak III, followed by I and II.

The kinetics of the hydrolysis of cyclic AMP by guinea pig ventricular PDE I, II and III were investigated by Lineweaver–Burk analysis and are summarized in Table 1. PDE III contained only a single form of the enzyme with a high affinity for cyclic AMP (K_m = 1.1 μM) in agreement with pre-

Table 1. Kinetic parameters of isolated guinea pig ventricular PDE peaks I, II and III

	K_m (μM)	V_{max} (pmoles/tube/10 min)	% Stimulation by calmodulin*
PDE I	2.9	35	233†
PDE II High affinity	13	133	328†
Low affinity	182	250	
PDE III	1.1	24	9

For Lineweaver–Burk analysis of cyclic AMP (substrate) hydrolysis by PDE I and III, the cyclic AMP concentration used ranged from 1 to 100 μM. Cyclic AMP concentration varied from 0.5 to 5.0 μM and from 10 to 300 μM, respectively, for Lineweaver–Burk analyses of the high- and low-affinity enzyme activities of PDE II. These kinetic experiments were performed in the absence of calmodulin.

* Five units of calmodulin plus 20 μM or 2 mM $CaCl_2$ were added to the enzyme incubation mixture containing 1 μM cyclic AMP as a substrate.

† Significant stimulation, $P < 0.05$ (DF ≥ 4), Student's *t*-test.

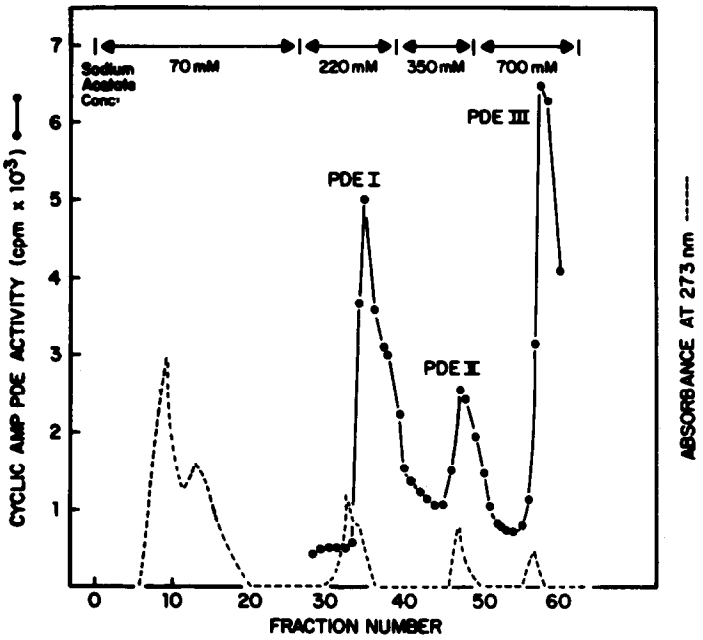

Fig. 2. Typical DEAE-cellulose chromatographic profile of guinea pig ventricular cyclic nucleotide PDE. Aliquots (50 μl) of eluates were assayed for enzyme activity using 1 μM cyclic AMP as the substrate. Fractions 34–38, 46–50 and 57–62 were pooled, respectively, for peaks I, II and III. The major peaks were labeled PDE I, II and III according to the order of elution from the column.

vious studies employing other species [9, 17]. PDE I contained a high-affinity isozyme with a K_m value of 2.9 μM, whereas PDE II was a mixture of a "high" (K_m = 13 μM) and a "low" (K_m = 182 μM) affinity form of PDE. These data contrast with reported studies showing the presence of only a "low" affinity form (K_m, approximately 50 μM for cyclic AMP) for both PDE I and II isolated from hearts of other animal species [9, 19]. Calmodulin (5 Sigma units) in the presence of calcium (20–2000 μM) stimulated PDE I and II 2- to 3-fold but not PDE III (Table 1).

Effects of drugs on cyclic AMP hydrolysis by guinea pig ventricular PDE I, II and III. To evaluate the specificity of the newer cardiotonic drugs, effects of these agents on PDE I, II and III were examined using cyclic AMP (1 μM) as a substrate. Also two standard PDE inhibitors, aminophylline and MIX, were tested for comparison. Amrinone, milrinone, AR-L 115BS, MDL 17,043, RMI 82,249, aminophylline and MIX all inhibited PDE III activity in a concentration-dependent manner. Their IC_{50} values for the inhibition of PDE III are listed in Table 2. Potencies of the newer cardiotonic agents varied widely: MDL 17,043 was the most potent, and amrinone and AR-L 115BS the least potent. Other cardiotonic agents including ouabain and *l*-norepinephrine did not inhibit PDE III at concentrations up to 100 μM (results not shown). In marked contrast to PDE III, PDE I and II activities, as measured by cyclic AMP hydrolysis, were inhibited significantly by aminophylline and MIX but not by amrinone, AR-L 115BS, MDL 17,043 and RMI 82,249 (Table 3). Further, the inhibition of PDE I and II required higher concentrations of aminophylline and MIX than that of PDE III. An essentially similar result was obtained when effects of these agents on PDE I activity were measured using cyclic GMP as a substrate (Table 3). The calmodulin-stimulated activity of PDE I was not antagonized by any of the cardiotonic agents tested but was antagonized by trifluoperazine, a calmodulin antagonist (results not shown).

Table 2. Inhibition of guinea pig ventricular PDE III by positive inotropic drugs

Drug	No. of experiments	PDE III inhibition IC_{50}* (μM)
Aminophylline	6	147 ± 12
MIX	4	13 ± 4
Amrinone	5	238 ± 56
Milrinone	2	45
AR-L 115BS	3	225 ± 73
MDL 17,043	3	25 ± 4
RMI 82,249	3	66 ± 22

Each IC_{50} value represents the mean ± S.E.M. derived from two to six experiments. Each experiment involved two to four concentrations of test agent assayed in duplicate. Substrate (cyclic AMP) concentration used was 1 μM. PDE III (25 μl) hydrolyzed 16.2 ± 1.4 pmoles cyclic AMP/10 min under the assay condition.

* Concentration producing 50% inhibition.

Relationship between PDE inhibition and cardiotonic activity for newer cardiotonic drugs and standard PDE inhibitors. The newer cardiotonic drugs, and aminophylline and MIX produced a concentration-related increase in isometric contractile force of isolated guinea pig atria. The concentration required to augment contractile force by 750 mg

Table 3. Effects of positive inotropic drugs on cyclic AMP nucleotide hydrolysis by guinea pig ventricular PDE I and II

Drug	Concn (μM)	Cyclic AMP hydrolysis % Change PDE I	Cyclic AMP hydrolysis % Change PDE II	Cyclic GMP hydrolysis % Change PDE I
Aminophylline	100	−11	−14	−19*
	500	−42*	−45*	−59*
MIX	3	+2	−4	−11
	30	−45*	−49*	−60*
AR-L 115BS	50	+4	+5	+3
	300	+19	+5	−6
MDL 17,043	50	−25	−23	ND
	100	ND	ND	−4
	1000	−10†	ND	+10
RMI 82,249	100	−4	−9	−9
	500	−4	−16	−17†
Amrinone	300	+1	+2	ND
	1000	−8	−7	ND
Milrinone	100	ND	ND	−19†

Each value represents the mean of four to six determinations pooled from two separate experiments unless otherwise indicated. Substrate (cyclic AMP or cyclic GMP) concentration used was 1 μM. PDE I (50 μl) hydrolyzed 21.4 ± 0.8 pmoles cyclic AMP/10 min (N = 6) and PDE II (50 μl) hydrolyzed 12.4 ± 0.6 pmoles cyclic AMP/10 min (N = 4) under the assay condition. PDE I and II used for cyclic AMP hydrolysis were obtained from the same run of DEAE-cellulose chromatography. PDE I used for cyclic GMP hydrolysis was obtained from another run. PDE (50 μl) hydrolyzed 25 ± 1 pmoles cyclic GMP/10 min (N = 7). In a separate experiment, the same batch of PDE I (50 μl) hydrolyzed 36 (two determinations; 34.4 and 37.6) pmoles cyclic AMP/10 min. ND = not determined.

* Significant inhibition at $P < 0.05$ (DF ≥ 6), Student's t-test.

† Results (two determinations) from a single experiment.

(EC_{750}), i.e. an approximately 75% increase, was used as an index of potency. The EC_{750} value of MDL 17,043 was obtained from single dosing rather than multiple cumulative dosing experiments due to attenuated response of atria to multiple doses of this drug. The EC_{750} values (an average value of two to eight separate experiments) of aminophylline, MIX, amrinone, milrinone, AR-L 115BS, MDL 17,043 and RMI 82,249 were: 623 ± 144, 36, 1530 ± 447, 110 ± 28, 56 ± 10, 572 and 459 ± 185 μM respectively ([25] and present results). Most of the agents

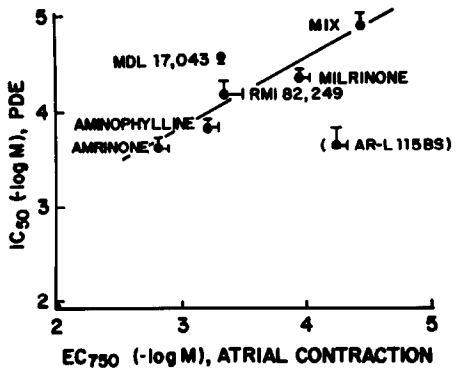

Fig. 3. Relationship between cyclic AMP PDE III inhibition and positive inotropic potency for cardiotonic agents. Correlation coefficient values were determined by linear regression analysis of respective IC_{50} and EC_{750} values of test agents. Each point with vertical and horizontal bars represents the mean values of IC_{50} and EC_{750} with their respective standard errors of the means.

examined inhibited PDE at lower concentrations than those required to increase contractile force ($IC_{50} < EC_{750}$). An exception was AR-L 115BS which exhibited a lower potency in inhibiting PDE than augmenting the atrial contractile force. To examine the relationship between PDE III inhibition and inotropic activity, the correlation coefficient (r) for these two parameters was calculated. If all the agents (seven) were included in the analysis, an r value of 0.41 ($P < 0.35$) was obtained. However, if AR-L 115BS was excluded from the calculation on the basis of its lower potency on PDE III with respect to its inotropic potency, a good correlation ($r = 0.80$, $P < 0.05$) between PDE III inhibition and inotropic activity was obtained for the remaining six compounds (Fig. 3).

Effects of drugs on cyclic AMP formation in guinea pig ventricular homogenates. Drug effects on net cyclic AMP formation were studied in broken cell preparations. The accumulated cyclic AMP represents the difference between synthesis and degradation since the guinea pig heart homogenates contain both adenylate cyclase and PDE. All of the test drugs produced concentration-dependent increases in cyclic AMP. The most potent agents were milrinone and MIX. The least potent was AR-L 115BS (Table 4). Likewise, amrinone and AR-L 115BS elevated cyclic AMP in guinea pig atrial homogenates, suggesting similar actions of these agents in ventricular and atrial preparations (results not shown). Forskolin, a direct stimulator of adenylate cyclase, also markedly increased cyclic AMP levels. The increases in cyclic AMP could have resulted from stimulation of adenylate cyclase, inhi-

Table 4. Influence of positive inotropic drugs on accumulation of cyclic AMP generated from ATP in homogenates of guinea pig ventricle

Drug	Concn (μM)	Cyclic AMP formation (pmoles/mg protein/10 min)	Percent increase
Basal		48 ± 5	
Aminophylline	100	178 ± 9*	271
	1000	333 ± 12*	594
MIX	10	246 ± 8*	412
	100†	490 ± 36*	920
Amrinone	100	168 ± 5*	250
	1000	319 ± 29*	565
Milrinone	10	208 ± 16*	333
	100	267 ± 26*	456
AR-L 115BS	50	72 ± 6*	50
	100†	120 ± 8*	150
MDL 17,043	10	139 ± 3*	190
	100†	182 ± 19*	279
RMI 82,249	100	172 ± 7*	258
	1000†	217 ± 20*	352
Forskolin	10	468 (458, 478)	875

Each value represents the mean ± S.E.M. of six determinations from two separate experiments except for the control (twelve determinations) and forskolin (two observations). The reaction mixture did not contain a cyclic AMP phosphodiesterase inhibitor.

* Significant increase, $P < 0.05$ (DF = 10), Student's t-test.

† DMSO (1%) was used as a vehicle. This concentration increased cyclic AMP level by 56%, and this value was subtracted from drug effects.

Table 5. Effects of inotropic drugs on adenylate cyclase activity in guinea pig ventricular homogenates

Drug	Concn (μM)	Adenylate cyclase activity (% change)
Isoproterenol	1	+57 ± 5*
	10	+57 ± 3*
	100	+80 ± 31*
Forskolin	0.1	+94 ± 6*
	1	+245 ± 21*
	10	+774 ± 74*
Histamine	10	+51 ± 21*
	100	+170 ± 20*
NaF	10,000	+149 ± 31*
Amrinone	300	+3 ± 4
	1000	−20 ± 4*
	3000	−47 ± 2*
AR-L 115BS	300	−10 ± 5
	1000	−1 ± 8
MDL 17,043	100	+3 ± 6
	300	−24 ± 10
RMI 82,249	300	−8 ± 6
	1000	−16 ± 12
Milrinone	100	−3 ± 2
	300	−18 ± 2*

Each value represents the mean ± S.E.M. of three to nine determinations (one to three separate experiments). The mean basal adenylate cyclase activity for three separate experiments was 688 ± 34 pmoles cyclic AMP/mg protein/10 min. No significant vehicle (DMSO) effects were observed. The reaction mixture contained 1 mM MIX as a cyclic AMP PDE inhibitor. An addition of 1 mM aminophylline did not further increase cyclic AMP formation.

* Significant changes from basal activity, $P < 0.05$ (DF ≥ 10), Student's t-test.

bition of PDE, or from both. To distinguish among these possibilities, effects of drugs on cyclic AMP formation were examined in guinea pig ventricular homogenates under conditions where cyclic AMP degradation was prevented by addition of a PDE inhibitor (1 mM MIX). In pilot studies, this concentration of MIX inhibited PDE activity completely in the homogenates. Forskolin and NaF, both direct stimulators of adenylate cyclase, stimulated adenylate cyclase activity under these conditions as indicated by cyclic AMP accumulation (Table 5). Isoproterenol and histamine also stimulated the cyclase activity. In contrast to these agents, amrinone, AR-L 115BS, MDL 17,043, RMI 82,249 and milrinone failed to stimulate adenylate cyclase activity significantly (Table 5). In fact, amrinone and milrinone inhibited the basal adenylate cyclase activity at higher concentrations.

Effects on cyclic AMP levels in intact cell preparations. Amrinone, milrinone and MIX produced concentration-dependent increases in cyclic AMP levels in guinea pig ventricular slices (Table 6). MDL 17,043 and RMI 82,249 also significantly increased cyclic AMP levels (Table 6). The effects of amrinone, milrinone and MIX were obtained at concentrations within their inotropic concentration range. However, AR-L 115BS at the relatively high concentration of 1 mM failed to significantly increase cyclic AMP levels (Table 6). Forskolin and isoproterenol also increased cyclic AMP levels (Table 6). In a separate series of experiments, the abilities of AR-L 115BS and other newer inotropic agents to augment forskolin-induced cyclic AMP increase were assessed in guinea pig ventricular slices. Amrinone, MDL 17,043

Table 6. Effects of inotropic drugs on cyclic AMP levels in guinea pig ventricular slices

Drug	Concn (mM)	No. of slice preparations	Cyclic AMP (% increase)
MIX	0.01	3	31 ± 22
	0.1	3	140 ± 22*
	0.5	12	247 ± 31*
Amrinone	0.1	14	20 ± 7
	0.5	4	53 ± 13*
	1.0	4	100 ± 21*
Milrinone	0.01	3	53 ± 9*
	0.1	3	119 ± 38*
	1.0	6	309 ± 53*
AR-L 115BS	1.0	9	31 ± 16
MDL 17,043	1.0	4	66 ± 16*
RMI 82,249	1.0	14	50 ± 7*
Forskolin	0.01	4	>3900*
Isoproterenol†	0.0001	4	134 ± 21*
	0.001	4	198 ± 4*

Each value (% increase) represents the mean ± S.E.M. of three to fourteen preparations or determinations pooled from one to four separate experiments. Control cyclic AMP level was 3.8 ± 0.8 pmoles/mg protein for fourteen experiments except for the experiment involving isoproterenol in which cyclic AMP level was elevated due to a 0.5 mM concentration of MIX included in the incubation medium. Neither the vehicle (2.5% DMSO) nor substitution of 0.6 mM EGTA with 0.8 mM $CaCl_2$ significantly affected basal cyclic AMP level and drug-induced cyclic AMP increases.

* Significant change from control cyclic AMP level, $P < 0.05$, Student's *t*-test.

† In the experiment involving isoproterenol, the final incubation medium contained 0.5 mM MIX and 0.8 mM $CaCl_2$ in place of 0.6 mM EGTA. The control cyclic AMP level (four determinations) was 14.5 ± 1.7 pmoles/mg protein.

and RMI 82,249 potentiated the forskolin-induced rise in cyclic AMP. However, AR-L 115BS did not augment the forskolin effect (Table 7).

DISCUSSION

These studies address the role of specific inhibition of cardiac cyclic nucleotide PDE isozymes and cyclic AMP elevation in the positive inotropic action of five new cardiotonic agents. The results of our studies suggest that inhibition of cardiac PDE III and subsequent elevation of cyclic AMP levels are responsible for the cardiotonic effects produced by amrinone, milrinone, MDL 17,042 and RMI 82,249 but not AR-L 115BS.

Amrinone, milrinone, AR-L 115BS, MDL 17,043, RMI 82,249 and two standard PDE inhibitors, aminophylline and MIX, all inhibited cyclic AMP hydrolysis by guinea pig ventricular PDE III in a concentration-dependent manner. MDL 17,043 and milrinone were two of the most potent agents among the new inotropic agents tested. MDL 17,043 was nine times more potent than amrinone. A similar relative potency was observed in previous studies employing dog and guinea pig ventricular PDE III [9, 17]. The IC_{50} value of AR-L 115BS is similar to those for heart crude PDE and guinea pig ventricular PDE III [15, 17, 26]. However, the relative potency of milrinone and MDL 17,043 differs from that of a previous report on guinea pig ventricular PDE III in which milrinone was more potent than MDL 17,043 [17]. The reason for this difference is not known.

In marked contrast to their effective inhibition of PDE III, amrinone, AR-L 115BS, MDL 17,043 and RMI 82,249 failed to significantly inhibit cyclic AMP hydrolysis by PDE I and II or cyclic GMP hydrolysis by PDE I. Only the standard PDE inhibitors MIX and aminophylline were able to inhibit cyclic AMP hydrolysis by PDE I and II. Similar to these findings, MDL 17,043 and amrinone were shown to inhibit only PDE III but not PDE I from dog heart [9]. In addition, amrinone, milrinone, AR-L 115BS, MDL 17,043 and RMI 82,249 lacked calmodulin antagonist activities as indicated by their inability to antagonize the basal as well as the calmodulin-stimulated PDE I (cyclic AMP hydrolysis) activity. In a previous report, MDL 17,043 and amrinone also did not inhibit the calmodulin-stimulated dog cardiac PDE I activity [9]. These data indicate that amrinone, milrinone, MDL 17,043, RMI 82,249 and AR-L 115BS are selective inhibitors of cardiac PDE III. In most cases, drug concentrations needed to inhibit PDE III were lower than those producing a positive inotropic effect in isolated guinea pig atria, i.e.

Table 7. Effects of inotropic drugs on forskolin-induced increase in cyclic AMP levels of guinea pig ventricular slices

Drug	Concn (mM)	Cyclic AMP level (pmoles/mg protein)
None (2.5% DMSO)		11 ± 2
Forskolin	0.001	58 ± 3*
Forskolin + amrinone	0.001 + 1	136 ± 14†
Forskolin + MDL 17,043	0.001 + 1	154 ± 12†
Forskolin + RMI 82,249	0.001 + 1	229 ± 37†
Forskolin + AR-L 115BS	0.001 + 0.5	42 ± 7*‡

Each value represents the mean ± S.E.M. of three preparations. Test drug was added together with forskolin to the medium containing slices and incubated for 10 min at 37°.

* Significant increase over the no drug control, $P < 0.05$ (DF = 4), Student's *t*-test.

† Significant increase over the forskolin value, $P < 0.05$ (DF = 4), Student's *t*-test.

‡ No significant change from the forskolin value.

$IC_{50} < EC_{750}$. An exception was AR-L 115BS. A greater concentration of this agent was required for inhibition of PDE III than for an inotropic effect, suggesting that PDE III inhibition alone may not account for its inotropic activity. To examine relationships between PDE III inhibition and inotropic activity, the correlation coefficient for these two parameters was calculated. No significant correlation ($r = 0.40$, $P < 0.35$) was obtained for all seven agents. If AR-L 115BS is excluded from the calculation on the basis of its lower PDE III inhibitory potency compared with its inotropic potency, a good correlation ($r = 0.80$, $P < 0.05$) between PDE III inhibition and inotropic activity was obtained for the remaining six agents. A similar good correlation ($r = 0.89$) was reported for six agents including amrinone, milrinone, AR-L 115BS and MDL 17,043 between PDE III inhibition and inotropic activity which were determined using guinea pig enzyme and anesthetized dogs [17].

Cyclic AMP PDE inhibitory activities of test drugs were also examined using guinea pig ventricular homogenates which possess both cyclic AMP-generating systems and cyclic AMP PDE. All the agents tested increased cyclic AMP levels in the homogenates in a concentration-dependent manner. With the exception of AR-L 115BS, all other agents produced more than a 200% increase in net cyclic AMP formation at concentrations below their respective inotropic EC_{750} values. These observations suggest that the prevailing form of PDE in the guinea pig ventricular homogenates is similar to the isolated PDE III rather than PDE I or II, since the new cardiotonic agents inhibited only PDE III. None of the new cardiotonic agents increased cyclic AMP levels in the presence of a PDE inhibitor, indicating that they are not activators of adenylate cyclase.

An agent which inhibits isolated PDE or cyclic AMP breakdown in the broken cell preparation may not be able to raise intracellular cyclic AMP levels in an intact tissue due to poor cell penetration. Therefore, effects of drugs on cyclic AMP accumulation were examined in relatively intact guinea pig ventricular slices. Amrinone, milrinone, RMI 82,249, MDL 17,043 and MIX significantly increased cyclic AMP levels. Forskolin and isoproterenol also produced increases in cyclic AMP levels in agreement with previous studies [22, 27]. However, AR-L 115BS did not produce a significant increase at a concentration four times its IC_{50} for PDE III. This observation is in contrast to a previous report showing a small significant elevation of cellular cyclic AMP levels by AR-L 115BS in rabbit papillary muscles [15]. However, the concentration required (1 mM) was much greater than its inotropic concentration ($EC_{750} = 56\ \mu M$). Further, AR-L 115BS could potentiate isoproterenol-stimulated increases in contractility of isolated papillary muscle at a concentration which failed to raise cyclic AMP [15]. Although some drugs were tested at only a single concentration, it is clear that amrinone, milrinone, MDL 17,043 and MIX were effective in their inotropic concentration range. Effects of AR-L 115BS and other inotropic agents were examined on the forskolin-induced cyclic AMP rise in guinea pig ventricular slices. In this stimulated condition cyclic AMP turnover should increase markedly, thus magnifying the effect of a PDE inhibitor. While amrinone, MDL 17,043 and RMI 82,249 potentiated the forskolin-induced cyclic AMP increase, again AR-L 115BS failed to augment the forskolin effect. The lack of an AR-L 115BS effect on cyclic AMP levels might result from its poor penetration into cells. Recent reports [13, 14, 28] demonstrated elevation of cyclic AMP levels in the isometrically contracting papillary or ventricular muscles from guinea pig, rabbit and dog hearts after addition of amrinone despite the negative results of earlier studies [8, 12]. More recently, milrinone and MDL 17,043 were found to increase cyclic AMP levels in the paced papillary muscles from dog or guinea pig hearts [3, 29]. Amrinone and milrinone also potentiated the isoproterenol-induced cyclic AMP rise and increased contractile force in the isolated contracting dog ventricular muscles [14, 15]. Detailed studies by Endoh *et al.* [14] and Honerjager *et al.* [13] indicated a close association between cyclic AMP elevation and increased contractility produced by amrinone in the isometrically contracting, isolated guinea pig and dog papillary or ventricular muscles.

The newer cardiotonic agents examined in this study exhibited a better separation of cardiotonic and chronotropic effects [5–7, 30] compared with standard PDE inhibitors such as methylxanthines or catecholamines [1, 31]. This may be due to a more discrete elevation of cyclic AMP levels in a specific compartment which is associated with PDE III. There is, in fact, some evidence supporting compartmentation of cyclic AMP and selective activation of cyclic AMP protein kinases in cardiac muscle [32].

In conclusion, the present study shows that amrinone, milrinone, MDL 17,043 and RMI 82,249 specifically inhibited the cardiac cyclic AMP PDE III isozyme in their inotropic concentration range and elevated cyclic AMP levels by inhibiting cyclic AMP breakdown rather than through stimulation of cardiac adenylate cyclase. Standard PDE inhibitors such as MIX and aminophylline were not specific inhibitors of PDE III since they also inhibited other PDE isozymes. These results together with previous findings suggest that the cardiotonic effects of these drugs are related to the elevation of intracellular cyclic AMP levels in the intact cardiac tissue. The present study also suggests that changes in cyclic AMP metabolism are not the primary mechanism in the positive inotropic effect of AR-L 115BS.

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