Role of intracellular sodium in the regulation of intracellular calcium and contractility. Effects of DPI 201-106 on excitation-contraction coupling in human ventricular myocardium.

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Research Article

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Role of Intracellular Sodium in the Regulation of Intracellular Calcium and Contractility

Effects of DPI 201-106 on Excitation-Contraction Coupling in Human Ventricular Myocardium

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Abstract

Experiments were performed to investigate the mechanism of action of DPI 201-106 on human heart muscle. In both control and myopathic muscles, DPI produced concentration-dependent increases in action potential duration, resting muscle tension, peak isometric tension, and duration of isometric tension. These changes were associated with increases in resting intracellular calcium and peak calcium transients as measured by aequorin. At higher concentrations of DPI, a second delayed Ca^{2+} transient (L') appeared. L' was inhibited by tetrodotoxin and ryanodine, suggesting that DPI acts at both the sarcolemma and the sarcoplasmic reticulum. DPI toxicity was manifested by after-glimmers and after-contractions reflecting a Ca^{2+}-overload state: DPI effects were mimicked by veratridine, a Na^+ channel agonist, and reversed by tetrodotoxin, yohimbine, and cadmium, Na^+ channel antagonists. These results suggest that DPI acts primarily as a Na^+ channel agonist. DPI may produce an increase in intracellular Ca^{2+} by increasing intracellular Na^+ and altering Na^+-Ca^{2+} exchange across the sarcolemma. DPI may also increase intracellular Ca^{2+} by directly altering sarcoplasmic reticulum Ca^{2+} handling.

Introduction

DPI 201-106 (4-[3-(4-diphenyl-methyl-1-piperazinyl)-2-hydroxypropoxy]-1H-indole-2-carbonitrile),[1] a piperazyinyl-indole, has been reported to act as a cardioactive agent with a novel mechanism of positive inotropic action. This agent does not stimulate histamine receptors, α-adrenoceptors, or β-adrenoceptors and does not produce liberation of catecholamines (1, 2). DPI does not increase cAMP levels nor does it interfere with sodium-potassium ATPase activity (1). However, DPI 201-106 has been demonstrated to be a sodium channel agonist (2–4). It has been suggested that the positive inotropic effect of this drug results from its sodium channel agonistic properties (2, 5, 6). DPI also has been reported to exert a positive inotropic effect by increasing cardiac myofilament sensitivity to calcium (2, 6, 7). The effects of DPI on excitation-contraction coupling have been studied in animal models but have not previously been reported in human ventricular myocardium.

Positive inotropic agents are thought to exert their cardiotonic effects by (*a*) increasing intracellular calcium concentration, (*b*) increasing Ca^{2+} sensitivity of the myofilaments, or (*c*) altering the response of myofilaments to calcium activation of troponin C (8). Intracellular calcium concentration can in turn be affected by intracellular sodium concentration. Inhibition of the Na/K ATPase activity with cardiac glycosides results in an increase in $[Na^+]_i$ and an increase in $[Ca^{2+}]_i$ via the sodium-calcium exchange mechanism. Sodium channel agonists also increase $[Na^+]_i$ and consequently increase the $[Ca^{2+}]_i$. Therefore, changes in the sodium and calcium electrochemical gradients can alter intracellular calcium concentration.

The purpose of this study was to investigate the mechanism of action of a novel inotropic agent, DPI 201-106, in human cardiac muscle. The effects of this agent on cardiac contractility, intracellular Ca^{2+} handling, and electrophysiological properties in both normal and myopathic myocardium are described. Previous studies have shown that changes in the amplitude and time course of the intracellular calcium transients in cardiac muscle correspond closely to known drug actions at the subcellular level (9, 10). Moreover, such studies have been very useful in delineating the mechanisms of actions of new inotropic agents (8, 11). Our results indicate that the positive inotropic effect of DPI 201-106 is related to an increase in intracellular calcium ($[Ca^{2+}]_i$) in both control and myopathic human myocardium. DPI effects on the amplitude and time course of the Ca^{2+} transient, resting levels of calcium, and action potential parameters are similar to those produced by agents that are known to increase sodium conductance such as veratridine, but different from inotropic agents that act by other mechanisms, such as BAY K 8644, a calcium channel agonist, and increased extracellular calcium ($[Ca^{2+}]_o$). Additional toxic effects that occur with high concentrations of DPI can be explained on the basis of the development of a calcium-overload state.

Methods

Tissue preparation. Informed consent was obtained from all heart transplant recipients and from the families of all prospective heart donors. Hearts that were not usable for transplantation were obtained from organ donor patients and used as controls (n = 14). Muscles were obtained from patients undergoing cardiac transplantation due to end-stage heart failure (n = 13). 9 of 13 muscles were obtained from patients with idiopathic cardiomyopathy and 4 were from patients

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1. *Abbreviations used in this paper:* DPI 201-106, 4-[3-(4-diphenyl-methyl-1-piperazinyl)-2-hydroxypropoxy]-1H-indole-2-carbonitrile.

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with ischemic cardiomyopathy. Organ bath studies revealed no significant differences between control and myopathic groups in inotropic effect for drugs whose mechanism was cAMP-independent. The clinical course of all donors was reviewed and the anatomy and histology compared for control versus myopathic groups as previously described (13).

At the time of excision, the hearts were placed in an oxygenated physiologic salt solution at room temperature. The composition of the salt solution was as follows (in millimolars): 120, NaCl; 5.9, KCl; 11.5, glucose; 25, $NaHCO_3$; 1.2, $NaH_2PO_4 \cdot H_2O$; 1.2, $MgCl_2 \cdot 6H_2O$; and 2.5, $CaCl_2$. The solution was equilibrated with a mixture of 95% O_2 and 5% CO_2 to a pH of 7.4. The hearts were rapidly transported to the laboratory, where a suitable muscle was dissected and placed into a light collecting apparatus (14). 26 of 27 muscles were from the right ventricle; one myopathic trabecula was from the left ventricle. For control and myopathic muscles, mean fiber diameters were 1.0±0.1 and 1.2±0.1 mm; mean lengths were 8.1±0.8 and 8.9±0.7 mm, respectively. Experiments were performed at 30°C. Each muscle was attached to a Statham force transducer (Gould, Inc., Cleveland, OH) for recording isometric tension development and stimulated to contract at 0.33 Hz unless otherwise noted. Square wave pulses of 5 ms duration were used to deliver threshold voltage through a punctate platinum electrode located at the base of the muscle. After maximum active tension was obtained by stretching the muscle to L_{max}, the muscle was allowed to equilibrate for 1 h.

Cardiac muscle was chemically loaded with aequorin as described elsewhere (15). The light emitted by aequorin was detected with a photomultiplier tube (9635QA; Thorn EMI, Gencom Inc., Fairfield, NJ). Light and tension responses and the stimulus artifact were recorded simultaneously on both magnetic tape recorder (FM model 3964A; Hewlett-Packard Co., Palo Alto, CA) and chart strip recorder (model 2107-4490; Gould Inc., Recording Systems Div., Cleveland, OH). Light signals are reported in amperes of anodal current. Isometric contractions are reported in grams.

Cumulative concentration-response relationships were determined for each agent. With the exception of BAY K 8644 and DPI, all agents were dissolved in distilled water. BAY K 8644 and DPI were dissolved in DMSO; similar amounts of DMSO alone produced no inotropic effect on the muscle. The maximum dose of DPI studied was limited by the insolubility of the drug at high concentrations. To obtain calcium concentration-response curves, a phosphate-free salt solution was used and calcium added in incremental concentrations up to 16 mM.

Because it is possible for drugs to interact directly with aequorin and thereby alter the luminescent reaction or the sensitivity of aequorin to calcium (14), each of the drugs used in these experiments was tested in vitro as described by Blinks et al. (16). Over the concentration range used in these experiments, none of the drugs affected the intensity or time course of aequorin luminescence.

Peak amplitudes of light and tension were measured. Time course measurements for light and tension responses were made from the onset of the stimulus artifact to peak light and tension. Times to 50 and 80% decline from peak light and peak tension were also measured.

The aequorin light signal was calibrated in terms of absolute $[Ca^{2+}]_i$ by the method of Allen and Blinks (17). In brief, light levels recorded during the experiment are expressed as L/L_{max} (fractional luminescence). Fractional luminescence is then converted to $[Ca^{2+}]_i$ by use of an appropriate in vitro calibration curve (18).

Electrophysiology. Because of the technical difficulty in simultaneously recording light signals and action potentials, action potentials were obtained in muscles from the same hearts and handled in a similar fashion to the aequorin-loaded preparations. Muscles were mounted horizontally in a bath and superfused with physiologic salt solution maintained at 30°C. One end of the muscle was immobilized by a Plexiglas clip. The other end was fastened to a force transducer (Statham) and stretched to L_{max}. The muscle was stimulated to contract at a frequency of 0.33 Hz unless otherwise noted with a punctate electrode that delivered 0.5–1-ms pulses at threshold voltage. Action potentials were recorded using standard glass microelectrodes (resistance 10–40 MΩ) filled with 3 M KCl. Signals were amplified through an A-M Systems high impedance electrometer (model 1600; A-M Systems, Everett, WA) and displayed on an oscilloscope (model 03830; Hewlett-Packard Co.) and chart strip recorder (model 2107-2202; Gould, Inc.) with simultaneous display of muscle tension. The maximal upstroke velocity of the action potential ($\dot{V}_{max}$) was determined with an analogue differentiator (DDT; World Precision Instruments, Inc., New Haven, CT). The resting membrane potential and the amplitude of the action potential were determined. The duration of the action potential was measured at 50 and 80% repolarization (APD_{50} and APD_{80}).

Chemicals. The following drugs and chemicals were used: DPI 201-106 (Sandoz Ltd., Basel, Switzerland), veratridine (free base), tetrodotoxin, cadmium chloride, verapamil, yohimbine HCL (Sigma Chemical Co., St. Louis, MO), ryanodine (Merck, Sharpe, and Dohme, West Point, PA), calcium chloride (British Drug Houses Chemicals Ltd., Poole, England), and BAY K 8644 (Pfizer Chemicals Div., Hoffman Estates, IL). The aequorin used in these experiments was purchased from the laboratory of Dr. J. R. Blinks at the Mayo Foundation in Rochester, MN. The concentration of each drug or chemical is expressed as final bath concentration.

Statistics. The *t* test for paired and unpaired data was used for statistical analysis. Two-way analysis of variance using repeated measures and one-way analysis of variance with repeated measures were used when appropriate. Mean and SEM are reported. A P value ≤ 0.05 was considered significant.

Results

Effects of drugs on the aequorin light signal and tension response. The effects of extracellular calcium, BAY K 8644, DPI, and veratridine on aequorin light signals and tension responses of trabeculae carneae from control hearts are illustrated in Fig. 1; and the effects of DPI and veratridine in myopathic trabeculae are illustrated in Fig. 2. In both control and myopathic muscles, the positive inotropic effect of each of these drugs was associated with an increase in peak $[Ca^{2+}]_i$ as reflected in the increased amplitude of the aequorin light signal (i.e., intracellular Ca^{2+} transient).

The addition of BAY K 8644 or increased $[Ca^{2+}]_0$ did not produce an increase in resting calcium or resting tension ($n = 2$ and 14, respectively). However, in myopathic tissue increased $[Ca^{2+}]_0$ did produce an increase in resting calcium and an increase in resting tension (8 of 10 muscles).

In both control and myopathic muscles, higher concentrations of DPI produced an increase in resting calcium and the appearance of a second component in the calcium transient (L'). DPI also produced a prolongation of isometric contraction. Veratridine had effects similar to DPI on the calcium transient, resting calcium level, resting tension, and isometric contraction duration (control, $n = 4$; myopathic, $n = 2$). These effects are in direct contrast to the effects of increased $[Ca^{2+}]_0$ and BAY K 8644.

Muscle exposure to increased extracellular calcium in the presence of DPI: effect on aequorin light signal and tension response. To determine the dependence of DPI action on the level of intracellular calcium, we studied the effects of increased extracellular calcium. Fig. 3 illustrates the result of increasing extracellular calcium in control and myopathic muscles exposed to a maximally effective concentration of DPI. In marked contrast to the effects of increasing $[Ca^{2+}]_0$ in the absence of DPI, increasing concentrations of extracellular calcium in the presence of DPI prolonged the time course of the calcium transient and the isometric contraction. Increasing

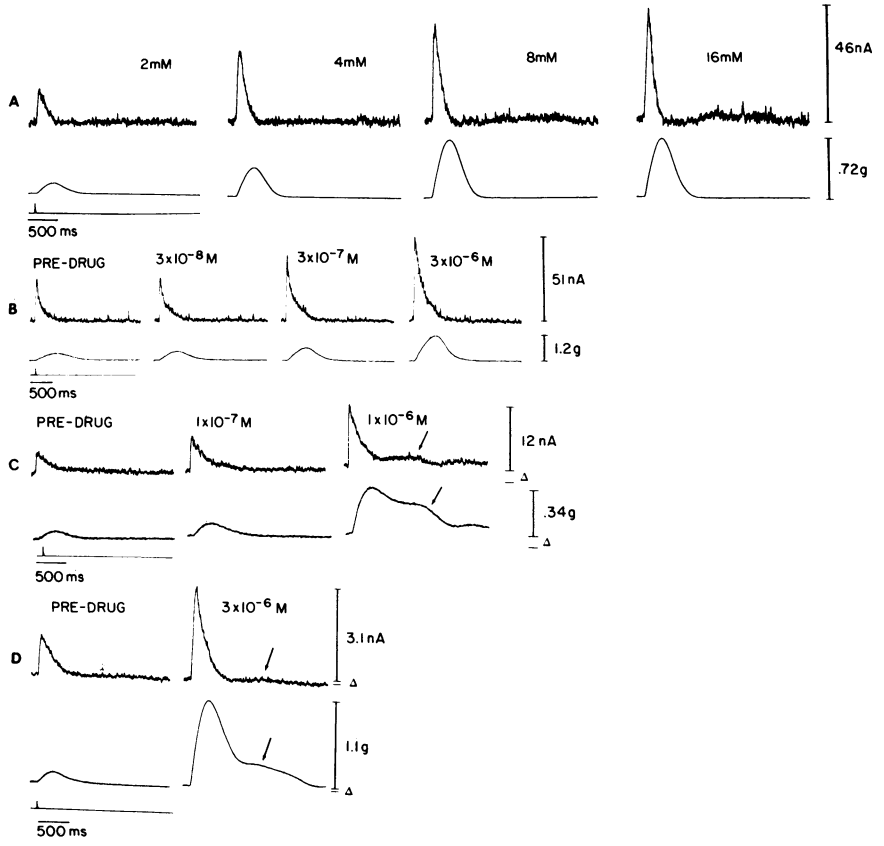

Figure 1. Effects of (*A*) increased $[Ca^{2+}]_0$, (*B*) BAY K 8644, (*C*) DPI, and (*D*) veratridine on the aequorin light signal (upper noisy trace) and tension response (middle smooth trace). Stimulus frequency was 0.33 Hz; temperature was 30°C. For this and subsequent figures, the bottom trace in *A–D* is the stimulus artifact. The aequorin signal amplitude (i.e., light) is expressed as nA of anode current recorded from a photomultiplier tube; tension is expressed in grams. Δ indicates change in resting tension and calcium from predrug control. *A–D* represent the average of 20–100 responses obtained at steady state. Arrow denotes L′ in the light trace and second component of relaxation in the tension trace.

$[Ca^{2+}]_0$ also produced an increase in resting $[Ca^{2+}]_i$ and resting tension. In addition, a delayed after-glimmer and associated after-contraction appeared. These toxic effects, which were observed in 14 experiments, reflect a calcium-overload state.

Effect of DPI on sodium-calcium exchange. The appearance of a unique component in the calcium transient, L′, in the presence of DPI (Figs. 1–3) prompted investigation of the possibility that L′ might arise from alterations in sodium-calcium exchange. To facilitate this investigation, we inhibited the contribution of calcium handling by the sarcoplasmic reticulum. As illustrated in Fig. 4, ryanodine in the presence of increasing extracellular calcium resulted in an increase in resting calcium, resting tension, increased amplitude of the calcium transient, and prolongation of the isometric contraction. The calcium transient had a short duration and the peak preceded the associated peak isometric contraction. Addition of

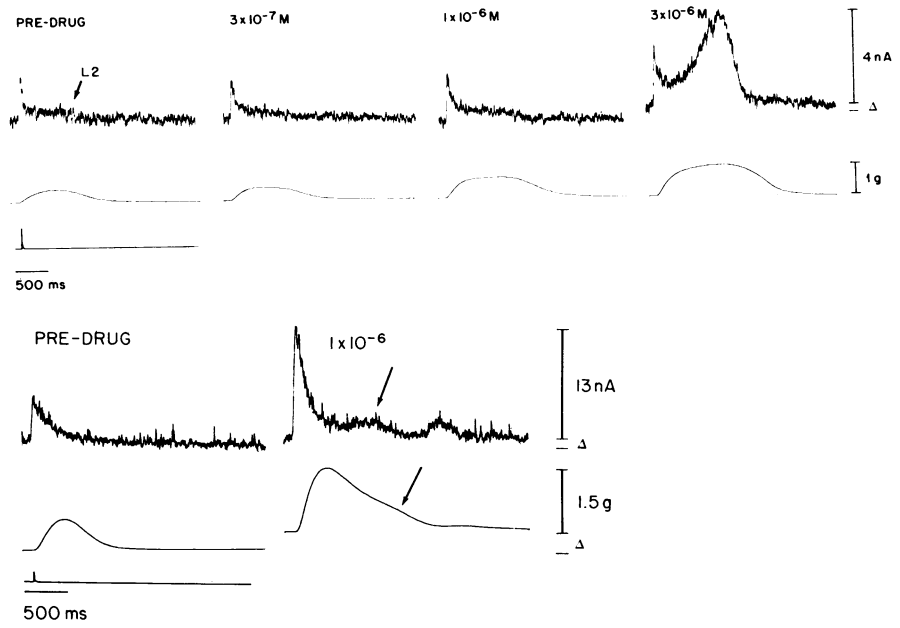

Figure 2. Effects of DPI (*top*) and veratridine (*bottom*) on a myopathic muscle. Δ indicates change in resting calcium and tension. The arrow indicates L′ in the light trace and second component of relaxation in the tension trace.

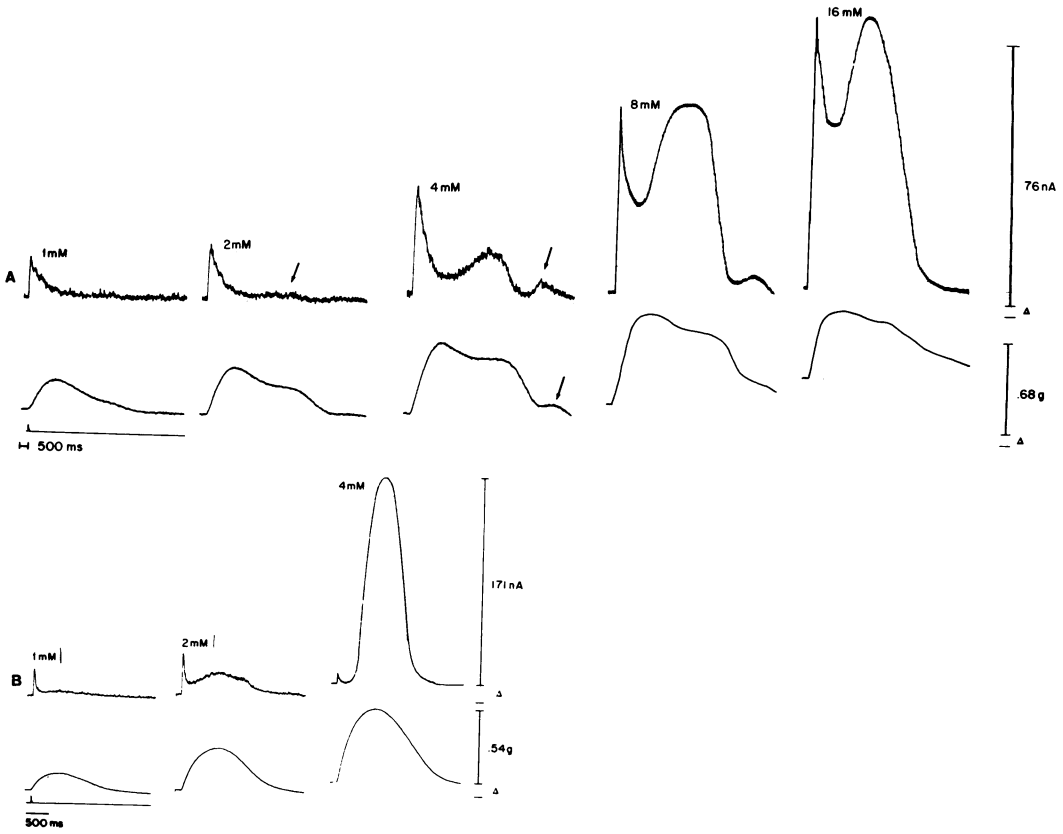

Figure 3. Effect of increased extracellular calcium on the light and tension responses in the presence of 1×10^{-6} M DPI. (*Top*) Control muscle; (*bottom*) myopathic muscle. Δ denotes change in resting tension and calcium. Calcium concentration is in millimolars. Arrows indicate delayed after-glimmer and associated after-contraction.

verapamil has been shown to abolish the calcium transient that remains after the addition of ryanodine (12). As illustrated in Fig. 5, the second component of muscle tension did not appear when a maximally effective concentration of DPI (3×10^{-6} M) was added to a maximally effective concentration of ryanodine (3×10^{-6} M). The positive inotropic effect of DPI, however, persisted and was associated with the appearance of L'. L' demonstrated a prolonged time course that was coincident with the corresponding isometric contraction. Addition of increasing concentrations of $[Ca^{2+}]_o$ resulted in increases in the amplitude of L', resting calcium, and tension. Subsequent addition of tetrodotoxin eliminated L'. The addition of verapamil did not affect the time course of L'. Similar results were obtained in myopathic and control muscles. Using the method of fractional luminescence, calculated values for the peak amplitude of the calcium transient and resting calcium in the presence of DPI, ryanodine, and 16 mM calcium were 630 and 2,000 nM, respectively.

Action potential durations were increased in the presence of ryanodine and further increased in the presence of DPI (see Fig. 12). The addition of 8 mM Ca^{2+} resulted in still further prolongation of action potential duration.

Effects of DPI on action potential parameters. DPI has been reported to prolong the action potential in animal species. This

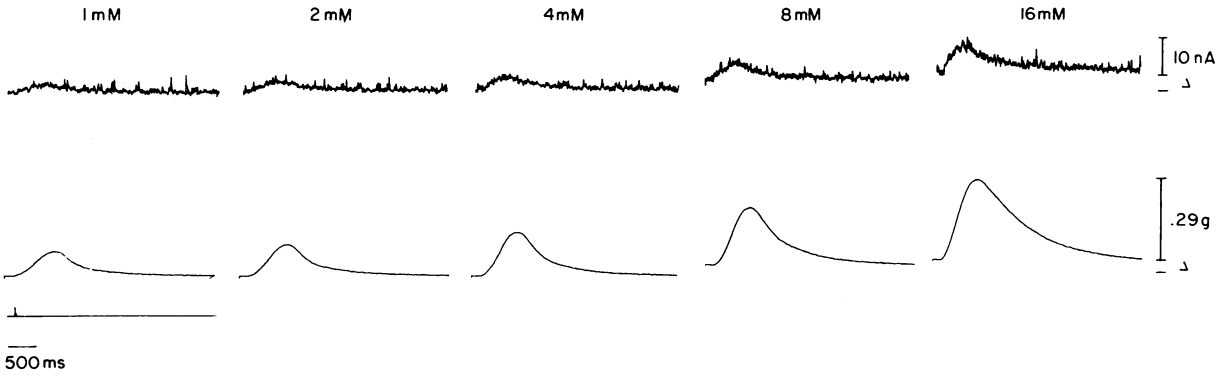

Figure 4. The effect of increasing calcium concentration in the presence of 3×10^{-6} M ryanodine. Calcium concentration expressed in millimolars. In the presence of ryanodine, a small component of the calcium transient remains in myopathic tissue (2). Δ denotes change in resting tension and calcium.

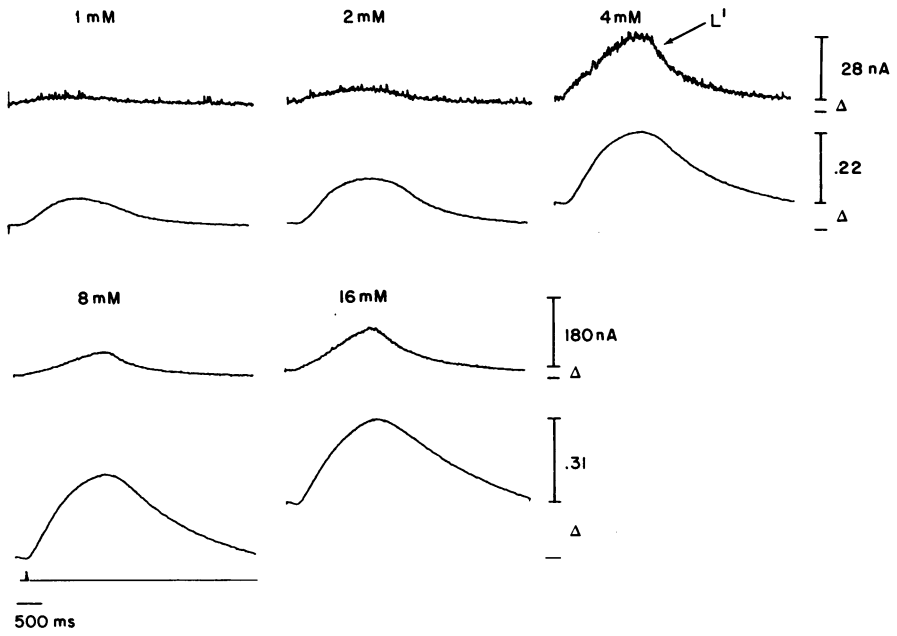

Figure 5. The effect of increasing calcium concentration (in millimolars) in the presence of 3×10^{-6} M ryanodine plus 3×10^{-6} M DPI. Δ denotes change in resting tension and calcium. The calcium transient is labeled L' because only the late component is present. The rapid initial component is suppressed by ryanodine. Note that time course of calcium transient and tension closely parallel one another.

effect has been attributed to its action as a sodium channel agonist. Table I demonstrates the effects of DPI on action potential parameters. DPI did not have statistically significant effects on resting membrane potential, action potential amplitude, or $\dot{V}_{max}$. DPI did, however, prolong action potential duration in both control and cardiomyopathic tissue in a manner similar to veratridine (Fig. 6). The effects of DPI and veratridine on action potential duration and muscle tension were reversed by the addition of tetrodotoxin or yohimbine (19).

The effects of DPI were concentration dependent in both control and myopathic tissues. Prolongation of action potential duration and muscle tension occurred at lower concentrations of DPI than did an increase in peak muscle tension.

Frequency dependency of DPI. The actions of many agents on sarcolemmal ion channels are frequency dependent. We therefore investigated the effect of varying stimulation frequency on the action of DPI. Fig. 7 illustrates the effect on the calcium transient, amplitude of L', and resting tension as the frequency of stimulation was varied. At a stimulation frequency of 1 Hz, the calcium transient and tension responses were abbreviated (Fig. 7, *inset*). The calcium-tension relationship with DPI closely approximated the calcium-tension relationship at slower frequencies, but diverged from this relationship at 1 Hz frequency. As the stimulation frequency was decreased, amplitude of the peak calcium transient and L' decreased along with resting calcium and peak tension response.

Effect of drugs on time course of the aequorin light signal and tension response. Time course changes in the aequorin light signal and associated tension response may reflect alterations in intracellular calcium mobilization and utilization. Increasing $[Ca^{2+}]_o$ and addition of BAY K 8644 did not change the time course of the aequorin light signal or corresponding tension response. DPI either prolonged the time course of the calcium transient (i.e., caused appearance of L'), or had no effect on the transient time course. However, DPI always prolonged the corresponding isometric twitch. Veratridine had similar effects to DPI. Fig. 8 illustrates that exposure to DPI could produce an increase in resting calcium and a markedly prolonged isometric twitch without affecting the time course of the calcium transient. This finding suggests that DPI-induced increases in resting calcium levels are alone sufficient to

Table I. Action Potential and Isometric Tension Parameters

Conc $n(\dot{V}_{max}$/other)	APA	APD_{50}	APD_{80}	RMP	RT_{50}	RT_{80}	$\dot{V}_{max}$
	mV	*ms*		*mV*	*ms*		*V/s*
Predrug (19/38)	94±2	360±8	450±8	−74±1	450±6	570±18	112±4
3×10^{-8} M (4/6)	98±3	410±8	500±8	−78±1	440±8	580±8	97±10
1×10^{-7} M (6/16)	92±2	430±15*	530±15*	−74±2	490±15*	650±15	119±3
3×10^{-7} M (17/30)	93±2	630±19*	710±19*	−74±1	530±22*	730±31*	108±4
1×10^{-6} M (5/7)	104±2	740±27*	810±27*	−74±1	600±40*	860±54*	117±5

APA = action potential amplitude; APD_{50} and APD_{80} = action potential duration at 50 and 80% repolarization, respectively; RMP = resting membrane potential; RT_{50} and RT_{80} = time to 50 and 80% relaxation; $\dot{V}_{max}$ = maximal upstroke velocity. All measurements are listed as mean±SE. * Significant difference at $P < 0.05$ compared with predrug value.

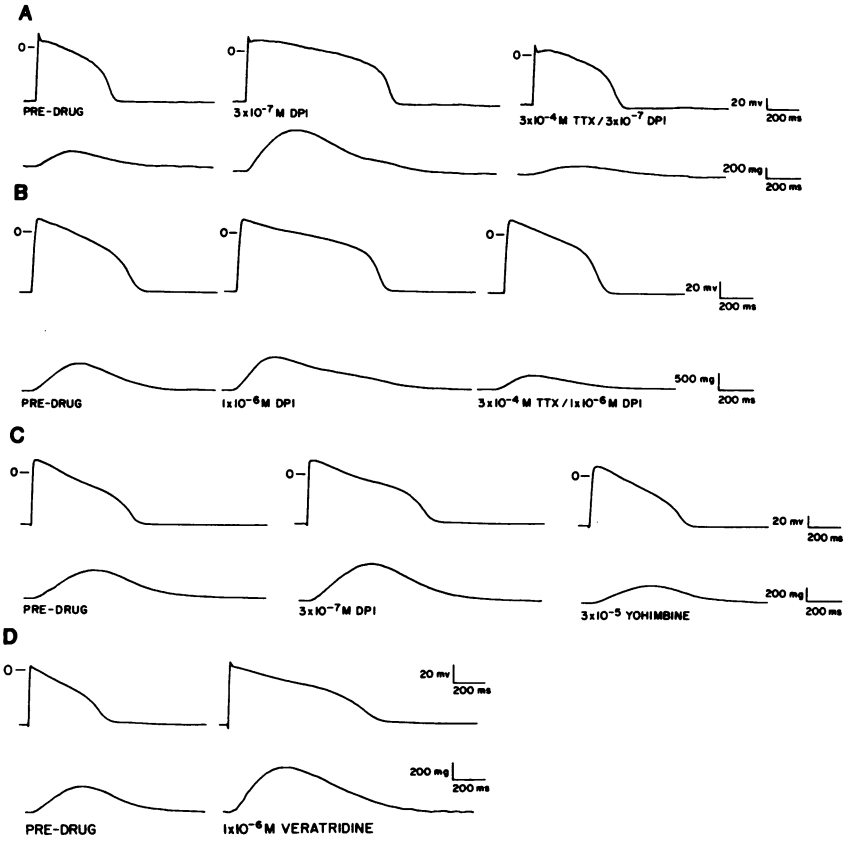

Figure 6. Effect of tetrodotoxin on the action potential in the presence of DPI in control (*A*) and myopathic (*B*) tissue. In both preparations, tetrodotoxin (TTX) decreased action potential duration to approximately control values. There was a corresponding decrease in peak tension and duration of tension in the presence of TTX. Addition of TTX to DPI did not significantly change $\dot{V}_{max}$. Yohimbine effects on action potential duration appeared similar to TTX effects (*C*). *D* illustrates similar prolongation of action potential duration in the presence of veratridine. Note that action potential duration in the absence of DPI is greater in cardiomyopathic muscle than in normal muscle as previously reported (12).

produce the increase in resting tension and contraction prolongation observed with DPI. Table II compares the time courses of the calcium transient and muscle tension in response to DPI in control and cardiomyopathic tissues.

Effects of DPI on isometric contractions. Table III demonstrates the effect of DPI, veratridine, tetrodotoxin, yohimbine, and cadmium on the time course of the isometric twitch in four control muscles. In each muscle, DPI and veratridine prolonged the contractile response. Subsequent exposure to the sodium channel antagonists tetrodotoxin and yohimbine reversed this effect. Cadmium, a calcium channel and sodium channel antagonist similarly reversed the time course changes. In all cases, the time course of the isometric twitch shortened to levels that were less than or equal to pre-DPI or preveratridine levels. There was a concomitant diminution of the amplitude of the peak calcium transient and L′ and a decrease in resting intracellular calcium. Experiments with tetrodotoxin and yohimbine alone resulted in only a slight decrease in the peak calcium transient and tension response. Therefore, some of DPI's effects are secondary to its action as a sodium channel agonist.

Fig. 9 illustrates the effect of yohimbine on the intracellular calcium transient, resting calcium, resting tension, and tension prolongation in the presence of veratridine or DPI and increased $[Ca^{2+}]_o$. In the presence of veratridine, yohimbine decreased resting calcium level, resting tension, and amplitude of the calcium transient and peak isometric tension (n = 6). In the presence of high $[Ca^{2+}]_o$ and DPI, yohimbine had a similar effect on resting calcium and resting tension but produced a transient increase in peak tension and the peak calcium transient (Fig. 10). Experiments with tetrodotoxin in the presence of high concentrations of DPI revealed a similar initial increase in peak calcium and tension (n = 10). Together, these experiments illustrate that the sodium channel effects of DPI are reversed by sodium channel antagonists and that an increase in intracellular calcium is necessary for DPI action.

Fig. 11 illustrates the effect of ryanodine on tissue treated with DPI. Ryanodine decreased the peak aequorin signal and the amplitude of DPI-induced L′ and abolished the second component of the isometric contraction. These data indicate that exposure to DPI affects sarcoplasmic reticular calcium handling either through direct action on the sarcoplasmic reticulum or indirectly as a consequence of intracellular calcium overload. Fig. 11 also illustrates the combined effect of tetrodotoxin and ryanodine on a muscle that had been exposed to DPI. The addition of tetrodotoxin abolished the calcium transient remaining after exposure to ryanodine.

Discussion

Interpretation of aequorin light signals. The aequorin signal from control human heart muscle consists of a single component that rises to a peak and declines before the corresponding contractile response. This is consistent with current understanding of excitation-contraction coupling in the heart (20–23). The peak of the aequorin signal can be used to measure the amount of Ca^{2+} released by the sarcoplasmic reticulum (24). Previous experiments have shown that the descending phase of the calcium transient predominantly reflects resequestration of calcium by the sarcoplasmic reticulum (10, 11).

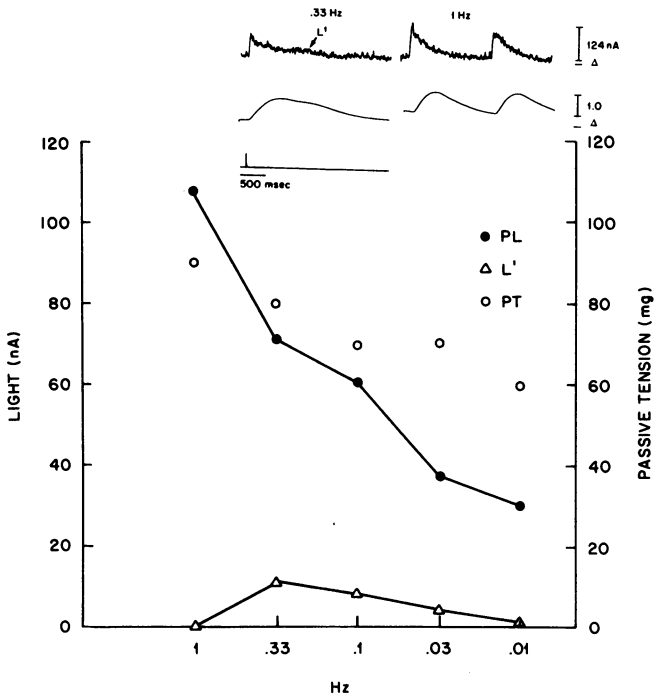

Figure 7. Effect of varying the frequency of stimulation on the peak light (PL) and time course of the calcium transient, amplitude of L' and passive tension. *Inset,* 0.33 and 1 Hz. Note the abbreviation of both the light and tension time course with increasing frequency.

We have previously reported that in contrast to control muscles, Ca^{2+} transients and isometric contractions of muscles from failing human hearts are markedly prolonged (12). The calcium transients also typically exhibit two distinct components (L_1 and L_2). The first component (L_1) appears to arise from sarcoplasmic reticular Ca^{2+} release, whereas L_2 reflects dysfunction of both the sarcoplasmic reticulum and the sarcolemma.

Effects of inotropic agents on the calcium transient and tension response. Increases in extracellular calcium or the addition of BAY K 8644 produced an increase in the peak calcium transient and corresponding tension response. In the presence of increased $[Ca^{2+}]_0$, there is an increased Ca^{2+} gradient across the sarcolemma and subsequent increased Ca^{2+} influx during depolarization. Increased $[Ca^{2+}]_0$ also reduces the efflux of calcium via sodium-calcium exchange across the sarcolemma (25). This results in an increase in intracellular calcium that becomes available for loading of intracellular stores (e.g. the sarcoplasmic reticulum). BAY K 8644 exerts its effect by promoting calcium influx through the slow calcium channels (26, 27) to increase the calcium available for loading of intracellular stores.

Veratridine and DPI also increased the peak calcium transient. Veratridine has been reported to act as a sodium channel agonist (28). DPI has similarly been reported to act as a sodium channel agonist (2–5). The sodium channel agonistic effect results in prolonged sodium conductance. As a consequence, there is intracellular sodium loading that, by affecting sodium-calcium exchange, causes an increase in intracellular calcium. An increase in intracellular calcium can result in enhanced I_{si} (slow inward current) and further calcium loading (29). The increase in intracellular calcium results in an increase in the peak calcium transient.

Effect of inotropic agents on resting intracellular calcium levels and resting tension. Increases in resting intracellular calcium can influence tension in several ways: by altering the magnitude of the slow inward current, by altering Ca^{2+} loading of the sarcoplasmic reticulum, and/or by activating myofilament cross-bridges to produce tonic tension. In control human myocardium exposed to BAY K 8644 and increased extracellular calcium, there was no increase in resting intracellular calcium or tension. However, in myopathic myocardium, there was an increase in resting calcium and tension in the presence of high extracellular calcium. The inability of myopathic tissue to handle increased transsarcolemmal calcium flux has been previously reported (12).

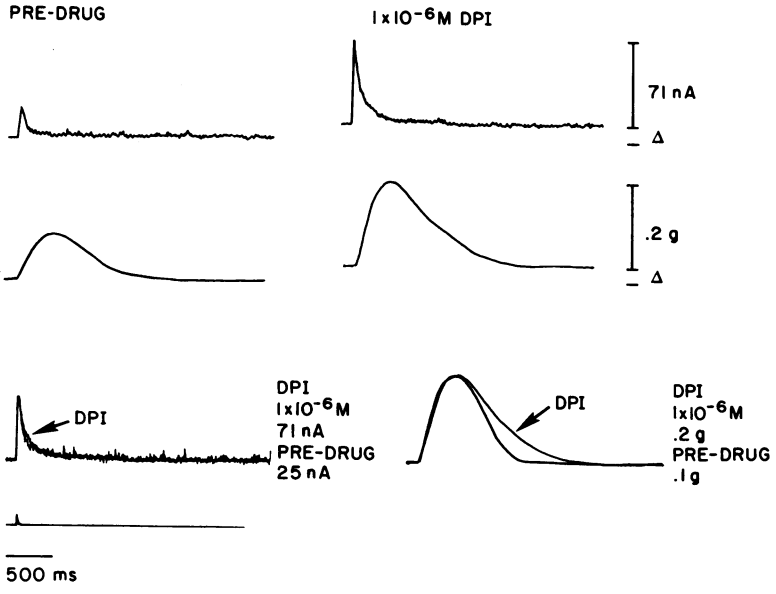

Figure 8. (*Top*) Increase in resting calcium and tension. (*Bottom*) Direct comparison of time courses. Light and tension responses have been amplified equally to allow direct comparison of time courses. Absolute light and tension responses for muscles pre-/postdrug exposure are indicated to the right of each panel. Δ denotes change in resting tension and calcium.

Table II. Effect of DPI 201-106 on the Time Course of Isometric Contraction and Aequorin Light

	Control					
	TPT	RT_{50}	RT_{80}	TPL	RL_{50}	RL_{80}
			ms			
Predrug	384±69	355±43	694±81	61±12	129±31	332±73
1×10^{-7} M	506±156	450±123	816±200	94±22	153±74	471±226
3×10^{-7} M	427±87	404±100	784±171	91±18	125±21	294±38
1×10^{-6} M	461±72	557±115	993±191	80±15	139±32	452±100
3×10^{-6} M	415±59	684±163	1,171±343	70±16	350±198	784±221*
1×10^{-5} M	614±128	962±383	1,962±79	101±26	192±66	444±145
	Myopathic					
	TPT	RT_{50}	RT_{80}	TPL	RL_{50}	RL_{80}
			ms			
Predrug	438±20	354±26	569±39	45±5	209±49	548±67
1×10^{-7} M	365±25	375±17	578±22	48±8	246±66	734±134*
3×10^{-7} M	471±35	443±86	659±101	52±7	214±58	664±126
1×10^{-6} M	555±83	595±76*	908±92*	43±7	155±37	856±168
3×10^{-6} M	579±106	680±79*	1,073±128*	51±6	402±200	1,020±222*

Patterns of changes in the Ca^{2+} transient and isometric contraction in response to DPI in control and myopathic human ventricular muscle. RL_{50}, RL_{80} = time to 50% and 80% decline in the calcium transient, respectively; RT_{50}, RT_{80} = time to 50% and 80% relaxation respectively; TPL = time to peak light; TPT = time to peak tension. All measurements are listed as mean±SE. * Significance at $P < 0.05$ compared with predrug value.

In the presence of veratridine and DPI, there was an increase in resting intracellular calcium and an associated increase in resting tension in control and myopathic human tissue. Furthermore, at higher concentrations of these agents, a second component (L') appeared in the calcium transient recorded from control muscles.

The increase in resting tension in the presence of DPI suggests that the intracellular calcium concentration achieved is above the threshold for activation. Sheu and Fozzard (30), using ion-sensitive electrodes to study ventricular muscle from sheep heart, reported a contraction threshold near 720 nM $[Ca^{2+}]_i$. This value is similar to that reported by Fabiato and Fabiato (31) in skinned cardiac fibers.

Table III. Effect of Sodium Channel Agonists and Antagonists on Isometric Contraction Duration

	TPT	RT_{50}	RT_{80}
		ms	
Predrug	279±26	331±43	677±84
DPI 3×10^{-6} M	355±31	562±113	823±120
VER 3×10^{-5} M	333±5	350±9	813±28
CAD 1×10^{-5} M	350±50	450±150	650±150
YOH 3×10^{-5} M	304±44	287±51	504±66
TTX 3×10^{-5} M	349±25	415±20	793±61
$n = 4$			

The effect of DPI and veratridine (VER) on the time course of the isometric twitch, reversal of the prolongation of isometric contraction produced by both agents is seen with addition of tetrodotoxin (TTX), yohimbine (YOH), and cadmium (CAD). RT_{50} = time to 50% relaxation; RT_{80} = time to 80% relaxation; TPT = time to peak tension. All measurements are listed as mean±SE.

Aequorin signals in these experiments were not translated into exact intracellular Ca^{2+} concentrations for comparison to pre-DPI levels for several reasons. In aequorin studies, the method for quantitation of intracellular calcium is the "fractional luminescence" technique (16). The elevated resting levels of calcium in the presence of DPI could possibly have resulted in aequorin consumption and complicated the quantitation of aequorin signals before and after the addition of DPI. Resting calcium concentration was increased in the presence of DPI and could not be accurately compared with pre-DPI control levels because DPI could not be washed out of muscle preparations. Despite these limitations, it is clear that veratridine and DPI both elevated resting calcium levels as detected by an increase in the resting aequorin signal.

Sodium-calcium exchange. The responses of human cardiac muscle exposed to DPI (3×10^{-6} M) and ryanodine (3×10^{-6} M) were unusual in that the time courses of calcium and tension closely paralleled one another (Fig. 5). Under these conditions the positive inotropic response of DPI remained prominent. Increased extracellular calcium concentrations resulted in an increase in peak calcium and tension and in resting calcium and tension. The time to peak and the overall time course of the calcium response and subsequent contraction were also prolonged. These results are in contrast to those obtained in the presence of ryanodine alone and in the presence of increased extracellular calcium.

The source of the contraction activator calcium therefore becomes an important question. There should be only minimal sarcoplasmic reticulum function in the presence of 3×10^{-6} M ryanodine. Addition of the calcium channel blocker,

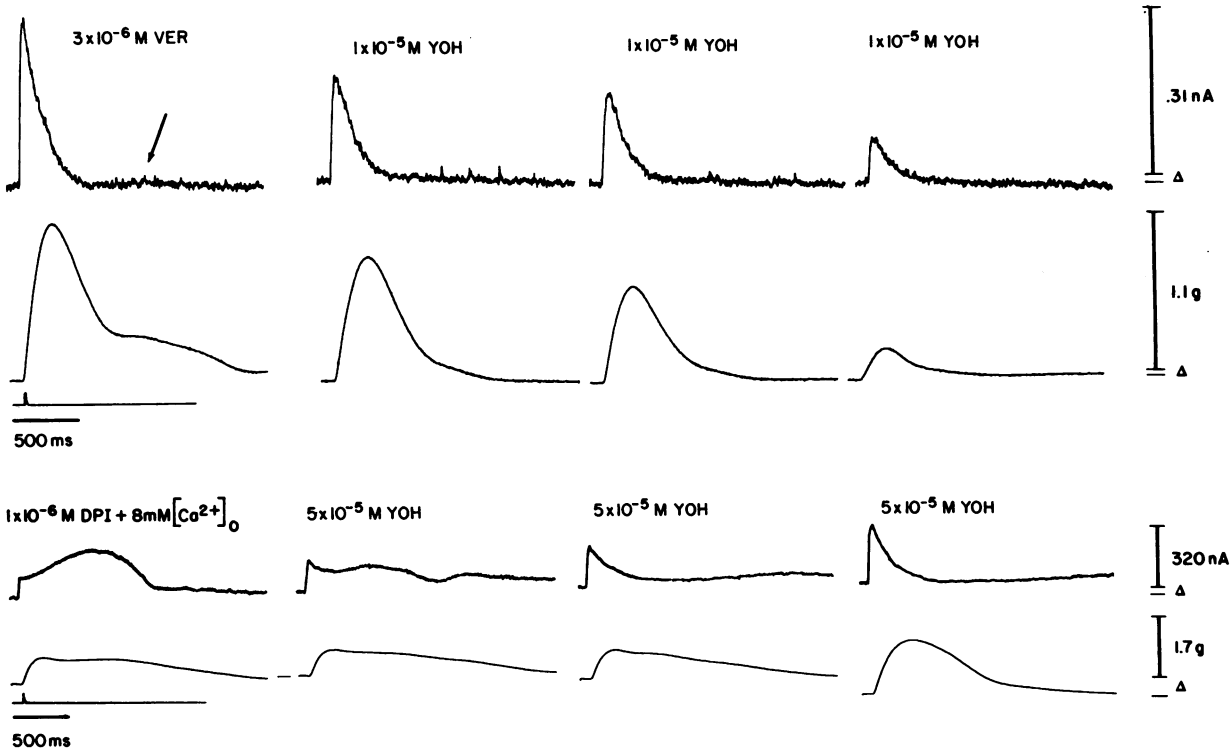

Figure 9. The effect of yohimbine on light and tension responses with time in the presence of veratridine (*top*) and DPI plus high calcium (*bottom*) in two control trabeculae. Δ denotes decrease in resting Ca^{2+} and tension upon addition of yohimbine.

verapamil, did not prevent the positive inotropic response. These results indicate that the activator calcium does not originate from either the sarcoplasmic reticulum or from calcium influx through voltage-dependent calcium channels. Another possible source, the sarcolemmal sodium-calcium exchanger, may well provide the activator calcium ion.

The sodium-calcium exchanger operating under control electrochemical gradients for sodium and calcium primarily removes calcium from the sarcoplasm. However, when there is a reduction in the sodium electrochemical gradient and/or an increase in the calcium electrochemical gradient, it is possible for the exchanger to move calcium into the cell. The ability of the exchanger to work in the reverse direction (i.e., move calcium into the cell and sodium out) has been demonstrated in guinea pig heart (32) and isolated myocytes (33, 34). Supporting evidence comes from experiments on squid axon, in which the calcium flux is altered and even reversed by alterations in the extracellular sodium concentration (35). Barcenas-Ruiz et al. (34) also demonstrated that intracellular calcium concentration is controlled by sodium-calcium exchange. As previously reported for other animal species (2, 5, 6), and as demonstrated here for human myocardium, DPI produces a positive inotropic response by increasing intracellular sodium concentration. This may in turn increase intracellular calcium concentration by reducing calcium efflux via the sodium-calcium exchanger.

To investigate the possibility that sodium-calcium exchange could provide the activator calcium in the presence of

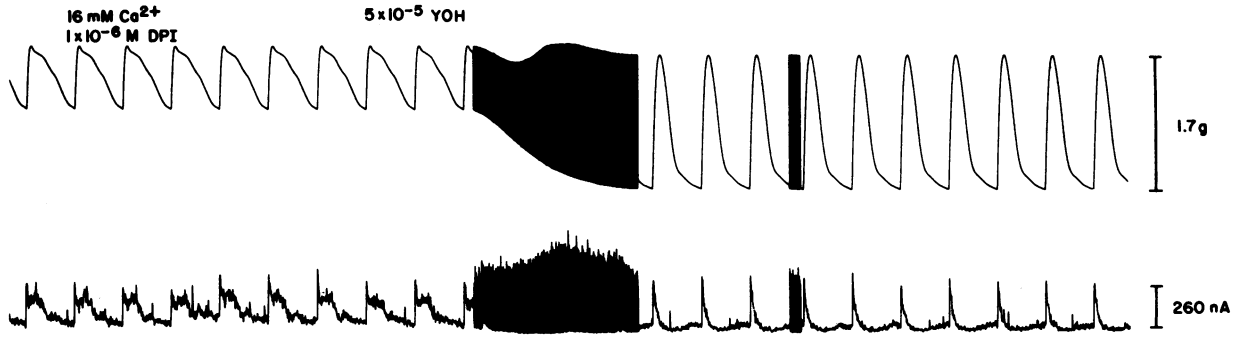

Figure 10. The effect of yohimbine in the presence of high $[Ca^{2+}]_0$ and DPI. The upper trace is tension; the lower trace is light (i.e., $[Ca^{2+}]_i$). Paper speed has been increased to show changes in $[Ca^{2+}]_i$ and tension on a beat to beat basis. Yohimbine abolished L', decreased resting calcium, and resting tension and increased active tension. Yohimbine also produced a transient increase in peak calcium transient.

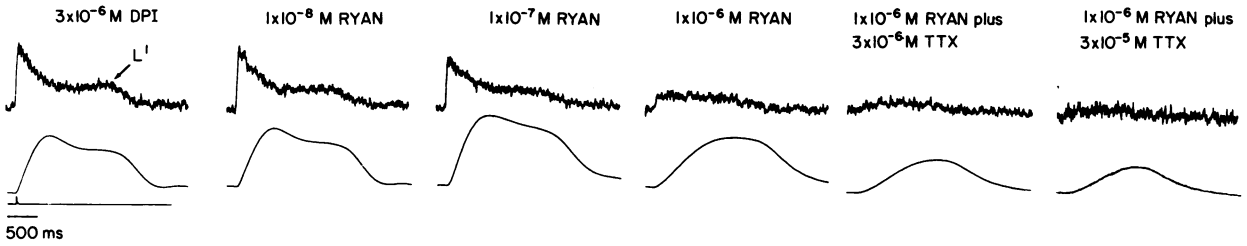

Figure 11. The effect of ryanodine and tetrodotoxin on the aequorin light signals and corresponding tension response in a myopathic trabecula after addition of DPI. The arrow denotes L', which was induced by DPI in this muscle.

DPI and ryanodine, we modeled sodium-calcium exchange. When the intracellular and extracellular calcium concentration and the extracellular sodium concentration are known, the intracellular sodium concentration can be predicted from the equation:

$$a^{i}\mathrm{Ca}(a^{0}\mathrm{Na})^{r} \exp - [(r-2)Vm\mathrm{F}/\mathrm{R}T] = a^{0}\mathrm{Ca}(a^{i}\mathrm{Na})^{r} \quad (1)$$

where r is the coupling factor, Vm is the membrane potential, F is the Faraday constant, R is the gas constant, and T is the temperature. For the physiologic salt solution used in these experiments and the measured intracellular calcium concentration, intracellular sodium concentration is predicted to be 19 mM at resting membrane potential given an r of 3.

Several mathematical models have been proposed to determine the current (I_{ex}) that could be generated by sodium-calcium exchange (36–40). All of these models are based on the assumption that the only energy sources are the sodium and calcium electrochemical gradients. One such equation, proposed by DiFrancesco and Noble, (40) is as follows:

$$I_{ex} = k\{\exp[c(r-2)\mathrm{VF}/2\mathrm{R}T]\}$$
$$\times ([\mathrm{Na}]_i^{r}[\mathrm{Ca}]_0) - \exp[(1-c)(r-2)\mathrm{EF}/2\mathrm{R}T]$$
$$\times ([\mathrm{Na}]_0^{r}[\mathrm{Ca}]_i)/[1 + d([\mathrm{Ca}]_i[\mathrm{Na}]_0^{r} + [\mathrm{Ca}]_0[\mathrm{Na}]_i^{r})] \quad (2)$$

where c is a variable for the position of the energy barrier and d and k are scale factors. This equation has been reported to closely reproduce the delayed second component of the slow inward current and the transient inward current (41, 42). Sodium-calcium exchange has been implicated as the source of both of these currents.

The electrogenic nature of sodium-calcium exchange is of major physiological importance in cardiac function. During the early plateau phase of the action potential, calcium may enter the cell (promoting contraction) via the sodium-calcium exchanger (hyperpolarizing current), whereas calcium efflux (promoting relaxation and a depolarizing current) will predominate during repolarization (34, 36, 43).

We assumed that the energy barrier was midway through the membrane and that the coupling factor was 3. The scaling variables from Eq. 2 were set to 0.2 for d and 0.001 for k. These values scale the current to an arbitrary level but to one that would be expected from a single myocyte. For this discussion, the resultant current polarity is more important than the magnitude. Substituting the intracellular and extracellular ion activities obtained in this study into Eq. 2, we calculated current-voltage relationships (Fig. 12). From these curves it can be determined that there is virtually no exchanger current at resting membrane potential. This indicates that at resting membrane potential, sodium-calcium exchange does not result in movement of calcium across the sarcolemma. Under these conditions a depolarization (upstroke of the action potential) would result in a hyperpolarizing exchanger current. This indicates that calcium is being moved into the myocyte while sodium is being removed. This calcium is available for initiating monocyte contraction. The greater the depolarization, the greater the influx of calcium. As the intracellular calcium concentration increases, the calcium electrochemical gradient will be reduced, resulting in reduced calcium influx.

At the new intracellular calcium concentration, the exchanger begins to remove calcium from the cell at potentials more negative than −40 mV. As the sarcolemma begins to repolarize, the exchanger will then remove the calcium, bringing about relaxation.

The above analysis demonstrates that sodium-calcium exchange could entirely mediate the intracellular calcium concentration, depending upon the membrane potential. The action potential could control the time course of the calcium transient and subsequent contraction through its effect on sodium-calcium exchange. Action potential duration in the presence of DPI and ryanodine is prolonged. The action potential illustrated in Fig. 12 repolarizes through −40 mV at the peak of the contraction. This was the calculated reversal potential for sodium-calcium exchange. Calcium influx thus would have continued until that point, producing contraction. With further repolarization, calcium efflux would begin, resulting in relaxation.

Effect of inotropic agents on action potential parameters. As has been reported for other mammalian species, the action potential in human myocardium was markedly prolonged in the presence of DPI or veratridine (2, 3, 5, 44). It has been argued that DPI acts mainly by modifying sodium channels, because tetrodotoxin in the presence of DPI normalizes the shape of the action potential and abolishes the DPI effect on muscle tension (2). Experiments using patch clamp techniques have shown that DPI prolongs the open state of sodium channels or delays sodium channel inactivation (4, 5). Our data support the hypothesis that DPI acts as a sodium channel agonist to produce prolongation of the action potential. Prolongation of the action potential was reversed by the addition of yohimbine and tetrodotoxin. Verapamil, which blocks slow inward current in mammalian myocardium (45), slightly abbreviated the action potential but did not return it to pre-DPI values. Also, as reported for other mammalian species, DPI does not affect resting membrane potential or action potential amplitude.

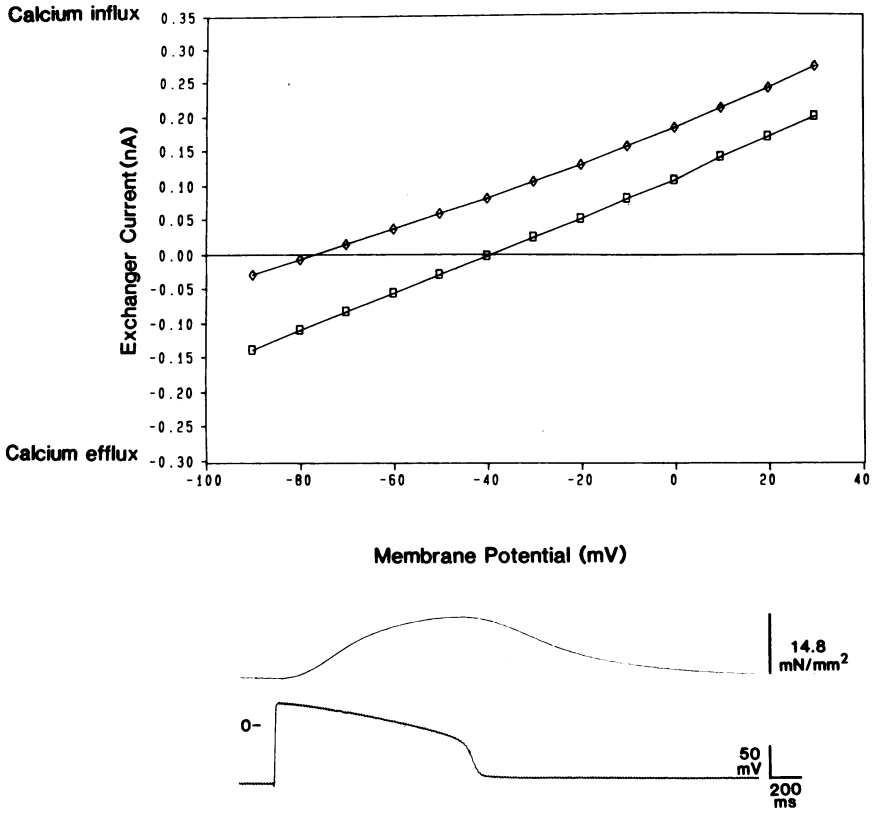

Figure 12. Calculated current-voltage relationship for sodium-calcium exchange (*top*). Calculated from the resting ionic concentrations (◇) and from the ionic concentrations at the peak intracellular calcium concentration (□). The coupling factor was set to 3. A positive current (hyperpolarizing) results in calcium influx, whereas a negative current (depolarizing) results in calcium efflux. (*Bottom*) Tension and membrane voltage in the presence of DPI (1×10^{-6} M), 8 mM $[Ca^{2+}]_o$, and ryanodine (3×10^{-6} M).

That the peak plateau amplitude of the action potential was not altered by DPI suggests that DPI does not primarily act on calcium channels to increase calcium current. BAY K 8644 has been previously reported to prolong action potential duration and calcium channel open time (46–48). In this study, BAY K 8644 did not affect the time course of the isometric contraction and associated calcium transient. This supports our conclusion that the prolongation of the calcium transient and contraction were not due to an increase in calcium channel open time (49). These findings are consistent with the hypothesis that DPI acts primarily on sodium, not calcium, channels.

Frequency dependency of DPI. The effects of DPI appear to be frequency dependent. The duration of the action potential, calcium transient, and corresponding tension response were abbreviated at a 1-Hz stimulation rate. Tension duration and L′ amplitude were also decreased at stimulation frequencies of 0.03 Hz. In the presence of varying frequencies, the peak calcium-tension relationship approximated the calcium concentration response curve at < 1 Hz frequency of stimulation in the presence of DPI. This indicates that the shift in the peak calcium-tension relationship in the presence of DPI may reflect the amount of intracellular calcium available at the level of the myofilaments as opposed to a change in the sensitivity of the myofilaments to calcium. Effects of other sodium channel agonists such as veratridine are also frequency dependent (28).

The observed abbreviation of tension time course at lower stimulation frequency may be due to reduced sodium loading and enhanced sodium-calcium exchange. Lower frequency of stimulation could also enhance sequestration of calcium by intracellular stores i.e., the sarcoplasmic reticulum. This might explain the lack of postrest potentiation of contractile force until after 10–20 contractions have occurred (5). At increased frequencies of stimulation, there is enhanced uptake by the sarcoplasmic reticulum, possibly due to a calmodulin-mediated effect and/or activation of calcium-dependent protein kinases that have synergistic effects. There is also enhanced dissociation of calcium from the myofilaments. The result is an increased loading of the sarcoplasmic reticulum and subsequent positive inotropic effect (3, 5).

Effects of drugs on the time course of the aequorin light signal and tension response. We have previously reported that increases in extracellular calcium do not affect the time course of the calcium transient and corresponding tension response in control human myocardium (12). Similarly, exposure to BAY K 8644 did not affect the time course of the calcium transient and corresponding tension response in control human muscle. In contrast to agents that increase sarcolemmal calcium flux, veratridine and DPI either had no effect on the time course of the calcium transient or caused prolongation of the calcium transient and the appearance of a second component, L′. L′ appeared to arise in part from the action of DPI on sodium-calcium exchange. L′ also appeared to reflect altered calcium handling by the sarcoplasmic reticulum. Associated with the appearance of L′ was a marked prolongation in the tension response. The tension response in the presence of veratridine and DPI consisted of two distinct relaxation phases, an early relaxation phase followed by a second slower phase. The addition of DPI in the presence of a maximally effective dose of ryanodine resulted in a positive inotropic effect and a prolonged relaxation without the occurrence of a second slower

phase of relaxation. DPI's effects on the time course of the isometric contraction could be reversed by sodium channel antagonists cadmium, yohimbine, and tetrodotoxin.

DPI effects on L′ did not appear to be secondary to DPI effects on sarcolemmal slow Ca^{2+} channels. L′ was affected by exposure to ryanodine. After the addition of ryanodine, only a portion of L′ remained. If this remaining component reflected slow inward current, one would expect addition of ryanodine to increase its amplitude, as ryanodine has been reported to increase the slow inward current in cardiac tissue (50). In addition, DPI has been reported to inhibit, rather than augment, calcium conductance through voltage-dependent channels (5, 51). Further, DPI has been reported to inhibit [^{3}H]nitrendipine binding to cardiac membranes at concentrations > 10^{-7} M (50).

L′ may in part arise from $[Na^+]_i$-dependent Ca^{2+} influx (52–54). This system, which exists in the cardiac muscle sarcolemma, would favor Ca^{2+} influx during depolarization. Such a system should be inhibited by sodium channel antagonists, and indeed, in these experiments, addition of tetrodotoxin completely abolished the remaining calcium transient.

That veratridine and DPI had similar effects on the time course of the calcium transient and isometric tension (44, 55) argues strongly that DPI exerts its effects primarily through its action as a sodium channel agonist. DPI effects also were reversed by known sodium channel antagonists, yohimbine and tetrodotoxin, and a calcium channel and sodium channel antagonist, cadmium. Tetrodotoxin blocks sodium channels; however, tetrodotoxin can reduce action potential duration. The reversal of DPI effects on action potential and isometric tension parameters might therefore be explained on the basis of a direct action of tetrodotoxin. This is unlikely, however, because yohimbine similarly blocks sodium channels but does not change action potential duration (19). Cadmium on the other hand probably reverses DPI effects by blocking both sodium and calcium channels.

Sensitivity of the myofilaments to calcium. DPI has been reported to increase the sensitivity of myofilaments to calcium (2, 6). This could account for its effects on cardiac muscle contraction: positive inotropy and prolongation of tension. It is difficult to address the question of whether an increase in sensitivity of the myofilaments to calcium exists when the agent involved elevates resting calcium and causes the appearance of L′. However, DPI effects on calcium transients are qualitatively similar in control and myopathic myocardium. Therefore, a comparison between control and myopathic tissue appears appropriate. To determine whether there is an increase in myofilament sensitivity to calcium, we have compared aequorin data with data from skinned fiber preparations from the same hearts (56).

Toxic effects of DPI. Toxic effects of DPI were noted at higher drug concentrations and in the presence of high extracellular calcium. These consisted of after-glimmers in the aequorin signals, after-contractions in the tension response and an increase in resting calcium and tension. It has been previously reported that after-contractions occur with toxic doses of most positive inotropic agents that increase intracellular calcium (57–59). Experimental evidence suggests that after-glimmers and after-contractions are due to oscillatory release of calcium by the sarcoplasmic reticulum (59). This hypothesis is supported by the fact that ryanodine and caffeine inhibit these toxic responses. In the presence of ryanodine, DPI, alone and in the presence of high extracellular calcium, did not produce the second slow component of relaxation.

High concentrations of DPI (1×10^{-5} M) alone produced a decrease in the amplitude of the light signal, i.e., a caffeine-like effect and a decrease in peak tension in the presence or absence of increased extracellular calcium. The mechanism of this effect is not clear, but may reflect decreased release of Ca^{2+} from the sarcoplasmic reticulum or oscillations of calcium release by the sarcoplasmic reticulum. The overshoot of the calcium transient after addition of yohimbine could reflect the reversal of the calcium overload state as reflected by the decrease in the resting $[Ca^{2+}]_i$.

The prolongation of the action potential with DPI may have clinical significance in that such prolongation could result in a "long Q-T syndrome." The occurrence of prolonged Q-T intervals is associated with the development of malignant arrhythmias such as *torsades de pointes.*

Effect of DPI on excitation-contraction coupling in human myocardium. Cytoplasmic free Ca^{2+} appears to be regulated by transport mechanisms in the sarcolemma, the sarcoplasmic reticulum, and the mitochondria. Contraction in cardiac muscle is influenced by Na^+ and Ca^{2+} electrochemical gradients, which suggest that the sodium-calcium exchange system has a role in the regulation of excitation-contraction coupling.

DPI is an inotropic agent that prolongs the action potential and increases intracellular calcium via its sodium channel agonistic properties. DPI appears to have similar effects on human control and myopathic myocardium. The positive inotropic effect can be explained primarily on the basis of increased sodium loading and its resultant effect on the sodium-calcium exchanger to increase intracellular calcium. The appearance of an L′ component in the calcium transient of myocytes exposed to DPI reflects additional calcium influx via the sodium-calcium exchanger and from faulty calcium handling by the sarcoplasmic reticulum. The increase in intracellular calcium produces a markedly prolonged contractile response and positive inotropy. DPI may also produce positive inotropy by increasing sensitization of the myofilaments to calcium (56).

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