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# CALCIUM ANTAGONIST BINDING SITES IN FAILING AND NONFAILING HUMAN VENTRICULAR MYOCARDIUM

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**Abstract**—Studies in myopathic hamsters have described an increase in calcium antagonist binding sites, which is presumably associated with an increase in the number of calcium channels. Such an abnormality might predispose the heart to further myocardial damage from calcium overload. We tested the hypothesis that calcium antagonist binding sites are increased in human idiopathic dilated cardiomyopathy by examining [$^3$H]PN 200-110 and [$^3$H]nitrendipine binding in membranes prepared from nonfailing controls and severely failing ventricles with idiopathic dilated cardiomyopathy. Despite the fact that $\beta$ receptor density was decreased by 50% in failing hearts (iodocyanopindolol $B_{max}$ 84.4 ± 8.9 fmol/mg protein in nonfailing hearts vs 42.9 ± 3.2 fmol/mg in failing hearts, $P < 0.01$), dihydropyridine calcium antagonist binding sites were not reduced significantly by heart failure. Maximum binding of [$^3$H]PN 200-110 was 92.9 ± 19.4 fmol/mg protein in membranes derived from failing ventricles, and 93.5 ± 17.4 fmol/mg in membranes derived from nonfailing ventricles (P = NS); values for [$^3$H]nitrendipine maximum binding were similar to those for [$^3$H]PN 200-110 and also were not reduced significantly in failing ventricles. Additionally, the dissociation constants ($K_D$) for [$^3$H]nitrendipine and [$^3$H]PN 200-110 were not significantly different in failing and nonfailing heart. We conclude that dihydropyridine calcium antagonist binding sites are not altered significantly in the failing human left ventricle with idiopathic dilated cardiomyopathy.

The underlying mechanism of the progressive heart muscle degeneration seen in idiopathic cardiomyopathy is not well understood [1]. One suggested hypothesis is that intracellular calcium overload [2] causes cellular dysfunction or death by exhausting energy stores and subsequently disrupting cell membrane integrity [3]. Recently there has been considerable interest in the role of calcium channels in this process, as calcium antagonists can delay or prevent the development of heart failure in a hamster strain afflicted with a hereditary cardiomyopathy [4]. In this model, Wagner *et al.* [5] described an excess of dihydropyridine binding sites, which are closely associated with calcium channels. This same group has shown recently an increase in dihydropyridine binding sites in human atria from hearts with hypertrophic cardiomyopathy compared to hearts with other abnormalities [6]. If calcium channels were increased in failing heart muscle, this alteration would predispose to calcium overload, and this might provide an explanation for the progressive nature of heart failure in most human subjects with cardiomyopathy [1, 7].

To investigate the importance of calcium channels in the pathophysiology of diffuse heart muscle disease in humans, we measured binding characteristics of two radiolabeled dihydropyridine calcium antagonists, [$^3$H]nitrendipine and [$^3$H]PN 200-110 in membranes prepared from nonfailing controls and failing human left ventricles with idiopathic dilated cardiomyopathy. To confirm that the failing hearts were undergoing the type of cell surface changes that have been demonstrated previously in heart failure, we measured $\beta$-adrenergic receptor density in both groups [8]. The results show that in contrast to the reduction in $\beta$-adrenergic receptor density, calcium antagonist binding sites were unaltered in endstage idiopathic cardiomyopathy.

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## MATERIALS AND METHODS

*Membrane preparation.* Human cardiac tissue was obtained from ten subjects with endstage biventricular heart failure secondary to idiopathic dilated cardiomyopathy at the time of heart transplantation and from nine organ donors with no evidence of cardiac dysfunction whose hearts were not used for transplantation due to blood type or size mismatch or late developing problems with the recipient. A 5-g aliquot of left ventricular free wall was dissected free of epicardial fat, endocardial fibrosis, chordae, and papillary muscles. The trimmed tissue was minced finely with scissors in ice-cold 10 mM Tris (pH 8.0), 1 mM ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA) and homogenized with a Polyton (Brinkmann Instruments) using three consecutive bursts of 3 sec at full speed. A crude membrane fraction was made by extracting the contractile proteins with 0.5 M KCl and then washing a 50,000 *g* pellet twice. The membranes were resuspended in 250 mM sucrose, 50 mM Tris (pH 7.5), 1 mM EGTA at a protein concentration of 5–10 mg/mL and were stored at −80°. This fraction has a 2- to 4-fold enrichment of sarcolemmal markers (adenylate cyclase, 5′-nucleotidase) compared to the original homogenate [8].

*Ligand binding studies.* Steady-state [$^3$H]PN 200-110 (New England Nuclear, Boston, MA) binding curves were measured using seven increasing concentrations of [$^3$H]PN 200-110 in the presence and absence of 1 μM nicardipine. The buffer was 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4), 2 mM $MgSO_4$, 1 mM EGTA and the final assay volume was 450 μL. Membrane protein concentrations were 1–2 mg/mL. The assay mixture was incubated for 50 min at 30°, and the assay was stopped by adding 5 mL of room temperature buffer followed by immediate vacuum filtering through 1-μm glass fiber filters. Steady-state [$^3$H]nitrendipine (New England Nuclear) binding was measured in 50 mM Tris (pH 7.5) in the presence and absence of 1 μM nicardipine. The membrane protein concentration was 1.5 to 2.5 mg/mL, and the assay mixture was incubated for 45 min at 30°. All other aspects of the protocol were as described for [$^3$H]PN 200-100 binding.

Association kinetics for [$^3$H]PN 200-110 were determined by adding cardiac membranes to 120 pM [$^3$H]PN 200-110 in the presence and absence of 1 μM nicardipine for increasing lengths of time. Dissociation kinetics were determined by incubating cardiac membranes with 120 pM [$^3$H]PN 200-100 for 50 min and then adding 1 μM nicardipine for various time intervals. Association kinetics for [$^3$H]nitrendipine were determined by adding cardiac membranes to 2 nM [$^3$H]nitrendipine in the presence and absence of 1 μM nicardipine. Dissociation kinetics were determined by incubating cardiac membranes with 2 nM [$^3$H]nitrendipine for 30 min and then adding 1 μM nicardipine for various time intervals.

[$^3$H]PN 200-110 nicardipine competition curves were measured in membranes by incubating 500 pM [$^3$H]PN 200-110 or 1.5 nM [$^3$H]nitrendipine with seventeen increasing concentrations of nicardipine for 50 min at 30°. Nicardipine was made up in an initial stock solution of 1 mM in absolute ethanol followed by serial dilution in buffer containing 1 mg/mL bovine serum albumin.

β-Adrenergic receptors were measured by [$^{125}$I]iodocyanopindolol (ICYP) specific binding using previously described methods [7]. Briefly, seven different concentrations of ICYP were incubated with 50–150 μg/mL of membrane protein for 120 min at 30° in a total volume of 450 μL. Nonspecific binding was determined with 1 μM (−)propranolol, and the buffer system was 150 mM NaCl, 20 mM Tris, 1 mM ascorbate (pH 7.5).

Protein concentration was measured by the method of Lowry *et al.* [9].

*Data and statistical analyses.* Binding parameters for the saturation binding, competition, and association/dissociation curves were determined by fitting the experimental data to the appropriate equations using nonlinear least squares regression analysis, as previously described [10].

For comparison of binding parameters between failing and nonfailing myocardium a two-tailed Student's *t*-test was used with $P < 0.05$ taken as statistical significance.

*Chemical supplies.* [$^3$H]PN 200-110, [$^3$H]nitrendipine and [$^{125}$I]iodocyanopindolol were purchased from New England Nuclear (Wilmington, DE). Nicardipine was a gift from the Syntex Corp. (Palo Alto, CA). (−)propranolol was a gift from Ayerst Laboratories (New York, NY). All other compounds were purchased from standard commercial suppliers.

## RESULTS

*Characterization of* [$^3$H]*PN* 200-110 *and* [$^3$H]*nitrendipine binding.* As shown in Fig. 1, binding of [$^3$H]PN 200-110 was rapid and reversible with an association rate constant, $k_1$, of 0.000884/min·nM and a dissociation rate constant, $k_2$, of 0.009/min. The dissociation constant ($k_2/k_1$) calculated from these data was 12 pM. Specific [$^3$H]PN 200-110 binding was linear between membrane protein concentrations of 250 and 2500 μg/mL ($r = 1.00$). As shown in Fig. 2, nicardipine displaced [$^3$H]PN 200-110 by simple competition (Hill coefficient near unity); the average dissociation constant, $k_D$, for nicardipine in the two grouped experiments shown in Fig. 2 was 2.32 ± 0.16 nM.

Binding of [$^3$H]nitrendipine was also rapid (steady-state achieved at 45 min) and reversible, with a $k_1$ of 0.060/min·nM, a $k_2$ of 0.086/min and a dissociation constant of 1.43 nM (data not shown). Specific [$^3$H]nitrendipine binding was linear between 125 and 2700 μg/mL of membrane protein ($r = 1.00$).

*Quantitative measurements of calcium antagonist binding sites and β-adrenergic receptors.* Representative saturation binding curves for ICYP, [$^3$H]PN 200-110 and [$^3$H]nitrendipine binding in membranes derived from the same nonfailing left ventricle are shown in Figs. 3–5. In nonfailing hearts, β-adrenergic receptor density as measured by maximum ICYP binding was 84.4 ± 8.9 fmol/mg protein, compared to 42.9 ± 3.2 fmol/mg protein in failing hearts ($P < 0.01$, Fig. 6). In contrast, the density of [$^3$H]PN 200-110 binding sites in membranes derived from failing ventricles was 92.9 ± 19.4 fmol/mg, a value nearly identical to that in nonfailing ventricles (93.5 ± 17.4 fmol/mg, P = NS, Fig. 6). In failing versus nonfailing ventricles there was also no difference in the [$^3$H]PN 200-110 dissociation constant, with a value of 110 ± 50 pM in nonfailing heart and 113 ± 26 pM in failing heart (P = NS, Fig. 7).

Similarly, the density of [$^3$H]nitrendipine binding sites was 96.7 ± 14.4 fmol/mg protein in nonfailing left ventricles and 89.7 ± 10.6 fmol/mg in membranes from failing heart (P = NS, Fig. 6). The dissociation constants for [$^3$H]nitrendipine were also not different, with an average value of 1.28 ± 0.27 nM in nonfailing heart and 0.83 ± 0.14 nM in failing heart (Fig. 7).

## DISCUSSION

Previous studies in a hamster model of cardiomyopathy have described a significant increase in dihydropyridine binding sites. Wagner *et al.* [5] reported that [$^3$H]nitrendipine binding is 50–100% higher in cardiomyopathic hamsters than in matched control hamsters. In subsequent studies using the same model, Kuo and co-workers [11] also found a similar increase in dihydropyridine binding sites, whereas Howlett and Gordon [12] found no change

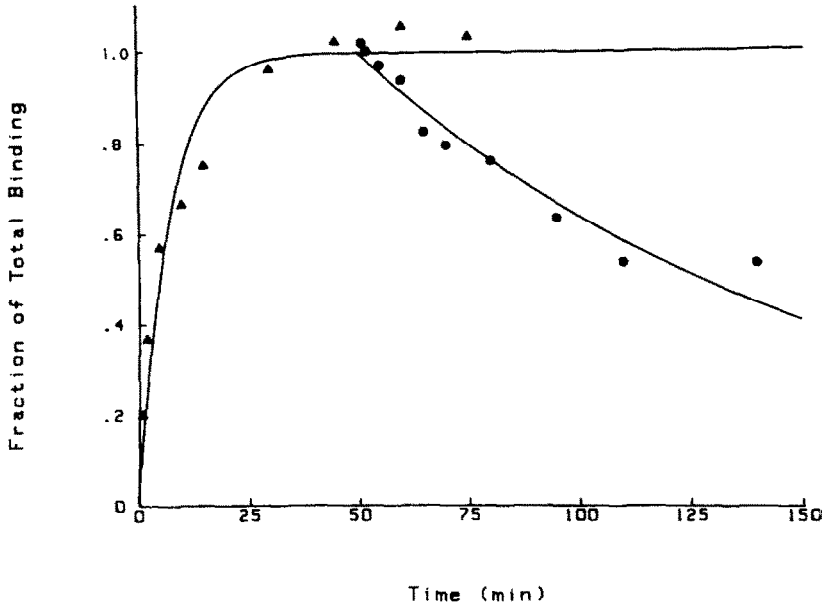


Fig. 1. Association and dissociation kinetics of [$^3$H]PN 200-110 in membranes derived from nonfailing human left ventricle. At 50 min 1 $\mu$M nicardipine was added to "chase" bound [$^3$H]PN 200-110, as described in Materials and Methods (see text for derived parameters). Key: (▲——▲) specific binding-association curve; and (●——●), specific binding-dissociation curve. Total binding was 2400 cpm.

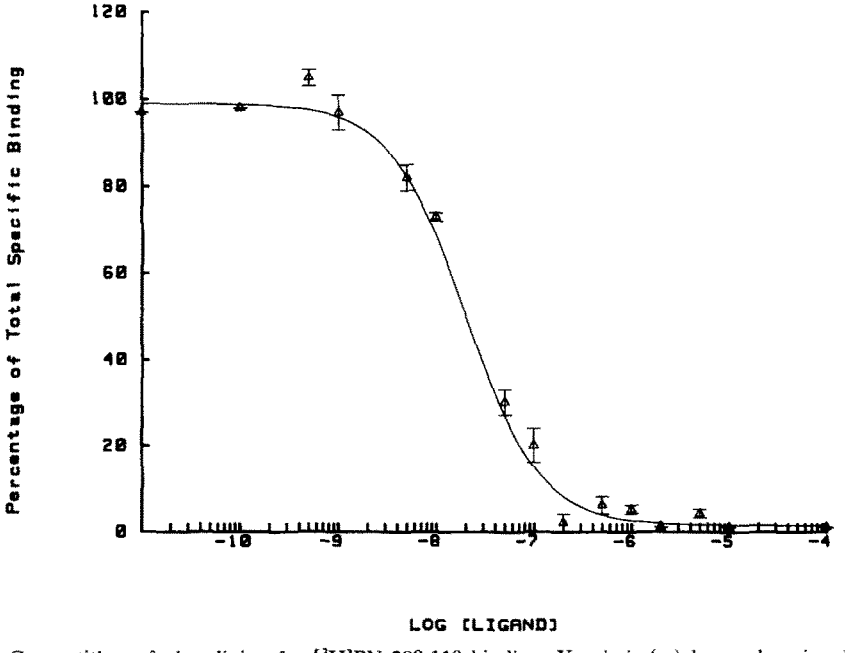


Fig. 2. Competition of nicardipine for [$^3$H]PN 200-110 binding. X axis is (−) log molar nicardipine concentration; see text for derived parameters and Materials and Methods for further experimental detail. Values are means ± range, N = 2. Maximum specific binding was 4470 cpm.

in calcium antagonist binding. Wagner *et al.* [6] reported recently a significantly higher number of dihydropyridine binding sites in the atria of patients with hypertrophic cardiomyopathy when compared to patients with other cardiac disorders.

We have measured calcium antagonist binding in membranes prepared from human left ventricle in individuals with heart failure secondary to idiopathic dilated cardiomyopathy, and compared the results to those in nonfailing hearts. Because different calcium antagonist ligands can sometimes give different results [12], we measured the binding of two dihydropyridine calcium antagonists, [$^3$H]nitrendipine and [$^3$H]PN 200-100. Both ligands showed no difference in the number of calcium antagonist binding sites in nonfailing organ donor control hearts and hearts

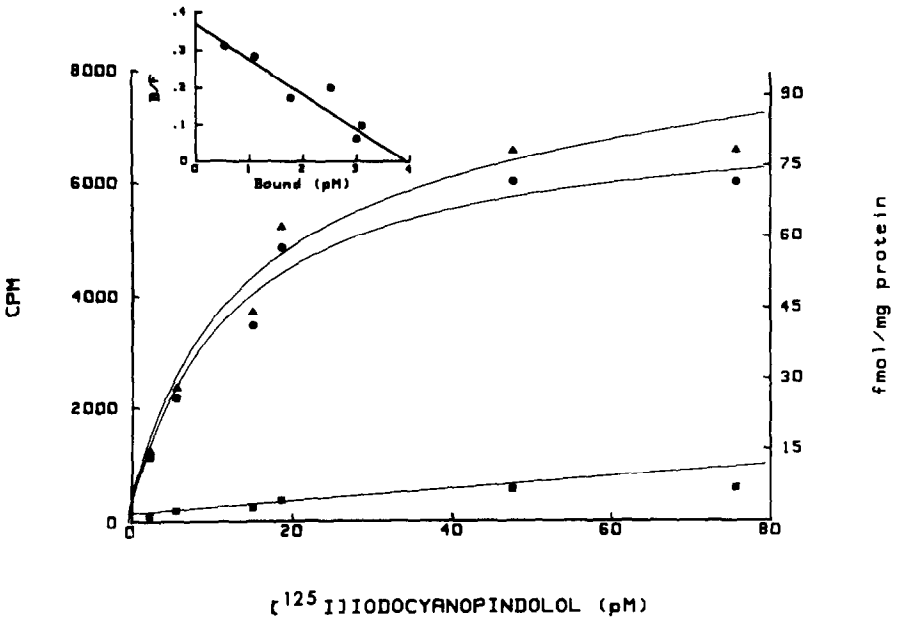


Fig. 3. [$^{125}$I]Iodocyanopindolol (ICYP) binding in membranes derived from a nonfailing left ventricle. Key: (▲——▲) total binding; (●——●) specific binding in the presence of 1 $\mu$M propranolol; and (■——■) nonspecific binding. Inset is plot of bound/free versus bound (Scatchard plot) for the specific binding data.

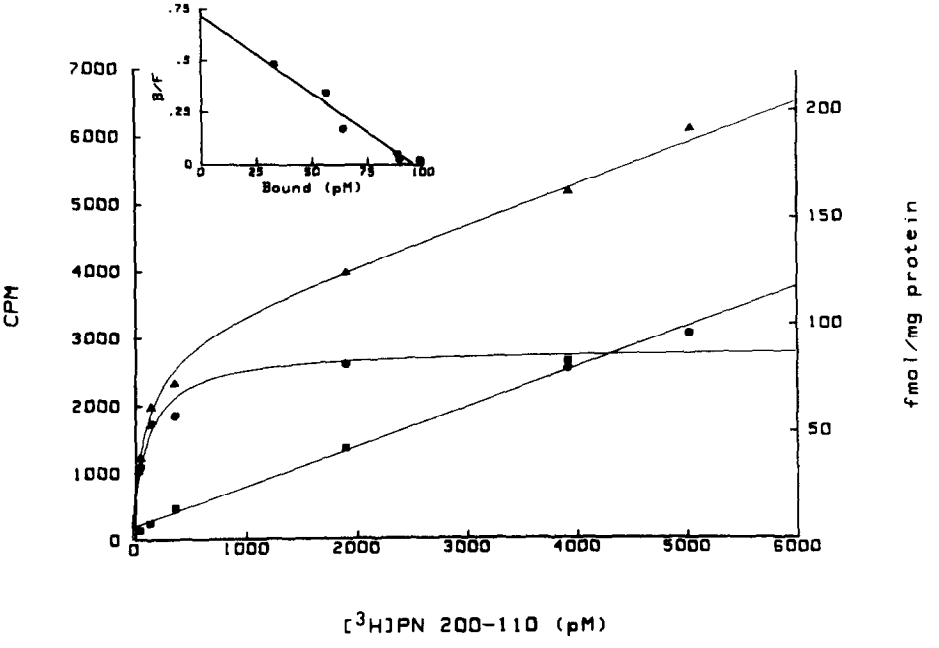


Fig. 4. [$^3$H]PN 200-110 binding in membrane preparation derived from the same left ventricle as in Fig. 3. Key: (▲——▲) total binding; (●——●) binding in the presence of 1 $\mu$M nicardipine; and (■——■) nonspecific binding. Inset is Scatchard plot of specific binding data.

with severe endstage failure. Additionally, no difference in ligand affinity was observed between failing and nonfailing left ventricles for either [$^3$H]nitrendipine or [$^3$H]PN 200-110. There was excellent agreement by the two ligands in the total number of dihydropyridine binding sites measured.

In contrast, we were able to demonstrate a large reduction in the number of $\beta$-adrenergic receptors in the myopathic left ventricles, with a 50% reduction relative to control. This is consistent with many previous reports on the effect of cardiac dysfunction on $\beta$ receptor density in human ventricular myocardium [7, 13–15]. This reduction in $\beta$ receptor density is evidence that the failing hearts did, in fact, have cell surface changes associated with severe heart failure.

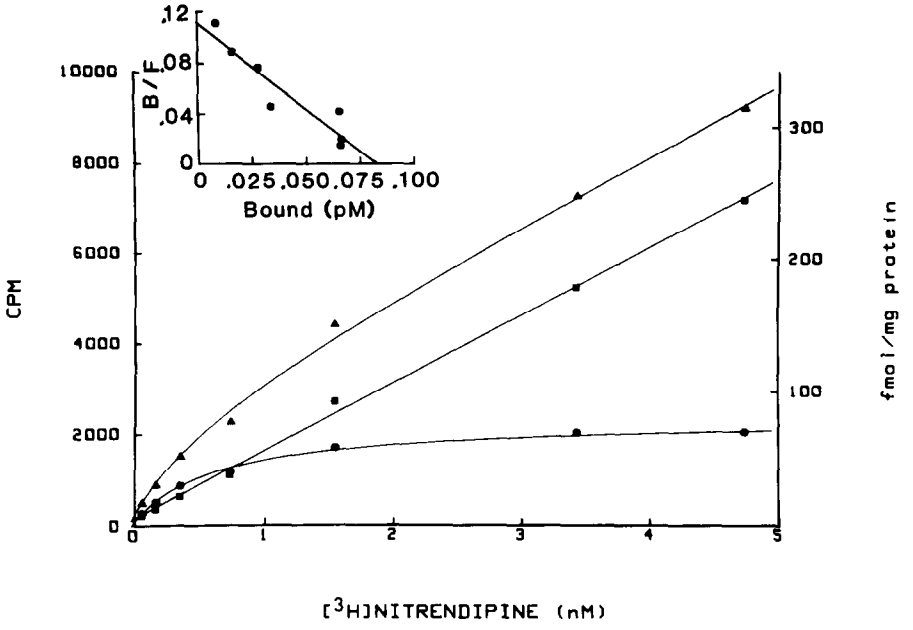


Fig. 5. [$^3$H]Nitrendipine binding in membranes derived from the same left ventricle as in Figs. 3 and 4. Key: (▲——▲) total binding; (●——●) specific binding in the presence of 1 $\mu$M nicardipine; and (■——■) nonspecific binding. Inset is Scatchard plot of specific binding data.

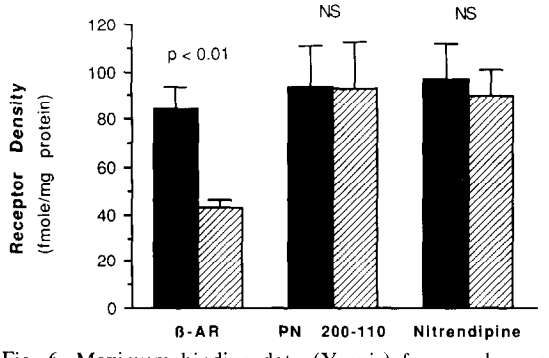


Fig. 6. Maximum binding data (Y axis) for membranes derived from nonfailing left ventricle (left-handed, closely thatched bars) and failing left ventricles (right-handed, open thatched bars). "$\beta$-AR" is maximum ICYP binding as a measurement of $\beta$ receptor density; PN-200 110 is [$^3$H]PN 200-110 maximum binding as a measurement of dihydropyridine receptor density. Nitrendipine is [$^3$H]nitrendipine maximum binding, as a second measurement of dihydropyridine receptor density. See text for actual values.

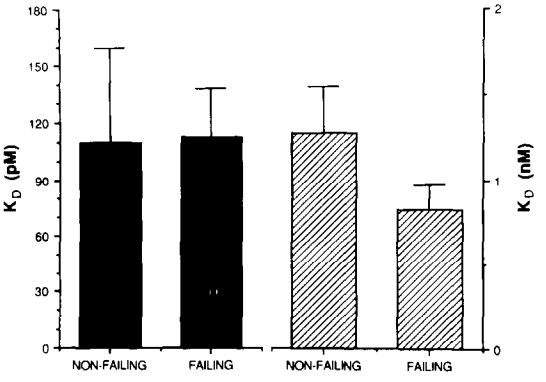


Fig. 7. Dissociation constants ($K_D$) for [$^3$H]PN 200-110 (two left-handed bars in pM) and [$^3$H]nitrendipine (two right-handed bars in nM) binding. See text for actual values.

The results of our study are supported by functional studies that show no difference in the maximum contractile response to calcium between failing and nonfailing isolated human right ventricular trabeculae [16, 17]. While there are many aspects to the regulation of myocardial contractility other than calcium influx through dihydropyridine sensitive calcium channels, these previous studies, together with our data, suggest that the failing human heart does not have a major defect in calcium channel-mediated calcium handling.

The apparent contradiction between the previously reported results in myopathic hamsters and our data in humans is perhaps not surprising. There are numerous differences in cardiac cell surface membrane pharmacology between human and animal models [18], and the cardiomyopathy observed in the hamster model is a genetic abnormality that may not be representative of idiopathic dilated cardiomyopathy in humans. As has been demonstrated on numerous occasions, care must be taken in extrapolating results from animal models of heart failure to the failing human heart [19, 20]. The results of Wagner and coworkers [6] in human hypertropic cardiomyopathy should probably not be compared with these results in idiopathic cardiomyopathy.

This study does not directly address the question of the importance of calcium overload in the development of heart muscle disease. It is possible that calcium overload does occur as a late manifestation of heart failure, but in human heart it seems unlikely

that it could be due to a relative excess of calcium channels.

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