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Chronotropic and inotropic actions of amrinone, carbazeran and isobutylmethyl xanthine: role of phosphodiesterase inhibition

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1 The chronotropic and inotropic effects of amrinone, carbazeran and 3-isobutyl-1-methyl xanthine (IBMX) were examined in isolated preparations of papillary muscle and right atria from rabbit heart. The effects of the drugs on cardiac phosphodiesterase and cyclic nucleotide content were also examined.

2 Amrinone (2.4×10^{-4} M–2×10^{-3} M), carbazeran (9.1×10^{-6} M–1.2×10^{-3} M), and IBMX (1.8×10^{-5} M–4.5×10^{-4} M) produced concentration-dependent positive inotropic responses of papillary muscle preparations, the rank order of potency being carbazeran = IBMX > amrinone. Sub-threshold positive inotropic concentrations of all three compounds potentiated the positive inotropic effects of isoprenaline; leftward shifts in the concentration-effect curves were 5 fold (IBMX), 11 fold (amrinone) and 46 fold (carbazeran).

3 Amrinone and IBMX produced concentration-dependent positive chronotropic responses in isolated right atria and showed a similar rate selectivity to isoprenaline, but carbazeran elicited a decrease in beating frequency. None of these drugs potentiated the positive chronotropic effects of isoprenaline.

4 Concentrations of amrinone, carbazeran and IBMX that produced similar positive inotropic responses were associated with different increases in papillary muscle cyclic AMP and cyclic GMP concentrations.

5 All three compounds inhibited right atrial and ventricular phosphodiesterase, with amrinone being the least potent. There was, however, a marked difference between the IC_{50} and EC_{50} values for phosphodiesterase inhibition and positive inotropy. In contrast the positive chronotropic effects of amrinone and IBMX were observed in the same concentration ranges that produced phosphodiestrease inhibition.

6 The results indicate that amrinone possesses a similar rate/force selectivity to isoprenaline and IBMX. In contrast, carbazeran exerts both positive inotropic and negative chronotropic effects. Phosphodiesterase inhibition and elevation of intracellular cyclic AMP concentration may be involved, at least in part, in the cardiac effects of these drugs.

Introduction

A variety of cardiotonic agents that exert their pharmacological effects principally through phosphodiesterase inhibition has been described (Farah *et al.*, 1984; Sharpe, 1984; Weishaar *et al.*, 1986). The rationale underlying the use of drugs of this type in the treatment of congestive heart failure is that phosphodiesterase inhibitors have the dual effect of stimulating myocardial activity and lowering pre- and after-load through vasodilatation (Sharpe, 1984; Farah *et al.*, 1984), consequently improving the pumping efficiency of the heart. Despite the intense activity in the development of these newer inotropes, the exact role of cyclic nucleotide generation in the effects of phosphodiesterase inhibitors remains controversial (see Van Belle, 1983; Bowman *et al.*, 1985). Furthermore, some observations have indicated that the relationship between tissue adenosine 3′:5′-cyclic monophosphate (cyclic AMP) concentration and

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cardiac contractility appears to be more complex than was originally assumed (Rodger & Shahid, 1984; England & Shahid, 1987).

It has also been proposed that the chronotropic effects of adrenoceptor agonists are mediated by cyclic nucleotides (Yamasaki *et al.*, 1974; Taniguchi *et al.*, 1977). However, an analysis of the literature indicates that the information available is sparse and relatively little is known about the role of cyclic nucleotides in mediating changes in the frequency of beating.

The aims of the present experiments were to compare and study further the inotropic and chronotropic effects of the two newer compounds, amrinone (Ward *et al.*, 1983) and carbazeran (1-butyl-3-[1-(6,7-dimethoxyquinazoline-4yl)piperidin-4-yl]area) (Smith *et al.*, 1987) with those of 3-isobutyl-1-methyl xanthine (IBMX) (Korth, 1978) in rabbit papillary muscles and spontaneously beating right atria. The effects of these compounds on tissue cyclic AMP and guanosine 3′:5′-monophosphate (cyclic GMP) concentrations and on cardiac phosphodiesterase were also examined.

Methods

Male New Zealand white rabbits (Ranch Rabbits, Sussex) were stunned with a blow to the back of the neck and exsanguinated. The thorax was opened and the hearts rapidly excised and placed in a dish containing warm oxygenated Krebs-Henseleit solution. Papillary muscles were removed from the right ventricle and suspended in an organ bath containing Krebs-Henseleit solution at 32°C. The solution was of the following composition (mmol l^{-1}): NaCl 118, KCl 4.7, $MgSO_4 7H_2O$ 1.2, KH_2PO_4 1.2, $CaCl_2 6H_2O$ 2.5, $NaHCO_3$ 25 and glucose 11.7 and bubbled with a mixture containing 95% O_2 and 5% CO_2. The papillary muscles were mounted vertically between two platinum wire electrodes such that the base of each tissue was in contact with the bottom electrode whilst the tendon end, attached to a force displacement transducer (Grass FTO3C) lay just beneath the upper electrode. Initially the tissues were stretched by adjusting the diastolic tension to 1 g and allowed to stabilize for 60 min (at driving frequency of 1 Hz with pulses of 1 ms duration at a votlage 50–100% above threshold from a Grass S88 stimulator). At the conclusion of this equilibration period, any fall off in diastolic tension was corrected by resetting back to 1 g and stimulation frequency unless otherwise stated, was lowered to 0.4 Hz before the addition of a drug.

In all the experiments that involved the construction of concentration-effect curves to isoprenaline, papillary muscles were first exposed to a maximally effective concentration (2×10^{-6} M) of the agonist. This was done because it is known that the position of the first isoprenaline concentration-effect curve differs substantially from subsequent curves (Clark & Poyser, 1977; Nicholson & Broadley, 1978). Following washout of isoprenaline, a concentration-effect curve was constructed to isoprenaline which was taken as the standard curve. Potential modifiers of isoprenaline responses were added for 20 min after the tissues had fully recovered from this standard isoprenaline response. A second concentration-effect curve to isoprenaline was then constructed in the presence of the modifier. Only one concentration of each drug (modifier) was tested on each preparation.

In experiments in which $CaCl_2$ was used as the inotropic agent, the frequency of stimulation was maintained at 1 Hz but extracellular Ca^{2+} concentration ($[Ca^{2+}]$) was lowered to 2.5×10^{-4} M. The tissues were allowed to equilibrate for 20–30 min until a new steady-state contraction had been established, before the addition of $CaCl_2$ in a cumulative manner. Cumulative concentration-effect curves to $CaCl_2$ were performed before and after treatment with amrinone. Results for both isoprenaline and $CaCl_2$ were expressed as percentages of the corresponding maximum control responses.

Papillary muscle twitch shapes were recorded on an oscilloscope and photographed. The following components of the isometric contraction curve were analysed: time to peak force, relaxation time and total twitch duration.

Chronotropic activity

The chronotropic action was determined using spontaneously beating right atria from male New Zealand white rabbit hearts. The preparations were suspended (diastolic tension 1.5–2.0 g) in Krebs-Henseleit solution as described for papillary muscle experiments. After a stabilisation period of 60 min, cumulative concentration-effect curves to the different drugs were constructed. In the case of isoprenaline the procedure was identical to that described for the papillary muscle experiments. The frequency of beating was measured using a tachograph and monitored on a Grass (Model 7; Quincy, Mass) recorder.

Cyclic nucleotide measurements

These measurements were made in rabbit papillary msucles according to the procedure described in detail previously (Rodger & Shahid, 1984). The cyclic nucleotide levels were measured at the peak of the positive inotropic responses.

Preparation of cardiac phosphodiesterase and measurement of activity

A fresh enzyme preparation was made for each experiment and all procedures were performed at 4°C. Fresh ventricles (1.5–2.5 g) or right atria (0.3–0.5 g), from male New Zealand White rabbit hearts, were trimmed free of fat and connective tissue and chopped into small pieces using scissors. The tissue was homogenised (Polytron; PT20 probe: 2 × 10 s bursts at speed settings 5 and 7) in 10 volumes of buffer A (pH 6.5) containing (mmol l^{-1}): Bis-Tris 20, dithiothreitol (DTT) 1, benzamidine 2, ethylenediaminetetra acetic acid (EDTA) 2, Na-acetate 50 and phenylmethanesulphonylfluoride (PMSF) 0.1. The PMSF was added just before homogenisation. The homogenate was filtered through two layers of cheesecloth and centrifuged at 12000 *g* for 10 min (Damon IEC M60 ultracentrifuge; rotor 410). The supernatant fraction was decanted and used as the source of phosphodiesterase. The pellet fraction was usually discarded. However, for some experiments it was resuspended (to remove soluble phosphodiesterases) in 40–50 ml of buffer A (using a hand held homogeniser). The washed membranes were recollected by centrifugation (12000 *g* for 10 min) and then resuspended in a small volume (8–10 ml) of buffer A, containing 250 mM sucrose. This was used as a source of membrane bound phosphodiesterase. The enzyme preparations were used within 2 h of homogenisation.

Phosphodiesterase activity was measured according to the method described by Methven *et al.* (1980) with minor modifications. The standard assay mixture (total volume 200 μl) contained 3 mM $MgCl_2$, 1 mM 5'-AMP or 5'-GMP, 1 μM ^{3}H-labelled/unlabelled cyclic nucleotide (~200,000 d.p.m. $nmol^{-1}$), 1 mM DTT, 0.05% (w/v) bovine serum albumin in 50 mM Tris-HCl buffer, pH 7.4. The concentration of cyclic nucleotide in stock solutions was determined spectrophotometrically. The reaction was started by the addition of enzyme, and samples were incubated at 37°C for 15 min in a shaking water bath (Grant Instruments) before termination by heat (3 min in aluminium blocks set at 105°C). ^{14}C-labelled cyclic AMP or cyclic GMP (20 μl; 10^4 d.p.m.) was added to each tube immediately before boiling to act as an internal recovery marker. Cyclic nucleotide in the boiled samples was separated from other labelled products by application to alumina columns (chromatographic alumina; 1.5 g dry wt, packed into Pasteur pipettes) which had been equilibrated with 10 ml buffer (0.4 M Tris HCl, pH 7.0). Each tube was washed with 0.2 ml of the same buffer and the washings were applied to the corresponding columns. The columns were allowed to drain directly into scintillation vials and were eluted with a further 2.8 ml (for cyclic AMP) or 3.8 ml (for cyclic GMP) of buffer. The eluted material was dissolved in Picofluor-30 scintillant (Packard) and counted on a Packard RCA 2000 liquid-scintillation spectrometer. The recovery of unchanged cyclic AMP and cyclic GMP was 70–80% and 60–70% respectively. Assays were performed in duplicate and were conducted in the linear range of the reaction, where less than 25% of the initial substrate was hydrolysed. The concentrations producing 50% inhibition of phosphodiesterase activity (IC_{50} values) were determined using an iterative non-linear regression curve fitting program on a PDP 11/44 computer.

Materials

The following substances were used: (±)-isoprenaline hydrochloride (Sigma), IBMX (Aldrich), amrinone, carbazeran and dantrolene were gifts from Sterling Winthrop (UK), Pfizer (UK) and Norwich Pharmacal Company (UK), respectively. Isoprenaline solutions were prepared in acidified (pH 3.5) saline to enhance stability. Carbazeran, amrinone and dantrolene were dissolved in saline, lactic acid (0.5 N) and propylene glycol, respectively, any further dilutions were made in Krebs-Henseleit solution. [8-^{3}H]-adenosine 3':5'-cyclic monophosphate (25–30 Ci $mmol^{-1}$), [8-^{3}H]-guanosine 3':5'-cyclic monophosphate (15–20 Ci $mmol^{-1}$), [U-^{14}C]-adenosine 3':5'-cyclic monophosphate, [U-^{14}C]-guanosine 3':5'-cyclic monophosphate were obtained from Amersham International (Amersham, Bucks, UK). Chromatographic aluminium oxide was obtained from May and Baker (UK). All other chemicals were obtained either from Sigma Chemical Co or BDH Chemicals (both of Poole, Dorset, UK) and were normally of Analar or equivalent grade.

Statistical evaluation

The results are presented as mean values ± s.e.mean. In isolated tissue experiments the EC_{50} values (the concentration of drug producing 50% of its own maximum response) were calculated, from the cumulative concentration-effect curve. The significance of differences between means was determined by Student's *t* test; a *P* value of less than 0.05 was taken as being significant.

Results

Effects on ventricular force and contraction shape

In these experiments, isoprenaline was used as a reference drug to determine the papillary muscle

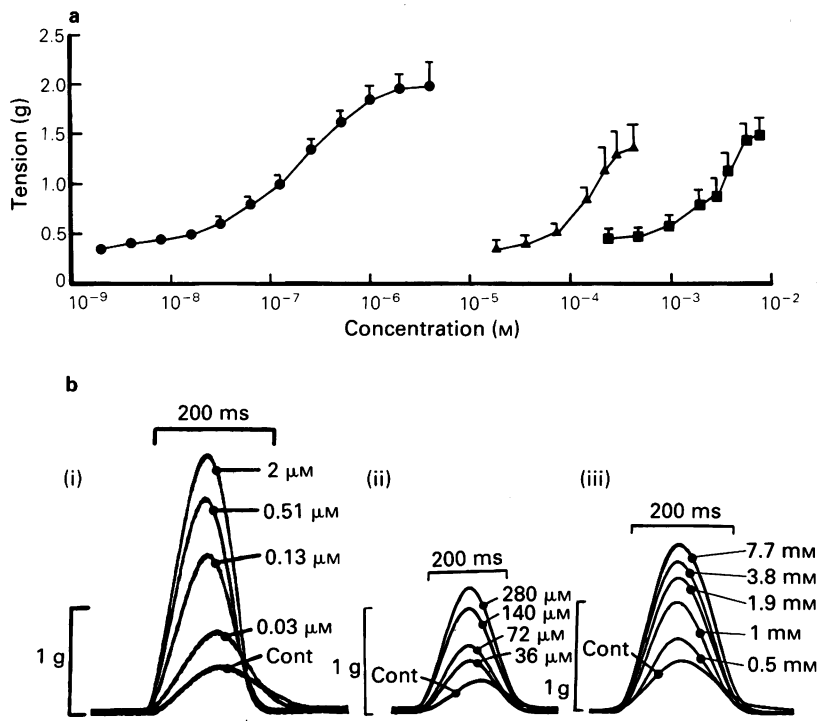

Figure 1 (a) Cumulative concentration-effect curves for the positive inotropic effects of isoprenaline (●), isobutylmethyl xanthine (IBMX, ▲) and amrinone (■) in electrically stimulated papillary muscles (0.4 Hz) of the rabbit. $n = 5$–7. Each point represents the mean and vertical lines indicate s.e.mean. (b) Concentration-related effects of (i) isoprenaline, (ii) IBMX and (iii) amrinone on papillary muscle twitch shape. Cont indicates the control contraction before addition of drug.

response to a full β-adrenoceptor agonist. Isoprenaline produced concentration-dependent increases in the force of contraction (Figure 1), which were rapid in onset (10–15 s) and reached peak effect within 60–180 s after each cumulative addition of the drug. Propranolol (10^{-7} M) competitively antagonised the effects of isoprenaline, producing a 15 fold rightward parallel shift of the concentration-effect curve, without significantly altering the maximum response. IBMX, amrinone (Figure 1) and carbazeran (Figure 2) also produced concentration-related positive inotropic responses. Compared to isoprenaline, the effects of these compounds were slow, the onset of action being 1–2 min after drug addition and 10–20 min elapsed before peak tension was achieved. Propranolol (1×10^{-7} M) did not significantly modify either the inotropic potency or the size of the maximum response produced by IBMX, amrinone or carbazeran, indicating that neither β-adrenoceptors nor noradrenaline release was involved. It is clear from the mean cumulative concentration-effect curves shown in Figures 1a and 2a that IBMX, amrinone and carbazeran were much less potent than isoprenaline at augmenting papillary muscle contractions. The EC_{50} values for the positive inotropic action are shown in Table 1 and indicate a rank order of potency of isoprenaline ≫ carbazeran = IBMX > amrinone. In addition to

Table 1 The cardiac stimulant effects of isoprenaline, isobutylmethyl xanthine (IBMX), amrinone and carbazeran.

	Chronotropic (μM)	*Inotropic* (μM)	*Chronotropic: inotropic ratio*
Isoprenaline	0.022 ± 0.004	0.35 ± 0.04	0.06
IBMX	9.5 ± 1.9	130 ± 20	0.07
Amrinone	295 ± 68	3400 ± 700	0.09
Carbazeran	NC	100 ± 20	NC

Each value is the mean EC_{50} ± s.e.mean, $n = 4$–7.
NC: negatively chronotropic.

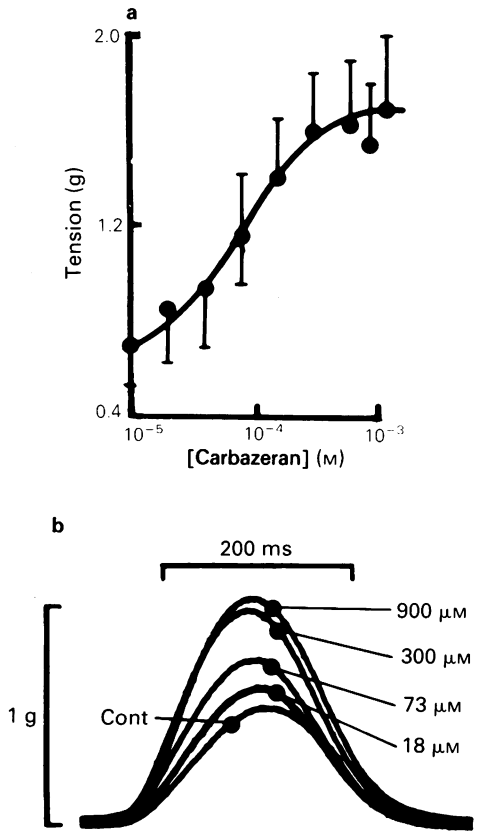

Figure 2 (a) Mean cumulative concentration-effect curve to carbazeran in papillary muscles electrically driven at 0.4 Hz. Each point represents the mean and vertical lines indicate s.e.mean. (b) Typical concentration-dependent effects of carbazeran on papillary muscle twitch shape. The stimulation frequency was 0.4 Hz and the control contraction is indicated by cont.

increasing force of contraction, both isoprenaline and IBMX stimulated the rate of tension generation (positive klinotropic action), reduced the time to peak force, accelerated the rate of relaxation (positive hisotropic action) and decreased total twitch duration in a concentration-dependent manner (Figure 1b). The effects of amrinone on papillary muscle twitch shape were similar to those of isoprenaline and IBMX (Figure 1b), although at higher concentrations ($>1 \times 10^{-3}$ M) the decrease in relaxation time was reduced. In contrast, carbazeran, whilst producing a positive klinotropic response and a reduction in total peak force, had a negative hisotropic action, which resulted in an increase in the total twitch duration (Figure 2b).

Threshold, non-inotropic, concentrations of all three drugs potentiated isoprenaline-induced positive inotropic responses. IBMX (4.5×10^{-6} M), amrinone (6×10^{-4} M) and carbazeran (3.6×10^{-5} M) produced 5, 11 and 46 fold parallel leftward shifts in the position of the isoprenaline concentration-effect curve, respectively. The positive inotropic agents ouabain and vanadate which exert their positive inotropic effects through cyclic AMP-independent mechanisms did not potentiate isoprenaline responses (data not shown).

The effect of amrinone (6×10^{-4} M) on the responsiveness of papillary muscles to [Ca^{2+}] is shown in Figure 3a. The EC_{50} values for Ca^{2+} pre- ($1.3 \pm 0.1 \times 10^{-3}$ M) and post- ($1.1 \pm 0.1 \times 10^{-3}$ M) amrinone are not significantly different, although the maximum response may have increased. Verapamil (10^{-6} M) did not significantly affect the cardiotonic action of amrinone (Figure 3b), which produced a maximum response similar to that obtained in the absence of the verapamil. The mean EC_{50} value for amrinone in these experiments was $1.5 \pm 0.2 \times 10^{-3}$ M. The negative inotropic effect of higher concentrations of verapamil (10^{-5} M) was, however, only partially reversed by amrinone. Possible effects of amrinone on Ca^{2+} mobilisation from intracellular stores were tested using dantrolene (2×10^{-5} M), which is known to inhibit Ca^{2+} flux across the sarcoplasmic reticulum (Morgan & Bryant, 1977). In the presence of dantrolene there was a significant 3 fold rightward shift in the concentration-response curve to amrinone (Figure 3c) (the EC_{50} values before and after dantrolene were $2.2 \pm 0.2 \times 10^{-3}$ M and $7.2 \pm 0.6 \times 10^{-3}$ M, respectively). Dantrolene at the concentration used in these experiments had no apparent effect on developed tension.

Chronotropic effects in isolated right atria

Isoprenaline produced concentration-dependent increases in beating frequency (Figure 4) with an EC_{50} value of 2.2×10^{-8} M. Thus, on a molar basis, it was 16 times more potent when compared to its positive inotropic action. The chronotropic effect was fast in onset (10–15 s) and reached peak effect in 2–3 min. The effect of isoprenaline was blocked by propranolol (10^{-7} M) which caused an 18 fold rightward shift in the concentration-effect curve. IBMX and amrinone also produced concentration-related positive chronotropic responses (Figure 5). These drugs were less potent than isoprenaline and the largest responses produced were 91% and 85% of the isoprenaline maximum for IBMX and amrinone, respectively. The chronotropic responses to IBMX and amrinone were not affected by propranolol. In

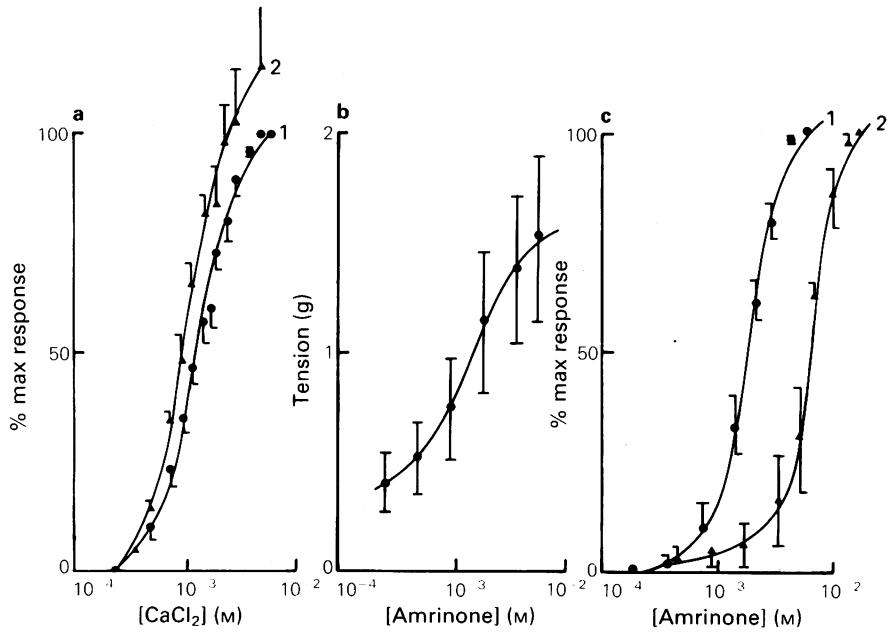

Figure 3 (a) Mean cumulative concentration-effect curves to $CaCl_2$ before (●, 1) and after (▲, 2) a 30 min incubation with amrinone (6×10^{-4} M) in papillary muscles stimulated at a frequency of 1 Hz. Mean ± s.e.mean basal tensions (at 2.5×10^{-4} M $CaCl_2$), before and after amrinone were 90 ± 19 mg and 100 ± 28 mg, respectively ($n = 5$). (b) Mean cumulative concentration-effect curve to amrinone in papillary muscles (0.4 Hz) in the presence of verapamil (10^{-6} M). Verapamil was allowed to act for 30 min before a concentration-response curve was obtained. Mean basal tensions before and after verapamil were 535 ± 160 mg and 335 ± 120 mg, respectively. The mean maximum tension induced by amrinone in the presence of verapamil was 1515 ± 385 mg ($n = 4$). (c) Mean cumulative concentration-effect curve to amrinone before (●, 1) and after (▲, 2) a 30 min incubation with dantrolene (2×10^{-5} M) in papillary muscles (stimulated at a frequency of 0.4 Hz). Mean basal tensions before and after dantrolene were 365 ± 85 mg and 285 ± 65 mg, respectively. The mean ± s.e.mean tension induced by amrinone in the presence of dantrolene was 1350 ± 325 mg ($n = 4$). Each point represents the mean and vertical lines show s.e.mean.

contrast, carbazeran had only a minor positive chronotropic action at low concentrations (23% increase at 3.6×10^{-5} M) and a negative chronotropic action at higher ($> 10^{-4}$ M) concentrations. The latter effect was not significantly modified by atropine (10^{-6} M) indicating the lack of involvement of acetylcholine release. Thus IBMX and amrinone show a clear selectivity for chronotropic activity (approximately the same order of magnitude as isoprenaline), whereas carbazeran was selective for inotropic activity at concentrations below 10^{-4} M (Figure 5, Table 1).

There was no significant parallel shift in the isoprenaline concentration-response curve in the presence of IBMX (2.3×10^{-6} M), carbazeran (3×10^{-5} M) or amrinone (3×10^{-5} M), but carbazeran produced a significant reduction (11%) in the maximum chronotropic response to isoprenaline (Figure 4).

Effects on cyclic nucleotide levels

From the experiments described above, concentrations of carbazeran, IBMX and amrinone producing approximately equieffective inotropic responses were selected for determining the effects of these drugs on cyclic nucleotides. Each concentration of drug was allowed to reach its peak effect (20 min) before papillary muscles were frozen rapidly in liquid nitrogen and analysed for cyclic nucleotide content. The tension and cyclic nucleotide results for these experiments are summarised in Table 2. It is clear that the positive inotropic effects of all three compounds, at the concentrations tested, were associated with concentration-dependent increases in both cyclic AMP and cyclic GMP levels. IBMX and carbazeran both produced similar elevations in cyclic AMP (~4 fold) and cyclic GMP (2–3 fold) content, whereas amrinone produced smaller increases in both cyclic

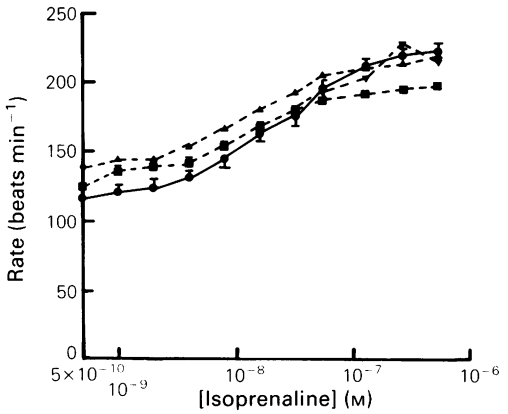

Figure 4 The chronotropic effects of isoprenaline, in rabbit isolated spontaneous right atria, in the absence (●) and presence of isobutylmethyl xanthine (2.3×10^{-6} M; ▲), amrinone (3×10^{-5} M; ▼) and carbazeran (3×10^{-5} M; ■). Each point represents the mean ($n = 4$–6) and vertical lines show s.e.mean.

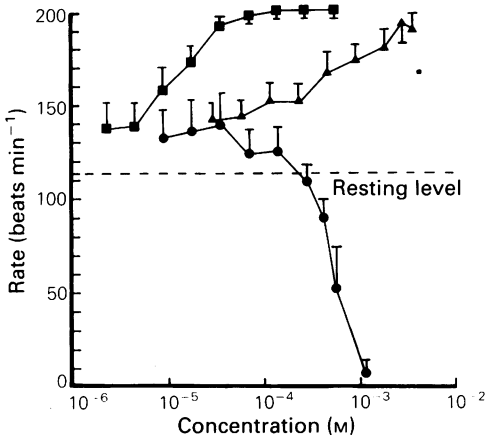

Figure 5 The chronotropic effects of isobutylmethyl xanthine (■), amrinone (▲) and carbazeran (●) in rabbit isolated spontaneously beating right atria. The basal rate was 113 ± 5 beats min^{-1} and each point is the mean of 4–6 determinations. Vertical lines show s.e.mean.

nucleotides. For all three compounds there was a good correlation between the tension response and the absolute values of both cyclic AMP and cyclic GMP level. However, there was no clear relationship between contractile force and the ratio of cyclic AMP to cyclic GMP. A supramaximal concentration of IBMX (4.5×10^{-3} M), which did not elicit any further increase in the maximum developed tension, produced a further increase in the levels of both cyclic AMP (6.66 ± 0.95 pmol mg^{-1}) and cyclic GMP (0.48 ± 0.05 pmol mg^{-1}).

Effects on phosphodiesterase activity

The distribution of phosphodiesterase activity in the rabbit ventricle is shown in Table 3. It is clear that a

Table 2 Effects of isobutylmethyl xanthine (IBMX), carbazeran and amrinone on tension responses and cyclic nucleotide levels in rabbit isolated papillary muscles

Drug	*Concentration* (mol l^{-1})	*Tension* (mg)	*Cyclic AMP* (pmol mg^{-1} tissue)	*Cyclic GMP* (pmol mg^{-1} tissue)	*Ratio Cyclic AMP: cyclic GMP*
IBMX	Solvent control	317 ± 56	0.63 ± 0.09	0.055 ± 0.008	11.5
	4.5×10^{-5}	517 ± 103	1.11 ± 0.10	0.082 ± 0.017	13.5
	2.3×10^{-4}	779 ± 208	1.42 ± 0.17	0.117 ± 0.018	12.1
	4.5×10^{-4}	1067 ± 152	2.36 ± 0.20	0.135 ± 0.021	17.5
			($r = 0.97$)	($r = 0.95$)	
Carbazeran	Solvent control	358 ± 26	0.60 ± 0.06	0.021 ± 0.002	28.6
	1.8×10^{-4}	565 ± 62	1.42 ± 0.20	0.049 ± 0.009	28.9
	9×10^{-4}	954 ± 141	2.03 ± 0.29	0.067 ± 0.010	30.3
	1.8×10^{-3}	1200 ± 110	2.47 ± 0.31	0.073 ± 0.008	33.8
			($r = 0.98$)	($r = 0.94$)	
Amrinone	Solvent control	303 ± 60	0.74 ± 0.06	0.039 ± 0.009	19
	6×10^{-4}	314 ± 90	0.77 ± 0.08	0.036 ± 0.007	21.4
	3×10^{-3}	882 ± 206	1.22 ± 0.09	0.079 ± 0.006	15.4
	6×10^{-3}	1250 ± 109	1.47 ± 0.14	0.102 ± 0.010	14.4
			($r = 0.98$)	($r = 0.98$)	

Each value is the mean ± s.e.mean, $n = 4$–5.
r values are for comparison between aboslute cyclic nucleotide and tension responses.

Table 3 Distribution of phosphodiesterase (PDE) activities in rabbit heart ventricle

Fraction	*Cyclic AMP-PDE activity* (pmol min^{-1} mg^{-1} protein) *Control*	*+ Ca/calmodulin*
Homogenate	265 ± 7.2 (100)	315 ± 2.4 (19)
Soluble (S1)	677 ± 11 (64)	948 ± 59 (40)
Membrane (P1)	112 ± 1 (32)	114 ± 0.8 (2)

* Values in parentheses represent % of total PDE activity.
† Values in parentheses represent % stimulation of PDE activity by addition of Ca^{2+} (20 μM) and calmodulin (0.3 μg).

large proportion (64%) of the total phosphodiesterase activity appears in the soluble fraction which is stimulated (by 40%) on the addition of Ca^{2+} (20 μM) and calmodulin (0.3 μg). However, there is a significant amount (32%) of phosphodiesterase activity which appears to be membrane bound. The membrane-bound phosphodiesterase (memb-PDE) was not activated by Ca/calmodulin. IBMX, amrinone and carbazeran produced concentration-dependent inhibition of cyclic AMP hydrolysis by ventricular phosphodiesterases (Figure 6) with mean (± s.e.mean) IC_{50} values of 4.1×10^{-6} M ($\pm 4 \times 10^{-7}$ M), 1.83×10^{-4} M ($\pm 3 \times 10^{-5}$ M) and 4.1×10^{-6} M ($\pm 1.2 \times 10^{-6}$ M), respectively. All three compounds displayed selectivity (2 to 43 fold) for inhibition of cyclic AMP hydrolysis when compared to their effects against cyclic GMP (mean IC_{50} values for IBMX, amrinone and carbazeran were 5.7×10^{-5} M ($\pm 1.3 \times 10^{-6}$ M), $>2.5 \times 10^{-4}$ M and 1.71×10^{-4} M ($\pm 2 \times 10^{-5}$ M) respectively). IBMX and carbazeran were approximately equipotent, and both compounds were 46 times more potent than amrinone at inhibiting cyclic AMP hydrolysis. IBMX was 3 times more potent than carbazeran at inhibiting cyclic GMP hydrolysis; amrinone was the least potent, producing 10–20% inhibition at the highest concentration tested. Thus the rank order of potency for inhibition of cyclic AMP hydrolysis was IBMX = carbazeran ≫ amrinone, and for cyclic GMP hydrolysis IBMX > carbazeran > amrinone. Although a low speed supernatant fraction was used as the source of the enzyme in these experiments, it is possible that the poor potency of amrinone may be due to missing memb-PDE. The results shown in Figure 7a indicate that IBMX, carbazeran and amrinone produced concentration-dependent inhibition of cyclic AMP hydrolysis by memb-PDE. Amrinone, however, was still the least potent (IC_{50}: $8.6 \pm 1.6 \times 10^{-5}$ M) when compared to IBMX (IC_{50}: $7.7 \pm 1.5 \times 10^{-6}$ M) and carbazeran (IC_{50}: $3.2 \pm 0.20 \times 10^{-6}$ M). Carbazeran was more potent than IBMX, giving a rank order potency of carbazeran > IBMX ≫ amrinone. The inhibitory effect of amrinone on memb-PDE was more marked than that obtained on the soluble phosphodiesterase.

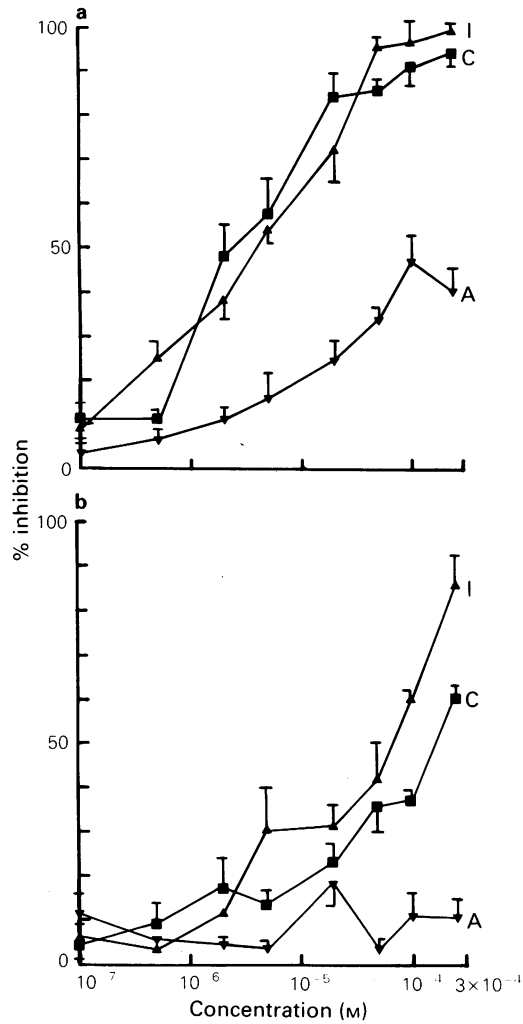

Figure 6 The inhibitory effects of carbazeran (■, C), isobutylmethyl xanthine (▲, I) and amrinone (▼, A) on (a) cyclic AMP hydrolysing and (b) cyclic GMP hydrolysing phosphodiesterase in a 12,000 *g* supernatant fraction from rabbit heart ventricles. Each point represents the mean from 4–6 different enzyme preparations. Vertical lines show s.e.mean.

To parallel the experiments examining the chronotropic actions of IBMX, amrinone and carbazeran, the effects of these compounds on right atrial phosphodiesterase were also analysed. All three drugs

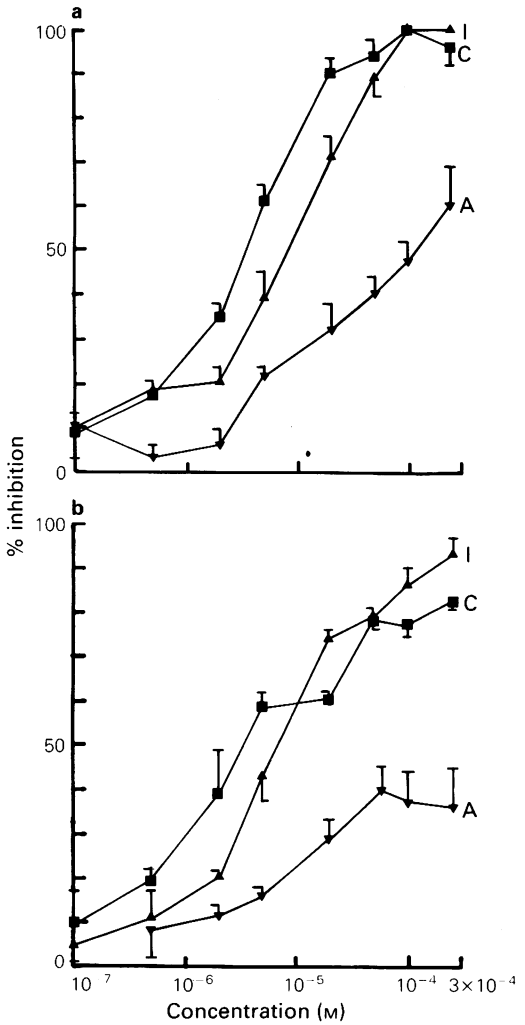

Figure 7 The inhibitory effects of carbazeran (■, C), isobutylmethyl xanthine (▲, I) and amrinone (▼, A) on cyclic AMP hydrolysis by (a) membrane-bound phosphodiesterase prepared from rabbit heart ventricles and (b) rabbit right atrial phosphodiesterase. See Methods for detailed description of the procedure for preparation of phosphodiesterases. Each point represents the mean of 4–6 experiments on different enzyme preparations. Vertical lines show s.e.mean.

produced concentration-dependent inhibition of cyclic AMP hydrolysis by right atrial phosphodiesterase (Figure 7b). Carbazeran (IC_{50}: $5.7 \pm 1.8 \times 10^{-6}$ M) and IBMX (IC_{50}: $7.6 \pm 0.4 \times 10^{-6}$ M) were approximately equipotent whilst amrinone was at least 30 times less potent, producing 35% inhibition at the top concentration tested. Thus, the rank order of potency was carbazeran = IBMX > amrinone.

Discussion

Amrinone, carbazeran and IBMX all produced positive inotropic responses in rabbit isolated heart preparations that were associated with increases in intracellular cyclic AMP and cyclic GMP concentrations. All three drugs were shown to inhibit cardiac phosphodiesterase activity, indicating that this may contribute to the mechanism underlying their positive inotropic effects.

The relatively slow onset of action of these drugs is indicative of an intracellular site of action. The effects of IBMX on ventricular twitch shape were qualitatively similar to those of isoprenaline, which is in agreement with results obtained in guinea-pig papillary muscles (Korth, 1978). The maximum positive inotropic effect, elicited by IBMX was smaller, despite a larger increase in cyclic AMP, when compared to published data for isoprenaline in rabbit papillary muscles (Schumann *et al.*, 1975; Rodger & Shahid, 1984). Amrinone, although not as potent as IBMX, mimicked the effects of the β-adrenoceptor agonists at low concentrations but prolonged relaxation time at higher concentrations, suggesting the involvement of other (cyclic AMP-independent) mechanisms. It is possible that amrinone may also directly enhance Ca^{2+} release from intracellular stores. This view is supported by the observation that the positive inotropic effects of amrinone were inhibited by dantrolene. A mixed profile of mechanism of action for amrinone has also been proposed on the basis of experiments in guinea-pig and canine cardiac tissues (Honerjager *et al.*, 1981; Endoh *et al.*, 1982).

Both IBMX and amrinone elicited positive chronotropic responses. These observations are consistent with the results of Kodama *et al.* (1983), who showed that amrinone increased the spontaneous activity of rabbit sinus node pacemaker cells, and are in accord with the hypothesis that cyclic AMP mediates positive chronotropic responses. In contrast to IBMX and amrinone, carbazeran displayed a substantially different profile in that it did not reduce ventricular relaxation time and it produced a negative chronotropic effect, despite producing generalised phosphodiesterase inhibition in both atrial and ventricular tissues. Thus, in contrast to IBMX and amrinone, carbazeran was force-selective but only at concentrations below 10^{-4} M. The bradycardic effects of carbazeran are unusual for a phosphodiesterase inhibitor but are in agreement with the results obtained by Smith *et al.* (1987). It is conceivable that at higher concentrations ($>10^{-4}$ M) carbazeran exerts direct depressant effects to reduce

activity of S-A nodal cells. Although the exact reasons for this difference are not clear, the pharmacological profile displayed by carbazeran may be more appropriate for the management of congestive heart failure. Another difference between atrial and ventricular effects of these compounds was the lack of potentiation of isoprenaline-induced positive chronotropy. This suggests that either cyclic AMP generation is irrelevant to the positive chronotropic effects of isoprenaline or that the phosphodiesterase predominant in atrial sinus-node cells is relatively insensitive to amrinone, carbazeran and IBMX at the concentrations tested. Elucidation of the differences in the sinus-node phosphodiesterases and those present in ventricular myocardium may aid the development of force-selective inotropic drugs, although microcompartmentalisation of the enzyme(s) may make this difficult.

Amrinone was markedly less potent than IBMX and carbazeran at inhibiting ventricular phosphodiesterase activity, the rank order of potency being the same as for the positive inotropic effect. Furthermore, the effects of the drugs on tissue cyclic nucleotide levels and on isoprenaline-induced positive inotropy are also consistent with their relative potency at inhibiting cardiac phosphodiesterase. The IC_{50} value for amrinone is in reasonable agreement with previously published data (Honerjager *et al.*, 1981; Endoh *et al.*, 1982). These data strongly suggest that phosphodiesterase inhibition and cyclic AMP mediate the positive inotropic effects of the compounds studied. However, there is a large difference between the IC_{50} and EC_{50} values for phosphodiesterase inhibition and positive inotropy. Concentrations of drugs required to elevate tissue cyclic nucleotide concentrations in intact cells are far in excess of those causing phosphodiesterase inhibition in broken cell preparations. These observations suggest that basal phosphodiesterase activity in intact active papillary muscles may be much higher than that obtained in broken cell preparations, so that larger drug concentrations are required for significant inhibition of phosphodiesterase. Poor penetration of drugs in intact cells is unlikely to be the cause of these differences since low, non-inotropic concentrations of the drugs potentiated the effects of isoprenaline. Thus, the phosphodiesterase inhibitory effect can be observed under conditions of increased cyclic AMP production by isoprenaline. This observation suggests that the phosphodiesterase inhibitory effects of these compounds (at low concentrations) in intact tissue preparations are only apparent under conditions of stimulated cyclic AMP production, which would also be associated with increased phosphodiesterase activity. Under non-stimulated conditions, there would be sufficient phosphodiesterase reserve to cancel any inhibitory effect of the compounds. There is also a lack of correlation between the effects of tissue cyclic GMP concentrations and the rather weak inhibition of cyclic GMP hydrolysis. The exact function of cyclic GMP in the cardiac effects of amrinone, carbazeran and IBMX remains undefined. Indeed, the effects of phosphodiesterase inhibitors on cardiac cyclic GMP metabolism have not in general been intensely studied. It is conceivable that increases in cyclic GMP may limit the inotropic/chronotropic effects of phosphodiesterase inhibitors since cyclic GMP has been shown to counteract the effects of cyclic AMP under certain circumstances (Nawrath *et al.*, 1981; Dickson *et al.*, 1986).

The mechanisms by which IBMX and carbazeran produced such large increases in cyclic AMP at equieffective inotropic concentrations when compared to isoprenaline (Rodger & Shahid, 1984) or other documented phosphodiesterase inhibitors (Farah *et al.*, 1984) are not clear. Cyclic nucleotide phosphodiesterases are known to exist in multiple molecular forms which differ in their sensitivities to inhibitors as well as in their physical, regulatory and kinetic properties (Beavo, 1988). Cardiac ventricular tissue contains at least four phosphodiesterase subtypes (Reeves *et al.*, 1987; Beavo, 1988). Thus it is possible that IBMX and carbazeran may inhibit ventricular phosphodiesterases in a non-selective fashion to produce an exaggerated cyclic AMP response. In contrast, the effects of selective phosphodiesterase inhibitors may be restricted to one type of phosphodiesterase involved in regulating cyclic AMP concentration, in a functionally important compartment such as the sarcoplasmic reticulum (Manganiello, 1987). Amrinone was slightly more potent at inhibiting memb-PDE, which may reflect differential effects on phosphodiesterase subtypes. Furthermore, the observation that a supramaximal concentration of IBMX which produced no increase in contractile force, but still further elevated cyclic nucleotide levels, suggests that the maxima for tension and cyclic nucleotide responses are different. This emphasises the view that there is no general relationship between gross tissue cyclic AMP concentration and contractile force. One suggestion proposed is that there may be distinct intracellular 'pools' of cyclic AMP mediating different responses (Brunton *et al.*, 1981; Rodger & Shahid, 1984; England & Shahid, 1987). Cyclic nucleotides mediate many cellular responses each of which may involve different phosphodiesterase isoenzymes and/or phosphodiesterases in different compartments of the cell.

In conclusion, the data indicate that amrinone possesses a similar cardiac rate/force selectivity to isoprenaline and IBMX. This is not the case for carbazeran which exerts both positive inotropic and negative chronotropic effects. Increases in intracellu-

lar cyclic AMP concentration caused by phosphodiesterase inhibition are clearly associated with the positive inotropic effects of amrinone, carbazeran and IBMX. However, the exact mechanisms underlying the chronotropic effects of these compounds are not clear.

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