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European Journal of Pharmacology 321 (1997) 201–208

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Mechanisms of positive inotropic action of flosequinan, hydralazine, and milrinone on mammalian myocardium

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Received 15 October 1996; accepted 26 November 1996

Abstract

Flosequinan is an arterial and venous dilator that also has a positive inotropic effect at relatively higher doses. The purpose of this study was to determine the mechanism of this positive inotropic effect in ferret papillary muscles loaded with the Ca^{2+} indicator, aequorin. Over the range of doses from 10^{-6} to 10^{-3} M, flosequinan produced a $61 \pm 9\%$ increase in peak tension that was accompanied by a corresponding increase in $[Ca^{2+}]_i$. This positive inotropic effect was not selectively blocked by addition to the perfusate of procaine 0.6 μM, tetrodotoxin 10^{-6} M or by verapamil, 5×10^{-8} M. In contrast, the positive inotropic effect of flosequinan, but not milrinone or hydralazine, was potentiated by prior addition of ouabain 3 nM to enhance intracellular Ca^{2+} via reduction of the Na^+/Ca^{2+} exchange. Moreover, antagonists of Na^+/Ca^{2+} exchange, including cadmium 10 μM, amiloride 600 μM and choline substitution for 1/3 Na^+ in the perfusate, blocked the response to flosequinan but not hydralazine or milrinone. These results indicate that flosequinan produces a positive inotropic effect by reduction of Na^+/Ca^{2+} exchange in mammalian myocardium. Moreover, flosequinan has the potential to interact synergistically with other positive inotropic agents such as digoxin that affect Na^+/Ca^{2+} exchange by direct or indirect actions.

Keywords: Na^+/Ca^{2+} exchange; Ca^{2+}, intracellular; Heart failure; cAMP

1. Introduction

Drugs that vasodilate the arterial and venous vascular beds have an established role in the treatment of symptomatic heart failure (Cohn, 1991). Flosequinan (Elborn et al., 1989; Cowley, 1991), hydralazine (Cohn et al., 1986) and milrinone (Colucci, 1991) are three drugs with vasodilator actions that have been used for this purpose in the United States and/or Europe. Each of these drugs appears to act by a different cellular mechanism to produce smooth muscle relaxation. Milrinone is a well-known phosphodiesterase inhibitor that has been shown to decrease intracellular Ca^{2+} levels and myofilament Ca^{2+} sensitivity in vascular smooth muscle preparations (Benotti et al., 1985; Alousi and Johnson, 1986; Silver et al., 1988; Resnick et al., 1991). The mechanism of vasodilator action of hydralazine is unknown, but may involve formation of nitric oxide and activation of guanylate cyclase with cyclic GMP formation (Kruszyna et al., 1987; Rudd and Blaschke, 1985; Kreye, 1984; Ishii et al., 1979). In this regard, Spokas et al. (1983) have shown that hydralazine-induced vasodilation is, in part, endothelium-dependent. Hydralazine may also affect intracellular Ca^{2+} mobilization (Rudd and Blaschke, 1985; Kreye, 1984) and phosphorylation processes (Jacobs, 1984) in some vascular beds. The mechanism of action of flosequinan is unknown, although some experimental evidence suggests that it may affect the inositol triphosphate (IP_3)-diacylglycerol system in smooth muscle (Resnick et al., 1991; Yates, 1991). Because of the differences in mechanism of action, the effect of each agent may vary depending upon the factors responsible for maintaining vascular tone in a particular patient. In addition, the effects of each of these agents may be additive with other vasodilator drugs acting by different mechanisms (Cohn et al., 1991).

Besides their actions on vascular smooth muscle, flosequinan, hydralazine and milrinone have been shown to produce positive inotropic effects on the heart in concentrations that overlap or only slightly exceed the clinically utilized therapeutic range (Yates, 1991; Corin et al., 1991; Leier et al., 1980; Ludmer et al., 1986; Greenberg and

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PII S0014-2999(96)00946-6

Touhey, 1990; Azuma et al., 1987). This is most easily documented for milrinone, which has been dubbed an 'inodilator', since its vasodilator and inotropic doses are similar (Opie, 1986). In fact, results of the PROMISE trial indicate that milrinone may decrease survival in patients with failure due to its effects on the heart, which presumably occurs via phosphodiesterase inhibition and elevated intracellular levels of cyclic AMP (cAMP) in the myocardium (Packer et al., 1991). Hydralazine has been reported to produce a positive inotropic effect in the clinically utilized range of doses that can be partially inhibited by β-adrenoceptor antagonists, and is due at least in part to reflex activation of the autonomic nervous system (Azuma et al., 1987; Saegusa et al., 1986). Therefore, to some extent, the mechanism of inotropic action of hydralazine is similar to milrinone, in that it predominantly involves elevation of intracellular levels of cAMP (Earl et al., 1986). There is evidence that a direct inotropic effect can occur at high doses of hydralazine (i.e., $\geq 10^{-4}$ M) in some species, and that this effect is not mediated by cAMP-dependent mechanisms (Frodsham and Jones, 1992). Flosequinan produces a dose-related positive inotropic effect in some mammalian species, including man, that occurs by an unknown mechanism but does not appear to be related to changes in intracellular cyclic nucleotide levels (Yates, 1991; Corin et al., 1991; Perreault et al., 1992). Despite different cellular mechanisms, flosequinan, like milrinone, has been shown to adversely influence survival of patients with heart failure in selected doses (Binkley et al., 1994). Of interest, the positive inotropic effects of both milrinone and flosequinan on human myocardial tissue appear to be significantly reduced with the development of heart failure (Perreault et al., 1992; Feldman et al., 1987; Bohm et al., 1988); hydralazine has not been studied in this regard.

Results of the PROMISE trial emphasized the importance of the cardiac effects of drugs used to treat patients with heart failure (Packer et al., 1991). The present study was undertaken to delineate the cellular mechanisms of the positive inotropic effects of flosequinan, hydralazine and milrinone on mammalian myocardium.

2. Materials and methods

2.1. Tissue acquisition and initial preparation

Papillary muscles of 1.0 mm or less in diameter were excised from the right ventricles of hearts removed from adult male ferrets, 12–14 weeks of age, under chloroform anesthesia. The methods of preparation and instrumentation used in these studies have been described in detail (Gwathmey and Morgan, 1985; MacKinnon et al., 1988). After removal from the hearts, muscles were placed in baths containing bicarbonate-buffered physiological salt solution bubbled with a gas mixture of 95% O_2 and 5% CO_2 to pH 7.4. The experiments were performed at 30°C. At this temperature, muscle contraction in mammalian species is more stable than at 37°C and therefore 30°C is the standard in our laboratory. Stimulating electrodes were positioned at the base of muscles. Muscles were stimulated to contract at 3-s intervals with pulses of 5 ms duration and voltage at threshold +10%. Propranolol 6×10^{-7} M, phentolamine 1×10^{-6} M and atropine 2×10^{-6} M were present in the bathing medium to minimize the post-synaptic effects of catecholamine and acetylcholine release from autonomic nerve endings (Blinks, 1966; Perreault et al., 1990). An initial 2-h equilibration period was allowed during which muscles were gradually stretched to the length where maximal isometric force developed. Since it was possible to obtain up to four papillary muscles per ferret, our *n* refers to the number of papillary muscles, not the number of ferrets.

2.2. Intact-muscle studies

One group of muscles ($n = 66$) was used to characterize the actions of flosequinan, hydralazine and milrinone. Peak tension, time to peak tension and time from peak tension to 50% and 80% decline from peak tension were measured on chart strip paper which simultaneously recorded the tension response and the stimulus artifact at 100 mm s^{-1}. All measures were made under steady-state conditions.

In a subset of muscles ($n = 9$), aequorin was loaded by macroinjection, as described in detail elsewhere (Kihara and Morgan, 1989). Light signals (an index of intracellular calcium, $[Ca^{2+}]_i$) were recorded with a photomultiplier by means of a light collecting apparatus, of a design described by Blinks (1982). In order to obtain a satisfactory signal-to-noise-ratio, it was usually necessary to average successive signals (from 16 to several hundred, depending on the light intensity). Signal averaging was performed only after responses had reached the steady state. The light and tension responses and stimulus artifact were recorded simultaneously both on magnetic tape and on chart strip recording paper. Light signals were passed through a filter with a 10 ms time constant. The light signal was measured in nanoamperes of anodal current. The amplitude and time course of the aequorin light signal were analyzed in the same manner described above for tension.

All data were compared by unpaired or paired (when appropriate) Student's *t*-test or multiple sample comparison tests. Statistical significance was set at $P < 0.05$.

2.3. Drugs

The aequorin used in these experiments was obtained from the laboratory of Dr. J.R. Blinks (Friday Harbor, WA, USA); tetrodotoxin, procaine HCl, ouabain, propranolol HCl, atropine SO_4 and verapamil HCl were purchased from Sigma (St. Louis, MO, USA) and dissolved in

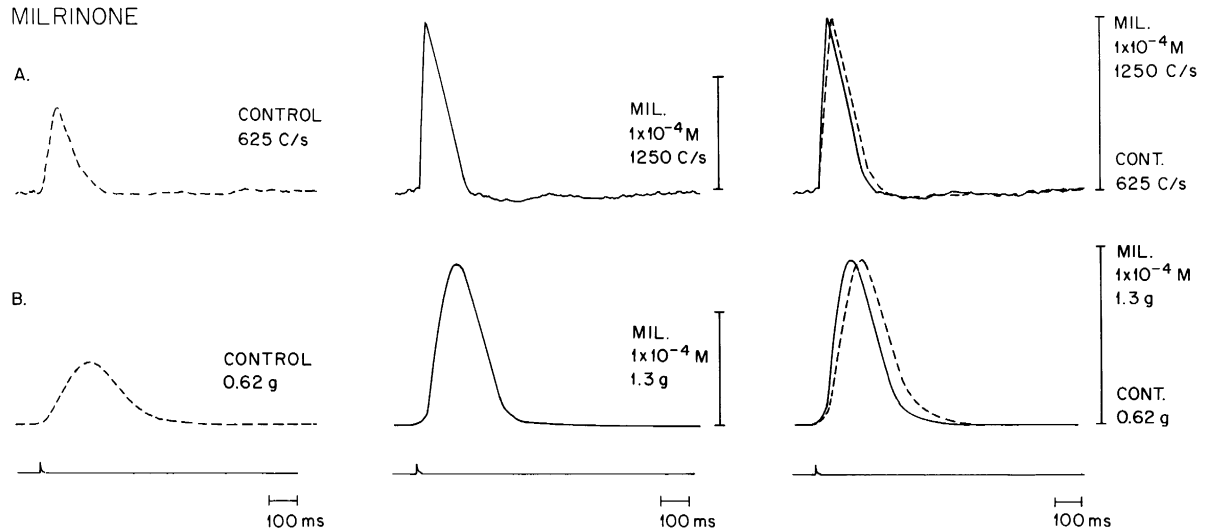

Fig. 1. Effects of milrinone on aequorin-loaded ferret papillary muscle. Upper A panels show Ca^{2+}; transient before (left) and after (middle) milrinone, expressed as counts/second of light emission (C/s). Lower B panels show isometric force in grams. A and B panels to far right show superimposed Ca^{2+} transients (left) and isometric twitches (right) before (broken line) and after (solid line) milrinone, with amplitudes electronically adjusted so that time courses can be directly compared. Stimulus artifact is lowermost tracing in each set.

aqueous solution. Flosequinan was generously supplied by Boots (Nottingham, UK); phentolamine mesylate and hydralazine HCl by Ciba-Geigy (Summit, NJ, USA); and milrinone by the Sterling-Winthrop Company (Rensselaer, NY, USA). A 10^{-2} M stock solution of flosequinan was prepared in dimethyl sulfoxide (DMSO; Sigma) and diluted with water; in the amounts added to the organ baths, the diluent had no detectable effect on muscle function. Milrinone was dissolved in a small amount of HCl and diluted with water. In the amounts added to the organ bath, this diluent did not shift the pH of the buffer or affect muscle function. Phentolamine was dissolved in water.

3. Results

Figs. 1–3 demonstrate the effects of milrinone, hydralazine and flosequinan on aequorin-loaded ferret papillary muscles. The presence of post-synaptic autonomic blockade with propranolol 6×10^{-7} M, phentolamine 10^{-6} M

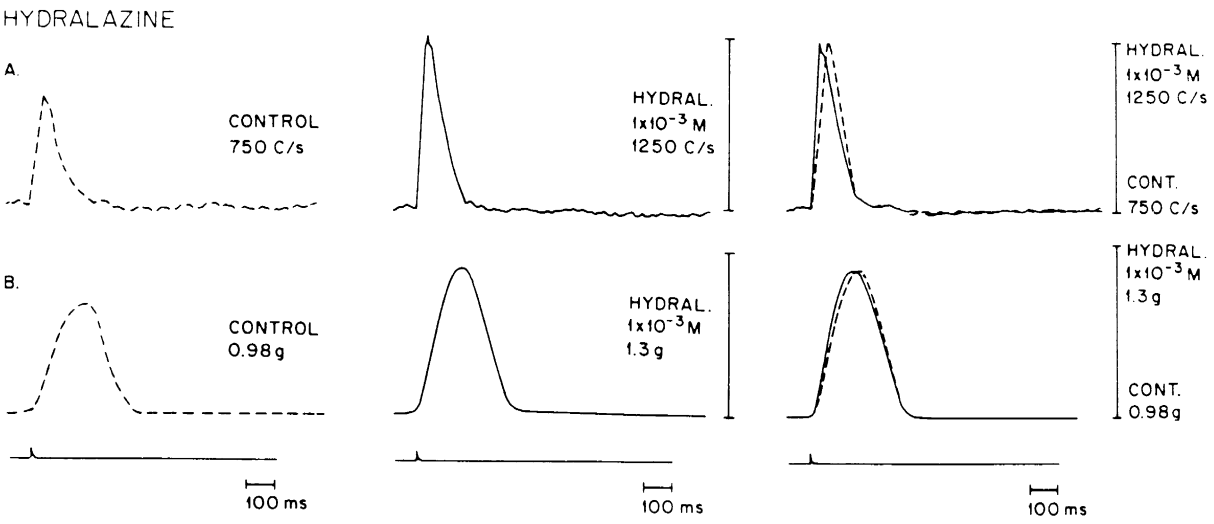

Fig. 2. Effects of hydralazine on aequorin-loaded ferret papillary muscle. Upper A panels show Ca^{2+}; transient before (left) and after (middle) hydralazine, expressed as counts/second of light emission (C/s). Lower B panels show isometric force in grams. A and B panels to far right show superimposed Ca^{2+} transients (upper) and isometric twitches (lower) before (broken line) and after (solid line) hydralazine, with amplitudes electronically adjusted so that time courses can be directly compared. Stimulus artifact is lowermost tracing in each set.

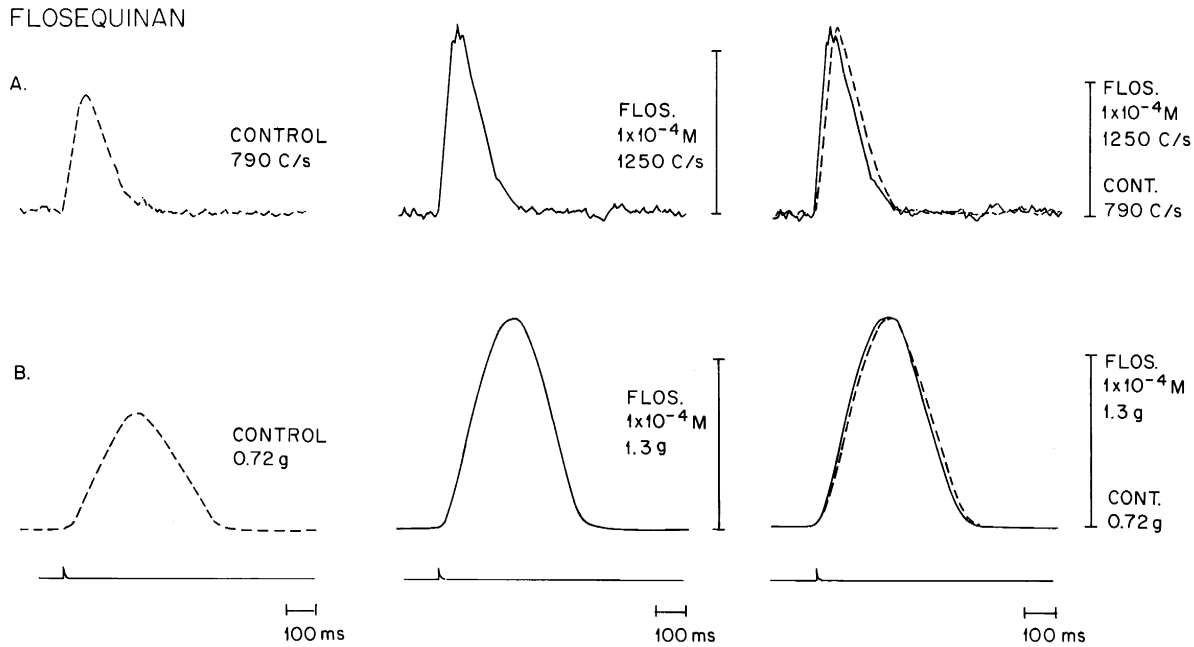

Fig. 3. Effects of flosequinan on aequorin-loaded ferret papillary muscle. Upper A panels show Ca^{2+}; transient before (left) and after (middle) flosequinan, expressed as counts/second of light emission (C/s). Lower B panels show isometric force in grams. A and B panels to far right show superimposed Ca^{2+} transients (left) and isometric twitches (right) before (broken line) and after (solid line) flosequinan, with amplitudes electronically adjusted so that time courses can be directly compared. Stimulus artifact is lowermost tracing in each set.

and atropine 2×10^{-6} M did not significantly influence the responses to milrinone, hydralazine or flosequinan under our experimental conditions (data not shown). Note that the positive inotropic effect of each drug was associated with an increase in the amplitude of the intracellular Ca^{2+} transient recorded with aequorin, demonstrating the Ca^{2+} dependency of this response. These figures also demonstrate that milrinone, and hydralazine (in higher doses), but not flosequinan, abbreviated the time course of the Ca^{2+} transient and tension response, indicating a differential effect of these agents on the relaxation properties of ferret papillary muscles.

Fig. 4 shows the effects of the Ca^{2+} channel antagonist, verapamil, 5×10^{-8} M on the inotropic responses to milrinone, hydralazine and flosequinan. This drug produced a $33 \pm 4\%$ decrease in isometric force development (40.39 ± 10.95 g/cm^2 to 27.06 ± 12.57 g/cm^2; $n = 17$; $P \leq 0.05$), indicating that the L-type Ca^{2+} channels are partially inhibited. Verapamil did not differentially affect the response to any of the three agents studied. These results indicate that none of these agents' inotropic response was due to an increase of intracellular Ca^{2+} by a presumed activation of the L-type Ca^{2+} channels.

Fig. 5 shows the effects of three different inhibitors of Na^+/H^+ and Na^+/Ca^{2+} exchange on the inotropic responses to milrinone, hydralazine and flosequinan. These interventions included amiloride 600 μM, cadmium 10 μM and substitution of one-third of the NaCl in the bathing medium with choline chloride. This dose of amiloride slightly increased peak tension $12 \pm 8\%$ (64.16 ± 13.28 g/cm^2 to 71.86 ± 14.44 g/cm^2; $n = 17$; $P < 0.05$); cadmium decreased peak tension by $35 \pm 5\%$ (53.76 ± 17.42 g/cm to 34.94 ± 16.59 g/cm; $n = 24$; $P = \leq 0.05$); and choline substitution did not significantly decrease peak tension by $2.7 \pm 9.8\%$ (54.49 ± 13.36 g/cm^2 to 53.02 ± 18.70 g/cm^2; $n = 18$; $P =$ ns). Note that none of these interventions affected the inotropic actions of milrinone or hydralazine compared to controls. In contrast, all three inhibitors markedly depressed the inotropic response to flosequinan. This clear difference indicates that flosequinan, but not hydralazine or milrinone, depends upon the integrity of the Na^+/H^+ and/or Na^+/Ca^{2+} exchange mechanisms in order to produce a positive inotropic effect.

Fig. 6 shows the effects of the Na^+ channel antagonists, tetrodotoxin 1 μM, procaine 0.6 mM and the Na^+/K^+-ATPase inhibitor, ouabain 3×10^{-7} M, on the inotropic responses to milrinone, hydralazine and flosequinan. This dose of tetrodotoxin decreased peak force by $16 \pm 2\%$ (60.84 ± 14.54 g/cm^2 to 52.45 ± 14.83 g/cm^2; $n = 22$; $P < 0.05$); procaine by $12 \pm 4\%$ (65.69 ± 17.76 g/cm^2 to 58.65 ± 15.41 g/cm^2; $n = 18$; $P < 0.05$); and ouabain increased peak force by $8 \pm 2\%$ (78.08 ± 7.11 g/cm^2 to 83.96 ± 9.46 g/cm^2; $n = 23$; $P < 0.05$). Neither tetrodotoxin nor procaine significantly altered the responses to milrinone, hydralazine or flosequinan compared to controls. However, note that ouabain markedly potentiated the inotropic response of flosequinan, which reached statistical significance at the 10 and 100 μM concentrations of the drug.

4. Discussion

4.1. Differential mechanisms of cardiac effects

The most important finding of this study is that the positive inotropic action of flosequinan appears to be related to an effect on Na^+/H^+ and/or Na^+/Ca^{2+} exchange. Fig. 5 shows that three different inhibitors of the Na^+/H^+ and Na^+/Ca^{2+} exchange mechanisms differentially affected the responses to hydralazine, flosequinan and milrinone (Kaczorowski et al., 1989; Philipson, 1985; Siegl et al., 1984). As expected, the inhibition of these mechanisms did not significantly affect the response to milrinone, a drug known to act via phosphodiesterase inhibition and elevation of intracellular levels of cAMP (Earl et al., 1986). Similarly, the response to hydralazine was not affected by these interventions, which seems reasonable when interpreted in light of reports that hydralazine, in the doses used clinically, predominantly affects cyclic nucleotide (i.e., cAMP and cGMP) generation (Jacobs, 1984). These results (shown in Fig. 5) must be interpreted in relation to the effects of the Na^+ channel antagonists (Fig. 6) and Ca^{2+} channel blockers (Fig. 4), which do not demonstrate a selective effect on the response to any of these three agonists. None of the interventions we employed (i.e., cadmium, amiloride, choline substitution for 1/3 Na^+ in the perfusate) are absolutely selective inhibitors of these exchange sites; all of the currently available agents and interventions that have been reported to affect the Na^+/Ca^{2+} or Na^+/H^+ exchangers have also been shown to have actions on other sites in the sarcolemma. These include the L-type Ca^{2+} channels and the voltage-dependent Na^+ channels (Sheu and Blaustein, 1991). However, if the mechanism of antagonism of flose-

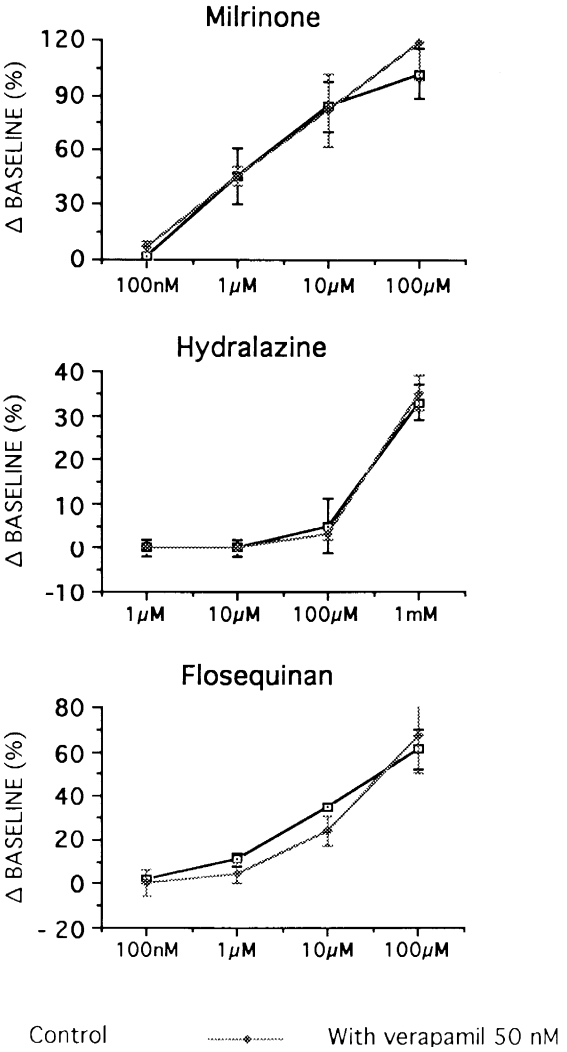

Fig. 4. Effects of the Ca^{2+} channel antagonist, verapamil, on the inotropic response to milrinone, hydralazine and flosequinan. Values equal mean ± S.E.

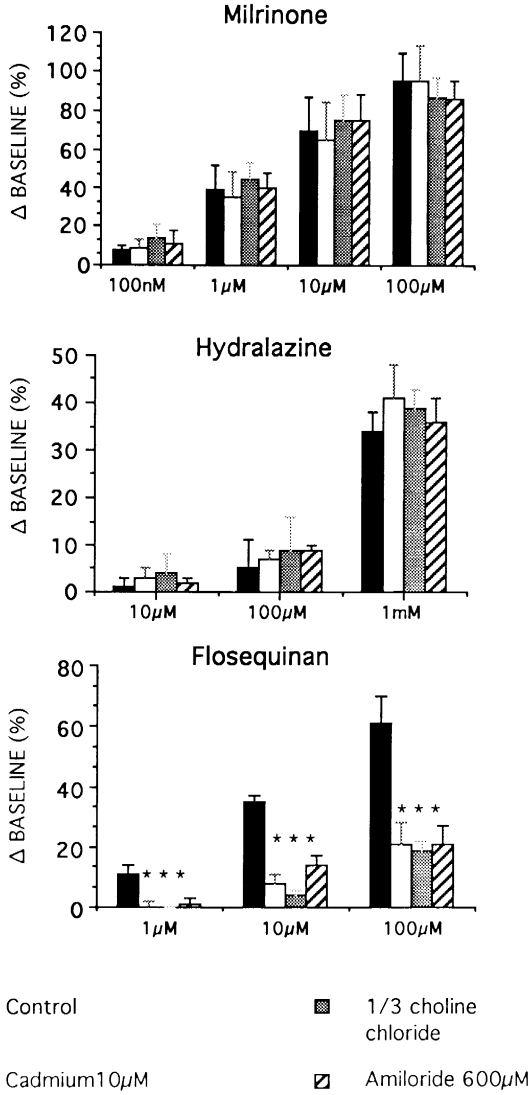

Fig. 5. Effects of three inhibitors of Na^+/H^+ and Na^+/Ca^{2+} exchange on the inotropic response to milrinone, hydralazine, or flosequinan. Values equal mean ± S.E.

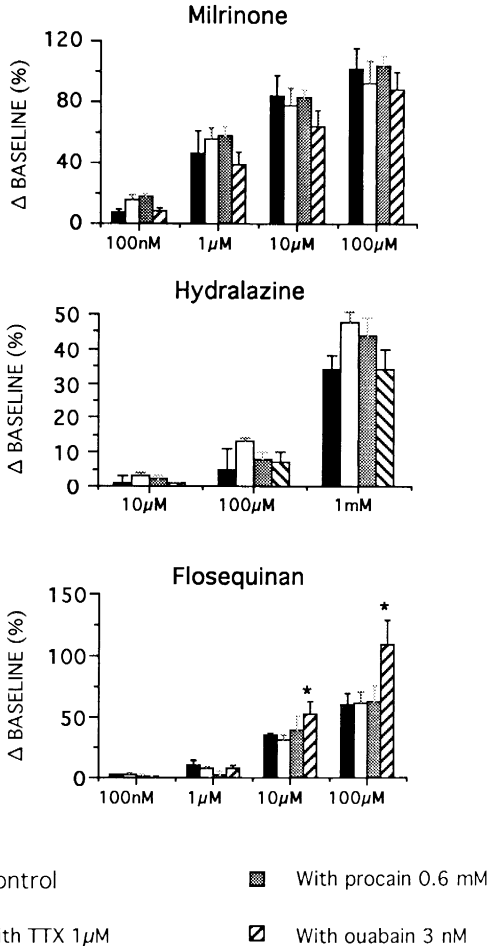

Fig. 6. Effects of Na^+ channel blockade with procaine and tetrodotoxin and Na^+/K^+-ATPase inhibition with ouabain on the inotropic response to milrinone, hydralazine and flosequinan. Values equal mean ± S.E.

quinan involved a nonspecific effect at either of these two sites, we would expect an equivalent depression of equi-inotropic concentrations of milrinone and hydralazine, as well. This was not observed, and our data allow us to conclude that a more specific agonist effect mediated by Na^+/Ca^{2+} and/or Na^+/H^+ exchange was occurring in the presence of flosequinan (Miao et al., 1992). Unfortunately, sufficiently selective agents are not currently available to allow us to distinguish which of these two sites is predominantly affected.

It is known that the Na^+/H^+ exchanger is a relatively low-capacity system, which nonetheless can produce a marked effect on contractility of the heart through modulation of intracellular pH and shifts in the force/Ca^{2+} relationship (Sheu and Blaustein, 1991). In most cases, such as with endothelin (Eid et al., 1989), α-adrenoceptor agonists (Gambassi et al., 1992) and κ-opioid agonists (Ventura et al., 1991), the exchanger has been implicated in mediating a positive inotropic response. However, it is possible that intracellular acidosis and negative inotropism could result from the inhibition of this exchange mechanism and an ensuing decrease in Ca^{2+} responsiveness from intracellular acidosis. Our current measurements, however, allow us to exclude such an effect with regard to flosequinan. Note that the positive inotropic action of this drug is associated with a dose-related increase in intracellular Ca^{2+}, as is also true for milrinone and hydralazine. In previous studies, we have not found convincing evidence for myofilament sensitization in response to flosequinan (Perreault et al., 1992). These results, coupled with evidence that the Na^+/Ca^{2+} exchanger is a high-capacity system that can transport large amounts of Ca^{2+} (Sheu and Blaustein, 1991), support the conclusion that the Na^+/Ca^{2+} exchanger is the more important site of action for flosequinan. However, the results are of an indirect nature and definitive support for this hypothesis must await the availability of selective antagonists of Na^+/Ca^{2+} versus Na^+/H^+ exchange.

Figs. 1–3 show the positive inotropic effects of hydralazine, milrinone and flosequinan. All three agents produced positive inotropic effects that were concentration dependent and associated with parallel increases in intracellular Ca^{2+} levels. In ferret papillary muscle, the relative potencies of these agents are milrinone > flosequinan > hydralazine. In contrast to hydralazine and milrinone, flosequinan did not abbreviate the time course of the Ca^{2+} transient or twitch, i.e., did not produce a positive lusitropic or relaxant effect. Abbreviation of the Ca^{2+} transient and twitch is characteristic of agents that act by increasing intracellular concentrations of cAMP (Morgan et al., 1986). This in turn results in increased phosphorylation of phospholamban and troponin I, which accelerates the decline in the Ca^{2+} transient and enhances diastolic relaxation (Katz, 1983; Morgan, 1991). In contrast, agents such as digoxin, which do not affect the cyclic nucleotide system, appear to produce little change in the time course of the Ca^{2+} transient or twitch (Morgan et al., 1986; Morgan, 1991). Since flosequinan appears to act by cAMP-independent mechanisms, the lack of enhancement of relaxation supports the hypothesis that flosequinan exerts its effects by cAMP-independent mechanisms.

4.2. Potential for interaction with other drugs and disease states

If our hypothesis concerning the effects of flosequinan on Na^+/Ca^{2+} exchange is correct, then it is reasonable to predict that the actions of these drugs would be potentiated by other agents that elevate intracellular Na^+, such as digitalis, which inhibits Na^+/K^+-ATPase. Fig. 6 shows that this appears to be the case. The minimally effective concentration of ouabain markedly potentiated the inotropic response to flosequinan. This is not only important with regard to the mechanism of action of flosequinan and supports our basic hypothesis, but also raises an interesting clinical question. The clinical studies that have been performed with flosequinan have indicated that a positive

inotropic effect cannot be demonstrated at concentrations within the usual therapeutic range (Yates, 1991). However, our results with digoxin suggest that the co-administration of this agent may produce a different effect than would be expected in the presence of flosequinan alone. The importance of this finding must await additional clinical evaluation.

It has also been established that disease states can affect the inotropic responses to flosequinan and milrinone. Both drugs lose their inotropic effectiveness in myocardium isolated from patients with heart failure (Perreault et al., 1992; Feldman et al., 1987; Bohm et al., 1988). Moreover, the degree to which this effect is lost appears to correlate with the severity of failure that is present; the inotropic response is essentially absent in myocardium from patients with the most severe degrees of heart failure (Morgan, 1991). It is also reasonable to speculate, in view of the above discussion regarding digoxin, that other cardiac disease states, such as hypoxia, which are associated with a rise in intracellular Na^+ levels (Neubauer et al., 1992), might be affected by the presence of flosequinan in the drug biophase. For example, elevated Na^+ might help to preserve the inotropic state of the heart during ischemia; whether or not this would become manifest as a beneficial or detrimental clinical effect also requires additional evaluation.

In conclusion, the mechanism of inotropic action of flosequinan appears to be different from that of hydralazine or milrinone. The precise mechanism by which flosequinan affects Na^+/Ca^{2+} and/or Na^+/H^+ exchange in the heart remains speculative. Ideally, any hypothesis explaining this effect must also account for the actions of this drug on vascular smooth muscle and why the cardiac inotropic effect is lost with the development of heart failure. Significantly, the mechanism appears to be independent of cAMP, which raises the important possibility that its side effects may be different from those of agents acting via this cyclic nucleotide. Moreover, flosequinan's potential for interaction with other commonly co-administered drugs, most especially the cardiotonic steroids, requires further testing.

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