Br. J. Pharmacol. (1993), **109**, 667–672

Cellular mechanism of the positive inotropic effect of hydralazine in mammalian myocardium

David G. Hurrell, Cynthia L. Perreault, Lin Miao, Bernard J. Ransil & [1]James P. Morgan

The Charles A. Dana Research Institute and the Harvard-Thorndike Laboratories of the Beth Israel Hospital and the Department of Medicine (Cardiovascular Division), Harvard Medical School, Boston, MA, U.S.A.

1 The purpose of this study was to elucidate the cellular mechanism of the positive inotropic effect of hydralazine, a vasodilator widely used for afterload reduction in patients with heart failure that has also been reported to have positive inotropic effects on the heart. After isolation, right ventricular papillary muscles from the ferret were maintained in bicarbonate-buffered salt solution (30°C). A concentration-response relationship was obtained for hydralazine (10^{-6} to 10^{-3} M). In order to mimic different levels of catecholamine release found in heart failure, we utilized two methods of stimulation: (a) threshold punctate pulses and (b) suprathreshold punctate stimulation with voltage approximately 10% above threshold.

2 In a first group of muscles ($n = 16$), a maximally effective concentration of hydralazine (10^{-3} M) increased peak isometric tension by $39 \pm 9\%$ ($P<0.05$). Doses lower than 10^{-5} M had no significant effect. The bioluminescent Ca^{2+} indicator, aequorin, was loaded into a subset of these muscles ($n = 7$). A significant increase in peak light (i.e., intracellular Ca^{2+}) developed, concurrently with an increase in peak tension ($38 \pm 5\%$ to $66 \pm 8\%$). This inotropic response was associated with a decrease in time to peak tension (ms), 221 ± 7 to 186 ± 5 ($P<0.05$), and time to peak light, 65 ± 4 to 52 ± 2 ($P<0.05$). These effects were markedly attenuated by pretreatment with autonomic blocking agents.

3 In a second group of muscles ($n = 12$), histamine was used to stimulate cyclic AMP production in the presence of propranolol. Hydralazine (3×10^{-4} M) led to a shift in the pD_2 (i.e. the negative log of the concentration of histamine producing 50% of the maximal response) from 6.1 ± 0.1 to 5.9 ± 0.1 ($P<0.05$), thus increasing the sensitivity of the muscles to histamine. Hydralazine also increased maximum tension from $160 \pm 77\%$ to $195 \pm 57\%$ ($P<0.05$) above baseline. Thus, hydralazine altered the potency and efficacy of histamine despite the presence of β-adrenoceptor blockade.

4 A third group of muscles were chemically skinned to examine the effects of hydralazine on myofilament Ca^{2+} responsiveness. Pretreatment of ferret papillary muscles with hydralazine (10^{-3} M) before skinning did not shift the force-pCa curve after skinning ($n = 16$). However, hydralazine added to previously skinned fibres desensitized the myofilaments, as indicated by a rightward shift of the force-pCa curve ($n = 12$). Maximum tension development was not changed.

5 The pharmacological effects of hydralazine are characteristic of inotropic drugs that act mainly via cyclic AMP; however, the increase in peak tension demonstrated with histamine in the presence of hydralazine also suggests an effect on cyclic AMP-independent second messenger pathways. These data are consistent with reports that large doses of hydralazine may increase cellular levels of cyclic AMP, as well as other second messengers, by direct cardiac and indirect neuronal mechanisms.

Keywords: Aequorin; calcium indicators; hydralazine; heart failure; calcium activation; histamine; inotropic agents

Introduction

Congestive heart failure is a disease that affects millions of people worldwide, and its associated mortality has gone nearly unabated despite modern therapy (Smith, 1985). Encouraging results have been reported in clinical trials using hydralazine and isosorbide dinitrate to prolong survival in patients with mild-to-moderate heart failure (V-Heft I and V-Heft II; Cohn *et al.*, 1986; 1991). While the major benefit of hydralazine is generally believed to be mediated through its vasodilator action, the drug may also elicit positive inotropic and chronotropic responses in both animal models and man (Gershwin *et al.*, 1967; Khatri *et al.*, 1977; Leier *et al.*, 1980). Moreover, a positive inotropic response to hydralazine can be demonstrated under conditions in which the rate of myocardial contraction is held constant (Rabinowitz, 1986; Azuma *et al.*, 1987). The mechanism of the inotropic effect of hydralazine remains incompletely defined, as does the role inotropy may play in the improved survival demonstrated in these trials. Unfortunately, the PROMISE trial, a large clinical study of 1088 patients, has demonstrated that the inotropic agent milrinone, a phosphodiesterase inhibitor, leads to increased mortality in patients with moderate-to-severe congestive heart failure (Packer *et al.*, 1991). Therefore, the mechanism by which an inotropic agent mediates its effect may be important in establishing its clinical utility.

Studies have suggested the positive inotropic effect of hydralazine is dependent upon the β-adrenoceptor and is completely abolished by propranolol and reserpine (Rabinowitz *et al.*, 1986; Rendig *et al.*, 1988). Specifically, Rabinowitz *et al.* (1986) and Saegusa *et al.* (1986) proposed that hydralazine stimulates release of catecholamines from the adrenergic nerve endings. Hydralazine may also inhibit phosphodiesterase activity in some concentrations (Ishii *et al.*, 1979). In total, this research suggests that hydralazine may exert its positive inotropic effect via an adenosine 3′:5′-cyclic monophosphate (cyclic AMP)-dependent mechanism. Additionally, cyclic AMP-independent mechanisms have also been suggested. Azuma *et al.* (1987) found the inotropic response was only partially inhibited by propranolol and concluded that hy-

[1] Author for correspondence at: Cardiovascular Division, Beth Israel Hospital, 330 Brookline Ave., Boston, MA 02215, U.S.A.

dralazine may act on the slow Ca^{2+} channels to stimulate Ca^{2+} influx.

The purpose of the present study was to define the mechanism of the positive inotropic response to hydralazine by elucidating its effect on intracellular calcium (Ca^{2+}) regulation, its interaction with both intrinsic catecholamines and histamine, and its direct effects on the myofilaments. Our results provide new information about the actions of hydralazine on intracellular Ca^{2+} modulation and myofilament Ca^{2+} responsiveness.

Methods

Muscle preparation

Papillary muscles of < 1.0 mm in diameter were excised from the right ventricle of adult male ferrets, 12–14 weeks old, under chloroform anaesthesia. The methods have been described in detail by Gwathmey & Morgan (1985); Mackinnon *et al.* (1988) and Kihara *et al.* (1989). The muscles were removed from the hearts and then placed in a physiological salt solution bubbled with a gaseous mixture of 95% O_2 and 5% CO_2 to pH 7.4. The experiments were performed at 30°C. The muscles were stimulated to contract at 0.33 Hz with pulses of 5 ms duration and threshold voltage applied via punctate electrodes at the base. An equilibrium period was allowed during which time the muscles were stretched until maximal isometric force developed (L_{max}). We used two stimulation protocols: (1) threshold punctate stimulation; and (2) suprathreshold punctate stimulation with voltage approximately 10% above threshold (Blinks, 1966). This level of voltage produces significant catecholamine release in the ferret papillary muscle preparation (Perreault *et al.*, 1990b) as a means to mimic the elevated catecholamine states found in heart failure. Since it was possible to obtain up to four papillary muscles per ferret, our n reflects the number of muscles, not the number of ferrets.

Intact muscle studies

Sixteen intact ferret papillary muscles were studied. As defined above, suprathreshold punctate stimulation was performed. Cumulative concentration-response relationships were obtained for hydralazine (10^{-6} to 10^{-3} M). The β-adrenoceptor antagonist, propranolol (6×10^{-7} M) was added to the organ bath and allowed to equilibrate. Of particular importance to the present study is the effect of this dose of propranolol on the release of catecholamines from sympathetic nerve endings in the papillary muscles used in our experiments. Blinks (1966) and Perreault *et al.* (1990) have demonstrated that the response to maximal sympathetic nerve stimulation via field pulses can produce the same maximal response as exogenously applied catecholamines. We have performed six additional experiments in which field pulses were applied at increasing voltage (2–12 V) until a maximal response was obtained. Propranolol (6×10^{-7} M) decreased the response to field stimulation at all voltages and depressed the maximal response by 5%. In the 2–4 V stimulation range (similar to the punctate voltage levels used to stimulate the muscles) no significant response to field pulses was observed with propranolol present in the bath. These data indicate that the dose of propranolol used in these experiments was adequate to minimize catecholamine release as a confounding variable with regard to interpreting our results. Hydralazine concentration-response relationships were then repeated. All measurements of peak tension were taken under steady-state conditions.

In a second set of fibres ($n = 7$) the bioluminescent Ca^{2+} indicator, aequorin, was loaded by macroinjection, as described in detail elsewhere (Kihara & Morgan, 1989). Light signals were recorded with a photomultiplier by means of a light collecting apparatus designed by Blinks (1982). Once steady state was obtained, it was necessary to average successive signals in order to obtain a satisfactory signal-to-noise ratio. The light signal was measured in nanoamperes of anode current and passed through a filter with a 10 ms time constant. Tension, light, and the stimulus artefact were recorded simultaneously on strip chart paper and magnetic tape. A concentration-response relationship was obtained for hydralazine in the absence and presence of propranolol (6×10^{-7} M), atropine (2×10^{-6} M), and phentolamine (1×10^{-6} M). Measurements were obtained for peak light and peak tension and time to peak light and tension.

In a third set of fibres ($n = 12$), we tested whether the inotropic response to hydralazine was independent of catecholamine release. We used histamine to stimulate cyclic AMP production via the H_2 receptor (Johnson, 1980), thus independently of catecholamine receptors. Muscles were allowed to reach baseline in the presence of propranolol (6×10^{-7} M) and a concentration-response relationship was obtained to histamine (10^{-8} to 10^{-5} M). This was repeated in the presence of hydralazine (3×10^{-4} M) plus propranolol (6×10^{-7} M).

Data analysis of intact muscles was conducted using the Wilcoxon Signed Rank Test to determine paired significance. Statistical significance was set at $P < 0.05$.

Skinned muscle studies

Muscles were chemically skinned by a 30 min exposure to a solution of the following composition (mM): imidazole 60, KCl 60, EGTA 5, $MgCl_2$ 7, K_2ATP 5, saponin 250 μg ml^{-1}, pH 7.1 at 21°C. Saponin in this concentration has been shown to remove both the sarcolemma and sarcoplasmic reticulum (Endo & Iino, 1980). The relaxing and activating solutions contained 10 mM EGTA and had a pMgATP of 2.5, pMg of 2.5, and a pH adjusted to 7.1 with KOH in the presence of 30 mM TES. The skinning, relaxing and activating solutions all contained creatine phosphokinase, 15 u ml^{-1}, and creatine phosphate, 12 mM, as an ATP regenerating system. The total salt concentrations necessary for obtaining the desired pCa, pMg, and pMgATP at a constant ionic strength of 0.16 M were calculated according to a programme developed by Fabiato & Fabiato (1979). The absolute stability constants used were as reported by Fabiato (1981). In order to study any sustained effect of hydralazine treatment, a group of muscles ($n = 16$) was pretreated with hydralazine (10^{-3} M) and allowed to reach baseline. Equilibrium was attained and these muscles were exposed to increasing calcium in a stepwise fashion, but without the further addition of hydralazine. In a second group of muscles ($n = 12$), skinning was performed first, followed by relaxation-activation cycles obtained by increasing the calcium in a stepwise fashion, with and without hydralazine (10^{-3} M). In half of these muscles a relaxation-activation cycle was performed first, followed by control. There were no significant differences noted in the pCa_{50}, F_{max}, or n obtained by this method.

Data analysis was performed by the Hill equation for the dependency of force on free calcium:

$$F = F_{max}\ ([Ca^{2+}]^n/Q + [Ca^{2+}]^n) \times 100\%$$

where Q is an effective dissociation constant (Segel, 1975), F if the developed force (i.e., isometric force), F_{max} is the maximal force developed (100%), and n is proportional to the slope of the force versus $\log[Ca^{2+}]$ curve when $F = 1/2F_{max}$, the calcium concentration required for 50% maximal force development (pCa_{50}) is given by: $pCa_{50} = -(\log Q)/n$ (Segel, 1975; Perreault *et al.*, 1990a).

The data were fitted to a modified Hill equation of the form:

$$F = F_{max}/1 + 10^{\log Q - n\log[Ca^{2+}]}$$

This calculation was performed using MLAB, a general non-linear regression package available in the NIH-sponsored PROPHET data analysis system. The curve fit parameters n and pCa_{50} were calculated for each individual curve from the above modified Hill equation and their means were compared by paired and unpaired t test where appropriate. Statistical significance was set at $P < 0.05$.

Drugs

Hydralazine, histamine, atropine, and propranolol were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A. and dissolved in water. Phentolamine was purchased from Ciba-Geigy Corp., Summit, N.J., U.S.A. Aequorin was purchased from Dr J.R. Blinks of Friday Harbor, Washington, U.S.A.

Results

Effects of hydralazine on the amplitude and time course of the contraction and Ca^{2+} transient

Table 1 presents the effects of increasing concentrations of hydralazine on the tension response and the $[Ca^{2+}]_i$ transient recorded with aequorin. In these experiments, the muscles were stimulated to contract at suprathreshold voltage to stimulate catecholamine release from the sympathetic nerve endings, which produced approximately a 10% increase in peak tension above levels produced by threshold punctate stimulation. Concentration-response relationships are presented for both peak tension and peak light in Table 1. Hydralazine (10^{-3} M) caused a $39 \pm 9\%$ increase in peak tension from baseline (2.5 ± 0.4 g to 3.5 ± 0.5 g; $P < 0.05$). This change in tension was associated with a significant increase in peak light. This increase in peak light is reflective of an increase in $[Ca^{2+}]_i$ transients in parallel with peak tension. A similar trend was present at lower concentrations, as well (Table 1), but did not reach statistical significance. Table 1 also provides data on the time courses for both peak tension and peak light. Both time to peak tension and time to peak light were signficantly abbreviated at maximally effective inotropic concentrations of hydralazine. Figure 1 displays an experimental recording of both the light and tension responses for control and hydralazine-treated muscles over several concentrations (10^{-5} to 10^{-3} M) and the paired response in the presence of autonomic blockers. With the addition of propranolol, phentolamine, and atropine as well as hydralazine, over several concentrations, produced no significant increase in peak tension or peak light and the time courses of tension and light were not altered.

Histamine stimulated inotropic effects of hydralazine

Following the observation that hydralazine increases the maximum isometric tension produced by suprathreshold stimulation, we explored whether this response was dependent upon catecholamine release. Propranolol alone has been shown to attenuate markedly the positive inotropic effects of hydralazine. Therefore, after β-adrenoceptor blockade with propranolol (6×10^{-7} M), histamine was added in increasing concentrations (10^{-8} to 10^{-5} M). Histamine stimulation in the presence of hydralazine (3×10^{-4} M) shifted the pD_2 (i.e. the negative log of the concentration of histamine producing 50% of the maximal response) from 5.9 ± 0.1 to 6.1 ± 0.1 ($P < 0.05$). It was also noted that the maximum response to histamine in control fibres was $160 \pm 23\%$ above baseline stimulation while hydralazine treatment (3×10^{-4} M) produced an additional increase to $195 \pm 17\%$ in the same muscles ($P < 0.05$). As shown in Table 1, the dose of hydralazine would not be expected to increase tension development of propranolol-blocked muscles in the absence of histamine stimulation.

Effects of hydralazine on myofilament Ca^{2+} responsiveness

To determine what effect, if any, hydralazine has on myofilament Ca^{2+} responsiveness, which includes calcium sensitivity, maximal calcium-activated force, and the slope of the calcium-force relationship, we examined the effects of the drug in skinned muscle preparations.

An experimental tracing is presented in Figure 2. Pretreatment with hydralazine did not shift the force-pCa relationship at the calcium concentration required for 50% maximal force development (pCa_{50}), nor did it significantly alter the maximal amount of force that a muscle could generate in the presence of calcium, F_{max} (Table 2). The Hill coefficient (n), which reflects the slope of the fit at pCa_{50}, was not altered by pretreatment with hydralazine. However, hydralazine (10^{-3} M) added after skinning had been performed, decreased the sensitivity of the myofilaments to calcium. The force-pCa curve was shifted to the right at the pCa_{50} ($P < 0.05$). While there was a similar trend in the pCa_{50} with 3×10^{-6} M hydralazine, it was not statistically significant (data not shown). The Hill coefficient (n) was not altered by hydralazine. Maximal calcium-activated force, F_{max}, also was not significantly altered in the presence of hydralazine. Figure 3 depicts curves obtained from the pCa_{50} and n as calculated from the modified Hill equation for all three sets of data.

Discussion

The most important findings of our study include: (1) the mechanism of the positive inotropic effect of hydralazine is

Table 1 Effects of hydralazine on aequorin-loaded ferret papillary muscles stimulated with suprathreshold punctate voltage

		Hydralazine (M)			
		0	10^{-5}	10^{-4}	10^{-3}
PT (%; $n = 16$)	Pre	59 ± 4	60 ± 4	68 ± 3	78 ± 3*
	Post	47 ± 3	44 ± 3	48 ± 3	52 ± 2
PL (%; $n = 7$)	Pre	38 ± 5	44 ± 8	45 ± 7	66 ± 8*
	Post	26 ± 4	24 ± 3	22 ± 4	25 ± 3
tPt (ms; $n = 7$)	Pre	221 ± 7	208 ± 10	208 ± 9*	186 ± 5*
	Post	196 ± 4	203 ± 6	204 ± 4	208 ± 5
tPL (ms; $n = 7$)	Pre	65 ± 4	60 ± 5	56 ± 3	52 ± 2*
	Post	56 ± 5	57 ± 5	57 ± 2	56 ± 3

Data shown are mean ± s.e.mean. % = % of maximal response to Ca^{2+}; absolute maximal response to hydralazine in the absence of adrenoceptor blockers was from 2.5 ± 0.4 g to 3.5 ± 0.5 g. PT = peak tension; PL = peak light; tPt = time to peak tension; tPL = time to peak light. Muscles were stimulated without (pre) and with (post) autonomic blocking agents.
*$P < 0.05$ compared to control. All muscles were stimulated with suprathreshold voltage via punctate electrodes at 0.33 Hz and bathed in 1 mM Ca^{2+} at 30°C.

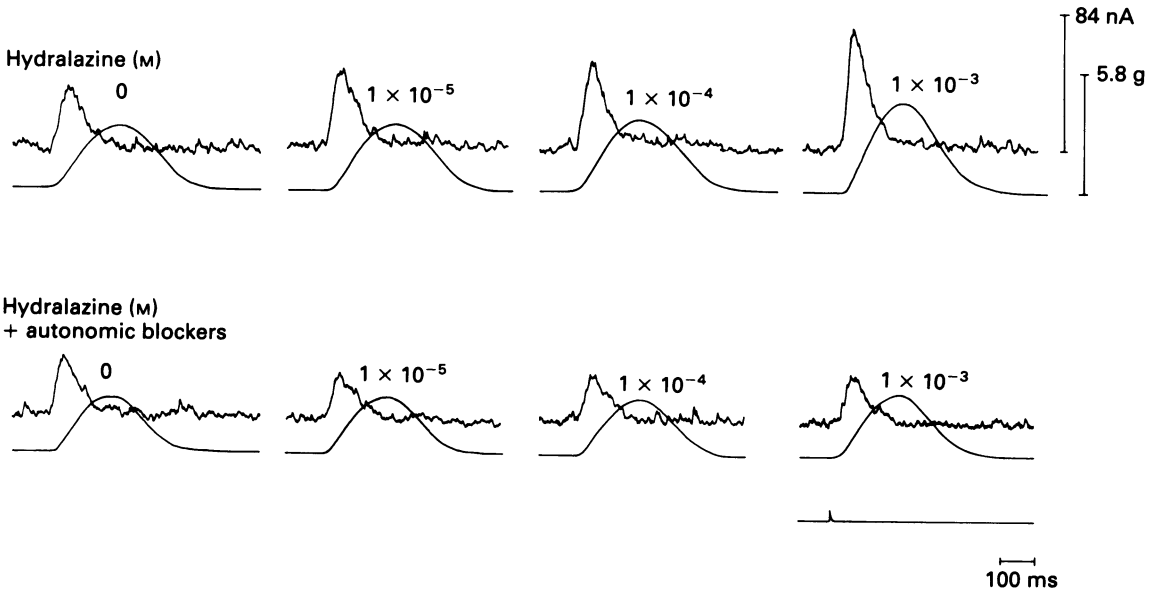

Figure 1 The effects of hydralazine alone and in the presence of autonomic blockers in an aequorin-loaded ferret papillary muscle. Muscle in the upper panels was treated with hydralazine alone, while that in the lower panels was treated with propranolol (6×10^{-7} M), atropine (2×10^{-6} M), and phentolamine (10^{-6} M), as well as hydralazine. The aequorin signal amplitude (i.e., light) is expressed as nA of current recorded from a photomultiplier; tension is expressed in grams (g). Each tracing represents the average of 30 responses obtained at steady state.

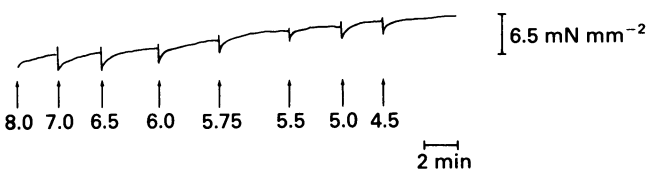

Figure 2 Representative experimental recording obtained from a saponin-skinned ferret right ventricular papillary muscle. Bottom arrows indicate pCa(− log[Ca^{2+}]) of buffer solution.

dependent on a concurrent rise in $[Ca^{2+}]_i$; (2) the positive inotropic effect of hydralazine is consistent with both cyclic AMP-dependent and independent mechanisms; (3) hydralazine has direct negative inotropic effects on the myofilaments.

Peak tension and peak light in ferret myocardium

Contraction-relaxation cycles in the heart are governed by cytosolic Ca^{2+} fluxes that are modulated by the sarcolemma and sarcoplasmic reticulum (Ruegg, 1988). Interventions that alter the strength of cardiac contraction produce changes in either the $[Ca^{2+}]_i$ concentration and/or in the Ca^{2+} requirements of the contractile apparatus. Cyclic AMP is a second messenger that regulates both effects through activation of a variety of cyclic AMP-dependent protein kinases (Opie, 1982). These kinases phosphorylate the sarcolemma and sarcoplasmic reticulum, as well as the contractile elements, which results in (1) increased transsarcolemmal influx of Ca^{2+}, (2) decreased Ca^{2+} responsiveness of the contractile elements, and (3) increased re-uptake of Ca^{2+} by the sarcoplasmic reticulum. Most inotropic agents used in clinical medicine produce an inotropic effect by increasing cellular levels of cyclic AMP. Since catecholamine levels are elevated in heart failure (Chidsey *et al.*, 1965), we used two stimulation protocols to study the effects of altered catecholamine levels on the inotropic response. Hydralazine demonstrated a positive inotropic response in intact fibres, which is similar to previous reports in both dogs and cats (Rabinowitz *et al.*, 1986). In our experiments, and in previous studies (Rabinowitz *et al.*, 1986), this effect was abolished by propranolol, suggesting that the mechanism may be related to noradrenaline release from the adrenergic nerve endings.

We studied the effects of hydralazine on cellular Ca^{2+} modulation with the bioluminescent Ca^{2+} indicator, aequorin, that emits light when it combines with free, ionized calcium. In mammalian myocardium, the aequorin light signal apears to reflect predominantly the release and re-uptake of Ca^{2+} by the sarcoplasmic reticulum (Morgan *et al.*, 1984;

Table 2 Effects of hydralazine on Ca^{2+} sensitivity (pCa_{50}), maximum force development (F_{max}), and Hill coefficient (n) in saponin-skinned ferret papillary muscles

		Hydralazine (10^{-3} M)	
	Control	*After skinning*	*Before skinning*
pCa_{50}	6.08 ± 0.06[1]	5.86 ± 0.06[1,2]	6.02 ± 0.04[2]
n	1.54 ± 0.27	1.23 ± 0.06	3.81 ± 1.58
F_{max}	3.47 ± 0.57	3.84 ± 0.53	4.22 ± 0.28
n	12	12	16

Data shown are mean ± s.e.mean. $[Ca^{2+}]_0 = 1.0$ mM.
[1]Control vs. After skinning $P < 0.05$; [2]After skinning vs. Before skinning $P < 0.05$.

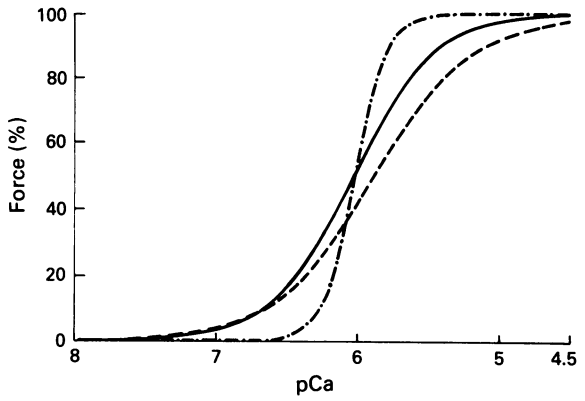

Figure 3 The effect of hydralazine on the force vs. calcium relation of skinned ferret right ventricular papillary muscles. The curves represent control fibres (—), hydralazine (10^{-3} M) treatment after skinning (– – –), and hydralazine (10^{-3} M) treatment before skinning (–·–·–). Force is plotted on the vertical axis as a percentage of maximal response to calcium. The horizontal axis denotes the pCa ($-\log[Ca^{2+}]$) of buffer solution. The curve parameters n and pCa_{50} were fitted to a modified Hill equation.

Morgan & Morgan, 1984; 1989). Moreover, the relative ratio of the amplitude of the light signal to a given increase in tension can be used as an index of myofilament Ca^{2+} responsiveness (Gwathmey & Morgan, 1985; Morgan & Morgan, 1984; 1989). Our data demonstrate the following: (1) hydralazine causes a simultaneous increase in peak light and peak tension indicating the mechanism of action depends upon a concurrent increase in $[Ca^{2+}]_i$; (2) the positive inotropic effect is associated with an abbreviated time to peak light and time to peak tension; (3) the positive inotropic response is associated with an increase in the ratio of the peak light to peak tension consistent with Ca^{2+} desensitization of the myofilaments. This combination of effects is characteristic of agents that act by increasing intracellular cyclic AMP concentrations (Morgan & Morgan, 1989). On the other hand, our data demonstrate no significant effect of hydralazine on peak light or peak tension in the presence of autonomic blockers. This blunted effect suggests that the inotropic response of hydralazine is either secondary to catecholamine release, and/or the response is facilitated by stimulation of the β-adrenoceptor-mediated production of cyclic AMP.

Effects of autonomic agents and varying stimulation protocols

To study further the possibility of an inotropic mechanism independent of catecholamine release and dependent upon cyclic AMP, muscles were stimulated with histamine in the presence of β-adrenoceptor blockade with propranolol to stimulate adenylate cyclase activity independent of the adrenoceptors. Without histamine stimulation, such β-blocked muscles would be expected to show little in the way of an inotropic response to hydralazine (see Table 1). However, in the presence of hydralazine, our data demonstrated a potentiation of the positive inotropic response to histamine with a leftward shift in the pD_2, again consistent with phosphodiesterase inhibition. Therefore, the positive inotropic effect of hydralazine may not be solely mediated by catecholamine release from the adrenergic nerve endings, but may occur in response to any manoeuvre that increases intracellular cyclic AMP levels. A second mechanism of positive inotropy is suggested by the increase in maximal tension found with hydralazine in histamine-stimulated muscles. Morad *et al.* (1978) have demonstrated that agents which are cyclic AMP-dependent decrease peak tension in maximally activated muscles, probably through desensitization of the myofilaments via phosphorylation of troponin I (Opie, 1983). Therefore, the observed increase in peak tension with histamine supports an additional mechanism of positive inotropy for hydralazine which is cyclic AMP-independent. However, it is important to note that these effects occurred in a dosage range generally considered to be above concentrations generally reached in the clinical setting and their relevance to man remains to be established.

Direct effects of hydralazine on the myofilaments

Inotropic agents that increase contractile force and act independently of changes in cyclic AMP have been demonstrated to act directly on the myofilaments, operating as sensitizing or desensitizing agents (Ruegg *et al.*, 1984; Perreault *et al.*, 1990a,b). In the present studies, pretreatment of intact fibres with hydralazine prior to skinning did not elicit an effect, indicating that hydralazine does not have a sustained effect on the contractile elements. This may be because hydralazine does not achieve high enough concentrations in the sarcoplasm of intact myocytes to exert a direct myocardial effect or because any such action is reversed by subsequent detergent treatment. However, hydralazine treatment of previously skinned fibres shifted the force-pCa_{50} curve to the right, consistent with a desensitizing effect on the cardiac myofilaments, without altering the force of maximal contraction (F_{max}). The cellular mechanism of this effect on skinned fibres is probably different from that observed in aequorin-loaded muscles since the sarcolemmal mechanisms required for cyclic AMP generation are removed by detergent skinning. In addition, the increased peak tension of histamine stimulated intact fibres in the presence of hydralazine is not due to changes of calcium binding mediated directly by myofilaments; rather, the increased calcium responsiveness is probably due to cytoplasmic or membrane alterations mediated by a second messenger system such as through activation of diacylglycerol (DAG) or phosphatidyl-inositol trisphosphate (IP_3), which have been removed by the skinning process. The precise mechanism responsible for the desensitizing, direct effects of hydralazine on the myofilaments remains speculative, but may involve an alteration in thin filament force regulation, such as a change in the affinity of troponin-C for Ca^{2+} as previously proposed for cocaine, a drug also known to act as a desensitizer (Perreault *et al.*, 1990b). Of interest is the fact that the direct and cyclic AMP-independent desensitizing effects of hydralazine on the myofilaments did not appear to be functionally important in ferret papillary muscles at any concentration of hydralazine we studied, since a negative inotropic effect was not observed. However, the significance of these effects may vary with species, the degree of activation of the muscle, or in the presence of disease states.

In summary, our findings suggest that hydralazine can produce a positive inotropic effect in mammalian myocardium. This response, measured as peak tension, is concentration-dependent and attenuated by β-adrenoceptor blockade. The increase in tension with hydralazine is mediated via an increase in $[Ca^{2+}]_i$, and both time to peak light and time to peak tension are abbreviated, consistent with a cyclic AMP-mediated effect. Further support to a cyclic AMP-dependent mechanism of hydralazine is demonstrated by a shift in the concentration-response curve for histamine in the presence of propranolol. An additional mechanism of positive inotropy which is cyclic AMP-independent is supported by an increase in peak tension in these same muscles. This effect is most probably mediated by a yet unidentified second messenger. In contrast, hydralazine acts as a direct desensitizer of myofilaments in skinned fibres in which the sarcolemma has been removed as a diffusion barrier to the drug, but this effect does not appear to be significant under the conditions of our intact muscle experiments. While recent clinical studies, such as the PROMISE

trial, suggest that increased cellular levels of cyclic AMP negatively impact survival, hydralazine appears to possess similar properties and yet has proven utility in mild-to-moderate heart failure. This may be explained by the lower potency of hydralazine compared to drugs like milrinone for producing a rise in cellular cyclic AMP, and/or by the potential importance of other cyclic AMP-independent mechanisms identified solely with hydralazine. Alternatively, the results of the PROMISE trial may not apply to all cyclic AMP-dependent inodilator agents.

The authors gratefully acknowledge Mr Jason Kravitz and Patricia Roche for their expert assistance in the preparation of this manuscript. This work was supported in part by a grant from the NIH (HL31117) and a Grant-in-Aid from the American Heart Association.

References

AZUMA, J., SAWAMURA, A., HARADA, H., AWATA, N., KISHIMOTO, S. & SPERELAKIS, N. (1987). Mechanism of direct cardiostimulating actions of hydralazine. *Eur. J. Pharmacol.*, **135**, 137–144.

BLINKS, J.R. (1966). Field stimulation as a means of effecting the graded release of autonomic transmitter in isolated heart muscle. *J. Pharmacol. Exp. Ther.*, **151**, 221–235.

BLINKS, J.R. (1982). The use of photoproteins as calcium indicators in cellular physiology. In *Techniques in Cellular Physiology, Part III.* ed. Baker, P.F. pp. 126/1–126/38. Ireland: Elsevier/North-Holland Scientific Publisher Ltd.

CHIDSEY, C.A., BRAUNWALD, E. & MORROW, A.G. (1965). Catecholamine excretion and cardiac stores of norepinephrine in congestive heart failure. *Am. J. Med.*, **39**, 442–451.

COHN, J.N., ARCHIBALD, D.G. & ZIESCHE, S. (1986). Effect of vasodilator therapy on mortality in chronic congestive heart failure: results of a Veterans Administration Cooperative Study. *N. Engl. J. Med.*, **314**, 1547–1552.

COHN, J.N., JOHNSON, M.S. & ZIESCHE, S. (1991). A comparison of enalapril with hydralazine-isosorbide dinitrate in the treatment of chronic congestive heart failure. *N. Engl. J. Med.*, **325**, 303–310.

ENDO, M. & IINO, M. (1980). Specific perforation of muscle cell membranes with preserved sarcoplasmic reticulum functions by saponin treatment. *J. Muscle. Res. Cell. Motil.*, **1**, 89–100.

FABIATO, A. (1981). Myoplasmic free calcium concentration reached during the twitch of an intact isolated cardiac cell and during calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned cardiac cell from the adult rat or rabbit ventricle. *J. Gen. Physiol.*, **78**, 457–497.

FABIATO, A. & FABIATO, F. (1979). Calculator programs for computing the compositions of solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *J. Physiol. (Paris)*, **75**, 463–505.

GERSHWIN, M.E. & SMITH, N.T. (1967). Mode of action of hydralazzine in guinea pig atria. *Arch. Int. Pharmacodyn.*, **170**, 108–116.

GWATHMEY, J.K. & MORGAN, J.P. (1985). The effects of milrinone and piroximone on intracellular calcium handling in working myocardium from the ferret. *Br. J. Pharmacol.*, **85**, 97–108.

ISHII, A., KUBO, K., DEGUCHI, T. & TANAKA, M. (1979). Inhibition of cyclic AMP phosphodiesterase activity by ecarazine, hydrochloride, hydralazine and their metabolites. *Yakagaku Zasshi*, **99**, 533.

JOHNSON, C.L. (1980). In *Pharmacology of Histamine Receptors.* ed. Ganellin, R. & Parsons, M. pp. 146–216. London: J. Wright & Sons.

KHATRI, I., USMURA, N., NOTARGIACOMO, A. & FREIS, E.D. (1977). Direct and reflex cardiostimulatory effects of hydralazine. *Am. J. Cardiol.*, **40**, 38–42.

KIHARA, Y. & MORGAN, J.P. (1989). A comparative study of three methods for intracellular loading of the calcium indicator aequorin in ferret papillary muscles. *Biochem. Biophys. Res. Commun.*, **162**, 402–407.

KIHARA, Y., GWATHMEY, J.K., GROSSMAN, W. & MORGAN, J.P. (1989). Mechanisms of positive inotropic effects and delayed relaxation produced by DPI 201-106 in mammalian working myocardium: effects on intracellular calcium handling. *Br. J. Pharmacol.*, **96**, 927–939.

LEIER, C.V., DESCH, C.E., MAGORIEN, R.D., TRIFFON, D.W., UNVERFERTH, D.V., BOUDOULAS, H. & LEWIS, R.P. (1980). Positive inotropic effects of hydralazine in human subjects: comparison with prazosin in the setting of congestive heart failure. *Am. J. Cardiol.*, **46**, 1039–1044.

MACKINNON, R., GWATHMEY, J.K., ALLEN, P.D., BRIGGS, B.M. & MORGAN, J.P. (1988). Modulation by the thyroid state of intracellular calcium and contractility in ferret ventricular muscle. *Circ. Res.*, **63**, 1080–1089.

MORAD, M., WEISS, J. & CLEEMAN, L. (1978). The inotropic action of adrenaline on cardiac muscle: does it relax or potentiate tension? *Eur. J. Cardiol.*, **7**, suppl., 53–62.

MORGAN, J.P., CHESEBRO, J.H., PLUTH, J.R., PUGA, F.J. & SCHAFF, H.V. (1984). Intracellular calcium transients in human working myocardium as detected with aequorin. *J. Am. Coll. Cardiol.*, **3**, 410–418.

MORGAN, J.P. & MORGAN, K.G. (1984). Calcium and cardiovascular function: intracellular calcium levels during contraction and relaxation of mammalian cardiac and vascular smooth muscle as detected with aequorin. *Am. J. Med.*, **77** (Suppl. 5A), 33–46.

MORGAN, J.P. & MORGAN, K.G. (1989). Intracellular calcium and cardiovascular function in heart failure: effects of pharmacologic agents. *Cardiovasc. Drugs Ther.*, **3** (Suppl. C), 959–970.

OPIE, L.H. (1983). Role of cyclic nucleotides in heart metabolism. *Cardiovasc. Res.*, **16**, 483.

PACKER, M., CARVER, J.R. & RODEHEFFER, R.J. (1991). Effect of oral milrinone on mortality in severe chronic heart failure. *N. Engl. J. Med.*, **325**, 1468–1475.

PERREAULT, C.L., BING, O.H.L., BROOKS, W.W., RANSIL, B.J. & MORGAN, J.P. (1990a). Differential effects of hypertrophy and failure on right versus left ventricular calcium activation. *Circ. Res.*, **67**, 707–712.

PERREAULT, C.L., HAGUE, N.L., RANSIL, B.J. & MORGAN, J.P. (1990b). The effects of cocaine on intracellular Ca^{2+} handling and myofilament Ca^{2+} responsiveness of ferret ventricular myocardium. *Br. J. Pharmacol.*, **101**, 679–685.

RABINOWITZ, B., PARMLEY, W.W., HAR-ZAHAV, Y., ELAZAR, E., BLUMLEIN, S., NERINSKY, R. & NEUFELD, H.N. (1986). Correlation between effects of hydralazine on force and on the adenyl cyclase system of ventricular myocardium in dogs and cats. *Cardiovasc. Res.*, **20**, 215–220.

RENDIG, S.V., SEGEL, L.D. & AMSTERDAM, E.A. (1988). Direct effects of hydralazine on cardiac contractile function, haemodynamics, and myocardial energetics in isolated myocardium. *Cardiovasc. Res.*, **22**, 322–328.

RUEGG, J.C. (1988). *Calcium in Muscle Activation*, pp. 1–285. New York: Springer-Verlag.

RUEGG, J.C., PFITZER, G., EUBLER, D. & ZEUGNER, C. (1984). Effect on contractility of skinned fibers from mammalian heart and smooth muscle by a new benzimidazole derivative, 4,5-dihydro-6[(2-4-methoxyphenyl)-1H-benzimidazole-5-yl]-5-methyl-3(2H)-pyridazinone. *Arzneim. Forsch. Drug Res.*, **34**, 1736.

SAEGUSA, K., FURUKAWA, Y. & CHIBA, S. (1986). Pharmacological analyses of hydralazine-induced cardiac action in intact dogs and isolated, blood-perfused canine atria. *J. Cardiovasc. Pharmacol.*, **8**, 614–620.

SEGEL, I.H. (1975). *Enzyme kinetics: Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme Systems.* pp. 360–361. New York: John Wiley and Sons, Inc.

SMITH, W.M. (1985). Epidemiology of congestive heart failure. *Am. J. Cardiol.*, **55**, 3A.

(Received June 11, 1992
Revised February 16, 1993
Accepted February 25, 1993)