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The density of ryanodine receptors decreases with pressure overload-induced rat cardiac hypertrophy

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We investigate the possibility that alterations in the calcium movements of the hypertrophied rat heart might involve sarcoplasmic reticulum (SR) ryanodine receptors. A decreased receptor density was observed with severe hypertrophy (0.26 ± 0.05 and 0.35 ± 0.06 pmol/mg protein and 170 and 366 receptors/μm^2 of SR in 50–80% hypertrophy and control, respectively); however, the total number of receptors per left ventricle was unchanged. The dissociation constant (0.7 nM) was similar in both hypertrophied and control left ventricles. Thus the decreased density of the ryanodine receptors may participate in altered calcium movements in hypertrophied rat heart.

Raynodine receptors; Hypertrophied rat heart

1. INTRODUCTION

In cardiac muscle, the key events in excitation-contraction (E-C) coupling comprise the calcium influx by the L-type calcium channel and the subsequent calcium release from the terminal cisternae of the sarcoplasmic reticulum (SR) [1]. Cardiac hypertrophy induced by pressure overload is an adaptational mechanism which is associated with a prolongation of the action potential duration [2], a delayed calcium transient [3] and a depressed contractility [4]. These modifications suggest that functional changes in E-C coupling might occur in the hypertrophied heart. Recent studies on the dihydropyridine (DHP) receptors [5] and calcium channels [6] during the compensated phase of hypertrophy demonstrated that a direct change in the calcium influx across the sarcolemma is not responsible for these functional alterations. Moreover, the calcium sensitivity of the myofibrils was unchanged [7] and thus cannot account for the depressed contractility of hypertrophied cardiac muscle. In contrast, the isomyosin shift from V1 to V3 might participate in the depressed contractility of hypertrophied rat heart [8] but not in other species where adult hearts contain almost exclusively V3 isomyosin. Another important alteration was recently reported in hypertrophied rat hearts demonstrating that both the density and activity of the SR Ca^{2+} ATPase decreased which probably contribute to the delayed relaxation [9].

We suggest that the calcium release from the SR which is directly involved in the increased intracellular concentration of ionized calcium [1] might also play a major role in the functional changes of the hypertrophied heart. In cardiac muscle, this release occurs through a channel which is generally referred to as the ryanodine receptor [10]. To test this hypothesis, we measured the density and affinity of the ryanodine receptors on crude sarcolemmal membrane preparations from rat left ventricles (LV) hypertrophied by pressure overload.

In this work, we reported the first observations that the density of ryanodine receptors is decreased by pressure overload without changes in the binding affinity. These results suggest that modifications in the SR calcium release could account, at least in part, for the alterations in E-C coupling seen with hypertrophy.

2. MATERIALS AND METHODS

2.1. *Aortic stenosis*

Male Wistar rats with body weights ranging from 180 to 220 g were anesthetized by intraperitoneal injection of 0.05 mg/g sodium pentobarbital. Cardiac hypertrophy was induced by an abdominal aortic constriction [5]. Compensated hypertrophy was stable after 2 weeks and was maintained for 2 months. The magnitude of hypertrophy was estimated one month after surgery by comparison of the LV weight/body weight ratio (LVW/BW) of operated animals with that of sham-operated animals. Only animals with a ratio ≥ 2.5 were selected for use in the binding studies. The sham-operated animals ($LVW/BW = 2.07 \pm 0.11$) underwent an identical procedure except that the hemoclip was not placed around the aorta. We checked that anatomical data of control and sham-operated animals of the same weight were not modified.

2.2. *Heart membrane preparation*

Hearts were quickly removed and immediately perfused with 5 ml of 0.15 M NaCl. The LV (including the septum) was excised and weighed. LV and septum (0.6–1.2 g) were minced into small pieces in

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the presence of buffer A (200 mM sucrose, 20 mM Tris-HCl, 0.4 mM $CaCl_2$, pH 7.0, with 1.1 μM leupeptin, 0.7 μM pepstatin, 0.7 μM aprotinin, 120 μM phenylmethane-sulfonyl fluoride, 1 mM iodoacetamide) and homogenized in 7 ml of buffer A/g wet weight tissue, using a Polytron PT20 (two bursts of 5 s at half maximal speed). The homogenate was filtered, diluted to 15 ml and centrifuged at 41 000 × g for 50 min in a Sorvall SS34 rotor. The resulting pellet was homogenized in ice cold buffer A. Aliquots of crude sarcolemmal fraction (6–8 mg protein/ml) were stored in liquid nitrogen. Protein content was determined by the method of Lowry et al. [11], using bovine serum albumin as a standard.

2.3. *Binding studies*

Characteristics of the ryanodine receptors were assessed by [^{3}H]ryanodine (60–95 Ci/mmol; New England Nuclear) binding using 12 concentrations (0.08–10 nM) of the ligand. Crude sarcolemmal fractions were diluted to 100 μg protein/ml and incubated at 37°C in a buffered medium containing 1 M KCl, 10 μM free-calcium, 50 mM PIPES-KOH (pH 7.4) [12] and proteolytic enzyme inhibitors. Unless otherwise specified, incubation times were 120 min. The medium was then automatically filtered through a Whatman GF-B filter in a Skatron apparatus. Filters were washed with 3 × 5 ml cold water. Non-specific binding was determined by addition of 5 μM ryanodine (Calbiochem) to the incubation medium. Radioactivity was counted after addition of 5 ml of scintillation fluid (Hionic fluor; Packard) in an LKB scintillation counter. We ensured that the specific binding was proportional to the protein concentration and that ligand concentrations always exceeded the receptor concentration. All incubations were performed in triplicate and the number of experiments were at least 3 per animal.

2.4. *Statistical analysis*

Estimates of equilibrium binding parameters (dissociation constant (K_d) and maximal density (B_{max})) were obtained from Scatchard plot analysis performed with the 'Ligand' program. Results are presented as mean ± SD. The statistical differences between mean values for the 2 groups were evaluated by Student's t-test. A value of $P<0.05$ was considered a significant difference between the 2 groups.

3. RESULTS

3.1. *Anatomical data*

Anatomical data of the sham-operated (designated as control) and experimental rats were shown in Table I. A significant increase in LV mass relative to body weight was obtained in 20% of the operated rats. Two groups of animals were selected: the first group had average hypertrophy of 30 ± 5% and the second one of 65 ± 15%. Whatever the indices (LVW or LVW/BW ratio) the degree of hypertrophy for the 2 experimental groups was statistically different from that of the control group.

3.2. *[^{3}H]Ryanodine binding*

In order to avoid a possible loss of membrane receptors during purification procedures, LV homogenates were centrifuged and the crude pellets used in the binding experiments. [^{3}H]Ryanodine bound in a reversible and saturable manner to this crude fraction and the time course of ryanodine binding at 1 nM was similar in preparations from control and hypertrophied LV (data not shown). Equilibrium was reached after 90 min and was stable for at least 3 h. The recovery of proteins and ryanodine receptors in the homogenates and the crude fractions from control and hypertrophied rat LV are shown in Table II; a total recovery of the ryanodine receptors was obtained in the crude fractions of the 2 groups.

The Scatchard analysis of specific [^{3}H]ryanodine binding revealed a single class of high affinity sites in both control and hypertrophied LV preparations (Fig.

Table I

General characteristics of sham-operated and operated rats

Condition	n	Body Wt (g)	LV Wt (mg)	LVWt/BWt (mg/g)
Control	7	335 ± 32	680 ± 60	2.07 ± 0.11
Hypertrophy				
25–35%	6	340 ± 31	890 ± 50***	2.62 ± 0.08***
50–80%	9	320 ± 29.5	1070 ± 105***	3.33 ± 0.24***

Values are means ± SD. The Student's t-test was done between control and hypertrophied group. ***$P>0.001$

Table II

Characteristics of the homogenates and pellets from control and hypertrophied rat left ventricles

Condition	n	Homogenate	Pellet		
		(mg protein/g LV)	(mg protein/g LV)	Protein yield (%)	Ryanodine receptor yield (%)
Control	7	166 ± 39	89.6 ± 22	53.8 ± 6.7	97 ± 13
Hypertrophy					
25–35%	6	162 ± 43	91.5 ± 21	56.5 ± 18.5	88 ± 17
50–80%	9	153 ± 45	91.8 ± 28	57.6 ± 3.08	103 ± 18

Ryanodine receptor yield has been calculated for each group from 4 experiments of specific binding at 4 nM ryanodine with 200 and 100 μg protein/ml for homogenates and pellets, respectively.

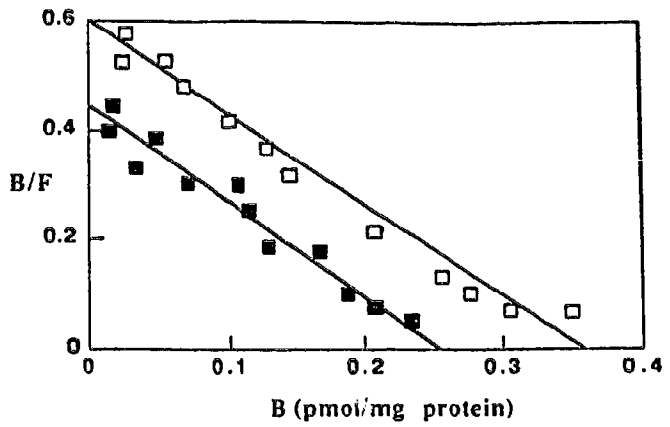

Fig. 1. Scatchard plot analysis of specific [^{3}H]ryanodine binding to crude fractions of LV from control (□) and 50-80% hypertrophied (■) rat hearts. Values represent mean of 3 experiments per crude fraction from 7 control and 9 hypertrophied animals. B = bound ryanodine; B/F = bound ryanodine/free ryanodine.

1). The non-specific binding of [^{3}H]ryanodine to crude fractions from control and hypertrophied LV was less than 20% at 10 nM ryanodine. The dissociation constant values were comparable for control and hypertrophied LV preparations (0.71 ± 0.36 and 0.62 ± 0.26 nM, respectively) (Table III). In contrast, the maximal number of [^{3}H]ryanodine binding sites tended to decrease in preparation of mildly hypertrophied LV and was significantly decreased in those from severely hypertrophied LV when compared with control LV (Table III). Due to the similar yields of protein and the total recovery of the ryanodine receptors in hypertrophied and control LV expression of the number of receptors per g of LV or per LV and comparison between hypertrophied and control LV were valid. The values in Table II demonstrate that the number of [^{3}H]ryanodine binding sites per LV remains similar in the 3 groups whereas the number of receptors per g of LV was significantly reduced with severe hypertrophy when compared to controls.

4. DISCUSSION

Alterations in E-C coupling and in calcium handling have been often reported for hypertrophied hearts [2,3]; however, it is not well established which of the functions involved in E-C coupling participate in those alterations. In this work, we observed a decrease in the density of the ryanodine receptors in severely hypertrophied rat LV. This result suggests that a decrease either in the amount or velocity of SR calcium release might occur which could account for the delayed calcium transient and/or the depressed contractility reported in the hypertrophied heart [3,4].

We estimate the number of ryanodine receptors of normal rat hearts per g of LV based upon the total receptor recovery in the preparation. Our results show that this number (26 pmol/g LV) was higher in the rat than in dog (20 pmol/g LV) [1] or rabbit (16 pmol/g LV, assuming that the protein yield of crude homogenate per g of LV is similar in the rat and the rabbit heart) [13]. If these receptors maintain similar properties among species, our data are consistent with the higher participation of the SR in the increase of intracellular free calcium in the rat relative to other species [14,15].

In moderate hypertrophy, no difference could be demonstrated between control and hypertrophied LV for the K_d, the association rate or the number of ryanodine receptors per mg of protein and per LV even though the LVW and LVW/BW ratio were significantly increased. These data are consistent with the previous observations showing unchanged Ca^{2+}-ATPase mRNA and protein accumulations in the SR of mildly hypertrophied rat LV [9].

In severe hypertrophy, the K_d and the association rate of the ryanodine for its receptor were unchanged and this finding might suggest that the same ryanodine receptor is expressed during hypertrophy. By contrast, the density of the ryanodine receptors was decreased by 26% (0.26 ± 0.05 and 0.35 ± 0.06 pmol/mg protein in hypertrophied and control LV preparations, respectively) but the total number per LV remains constant (23.9 ± 7.8 and 22.4 ± 6.2 pmol/LV in hypertrophied and control LV, respectively). To express and compare the density of the receptors in the control and hypertrophied myocyte, one might take into account the structural changes described by Anversa [6]. First, as hypertrophy occurs without cell division, the number of ryanodine receptors per hypertrophied myocyte remains constant. This number, according to the number of myocytes per LV (46×10^6) is 31.3 and 29.3 $\times 10^4$

Table III

Characteristics of the ryanodine receptors in crude microsomal fractions from control and hypertrophied rat left ventricles

Condition	n	K_d (nM)	B_{max} (pmol/mg protein)	B_{max} (pmol/g LV)	B_{max} (pmol/LV)
Control	7	0.71 ± 0.36	0.35 ± 0.06	31.3 ± 7.2	22.4 ± 6.2
Hypertrophy					
25-35%	6	0.62 ± 0.26	0.31 ± 0.04	26.6 ± 3.5	22.5 ± 1.8
50-80%	9	0.47 ± 0.21	0.26 ± 0.05***	24.0 ± 5.5*	23.9 ± 7.8

Experiments were performed 3 times on each fraction. Values are means ± SD. *$P<0.05$, ***$P<0.01$

receptors per hypertrophied and control myocyte, respectively. Second, according to (i) the localization of the ryanodine receptors in the junctional SR [17], (ii) the surface of the junctional SR in control rat myocyte which represented 1/10 of the total SR area [18] and (iii) the high increase in volume and surface of the SR (approximately two-fold for a 60% hypertrophy) [16] in the hypertrophied rat myocyte, the density of the ryanodine receptors can be estimated to 170 and 366/μm^2 in the hypertrophied and the control myocyte, respectively. This density is thus decreased by about 55% in the hypertrophied myocyte.

In the heart, recent studies on the SR calcium-induced release of calcium [19] have underlined the trigger role of the slow Ca^{2+} current in this mechanism. Therefore, we considered the ratio of the ryanodine and DHP receptors in the hypertrophied myocyte. We have previously shown that the number of DHP receptors [5] and the amplitude of the Ca^{2+} current [6] per hypertrophied myocyte is increased in such a way that the density of the receptors and of the current remains constant. The total number of DHP receptors calculated according to the morphological studies of Anversa [16] was estimated at 12×10^4 and 20×10^4 per control and hypertrophied myocyte, respectively; the mean receptor density on the total sarcolemma at 15/μm^2 in control and hypertrophied myocytes [20]. The ratio of the ryanodine receptor density to the DHP receptor density decreases from 24.4 in the control myocyte to 11.3 in the hypertrophied myocyte. Although the density of receptors is not indicative of channel activity, these data strongly suggest that the calcium release channel of the SR is a limiting parameter in the increase in intracellular free calcium during E-C coupling of the hypertrophied rat heart. It might also be emphasized that the ryanodine receptor density is decreased by the same order of magnitude as the density of another main SR protein, the Ca^{2+}-ATPase [12] which is responsible for the uptake of calcium during the relaxation. A decrease in these two functions could be the first sign of poor regulation of calcium movements and homeostasis in the compensated phase of hypertrophy.

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