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CALCIUM CHANNELS IN NORMAL AND DYSTROPHIC HAMSTER CARDIAC MUSCLE

[^{3}H]NITRENDIPINE BINDING STUDIES

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Abstract—Progressive cardiac cell necrosis in the dystrophic hamster may be related to intracellular calcium overload, particularly as necrosis is prevented by treatment with calcium channel antagonists. Calcium overload could arise as a consequence of an imbalance in calcium influx, efflux and/or sequestration. The possibility that increased numbers of calcium channels in myopathic cells leads to excessive calcium influx has been studied by assaying the number of [^{3}H]nitrendipine ([^{3}H]NTP) binding sites in cardiac muscle preparations. Crude homogenate and partially-purified ventricular muscle preparations from 60-day-old normal and genetically dystrophic hamsters were compared in this study. The results of equilibrium binding studies showed that, in both crude and partially-purified membrane preparations, the affinity and the maximum number of [^{3}H]NTP binding sites in normal muscle were not significantly different from those measured in dystrophic muscle. For the homogenate preparation, the K_D values were 0.07 ± 0.01 and 0.08 ± 0.01 nM for normal and dystrophic tissues, respectively, and the B_{max} values were 62 ± 6 and 73 ± 6 fmol/mg protein for normal and dystrophic preparations respectively. These data show that a simple increase in the number of [^{3}H]NTP binding sites is unlikely to account for calcium overload in the cardiomyopathic hamster.

In the genetically dystrophic hamster, the cardiomyopathy may be secondary to progressive necrotic changes [1]. Muscle cell necrosis in the muscular dystrophies, in turn, has been linked to intracellular calcium overload [2–5].

Calcium accumulation in myopathic cells could arise as a consequence of increased influx through sarcolemmal calcium channels, excessive release from intracellular calcium stores, or from defective calcium sequestration. The simplest explanation of calcium overload is that the number of voltage-sensitive calcium channels is increased in the dystrophic hamster heart. Such an explanation is suggested by the prevention of cardiomyopathy following chronic treatment with calcium channel antagonists [6, 7]; these compounds have a potent and selective ability to block the voltage-sensitive calcium channel in the cardiac sarcolemma [8].

To determine whether calcium overload in the dystrophic hamster heart results from excessive calcium influx through sarcolemmal calcium channels, we have conducted radioligand binding studies using a radiolabelled calcium channel antagonist, [^{3}H]nitrendipine ([^{3}H]NTP). Tritiated-NTP has been shown to bind, with high affinity and in a saturable manner, to the voltage-sensitive calcium channel of the cardiac sarcolemma [9–13]. These studies were conducted by comparing [^{3}H]NTP binding to crude muscle homogenates prepared from normal and dystrophic hearts. A crude homogenate preparation was chosen because membrane purification may result in variable protein yields [14, 15], and complicate comparisons between normal and diseased tissue. Using these techniques, we demonstrated that the number of [^{3}H]NTP binding sites was not increased in dystrophic hamster cardiac muscle.

During the course of these studies, Wagner *et al.* [16] published a report showing an increase in the number of [^{3}H]NTP binding sites in the brain and in cardiac, skeletal and smooth muscles of dystrophic hamsters. Therefore, we have made extensive comparisons of the data obtained using both our homogenate preparation and the partially-purified preparation of Wagner *et al.* [16]. In our studies, the results showed clearly that the number of [^{3}H]NTP binding sites was not increased in the cardiac muscle of the dystrophic hamster regardless of the preparative technique used. These findings are consistent with those reported recently [17] showing equivalent numbers of [^{3}H]NTP binding sites in skeletal muscle from human Duchenne dystrophy patients.

MATERIALS AND METHODS

Preparation. Fifty- to sixty-day-old dystrophic male Syrian hamsters (CHF 146) and genetically-matched control Syrian hamsters (CHF 148) were obtained from Canadian Hybrid Farms, Halls Harbour, Nova Scotia, Canada. In a few experiments, 30-day-old hamsters from the same source were used. Random-bred control Syrian hamsters were obtained locally from the University of Alberta breeding colony. The hamsters were weighed and then killed by cervical dislocation. The hearts were rapidly

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removed, weighed and washed in ice-cold buffer [0.25 M sucrose, 0.02 M 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), pH 7.8]. Following removal of the atria, the ventricles were finely minced and homogenized for two 10-sec bursts with a Brinkmann Polytron at half the maximal setting. The homogenate was then filtered through four layers of cheesecloth, diluted in 10 vol. (w/v) of sucrose/HEPES buffer, and either used immediately or frozen at −70°.

In a second set of experiments, the ventricles from pairs of age- and strain-matched hamsters were minced and homogenized for 15 sec in 50 mM HEPES buffer (pH 7.4 with Tris base). The homogenate was then filtered through four layers of cheesecloth. Half of this filtrate was diluted in 10 vol. of HEPES buffer and reserved for homogenate binding. As described by Wagner *et al.* [16], the other half was first centrifuged at 48,000 *g* for 15 min, and the pellet was washed twice and resuspended in 10 vol. of 50 mM Tris–HCl (pH 7.4 with Tris base) buffer prior to use.

Equilibrium binding assays. Using radioligand binding techniques similar to those described by Colvin *et al.* [18] for purified membrane preparations, we assayed the equilibrium binding of [^{3}H]NTP to hamster heart homogenates. The [^{3}H]NTP (77.4 Ci/mmol), at a radiochemical purity of >99%, was obtained from New England Nuclear and stored, light-protected, at −20°.

Binding was initiated by the addition of [^{3}H]NTP to about 0.5 mg of muscle protein to a final volume of 5 ml of 50 mM Tris–HCl (pH 7.4 with Tris base). Assays were performed, in duplicate, at labelled ligand concentrations from 0.005 to 0.5 nM (in a few experiments the labelled ligand concentration was increased to 2.6 nM). After 90 min of incubation in light-protected conditions at room temperature, the reaction was terminated by rapid vacuum filtration through Whatman GF/B filters and washed six times with 5-ml volumes of ice-cold Tris buffer [19]. We found that up to six filter washes substantially reduced non-specific binding without reducing specific binding (Fig. 1A).

In a second set of experiments, we used the techniques described by Wagner *et al.* [16] to assay [^{3}H]NTP binding to the resuspended pellet. Binding was conducted as described in the preceding paragraph with the following exceptions: the incubation time was 1 hr, and the filters were washed with three 4-ml aliquots of ice-cold NaCl (50 mM).

The protein concentration was determined, using a range of bovine serum albumin (BSA) standards, with the method of Lowry *et al.* [20]. Within the

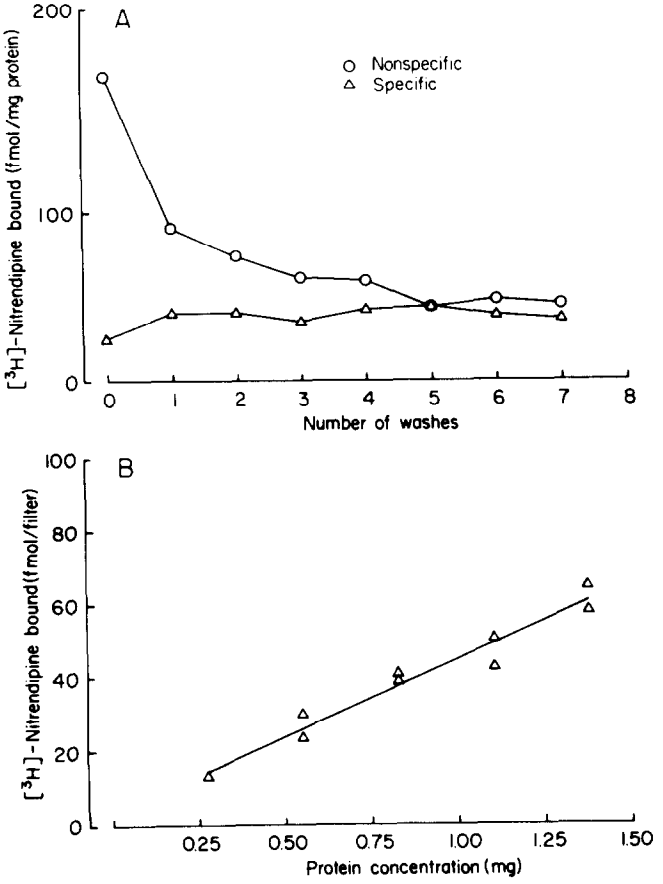

Fig. 1. (A) Effect of increasing the number of 5-ml volume filter washes with 50 mM Tris on the amounts of non-specific and specific binding. (B) [^{3}H]NTP bound versus protein, within the range of concentrations used in this study.

concentration range used in this study, the amount of specific [^{3}H]NTP binding increased linearly with the protein concentration (Fig. 1B) used in the assays.

Following the incubation step, 100-μl aliquots were removed from each tube for counting to determine the total amount of radioactivity present in each tube. The amount of [^{3}H]NTP bound was then subtracted from this value to yield the concentration of free [^{3}H]NTP in each tube.

Both the filters and the 100-μl aliquots were counted, at an efficiency of about 45%, using liquid scintillation techniques. Total and non-specific binding were determined in the absence or presence of either 20 nM unlabelled nifedipine or, in a few experiments, 500 nM nitrendipine (the nitrendipine was a gift of Miles Laboratories, Inc.). Subtraction of the non-specific binding from the total binding yielded the specific binding. The specific binding data were then fitted with a hyperbolic curve of the form:

$$B = \frac{B_{max}F}{F + K_D}$$

where B_{max} is the maximum number of binding sites, K_D is the dissociation constant, B is the amount of bound ligand, and F is the concentration of free ligand. The results were expressed as the mean ± SE; differences between means were assessed using either a student's t-test or analysis of variance.

Competition assays. Competition assays were conducted as described above with the following exceptions. [^{3}H]NTP (0.26 nM) was incubated with protein and increasing concentrations of unlabelled nifedipine. The data were expressed as a percentage of the maximum [^{3}H]NTP specifically bound, and the IC_{50} values (the concentration of unlabelled drug producing a 50% inhibition of [^{3}H]NTP binding) were computed using the logit transform method [21]. The IC_{50} values were transformed into K_i (the affinity constant for the unlabelled drug) values using the relationship:

$$K_i = \frac{IC_{50}}{(1 + [L]/K_D)}$$

where $[L]$ equals the concentration of labelled ligand used in the assay [22].

Histology. Cardiac muscle from 60-day-old normal and dystrophic hamsters was rapidly frozen in isopentane cooled in liquid nitrogen, sectioned at 10 μm, and stained with haematoxylin and eosin, using standard techniques.

RESULTS

Histologic and clinical measurements of disease. Figure 2 shows the characteristic focal lesions in the left ventricle which have been described previously in the cardiac muscle of the dystrophic hamster [23, 24]. These lesions, which correspond to regions of calcification [23], are seen as areas of dense stain surrounded by an annulus of infiltrating mononuclear cells in muscle sections that have been stained with hematoxylin and eosin. At 60 days of age, the lesions occur infrequently and are surrounded by apparently normal cardiac muscle cells.

Compensatory cardiac hypertrophy, which is an early sign of cardiac failure in dystrophic hearts [24], was not evident in the hearts of 60-day-old affected hamsters. As shown in Table 1, the dystrophic hearts were smaller than the hearts of control animals and corresponded directly with body weight. Thus, the ratios of heart weight to animal body weight, which may be used as an index of heart size for comparison, were not significantly different between normal and diseased groups (Table 1).

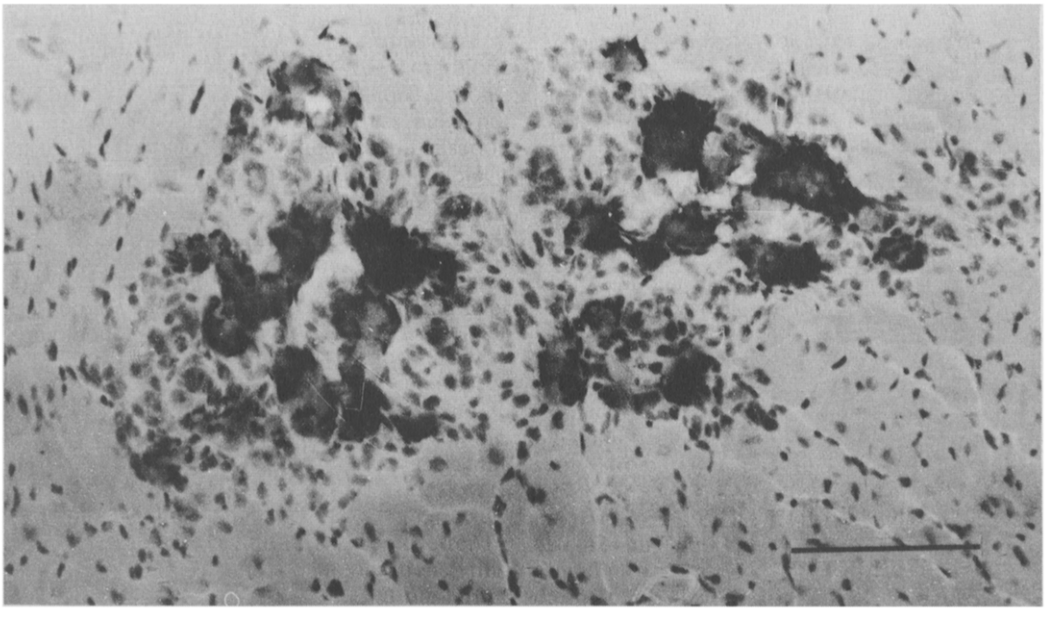

Fig. 2. Characteristic histopathologic signs of muscular dystrophy in dystrophic hamsters. In this photograph, focal lesions of the ventricular muscle (hematoxylin and eosin, calibration bar = 0.1 mm) are shown.

Table 1. Physical characteristics of normal and dystrophic hamsters

Hamsters	Animal weight (kg)	Heart weight (g)	Heart wt/animal wt (g/kg)
Normal	0.109 ± 0.004	0.36 ± 0.02	3.32 ± 0.12
Dystrophic	0.083 ± 0.006*	0.28 ± 0.02*	3.37 ± 0.18

Values are mean ± SE, N = five animals per group.
* Significantly different from genetically-matched control, df = 8, $P < 0.05$.

[^{3}H]NTP binding sites in normal and dystrophic heart. Tritiated-NTP binding to cardiac muscle tissue was saturable and of the same high affinity as previously reported [10, 25]. Figure 3A shows the total and non-specific [^{3}H]NTP binding measured in the absence and presence of 20 nM unlabelled nifedipine. Similar non-specific binding curves were obtained when a large excess of nitrendipine was used to displace specific binding of [^{3}H]NTP to muscle sites. Subtraction of the non-specific binding from the total binding yielded the specific binding which was saturable and occurred at a single, high-affinity site as shown in the saturation isotherm in Fig. 3B and in the single line in the Scatchard plot shown in Fig. 3C. The competition curve illustrated in Fig. 4 shows that nifedipine displaced [^{3}H]NTP at a single, high-affinity site ($K_i = 0.21 \pm 0.05$ nM; $\bar{X}$ ± SE, N = 4) and that the excess concentration of unlabelled nifedipine used in these studies was sufficient to displace all the specifically-bound [^{3}H]NTP.

A comparison of saturation isotherms and Scatchard plots of [^{3}H]NTP binding to normal and dystrophic cardiac muscle, shown in Fig. 5, indicates that the saturable, high-affinity specific binding was very similar. When hyperbolic curves were fitted to the pooled data of normal (Fig. 6A) and dystrophic (Fig. 6B) cardiac muscle, direct comparison (Fig. 6C) showed that neither the affinity nor the maximum number of [^{3}H]NTP binding sites in dystrophic muscle was significantly different from normal. The average values of the K_D (0.08 ± 0.01 nM; $\bar{X}$ ± SE, N = 7) and of the B_{max} (73 ± 6 fmol/mg protein) in dystrophic muscle were not statistically different from normal ($K_D = 0.07 \pm 0.01$ nM; $B_{max} = 62 \pm 6$ fmol/mg protein; df = 12, $P > 0.05$). Even when the concentration of [^{3}H]NTP was increased to

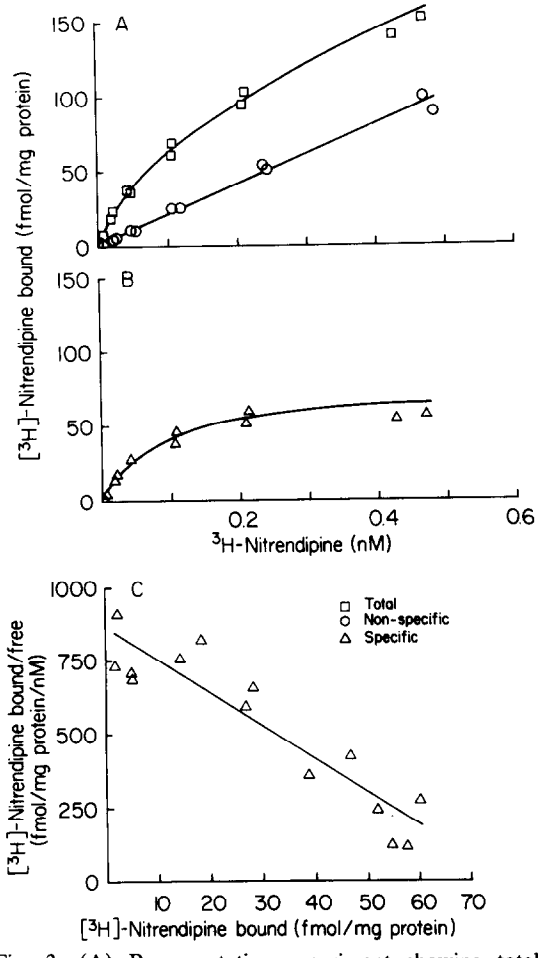

Fig. 3. (A) Representative experiment showing total (□——□) and non-specific (○——○) binding determined in the absence or presence of 20 nM unlabelled nifedipine. Subtraction of the non-specific binding from the total binding yielded the specific (△——△) binding. (B) Data of (A) fitted with a hyperbolic curve as described in Materials and Methods. (C) Scatchard plot of the specific binding data.

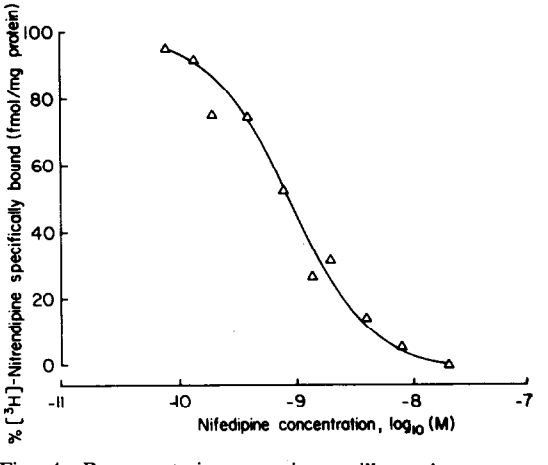

Fig. 4. Representative experiment illustrating a competition curve obtained using increasing concentrations of nifedipine to displace specifically bound [^{3}H]NTP (100% specific binding = 42 fmol/mg protein) in a normal muscle homogenate. The K_i values ($\bar{X}$ ± SE) were 0.21 ± 0.05 nM for normal muscle and 0.25 ± 0.05 nM for dystrophic muscle. These values are not significantly different (df = 5, $P > 0.05$).

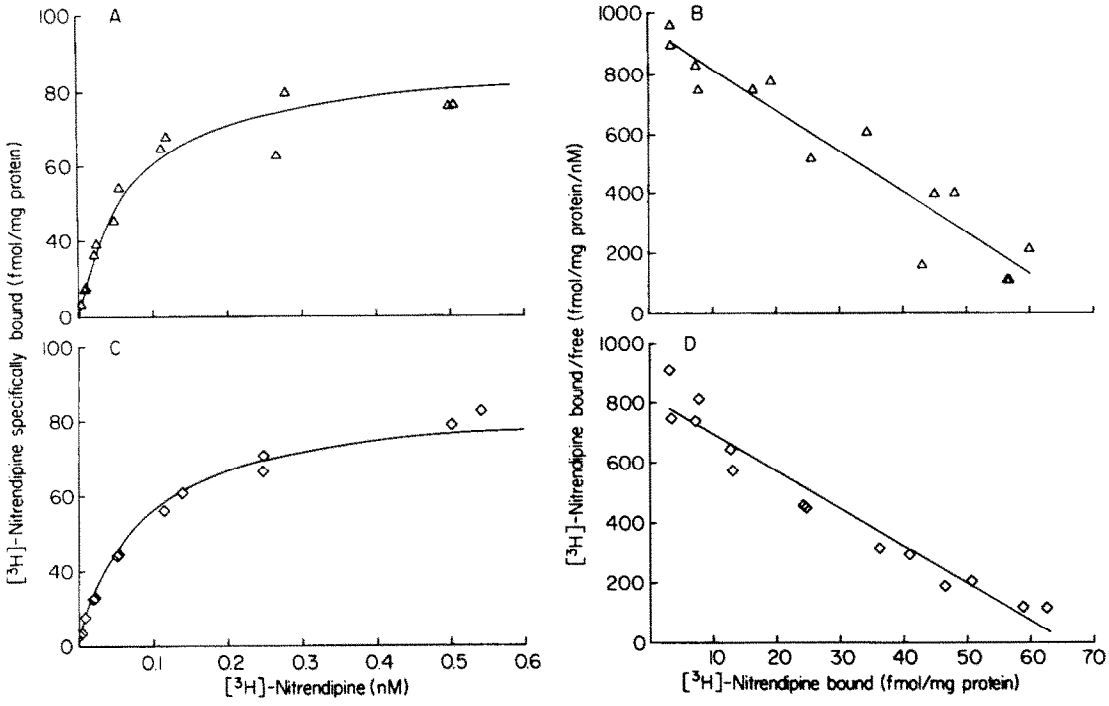

Fig. 5. Specific binding curves and Scatchard plots obtained in normal (A, B) and dystrophic (C, D) muscle.

2.6 nM, there was no evidence for a difference between normal and dystrophic preparations. Values from each experiment are compared with the mean values for K_D and B_{max} in Fig. 7 to illustrate further the extent to which the distributions overlap. Moreover, competition curves constructed using either normal (Fig. 4) or dystrophic heart homogenates were virtually identical; the average values of the K_i for nifedipine (0.21 ± 0.05 and 0.25 ± 0.05 nM for normal and dystrophic preparations, respectively) were not significantly different from normal ($df = 5$, $P > 0.05$), illustrating that competition by nifedipine is similar in normal and diseased muscle.

Thus, comparison of [³H]NTP binding to the cardiac muscle of dystrophic hamsters and genetically-matched controls shows that neither the affinity nor the number of the binding sites was altered in dystrophy. Even when random-bred hamsters were used as the control group, the K_D and the B_{max} were not significantly different from normal ($K_D = 0.08 \pm 0.01$ nM; $B_{max} = 80 \pm 6$ fmol/mg protein; $N = 8$, $df = 20$, $P > 0.05$).

In the study of Wagner *et al.* [16], almost twice as many binding sites were reported in dystrophic hearts when compared to random-bred controls. A partially-purified membrane preparation, however, was used to measure [³H]NTP binding in their study, yet our comparison of the K_D and B_{max} values determined using the same preparation [cf. Ref. 16, Methods] again showed that dystrophic muscle had the same number of binding sites as normal muscle. In an experiment in which both muscle homogenate and partially-purified preparations were prepared from the same 60-day-old normal or dystrophic hamster hearts, B_{max} values were 70 and 109 fmol/mg protein in dystrophic muscle (homogenate and pellet respectively) compared with the B_{max} values in normal muscle of 80 and 101 fmol/mg protein (homogenate and pellet respectively). There were no disease-related changes in the K_D. The B_{max} values per mg protein were higher, as expected, in the partially purified preparations, and there was no change in the K_D obtained using different preparative techniques. Similar results were obtained when we compared [³H]NTP binding to both homogenate and pellet preparations of the hearts of 30-day-old normal and dystrophic hamsters.

DISCUSSION

The calcium overload theory of the muscular dystrophies holds that muscle cell necrosis arises as a consequence of an increase in intracellular calcium levels. If this thesis obtains, one explanation of how intracellular calcium levels are increased is that the number and/or affinity of the voltage-sensitive calcium channel is altered. The data presented in this study clearly show that the number of [³H]NTP binding sites and the affinity of [³H]NTP binding were not altered in dystrophic hamster cardiac muscle. This finding is of considerable interest as it implies that calcium overload in the cardiomyopathic hamster is due either to a defect in another of the cellular systems involved in calcium homeostasis in the heart or to an alteration in the complexity of the calcium channel.

Our results are consistent with similar findings in human skeletal muscle [17] but quite different from the recent results of Wagner *et al.* [16] in which the number of [³H]NTP binding sites in the brain, heart

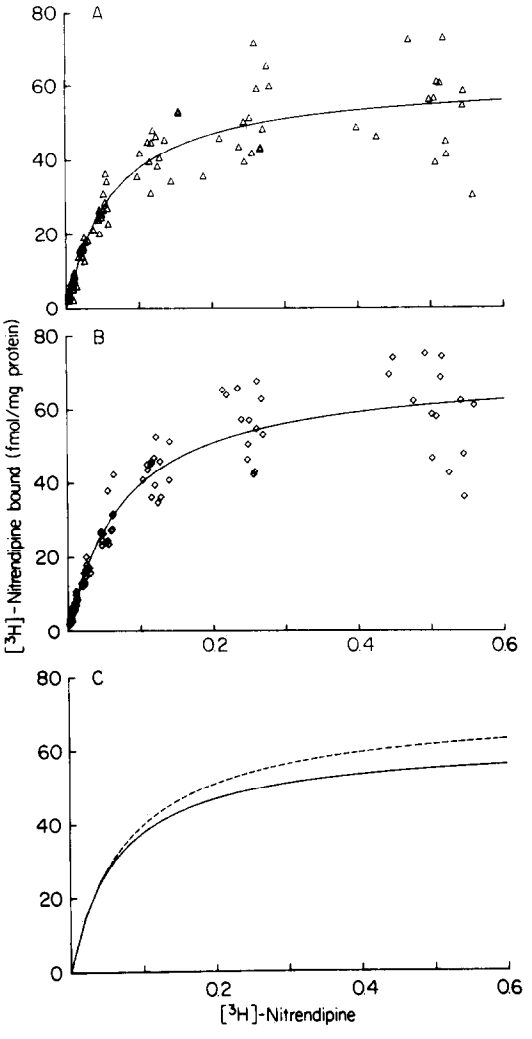

Fig. 6. Hyperbolic curves fitted through pooled specific binding data. (A) Binding data from normal homogenates. (B) Binding data from dystrophic homogenates. (C) A comparison of the curves from panels A and B. The K_D of pooled data from the normal hamsters was 0.06 nM and the B_{max} was 62 fmol/mg protein; the K_D of pooled data for the dystrophic group was 0.08 nM and the B_{max} was 71 fmol/mg protein.

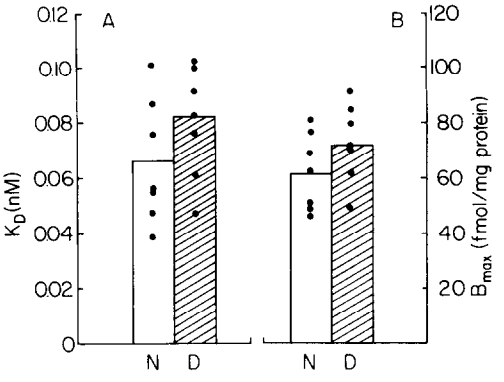

Fig. 7. Histogram illustrating the mean and range of K_D (A) and B_{max} (B) values obtained in individual experiments for normal (N) and dystrophic (D) preparations. The average values of K_D ($\bar{X} \pm SE$) were 0.07 ± 0.01 and 0.08 ± 0.01 nM for normal and dystrophic preparations, respectively, and the average values for B_{max} were 62 ± 6 and 73 ± 6 fmol/mg protein for normal and dystrophic preparations respectively.

and in smooth and skeletal muscles of the cardiomyopathic hamster were elevated significantly. The number of [^{3}H]desmethoxyverapamil binding sites was also increased in the brain but not in the heart of affected animals [16]. Several possibilities have been considered to account for the differences in these results. It is unlikely that the number of [^{3}H]NTP binding sites was too low to be resolved effectively in homogenate binding because we obtained similar results using homogenate and partially-purified preparations. Our results showed that the density of binding increased following partial purification to an equal extent in both normal and dystrophic muscle. Our results also showed that the hearts of dystrophic hamsters were smaller than controls, suggesting that the cardiac cells may be atrophied in diseased animals. If this were the case, the density of [^{3}H]NTP binding sites could actually be increased although the B_{max} values were apparently similar. This is unlikely, however, as there is no change in cardiac cell size in the dystrophic hamster heart [26]. We also considered the possibility that the difference in the B_{max} of [^{3}H]NTP binding to normal and dystrophic muscle, as reported earlier [16], actually reflected a strain difference rather than a difference between normal and diseased muscle. Hearts from random-bred genetically-unrelated hamsters were used by Wagner *et al.* [16] as controls. By contrast, we used cardiac muscle from genetically-matched normal hamsters to compare with affected muscle in dystrophic hamsters. This was a tenable hypothesis, as some "differences" between normal and genetically-diseased animals (e.g. dystrophic and spontaneously hypertensive animals) are actually strain differences [27, 28]. In our study, however, the density and affinity of [^{3}H]NTP binding sites in the cardiac muscle of a genetically-unrelated line of normal hamsters were similar to those in muscle from related pairs of normal and dystrophic hamsters.

Thus, it is difficult to reconcile the discrepancies between the results showing an increase in the number of [^{3}H]NTP binding sites in dystropic muscle [16] on the one hand, and the results showing no change obtained in our laboratory for the same preparation and by others [17] for human skeletal muscle on the other hand. The affinity of [^{3}H]NTP binding reported in our study also differs substantially from that reported by Wagner *et al.* [16]. Our results for normal cardiac muscle are at the lower end ($K_D = 0.07 \pm 0.01$ nM; $\bar{X} \pm SE$, $N = 7$) of the range typically reported, whereas the results of Wagner *et al.* [16] ($K_D = 0.60 \pm 0.13$ nM) are up to six times higher than the range of values usually reported [9, 10, 18, 25, 29, 30].

Although it is tempting to speculate that an increase in the number of high-affinity [^{3}H]NTP binding sites is the causative factor in hamster cardiomyopathy, the results of this study show clearly

that this is not the case. These data, however, do not discount the possibility that some defect in the calcium channel may still account for calcium accumulation in myopathic cells. The voltage-sensitive calcium channel contains a selectivity filter and voltage-dependent activation and inactivation gates [31]; defective channel gating may, then, be responsible for calcium overload. A consistent increase in both [^{3}H]NTP binding sites and [^{3}H]desmethoxyverapamil binding sites in the brains of dystrophic hamsters has been demonstrated and correlated with an increase in ^{45}calcium influx in brain synaptosomes [16]. These data suggest that some change in calcium channel activation or inactivation may be involved in the pathogenesis of hamster cardiomyopathy.

Further control of the number of active channels is effected by the cAMP-protein kinase system. Sperelakis and Pappano [32] have postulated that the calcium channel exists in either an inactive, dephosphorylated state or in an active, phosphorylated state; agents that elevate cAMP increase the number of phosphorylated channels by activating a protein kinase. Calcium accumulation in myopathic cells could also arise as a consequence of a defect in this regulatory pathway. The number of β-adrenoceptors in the cardiac muscle of the dystrophic hamster is increased [33, but cf. Ref. 16] and may lead to channel phosphorylation, an increase in number of active channels, and finally to calcium overload. Calcium influx through voltage-sensitive calcium channels is, however, only one source of cell calcium and may, in fact, trigger calcium release from the sarcoplasmic reticulum in cardiac muscle [34, 35]. Thus, a defect in the sarcoplasmic reticulum or the sarcolemmal sodium-calcium antiporter as recently reported by Makino *et al.* [36] could also account, at least in part, for calcium accumulation in dystrophic cells.

In summary, the results of the present study show that a simple increase in the number of voltage-sensitive calcium channels is unlikely to account for calcium overload in the cardiomyopathic hamster. If calcium overload indeed plays a central role in the development of muscle necrosis in this model of muscular dystrophy, it is likely to arise as a consequence of a defect in either the complexity of the calcium channel or another of the calcium regulatory systems.

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