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Molecular Properties of Excitation-Contraction Coupling Proteins in Infant and Adult Human Heart Tissues

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Excitation-contraction coupling (ECC) proteins in the human heart were characterized using human atrial tissues from different age groups. The samples were classified into one infant group (Group A: 0.2–7 years old) and three adult groups (Group B: 21–30; Group C: 41–49; Group D: 60–66). Whole homogenates (WH) of atrial tissues were assayed for ligand binding, $^{45}Ca^{2+}$ uptake and content of ECC proteins by Western blotting. Equilibrium [^{3}H]ryanodine binding to characterize the ryanodine receptor (RyR) of the sarcoplasmic reticulum (SR) showed that the maximal [^{3}H]ryanodine binding (B_{max}) to RyR was similar in all the age groups, but the dissociation constant (k_d) of ryanodine was higher in the infant group than the adult groups. Oxalate-supported $^{45}Ca^{2+}$ uptake into the SR, a function of the SR SERCA2a activity, was lower in the infant group than in the adult groups. Similarly, [^{3}H]PN200-110 binding, an index of dihydropyridine receptor (DHPR) density, was lower in the infant group. Expression of calsequestrin and triadin assessed by Western blotting was similar in the infant and adult groups, but junctin expression was considerably higher in the adult groups. These differences in key ECC proteins could underlie the different Ca^{2+} handling properties and contractility of infant hearts.

Keywords: Calsequestrin; Dihydropyridine Receptor; Junctin; Ryanodine Receptor; Sarcoplasmic Reticulum; SERCA; Triadin.

Introduction

Excitation-contraction coupling (ECC) in adult mammalian hearts involves a series of events in which depolarization of the plasma membrane permits the entry through dihydropyridine receptors (DHPR) of extracellular Ca^{2+}, which releases further Ca^{2+} through the Ca^{2+} release channels/ryanodine receptors (RyR) of the sarcoplasmic reticulum (SR). The efflux of Ca^{2+} through the RyR induces contraction of the cardiac muscle, and the subsequent relaxation is initiated by the reuptake of intracellular Ca^{2+} via the action of SR SERCA2a (Cannell *et al.*, 1995; Fabiato, 1983; Sun *et al.*, 1995). Several integral membrane proteins such as triadin and junctin, and the intralumenal protein, calsequestrin, can modulate Ca^{2+} release from the SR (Mackrill, 1999). Recently, evidence was presented for the presence of a quaternary complex consisting of RyR, triadin, calsequestrin, and junctin in the junctional SR (Lee *et al.*, 2004; Zhang *et al.*, 1997).

Considerable information is available concerning ECC in adult mammalian hearts, whereas much less is known in the case of immature hearts. Increased contractility (Ostadalova *et al.*, 1993) and speed of relaxation (Kaufman *et al.*, 1990) and the appearance of ryanodine-sensitive phasic contraction (Klitzner and Friedman, 1989) in postnatal development has been correlated with the levels of expression of ECC proteins in several animal models (Kaufman *et al.*, 1990; Park *et al.*, 1997; Seguchi *et al.*, 1986). It is now generally accepted that the role of the SR in ECC in the mammalian heart increases during and after the postnatal period (Blaustein and Lederer, 1999; Mahony, 1996; Ramesh *et al.*, 1995; 2001). How-

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Abbreviations: DHPR, dihydropyridine receptor; ECC, excitation-contraction coupling; RyR, ryanodine receptor; SERCA2a, sarco/endoplasmic reticulum Ca^{2+}-ATPase type 2a; SR, sarcoplasmic reticulum.

ever, no systematic study of ECC proteins in infant and adult human hearts has been reported, probably because of the difficulty of obtaining human heart tissue.

In the present study we compared the functions and expression levels of several key ECC proteins in infant and adult human atrial tissues. Infant atria had less DHPR, SERCA and junctin, whereas they had similar levels of RyR, calsequestrin and triadin to adult heart atria. Our findings suggest that the different levels of the key ECC proteins could be associated with the different Ca^{2+} handling behavior and contractility of infant hearts. In addition, the absence of significant differences in ECC coupling proteins between the different adult age groups suggests that the nature of ECC may not change significantly in this range of ages.

Materials and Methods

Preparation of human heart atria and whole homogenates (WH) Human heart tissues were obtained from discarded right atrial appendages during cannulation for cardiopulmonary bypass. 31 specimens were grouped into four age groups (Table 1). The samples were used according to the guidelines of Ajou University, College of Medicine. The specimens were frozen in liquid nitrogen immediately after extraction and kept in a deep freezer until use. They were homogenized twice for 20 s in 20 mM MOPS (pH 7.4), 1 M KCl, 1 μM leupeptin, 1 μM pepstatin, 1 μM aprotinin, 0.1 mM phenylmethylsulfonyl fluoride and 10 μg/mg trypsin inhibitor, using a Polytron PT 10 probe (Brinkmann) at a setting of 5 (Kim *et al.*, 1994). All homogenized samples were kept at −70°C.

[^{3}H]Ryanodine binding assay Equilibrium ryanodine binding assays were conducted as reported previously (Kim *et al.*, 1994) with minor modifications. Ryanodine binding by WH was assayed by incubating 200 μg of WH protein in 250 μl reaction mixtures containing 1 M KCl, 20 mM MOPS (pH 7.4), various concentrations of [^{3}H]ryanodine (54.7 Ci/mmol), 1 mM EGTA, and 0.98 mM $CaCl_2$, for 2 h at 37°C. To measure nonspecific binding, a 100-fold excess of unlabeled ryanodine (Calbiochem) was added. At the end of the incubation, 100 μl of 30% polyethylene glycol (PEG) in 50 mM Tris (pH 7.4) and 1 mM EDTA was added to each vial and incubated for 5 min at room temperature after vortexing. The vials were centrifuged for 5 min at 14,000 rpm in a microcentrifuge and the pellets were rinsed twice with 0.6 ml of the ryanodine binding buffer. One hundred μl of Soluene 350 (Packard Co.) was added to each vial, left at 70°C for 30 min, transferred to scintillation vials, and the radioactivity counted in 4 ml Picofluor (Packard Co.) with a liquid scintillation counter (Beckmann Co.).

Oxalate-supported Ca^{2+} uptake assays Human heart atria WH were pre-incubated for 4 min at 37°C in 50 mM KCl, 20 mM MOPS (pH 6.8), and 5 mM NaN_3, with or without 500 μM ryanodine (Park *et al*., 1999). The reactions were started by adding in rapid succession 5 mM MgATP, 5 mM potassium oxalate, and 0.2 mM $^{45}CaCl_2$. A prefilter (Gelman Co.) and a 0.45 μm Millipore filter were used successively to facilitate filtration. Samples of the filtrates made at various times were transferred to scintillation vials, and the radioactivity counted. The amount of Ca^{2+} in the SR was calculated by subtracting the radioactivity of filtrates from that of the reaction mixtures.

Table 1. Characteristics of human heart tissue specimens in the four age groups.

Group	Age	Sex	Disease	Assays
	2m	F	VSD	RB
	3m	F	TOF	RB
	3m	F	ECD	WB
	6m	F	MR	CU and PB
	7m	M	VSD	WB
A	1	M	VSD	RB and WB
	2	F	VSD	WB
	4	F	VSD	CU
	5	F	VSD	CU and PB
	7	M	VSD	PB
	21	M	ASD	RB
	25	F	VA	RB and WB
	26	F	ASD	CU, PB, and WB
B	26	F	MR, TR, and AR	CU and PB
	30	F	VSD	CU and PB
	30	M	MR	RB and WB
	41	F	CAOD	RB and WB
	42	M	AMI	PB and WB
	43[a]	M	CAOD	CU and PB
	43[a]	M	CAOD	RB
C	44	M	AD	CU and PB
	45	M	CAOD	RB
	46	F	CAOD	CU
	49	M	CAOD	RB and WB
	60	F	AS and AR	RB, PB, and WB
	60	M	CAOD	CU
	60	F	CABG	PB
D	61	M	CABG	RB and WB
	63	M	CABG	CU
	66[b]	M	CAOD	PB
	66[b]	M	CABG	CU and WB

[a] and [b], samples from different donors.
AD, aortic dissection; AMI, acute myocardial infa rction; AR, aortic regurgitation; AS, aortic stenosis; ASD, atrial septal defect; CABG, coronary artery bypass graft; CAOD, coronary artery occlusive disease; ECD, endocardial cushion defect; MR, mitral regurgitation; TOF, tetralogy of fallot; VA, variant angina; VSD, ventricular septal defect; CU, oxalate-supported Ca^{2+} uptake; PB, [^{3}H]PN200-110 binding; RB, [^{3}H]ryanodine binding; WB, Western blot.

[^{3}H]PN200-110 binding assay The number of DHPR in the tissue WH was assessed from the binding of [^{3}H]PN200-100, as described previously (Park *et al*., 1997). WH were incubated for 1 h at 23°C in 20 mM MOPS (pH 7.4), 10 μM $CaCl_2$, 5 nM [^{3}H]PN200-110. To measure nonspecific binding, a 1000-fold excess of nifedifine (Calbiochem Co.) was included. The reactions were stopped by adding PEG solution and the pellets were washed with 20 mM MOPS (pH 7.4) containing 10 μM $CaCl_2$. The radioactivity was measured as described for the [^{3}H]ryanodine binding assay.

Immunoblotting Atrial tissue WH (40 μg for calsequestrin and 100 μg for triadin and junctin) were run on 12% SDS-PAGE gel, and the proteins were transferred to nitrocellulose (NC) membranes (Schleicher & Schuell Co.). Immunoblotting with primary rabbit anti-calsequestrin, -triadin or -junctin antibodies prepared in-house was conducted following general protocols (Harlow and Lane, 1998). Band intensities were compared by densitometry using the "SigmaGel image" program (Version 1.0, Sigma Co.).

Materials [^{3}H]Ryanodine (54.7 Ci/mmol) and [^{3}H]PN200-110 (79.7 Ci/mmol) were obtained from NEN Co. and nonradioactive ryanodine and nifedifine were from Calbiochem Co. AP-conjugated anti-rabbit IgG, and all other chemicals were purchased from Sigma Co.

Miscellaneous Protein concentrations were determined by the method of Bradford (1976). The data are presented as means ± S.E. Statistical significance was evaluated by Student's unpaired *t*-test or ANOVA. P values < 0.05 were considered significant (* P < 0.05; ** P < 0.01; *** P < 0.005).

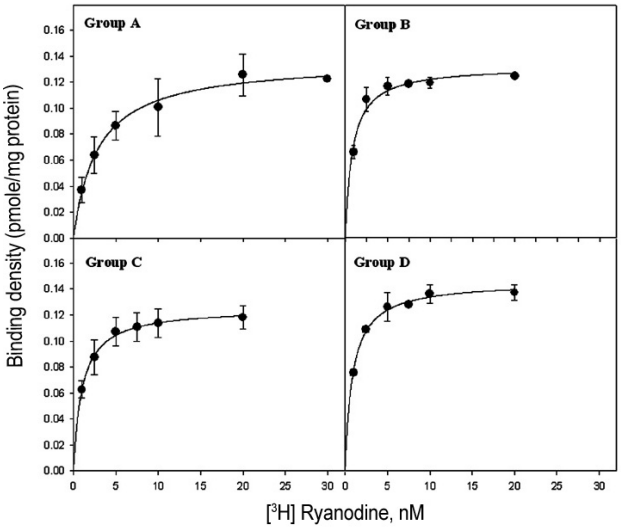

Fig. 1. Equilibrium [^{3}H]ryanodine binding to WH of human heart atrial tissues in the different age groups. [^{3}H]ryanodine binding was measured with 1–30 nM [^{3}H]ryanodine (54.7 Ci/mmol) in 250 μl reaction mixture containing atrial tissue WH (200 μg/250 μl), 1 M KCl, 20 mM MOPS, pH 7.4, 1 mM EGTA, 0.98 mM $CaCl_2$ for 2 h at 37°C. After removal of unbound [^{3}H]ryanodine by centrifugation at 14,000 rpm for 5 min in the presence of PEG, the pellets were solubilized with Soluene 350, and the radioactivity was counted. The binding parameters were calculated by iterative computer fitting using the equation $Y = B_{max} \cdot [X/(K_d+X)]$, where Y is the ryanodine bound to WH (pmol/mg protein), X is the ryanodine concentration (nM), and K_d is the dissociation constant of ryanodine (nM). Values are means ± S.E. N = 3 for Groups A, B and D, and 4 for Group C.

Results

Equilibrium [^{3}H]ryanodine binding to RyR in whole homogenates (WH) of infant and adult human heart atrial tissue Maximal ryanodine binding (B_{max}) and the dissociation constant of ryanodine (K_d) in the atrial tissues of the infant (A) and each of the adult groups (B–D) were calculated after assaying equilibrium [^{3}H]ryanodine binding to the WH (Fig. 1). B_{max} in the infant group was similar to those in the adult groups. On the other hand, the K_d value was significantly higher in the infant group, suggesting that the functional state but not the density of RyR in infant myocardium could be different from that in adult myocardium. It is interesting to note that B_{max} and K_d values were similar in all the adult groups (B–D).

Oxalate-supported Ca^{2+} uptake into SR in WH of infant and adult human atrial tissues To investigate the functions of SERCA2a in the atrial SR of the infant (A) and adult groups (B–D), oxalate-supported Ca^{2+} uptake was measured in the presence and absence of 500 μM ryanodine (Table 2). The presence of 500 μM ryanodine ensured that the rate of oxalate-supported Ca^{2+} uptake into the SR was a function of SERCA activity, because 500 μM ryanodine blocks Ca^{2+} efflux through the RyR. The rate of Ca^{2+} uptake into the SR in the presence of 500 μM ryanodine was lower in the infant group (A) than the adult groups (B–D), all of which had similar Ca^{2+} uptake rates (B–D). Furthermore, ryanodine sensitive Ca^{2+} uptake (calculated by subtraction of the Ca^{2+} uptake measured in the absence of ryanodine from that measured in the presence of ryanodine), was similar in all four age groups (Table 2), suggesting that the activity of the RyR parallels the maximal ryanodine binding capacity (B_{max}) shown in Fig. 1.

[^{3}H]PN200-110 binding to WH of infant and adult human heart atrial tissues The radiolabeled dihydropyridine analog, [^{3}H]PN200-110, has been widely used for measuring equilibrium dihydropyridine binding to DHPR (Glossmann and Ferry, 1985). The density of DHPR in the human heart atrial tissues was deduced from [^{3}H]PN200-110 binding to WH (Fig. 2). To obtain the maximal binding of [^{3}H]PN200-110, the experiments were conducted at

Table 2. Rates of oxalate-supported Ca^{2+} uptake in WH of human heart atrial tissues of the four age groups (unit: nmol/mg protein/min).

Group	With 500 μM ryanodine	Without 500 μM ryanodine	Ryanodine-sensitive Ca^{2+} uptake
A	3.041 ± 1.537*	1.888 ± 0.839*	1.15 ± 0.18
B	5.987 ± 1.215	5.114 ± 0.959	0.87 ± 0.29
C	5.897 ± 1.054	4.735 ± 0.775	1.16 ± 0.24
D	5.759 ± 1.448	4.692 ± 1.158	1.07 ± 0.19

The rates of oxalate-supported Ca^{2+} uptake were calculated by linear regression of Ca^{2+} uptake determined at 1, 2, 3, 5 and 7 min. Values are means ± S.E.

*, Significantly different from Group B, C or D ($P < 0.05$), according to one-way ANOVA test.

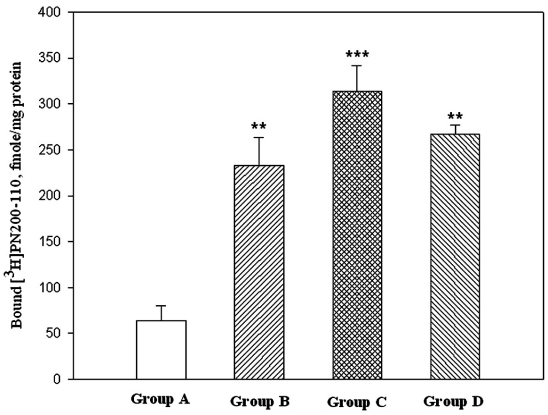

Fig. 2. [^{3}H]PN200-110 binding to WH of human heart atrial tissues in the different age groups. [^{3}H]PN200-110 binding was conducted in 250 μl reaction mixtures containing atrial tissue WH (100 μg protein/250 μl), 20 mM MOPS, pH 7.4, 10 μM $CaCl_2$, 5 nM [^{3}H]PN200-110 for 1 h at 23°C. To determine non-specific binding, a 1000-fold excess of unlabelled nifedifine was included. The reactions were stopped by the addition of PEG solution. After centrifugation at 14,000 rpm for 5 min, the radioactivity in the pellets was measured. ** Significantly different from Group A at $P < 0.01$ and *** at $P < 0.001$, according to one-way ANOVA. N = 3 for all groups.

5 μM [^{3}H]PN200-110, which is substantially higher than the reported K_d of [^{3}H]PN200-110 binding to human heart DHPR (e.g. 0.28 nM) (Glosmann and Ferry, 1985). The [^{3}H]PN200-110 binding values in the adult were higher than that in the infant group, suggesting that the infant myocardium is less responsive to extracellular depolarization stimuli. All the adult groups gave similar [^{3}H]PN200-110 binding values (Fig. 2).

Levels of calsequestrin, triadin and junctin in WH of infant and adult human heart atrial tissues The involvement of a quaternary protein complex consisting of

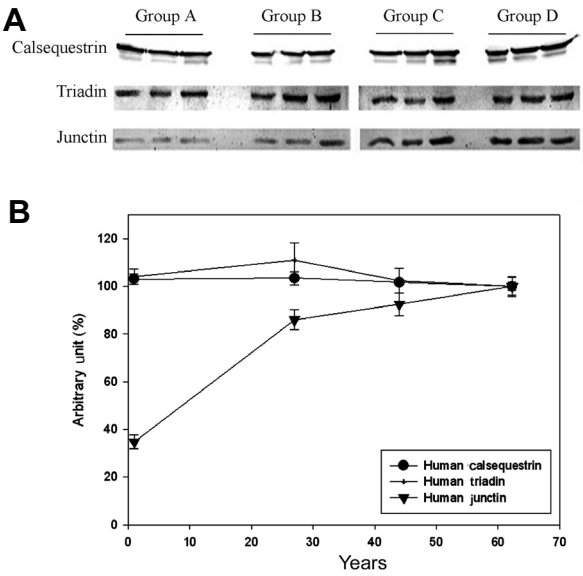

Fig. 3. Expression levels of several SR proteins in human heart atrial tissue WH in the different age groups. The levels of calsequestrin, triadin and junctin in WH of human heart atrial tissues in the four age groups were determined by immunoblotting after 12% SDS-PAGE and transfer to NC membranes (A). Band intensities (arbitrary units) quantified by densitometry (B). The apparent molecular weights of calsequestrin, triadin, and junctin were approximately 55, 43 and 26 kDa, respectively.

RyR, calsequestrin, triadin and junctin in ECC has been proposed (Lee *et al.*, 2004; Zhang *et al.*, 1997). In order to assess the role of the quaternary SR proteins in the infant and adult human hearts, the levels of calsequestrin, triadin, and junctin were determined by immunoblotting (Fig. 3). As shown in Fig. 3A, levels of calsequestrin (55 kDa) and triadin (43 kDa isoform) were similar in all the groups, whereas the level of junctin (26 kDa) was higher in the adult groups (B-D). A higher level of expression of junctin in older animals was also found in rat heart (data not shown). These results indicate that of the proteins of the quaternary complex, only junctin increases with age.

Discussion

In this report, we have identified differences and similarities between the E-C coupling proteins of infant and adult human heart tissues. The infant tissues had lower SERCA2a, DHPR and junctin, whereas the amounts of RyR, calsequestrin and triadin were similar at all ages. These differences between the key ECC proteins may be related to the reported differences in Ca^{2+} handling and force generation between neonatal and adult hearts (Kaufman *et al.*, 1990; Klitzmer and Friedman, 1989; Ostadalova *et al.*, 1993; Rowland and Gutgesell, 1995).

The use of heart samples from healthy people of differ-

ent ages to study SR proteins is extremely difficult. Therefore, in the present study we used samples of human heart tissue that had been removed during hospital cannulation procedures for cardiopulmonary bypass. Although the samples were derived from patients with various types of heart diseases (refer to Table 1), the location of the tissues sampled (atrial appendage), the nature of the diseases, and the relatively high number of samples (total 31) suggest that the apparent differences in ECC proteins are true age-related changes. We saw no clear signs of myocardial hypertrophy in the atrial appendages that could affect the expression of the SR proteins (Kim *et al*., 1994).

To examine the properties of the RyR in infant and adult heart tissues, we conducted equilibrium ryanodine binding studies (Fig. 1). The number of RyR in the infant and the adult groups did not differ, as evidenced by the similar B_{max} values. However, the apparently lower ryanodine binding affinity in the infant group suggests that the mechanism of regulation of the RyR is somewhat different in the infant and adult groups, since the frequency of RyR channel opening could alter the apparent ryanodine binding affinity (Meissner, 1994).

In animal models, it has been shown that the role of SR SERCA2a in sequestering cytosolic Ca^{2+} gradually increases, and that of the Na^{+}-Ca^{2+} exchanger decreases during maturation of heart the (Blaustein and Lederer, 1999). In the present study, we examined whether the participation of SERCA2a changes during the maturation of the human heart (Mahony, 1996). Our measurements of oxalate-supported Ca^{2+} uptake into the SR showed that the infant group had a lower Ca^{2+} uptake capacity than the adult groups (B-D), consistent with previous reports from animal experiments (Mahony, 1996). The lower contractile force generated in neonatal hearts may be related to the slower rates of Ca^{2+} uptake (Mahony, 1984).

The densities of DHPR, the key protein in the ECC process, were deduced from equilibrium [^{3}H]PN200-110 binding to the human atrial tissues. Binding was lower in the infant group than the adult groups, with no differences among the adult groups (B–D) (Fig. 2). This result is in agreement with the report that the level of DHPR mRNA increases during the transition from neonate to adult hearts (Qu, 2001).

A quaternary complex of calsequestrin, triadin and junctin together with RyR may regulate Ca^{2+} release in the SR (Jones *et al.*, 1995; Lim *et al.*, 2000; Mackrill, 1999; Zhang *et al.*, 1997). The levels of these three proteins in the atrial tissues were investigated by Western blotting (Fig. 3). The levels of calsequestrin and triadin did not change with age, whereas that of junctin was higher in the adult groups. In the light of the evidence that junctin is involved in regulating cytosolic Ca^{2+} homeostasis, as well as gene expression (Hong *et al.*, 2002), our data suggest that junctin is associated with maturation of the ECC during neonatal development. However, its role remains to be elucidated. It is important to note that a similar age-dependent increase in junctin expression was found in rat myocardium.

The constant activities and expression levels of the E-C coupling proteins from the twenties to the sixties (Figs. 1–3) suggest that there is no clearcut deterioration in cytosolic Ca^{2+} regulation over this age range. In a previous study of "senior" (mean age 46) and "elderly" (mean age 70) human hearts (Cain *et al*., 1998), it was found that the functional state of the human myocardium in the "elderly" was impaired and that SR SERCA2a and the ratio of SR SERCA2a to either phospholamban or calsequestrin was lower. Taken together, these findings suggest that additional physiological and biochemical alterations of ECC proteins may occur in later life.

We have demonstrated molecular differences in human heart ECC proteins between the four age groups with particular attention to a comparison between the infant and adult groups. The molecular differences in the key ECC proteins are interesting in relation to the age-related differences in Ca^{2+} handling and contractility found in the human myocardium. However, elucidation of the genetic regulation of the ECC proteins during the period of maturation must await further molecular studies such as analysis using DNA arrays (Kim *et al*., 2004).

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