ภาคผนวก

ประกอบด้วย manuscript จำนวน 2 เรื่องได้แก่

1. Age-related expressions of parvalbumin in the female rat heart
2. Influences of aging and long-term swimming exercise on the expression of parvalbumin in rat hearts

Age-related expressions of parvalbumin in the female rat heart

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Short title: parvalbumin in the female rat heart

ABSTRACT

Changes of parvalbumin (PV) expressions during the postnatal development of the female rat heart were investigated in order to determine if they correlated with the age-related changes of the heart function. Newborn, 3-month (young), 6-month (young adult) and 12-month (adult) female Wistar rat's heart were processed for immunohistochemistry and Western blotting assay. PV was detected, by both methods, in all age groups from newborn to 12-month old rats but was very low compared to that in EDL fibers. However, in the newborn rat heart, PV immunoreactivity did not fully fill the cytoplasm of the cardiac myocytes and the PV expression was low (60.14±9.98%) compared to the adult level. In contrast with 3-month to 12-month animals, strong PV immunoreactivity was detected throughout the cytoplasm of all cardiac myocytes and the expression of PV increased with increasing age: 3-month (76.90±9.75%), 6-month (86.60±10.69%) and 12-month (100%). Our study indicates that an increase of PV in the female rat heart with increasing age (from newborn to adult) may be associated with maintaining proper relaxation of the cardiac myocytes that is needed to cope with an increasing workload of the heart during body growth.

KEYWORDS: Female rat heart; Parvalbumin; Calcium-binding protein; Age-related postnatal development; Immunohistochemistry; Western blotting

INTRODUCTION

Parvalbumin (PV), is a low molecular weight protein (12 kDa) that belongs to the EF-hand family of calcium (Ca^{2+}) binding proteins. It has two metal binding sites, with high affinity for calcium and moderate affinity for magnesium[1]. PV is found in high concentration in fast-contracting skeletal muscle fiber[2] and its role there has been postulated to be a relaxing factor capable of removing Ca^{2+} ions from the myofibrillar Ca^{2+}-binding subunit of troponin, troponin C and then transporting the Ca^{2+} ions to the sarcoplasmic reticulum (SR)[3,4,5]. Due to its Ca^{2+} affinity, PV functions as an ATP-independent Ca^{2+} sink and enhances relaxation in skeletal muscle[6,7]. PV is therefore considered as a target protein for diastolic heart failure for which currently, there is no specific treatment [8]. Diastolic dysfunction is characterized by a prolonged relaxation that is typically the result of a decreased rate of intracellular Ca^{2+} sequestration[9].

PV was recently identified in rat heart tissue[10] and it has been suggested that PV is involved in mediating relaxation in cardiac myocytes[8, 11-14]. A mechanism by which PV mediates cardiac myocytes relaxation has been proposed by Coutu et al.[13]. He explained that at late diastole, intracellular Ca^{2+} levels are low and PV is mainly bound to Mg^{2+} whereas at systole, intracellular Ca^{2+} levels rapidly increase resulting in some of the metal-binding sites on PV switching from binding Mg^{2+} to binding Ca^{2+}. However, unbinding of Mg^{2+} from PV is relatively slow, thus Ca^{2+} released at stimulation is bound primarily to TnC leading to activation of cardiac myocytes contraction. At the end of systole, Ca^{2+} dissociates from TnC and binds to PV resulting in activation of cardiac myocytes relaxation. At mid-diastole to late diastole, intracellular Ca^{2+} levels decrease, the metal-binding sites on PV switching back from binding Ca^{2+} to binding Mg^{2+} again. Therefore, changes of parvalbumin levels may affect heart function. According to the work of O'Mahony et al.[15], women had proportionately more diastolic dysfunction heart failure than men, whereas men have more heart failure caused by systolic dysfunction than women[16]. Up to now, the underlying mechanisms remain to be clarified. The expression of PV may be associated. However, no information on the expression of parvalbumin in the female rat heart at different stages of maturation has been available. Therefore, this study aims to investigate expression of PV in the female rat heart at different stages of maturation (from newborn to adult). This may help to explain how the female cardiac response adapts to cope with the increasing workload of the heart during body growth. This data would have important clinical implications for the treatment of cardiovascular disease in women.

MATERIALS AND METHODS

Animals

Ten female Wistar rats at each of the following ages: newborn, 3-months-old (young), 6-months-old (young adult) and 12-months-old (adult) were obtained from the Animal Unit, Faculty of Science, Prince of Songkla University, Songkhla, Thailand. The maturity stage was assigned according to the work of Narayanon[17]. The experimental protocols described in the present study were approved and guided by the Animal Ethical Committee of the Prince of Songkla University for care and use of experimental animals. Rats were anesthetized by an intraperitoneal injection of 75 mg/kg pentobarbital sodium. Hearts were then removed and divided into two halves. Only the left and right ventricles were used in this study. The Extensor digitorum longus (EDL) muscle was also removed, due to it being known to have a high parvalbumin content[2] and divided into two halves, to be used as positive controls. The ventricles and EDL were processed for immunohistochemistry and Western blotting.

Immunohistochemistry

Tissues were fixed in 10% formalin and processed for paraffin wax embedding. Serial 5 μm-thick sections were cut and mounted on TESPA- coated slides. The immunohistochemistry method has been described previously[10]. Briefly, after deparaffinization and dehydration, the sections were incubated sequentially with 0.3% (v/v) Triton X-100 for 30 min, 3%(v/v) H_2O_2 in methanol for 30 min, 10% (v/v) normal horse serum (Vector Laboratories, Burlingham, CA) 60 min, and then with anti-parvalbumin mouse monoclonal antibody (Parv-19, Sigma) at a dilution of 1:1,000, for 48 h, at 4°C. The specificity of the antibody has been described previously[10, 18-20]. The sections were then incubated with biotinylated secondary anti-mouse IgG antibody (Vector Laboratories), at a dilution of 1:200, for 2 h, washed in 0.1M Tris phosphate buffer, pH 7.4, 3 times for 5 min and incubated for 2 h with avidin-biotin complex. Immunoreactive sites were revealed using the diaminobenzidine (DAB) chromogen-based visualization system (Vector Laboratories). Negative controls were performed by omitting the primary antibodies.

Western blotting

Rat hearts and EDL were freshly removed and washed with phosphate buffered saline. The heart and EDL cell lysates were prepared using a pestle with mortar and lysis buffer. The cell lysates were centrifuged at 14000 X g, 4°C for 10 min. The supernatant fractions were separated and the protein concentrations were determined by the DC protein assay (BIO-RAD, Laboratories, Hercules, CA, USA). 20 μg of varied age lysate samples were separated by SDS-

polyacrylamide gel (12%) electrophoresis and transferred onto nitrocellulose membranes. Blots were treated with anti-parvalbumin (Parv-19, Sigma) (1:1000) as primary antibody, followed by horse radish peroxidase conjugated anti-mouse IgG (1:5000) as the secondary antibody. The parvalbumin proteins were visualized using a chemiluminescence method (Amersham Bioscience). Band intensities were measured with a BIO-RAD model GS-700 Imaging Densitometer (Bio-Rad). Parvalbumin immunoreactivity of each group of animals was expressed as percentage of the signal measured in the adult (12-month old) animals. 10 repetitions of the experiment were performed. Therefore, the data were reported as means of the indicated number ± standard error mean (SEM).

Statistical analysis

Statistical analysis was performed by One-way ANOVA to determine whether there were any significant differences among multiple groups of data. When there was a significant difference, a Least-Significant-Difference test was performed to determine which of the individual groups were different. P-Values < 0.05 was considered significant.

RESULTS

PV immunoreactivity in skeletal muscle and heart tissue

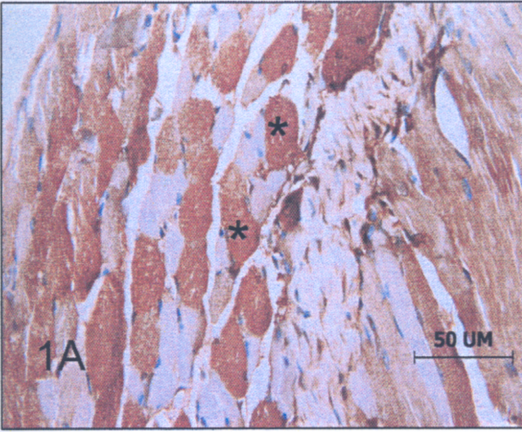
PV immunoreactivity was identified in the cytoplasm of EDL muscle fibers and heart tissue (Fig. 1A and B). Each fiber of EDL had different labeling intensities: strong, moderate and weak intensities (Fig. 1A). In the heart tissue (ventricular wall) composed of cardiac myocytes, connective tissue and blood vessels showing strong PV immunoreactivity was identified in cardiac myocytes whereas in blood vessels and fibroblast, no PV immunoreactivity were found (Fig. 1C and D). Identical PV immunoreactivity was observed in all cardiac myocytes (Fig. 1 B-D).

PV immunoreactivity in heart tissue at different ages

In the newborn, cardiac myocytes showed different features from those of the 3, 6 and 12-month groups. The newborn cardiac myocytes were spindle-shaped with a central and clear cytoplasm and PV immunoreactivity did not fully fill the cytoplasm (Fig. 2A). In contrast, the cardiac myocytes from 3-12 month old animals were cylindrical in shape, branched and exhibited striations in their cytoplasm. In addition, strong parvalbumin immunoreactivity was detected throughout the cytoplasm of all cardiac myocytes (Fig. 2B-D). The intensity of parvalbumin immunoreactivity was found to increase with increasing age (Fig. 2B-D). No PV immunoreactivity was identified in negative controls (data not shown).

Quantification of parvalbumin by Western blotting

Parvalbumin was detected in heart tissue of rats of all age groups. However, the expression of parvalbumin in the heart tissue was low compared to that of EDL (Fig. 3A). Densitometric quantification of the blots of parvalbumin revealed that there was a statistically significant difference in parvalbumin expression among different age groups ($p<0.05$). The expression of parvalbumin was low in the newborn rat heart ($60.14 \pm 9.98\%$) and then increased in hearts from 3-month ($76.90 \pm 9.75\%$), 6-month ($86.60 \pm 10.69\%$) and 12-month (100%) animals, as illustrated in Figure 3B. A significant increase of parvalbumin expression was detected in 6-month and 12-month compared to the newborn groups ($p<0.05$) (Fig. 3B).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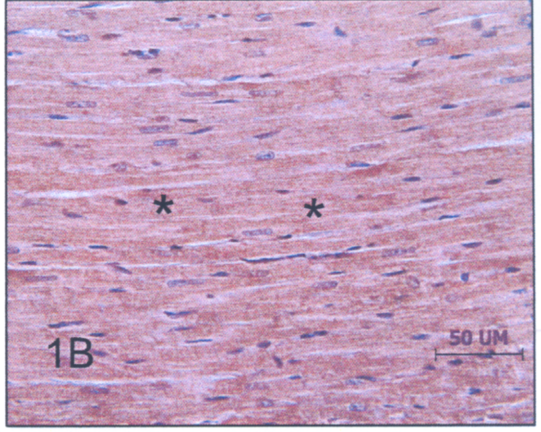
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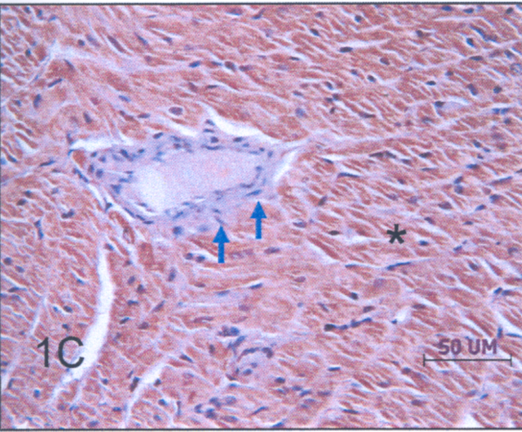
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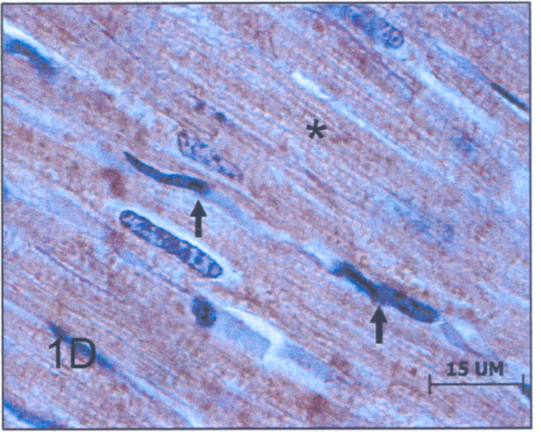
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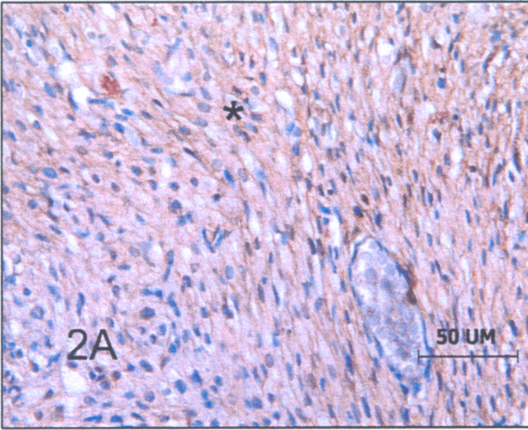
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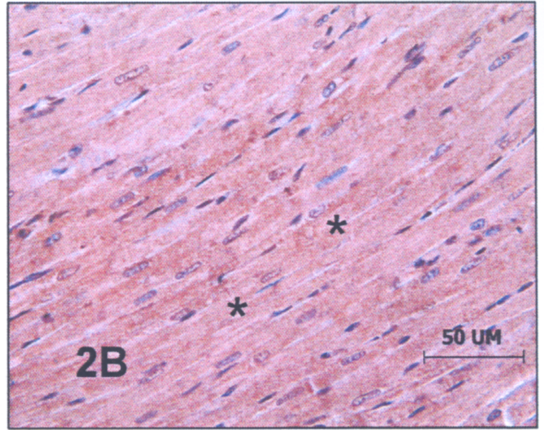
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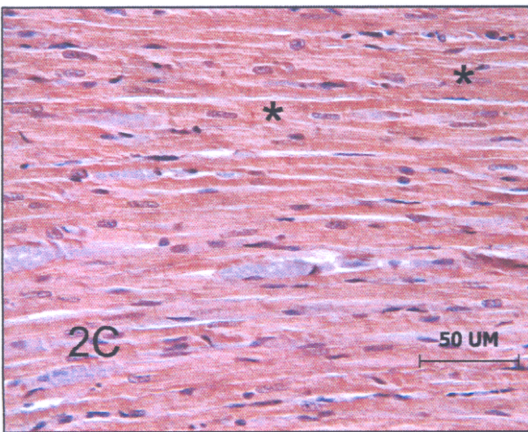
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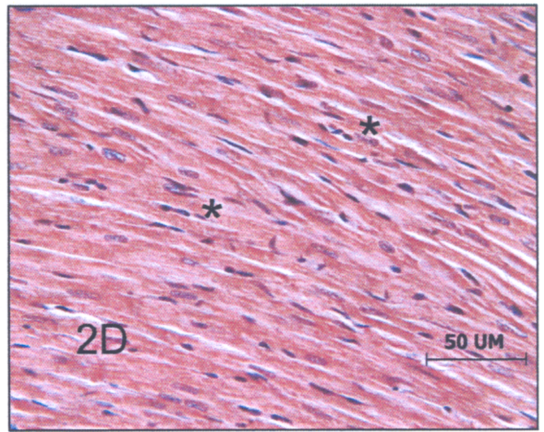
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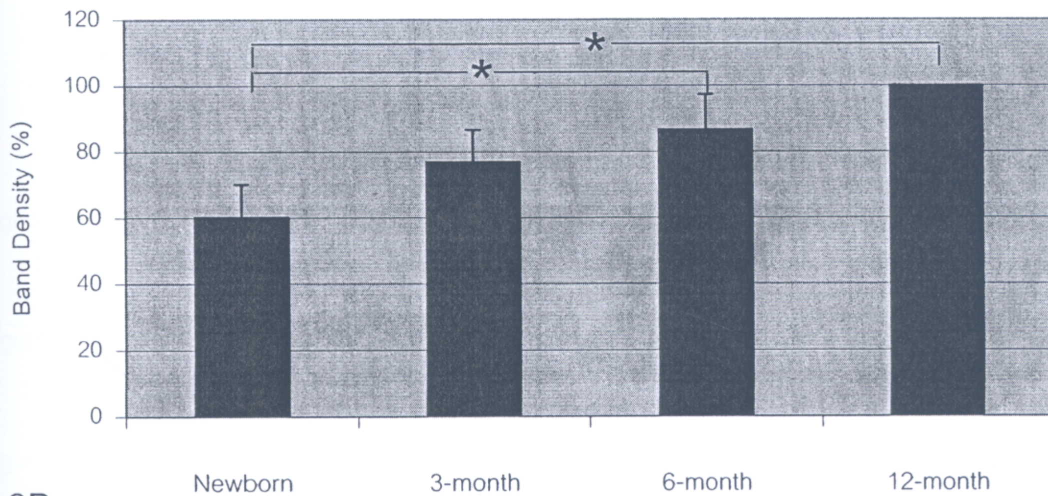
3A
EDL
Newborn
3-month
6-month
12-month


120
100
80
60
40
20
0
Band Density (%)
*
*
Newborn
3-month
6-month
12-month
3B

Legends to Figures

Figure 1. (A) 3-month old rat EDL section showing strong PV immunoreactivity in the cytoplasm of some muscle fibers (*).

(B) 3-month old rat heart section showing strong PV immunoreactivity in the cytoplasm of all cardiac myocytes (*).

(C) Cross section of 3-month old rat heart section showing strong PV immunoreactivity in the cytoplasm of all cardiac myocytes (*) and no PV immunoreactivity in blood vessel (blue arrows).

(D) Higher magnification of heart section showing strong PV in the cytoplasm of all cardiac myocytes and no PV immunoreactivity in fibroblasts (black arrows).

Figure 2. The PV immunoreactivity in the cytoplasm of cardiac myocytes (*) at different ages; (A) newborn, (B) 3-month, (C) 6-month and (D) 12-month rat hearts.

Figure 3A. Western blotting showing expression of PV in EDL and rat hearts at different ages: newborn, 3-month, 6-month and 12-month.

Figure 3B. Histogram showing densitometric quantitation of blots of PV in rat hearts at different ages: newborn, 3-month, 6-month and 12-month. * significantly different, $P<0.05$

DISCUSSION

Our results from Western blotting and immunohistochemistry identified a different pattern of PV expression and immunoreactivity between fast-twitch skeletal muscle fibers (EDL) and heart tissue. Our data demonstrated a high expression of PV in EDL that correlated to the previous study using HPLC (High performance liquid chromatography) that showed high concentrations of PV in EDL[2]. The high concentration of PV in EDL explains its ability to relax rapidly. This may be related to its functional ability. In the muscle that functions for fine movements, the contraction and relaxation process must be highly coordinated. Thus extensor muscles relax fairly rapidly when the flexor muscle contracts. However, each fiber of the EDL showed different intensities of PV immunoreactivity whereas all cardiac myocytes had a similar pattern of immunoreactivity in the cytoplasm, indicating that they all might contain a similar concentration of PV. If this is the case, then all activated cardiac myocytes may operate simultaneously and this may be important for rhythmic function, in that all contractile units have synchronized activity to produce an effective cardiac output.

In rat skeletal muscle, PV was identified 4 days after birth[21] whereas in this study, PV was detected in heart muscle at birth. Leberer and Pette[22] suggested that PV synthesis in rat skeletal muscle is related to their immediate use after birth. However, the heart was already functioning before birth, therefore, it will be interesting to study PV levels in embryonic heart to investigate its role.

As an approach to correct diastolic dysfunction, a PV skeletal muscle gene was transferred to cardiac myocytes. This resulted in an increased PV level in cardiac tissue and an enhanced heart relaxation performance[8,11-14]. With this technique an over-expression of PV in cardiac tissue and possible activation of the immune response may cause potential problems[23]. Thus, the application of this technique for treatment of DHF in humans remains controversial and perhaps a more effective technique is required to prevent possible adverse effects.

From our study, the low level of PV found in the newborn rat heart, may indicate an inefficient diastolic heart function in the newborn. This can be related to the work of Zhou et al[24] who found that the diastolic function matures 3 weeks after birth. In 3 to 12-month rat hearts all cardiac myocytes were mature, and parvalbumin immunoreactivity fully filled the cytoplasm of all cardiac myocytes. As judged from the intensity of parvalbumin immunoreactivity the expression of parvalbumin increased with increasing age. This may explain a higher Ca^{2+} uptake of the sarcoplasmic reticulum in adult compared to the neonatal heart[25] and this could explain an increase in load sensitivity of relaxation of the heart during maturation from the neonatal to adult

rat heart [26]. The up-regulation of PV expression from newborn to adult found in female rat hearts may be a fundamental adaptation of the heart to deal with an increasing workload during postnatal growth. It is likely that the up-regulation occurs in parallel with the increasing function of the neuroendocrine system, especially sex hormone. Thus, it is hypothesized that the PV up-regulation is associated with levels of sex hormone. Previously, it has been demonstrated that the protective effects of ovarian function on the cardiovascular system are largely mediated by 17β-estradiol[27]. According to the work of Cai et al.[28], PV expression in male rat skeletal muscle was down regulated during aging (in 18 and 24 month old). Furthermore, it has been shown that female rats enter menopause between ages 15 and 18 months[29]. This suggests that the down regulation may be sex hormone related. However, the animal used in the present study ranged only from newborn to 12 month old. Therefore, it is of interest to investigate the relationship between sex hormone and PV expression. Thus, the further study is underway to study the PV levels in ovariectomized rats or the rats entering menopause. Measurement of the PV expression in aging or ovariectomized animals could explain some mechanisms of diastolic heart failure in aging women.

ACKNOWLEDGEMENTS

This study was funded by a Thailand Research Funding (TRF) under MRG 4880155. We would like to thank Dr. Brian Hodgson for assistance with the English and Miss Pranom Intasaro for sample preparation.

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Influences of aging and long-term swimming exercise on the expression of parvalbumin in rat hearts

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Abstract

Parvalbumin (PV), a small (12 kDa) cytoplasmic calcium binding protein, has been implicated in mediating relaxation in cardiac myocytes. The influence of aging and exercise on the expression of PV in rat heart was investigated. Male Wistar rats at each of the following ages: 3, 6, 12 and 18-months were divided into sedentary and exercise groups. The exercise group had been trained to swim for 6 months. The hearts were processed for immunohistochemistry and Western blotting. The intensity of PV immunoreactivity (PV-ir) was strong in the 9 and 12-month hearts and decreased in the 18-month hearts. The smallest amount was in the 24-month rat heart when compared to those of the 9,12 and 18 month rat hearts. A significant decrease of PV expression was found at 18-months (49.05 ± 11.98) and 24-months (45.67 ± 4.64) compared to that of the 12-month rat heart (67.67 ± 15.12) ($P < 0.05$). The intensity of PV-ir was obviously stronger in the 9-month, 12-month and 18-month exercised rat hearts than those of the sedentary rat heart whereas, in the 24-month rat heart, PV-ir was slightly stronger in the exercised rat heart than that of the sedentary rat heart. A significant increase of PV expression was identified in the exercised rat heart compared to those of the sedentary rat heart in the 9-month (S= 62.35 ± 16.33, E = 83.20 ± 7.41),12-month (S= 67.67 ± 15.12, E= 87.46 ±15.47) ($P < 0.05$) and 18-month samples (S= 49.05 ± 11.98, E= 82.01 ± 21.11) ($P < 0.01$). Our data indicate that PV expression is down regulated in rat heart during aging. This may explain the diastolic dysfunction which has been predominantly found in the elderly. In addition, our data indicate that long-term swimming exercise could induce an increase of PV expression and this may explain the fundamental mechanism of exercise on improving the aging-induced decrease in cardiac myocyte relaxation.

Key words Parvalbumin, Calcium binding protein, Aging, Swimming, Rat heart

Introduction

Many studies have shown that physiological aging induces a decrease in cardiac functions (Rengo et al. 1991). In several mammalian species including humans, it has been demonstrated that aging is related to impaired cardiac relaxation (Weisfeldt 1975; 1980). In addition, prolonged relaxation is identified in senescent myocytes (Weisfeldt 1998). This impairment in diastolic function is a hallmark of congestive heart failure in the elderly (Haney et al. 2005). Diastolic heart failure represents one of the major causes of morbidity and mortality in the elderly population (O'Mahony et al. 2003; Kitzman 2002). It is identified by a prolonged relaxation resulting in impaired filling and a depression in stroke volume (Lorell 1991; Mandinov et al. 2000; Zile and Brutsaert 2002). According to the work of Gwathmey et al. (1987), it has been shown that in human cardiac tissue samples obtained from diastolic heart failure patients, the duration time required to remove calcium (Ca^{2+}) from the myoplasm is prolonged.

Parvalbumin (PV), a small (12kDa) cytoplasmic calcium binding protein, plays an important role as a relaxing factor in fast-twitch skelétal muscle by acting as a calcium sink to temporarily bind calcium before uptake by the sarcoplasmic reticulum (SR) (Muntener et al. 1995; Hou et al. 1993; Lannergren et al. 1993). This process has the additional advantage that it is a non- ATP dependent process (Gerday and Gillis 1976; Haiech et al. 1979; Gillis et al. 1982). PV is therefore a target protein for the treatment of human diastolic heart failure. Recent, research has identified PV in the heart tissue of various species: rat (Inaguma et al. 1991; Vongvatcharanon and Vongvatcharanon 2003; Vongvatcharanon et al. 2006), mouse, chicken, rabbit, pig (Vongvatcharanon et al. 2008) and it has been demonstrated that PV is implicated in mediating relaxation in cardiac myocytes (Coutu et al. 2003). In an aging rat, down regulation of PV expression has been identified in fast-twitch skeletal muscles (Cai et al. 2001). In addition, from previous studies, up regulation of PV expression has been demonstrated in the adult rat heart (Vongvatcharanon et al. 2006). However, there has been no information on PV expression in the rat heart during aging. This information may be useful for explaining the pathology of diastolic dysfunction which has been predominantly found in the elderly.

It is well known that chronic endurance exercise training improves cardiac functions (Barnard et al. 1980; Schaible and Schever 1985; Jin et al. 2000). Normally, diastolic filling is reduced in aging, however, exercise training was found to increase diastolic filling in healthy young and old men (Levy et al. 1993). The role of exercise training in improving diastolic function is unclear. It has been demonstrated that exercise training improves the aging-induced decrease in myocardial contraction and relaxation (Levy et al. 1993; Li et al. 1986; Starnes et al. 1983). In rat, swimming exercise has been demonstrated to induce cardiovascular responses especially, by inducing an increase in the left ventricular end-diastolic volume and much more than did a running exercise (Geenen et al. 1988). Therefore, the purpose of the present studies was to evaluate the

effect of aging and long-term swimming exercise on the expression of PV in the rat heart. The results from this study may explain the pathology of diastolic dysfunction in the elderly and may have an important clinical implication for preventing diastolic heart failure.

Materials and methods

Animals

Twenty male Wistar rats at each of the following ages: 3-months-old (young), 6-months-old (young adult), 12-months-old (adult), 18- months-old (middle aging) were obtained from the Animal Unit, Faculty of Science, Prince of Songkla University, Songkhla, Thailand. The maturity stage was assigned according to the work of Narayanon (1987). The experimental protocols described in the present study were approved and guided by the Animal Ethical Committee of the Prince of Songkla University for the care and use of experimental animals. All rats were given standard rat chow and tap water *ad libitum* and were housed at 23± 2^0 C on a 12 h dark and 12 h light cycle. The rats from each of the different age groups were divided into two groups, a sedentary group (n= 10) and an exercise group (n= 10).

Training program

The swimming protocol initially started at 5 min/day and was gradually increased by an additional 5 min/day. Swimming frequency was three days/week for a total duration of six month. The rats swam in groups of five animals, because it has been demonstrated that the intensity of swimming exercise was significantly raised by any interaction among the rats (Iemitsu et al. 2004), in a 60-cm-deep tub and a surface area of 2,830 cm^3 with water temperature maintained at 32-34^0 C, and were towelled dry, and kept warm by lighting after each exercise section. According to the work of Matsumoto et al (1996), a forced-swimming apparatus, a swimming pool with a pump (type C-P60H, Hitachi, Tokyo, Japan) that generated circulating currents, was developed to standardize the workload and reduce the swimming time for endurance swimming. Thus, after 2-month, the rats swam in a circulating water tank which had been modified for them. Measurement of the maximum swimming time was evaluated in each age group according to the work of Matsumoto et al (1996). Briefly, the rats were made to swim in groups of five at a time until fatigue, defined by the failure to rise to the surface of the water to breathe within a 7-s period. A different time for the forced swimming were applied to the different age groups. 20 min/day for 3 and 6 month old rats, 15 min/day for 12-month old rats and 10 min/day for 18 month old rats for a total duration of four months. At the end of the exercise period, the rats were 9, 12, 18 and 24 months old.

Tissue preparation

Rats were anesthetized by an intraperitoneal injection of 75 mg/kg of pentobarbital sodium. Hearts were then removed and divided into two halves. Only the left and right ventricles were used in this study. The Extensor Digitorum Longus (EDL) muscle was also removed, due to it being known to have a high parvalbumin content (Heizmann et al. 1982) and this was divided into two halves, to be used as positive controls. The ventricles and EDL were processed for immunohistochemistry and Western blotting.

Immunohistochemistry

Tissues were fixed in 10% formalin and processed for paraffin wax embedding according to routine protocols. Serial 5 μm-thick sections were cut by a microtome and mounted on TESPA-coated slides. The immunohistochemistry method has been described previously (Vongvatcharanon et al 2006; 2008). Briefly, after deparaffinization and dehydration, the sections were incubated sequentially with 0.3% (v/v) Triton X-100 for 30 min, 3%(v/v) H_2O_2 in methanol for 30 min, 10% (v/v) normal horse serum (Vector Laboratories, Burlingham, CA) 60 min, and then with anti-parvalbumin mouse monoclonal antibody (Parv-19, Sigma) at a dilution of 1:1,000, for 48 h, at 4°C. The specificity of the antibody has been described previously (Celio and Heizmann 1981; 1982; Celio et al. 1988; Vongvatcharanon et al 2006). The sections were then incubated with biotinylated secondary anti-mouse IgG antibody (Vector Laboratories), at a dilution of 1:200, for 2 h, washed in 0.1M Tris phosphate buffer, pH 7.4, 3 times for 5 min and incubated for 2 h with the avidin-biotin complex. Immunoreactive sites were revealed using the diaminobenzidine (DAB) chromogen-based visualization system (Vector Laboratories). Negative controls were performed by omitting the primary antibodies.

Western blotting

The Western blotting method has been described previously (Vongvatcharanon et al 2006). Briefly, rat hearts and EDL were freshly removed and washed with phosphate buffered saline. The heart and EDL cell lysates were prepared using a pestle with mortar and lysis buffer. The cell lysates were centrifuged at 14000 X g, 4°C for 10 min. The supernatant fractions were separated and the protein concentrations were determined by the DC protein assay (Bio-Rad, Thailand). 0.5 μg of lystate EDL sample and 20 μg of varied age lysate heart samples were separated by SDS-polyacrylamide gel (12%) electrophoresis and transferred onto nitrocellulose membranes. Blots were treated with anti-parvalbumin (Parv-19, Sigma) (1:1000) as the primary antibody, followed by horse radish peroxidase conjugated anti-mouse IgG (1:5000) as the secondary antibody. The parvalbumin protein bands (12 KDa) were visualized using a chemiluminescence method (Amersham Biosciences). Band intensities were measured with an Imaging Quant TL (v. 2003.03) Densitometer (Amersham Biosciences).

Statistical analysis

The data were reported as means of the indicated number ± standard error mean (SEM). Statistical analysis for comparing the expression of PV in the different age groups was performed by One-way ANOVA to determine whether there were any significant differences among multiple groups of data. When there was a significant difference, a Least-Significant-Difference test was performed to determine which of the individual groups had a different. P-Value and,< 0.05 was considered

significant. For comparing the expression of PV in the non exercise and exercise groups the T-test was performed.

Results

PV immunoreactivity (PV-ir) in the aging rat heart

PV-ir was identified in the cytoplasm of all cardiac myocytes and all cardiac myocytes in any sample had identical intensities of PV-ir (Fig. 1A).The intensity of PV-ir was strong in the 9-month and 12-month rat hearts (Fig. 1A and 2A). The intensity of PV-ir decreased in the 18-month rat heart (Fig. 3A) and the intensity of PV-ir was weakest in the 24-month rat heart compared to those of the 9,12 and 18 month samples (Fig. 4A).

PV immunoreactivity (PV-ir) in the sedentary and exercised rat heart

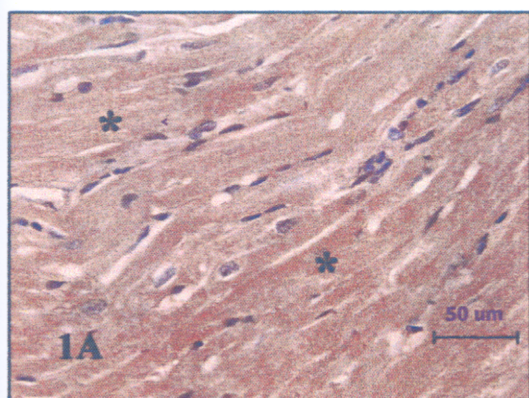
The intensity of PV-ir was obviously stronger in the 9-month, 12-month and 18-month exercised rat heart than those of the sedentary rat heart (Fig. 1A-B, 2A-B and 3A-B). whereas, in the 24-month rat heart, PV-ir was only slightly stronger in the exercised rat heart than that of the sedentary rat heart (Fig.4A and B).

Expression of PV in aging rat heart

The expression of PV was identified in EDL and the rat hearts in all age groups (Fig.5). The expression of PV was not significantly different between 9-month (62.35 ± 16.33) and 12-month (67.67 ± 15.12) samples. On the other hand, a significant decrease of PV expression was found in the 18-month (49.05 ± 11.98) and 24-month samples(45.67 ± 4.64) compared to that of the 12-month rat heart samples($P < 0.05$) (Fig.6). No significant difference of PV expression was observed in the 18-month and 24-month rat hearts.

Expression of PV in the sedentary and exercised rat heart

Expression of PV was higher in the exercised rat heart than in the sedentary rat heart in the 9-month, 12-month, 18-month and 24-month samples (Fig.5). A significant increase of PV expression was identified in the exercised rat heart compared to those of the sedentary rat heart in the 9-month (S= 62.35 ± 16.33, E = 83.20 ± 7.41),12-month (S= 67.67 ± 15.12, E= 87.46 ±15.47) ($P < 0.05$) and 18-month samples (S= 49.05 ± 11.98, E= 82.01 ± 21.11) ($P < 0.01$). No significant differences of PV expression were found in the 24-month sedentary and exercised rat hearts (S= 45.68 ± 4.65, E = 49.92 ± 9.67) (Fig.7).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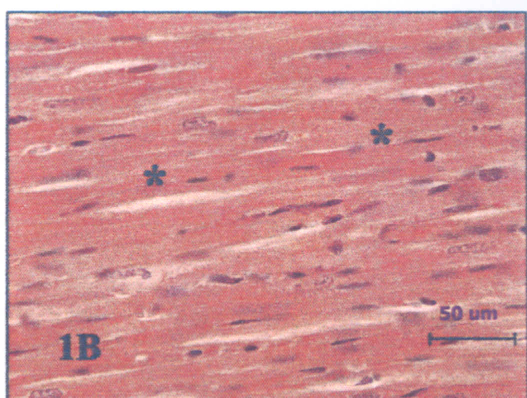
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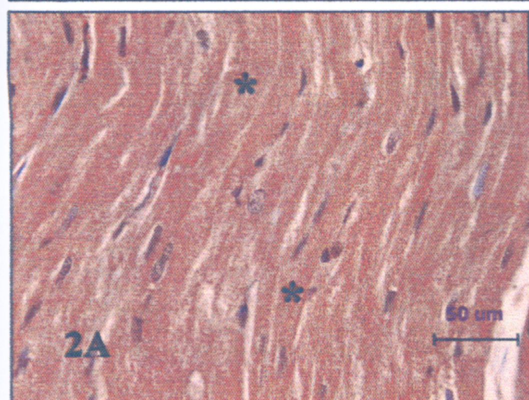
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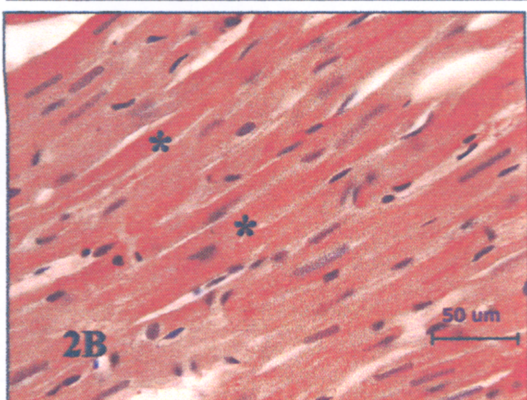
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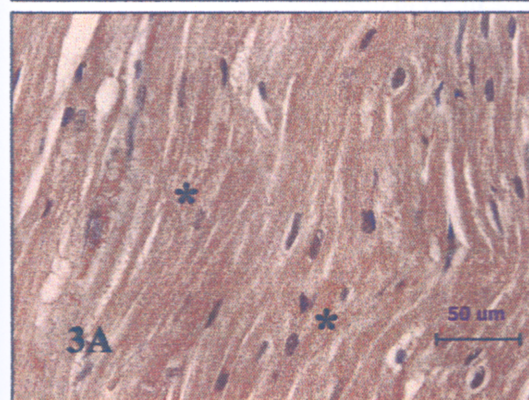
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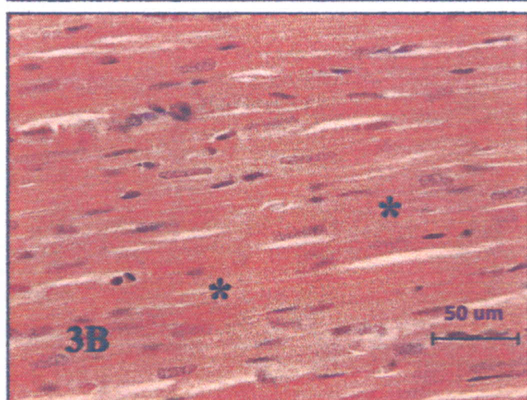
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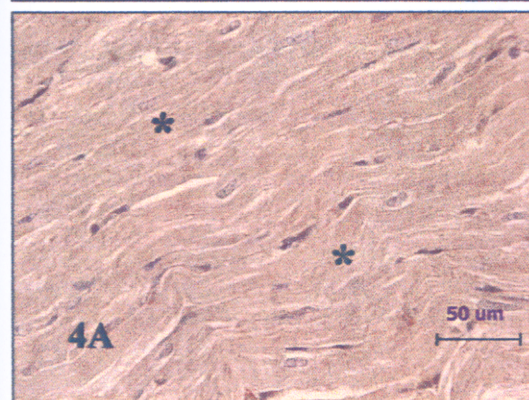
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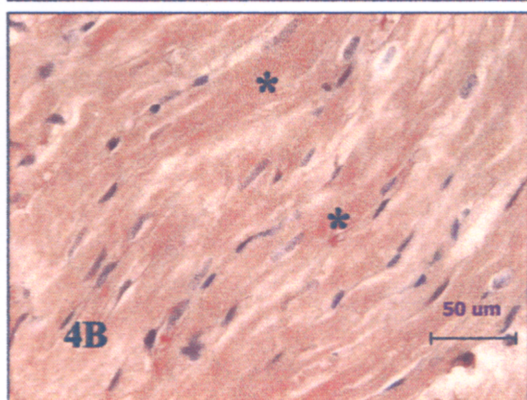
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Expression of PV in aging rat heart

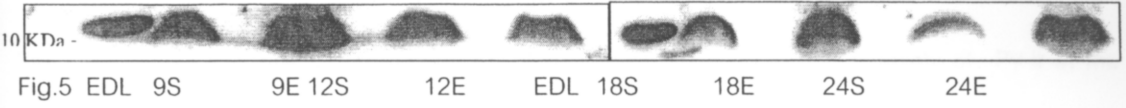

Fig.5 EDL 9S 9E 12S 12E EDL 18S 18E 24S 24E

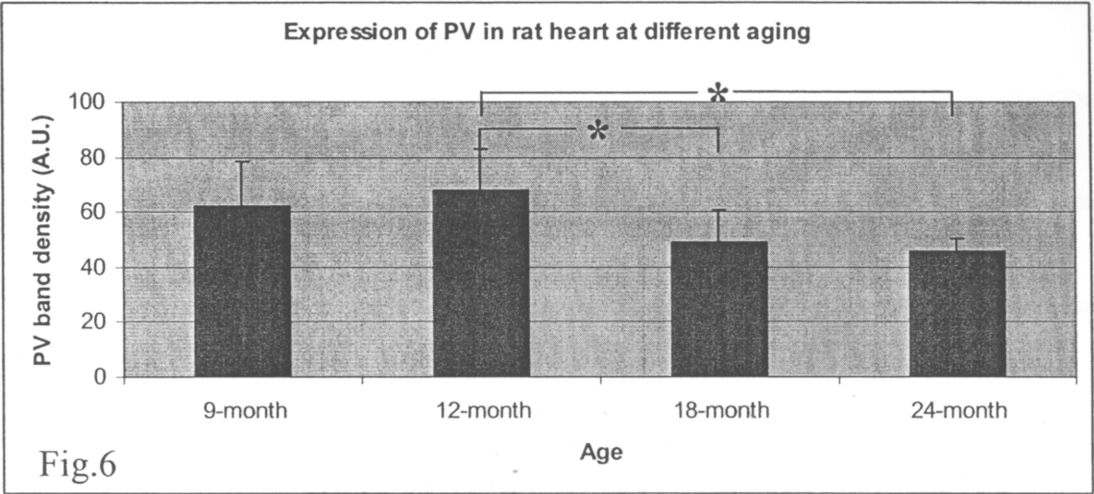

Fig.6

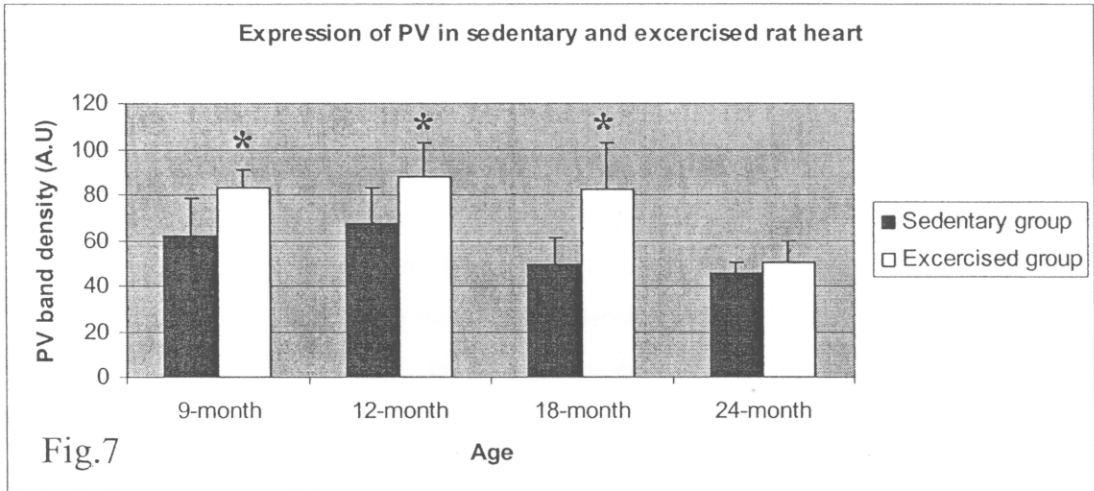

Fig.7

Legends to Figures

Figure 1. (A) 9-month sedentary rat heart section showing strong intensity of PV-ir in the cytoplasm of all cardiac myocytes(*).

(B) 9-month exercised rat heart section showing stronger intensity of PV-ir in cardiac myocytes(*).

Figure 2. (A) 12-month sedentary rat heart section showing strong intensity of PV-ir in the cytoplasm of all cardiac myocytes(*).

(B) 12-month exercised rat heart section showing stronger intensity of PV-ir in cardiac myocytes(*).

Figure 3. (A) 18-month sedentary rat heart section showing strong intensity of PV-ir in the cytoplasm of all cardiac myocytes(*).

(B) 18-month exercised rat heart section showing stronger intensity of PV-ir in cardiac myocytes(*).

Figure 4. (A) 24-month sedentary rat heart section showing weak intensity of PV-ir in the cytoplasm of all cardiac myocytes(*).

(B) 24-month exercised rat heart section showing slightly stronger intensity of PV-ir in cardiac myocytes(*).

Figure 5. Expression of PV in EDL and rat heart in the different groups.

Figure 6. Histogram showing the PV band density (A.U.= arbitrary unit) in different age groups. * significantly different at $P<0.05$, $n=6$

Figure 7. Histogram showing the PV band density (A.U.= arbitrary unit) in sedentary and exercised rat heart in the different age groups.

* significantly different, at $P<0.05$, ** significantly different, at $P<0.01$, $n=6$

Discussion

Expression of PV in aging rat heart

Our data demonstrated that the intensity of PV immunoreactivity decreased in middle age and aging rat hearts. In addition, our data showed the highest expression of PV in the 12-month rat heart and this was reduced at 18- and 24- months. The lowest PV expression was identified at 24-months, so indicating that down regulation of PV expression was found in the rat heart during aging. Our data agree with a previous study by Cai et al. (2001) who showed that PV was down regulated in rat fast-twitch skeletal muscle during aging. In an aging heart, it has been demonstrated that the cells are larger in the left ventricle because there is a drop out of individual cells with age. In humans between the age of 20 and 90, there is a 40% to 50% drop in the total number of nuclei and hence the number of cells in the cardiac myocytes (Olivetti et al. 1991). This indicated an age-associated drop out of individual cells with a compensatory hypertrophy of the remain cell (Weisfeldt 1998). The drop in the total number of cardiac myocytes may reflect down regulation of PV expression in the aging rat heart compared to the adult rat heart.

According to the function of PV as a relaxing factor in skeletal muscle, attempts have been performed to use PV for clinical purpose in the treatment of diastolic heart dysfunction. The PV gene from a rat fast-twitch skeletal muscle was transferred *in vivo* into rat cardiac myocytes to increase PV levels in the cardiac tissue (Wahr et al. 1999; Szatkowski et al. 2001; Coutu et al. 2003; Michele et al. 2004; Schimdt et al. 2004). After transfer of the PV gene, diastolic dysfunction in diseased cardiac myocytes was corrected (Wahr et al. 1999), the speed of heart relaxation performance was increased (Szatkowski et al. 2001) and the *in vivo* diastolic function of an aged myocardium was enhanced (Michele et al. 2004). However, with this technique, activation of an immune response and overexpression are considered as major potential problems (Senior, 2001). Therefore, the potential application of this technique for human therapeutic purpose is still controversial and more effective techniques need to be established in order to prevent possible adverse effects. As a consequence of PV gene transfer, it has been suggested that PV is involved in mediating cardiac myocyte relaxation and the mechanism of PV in mediating cardiac myocyte relaxation was proposed by Coutu et al. (2003). They suggested that in late diastole, intracellular Ca^{2+} levels are low and PV is mainly bound to Mg^{2+}. At systole, intracellular Ca^{2+} levels increase resulting in the metal-bind sites on PV turning from the binding of Mg^{2+} to binding Ca^{2+}. However, the process of unbinding Mg^{2+} from PV is slow, thus the Ca^{2+} released at stimulation would primarily bind to TnC leading to cardiac myocyte contraction. At the end of systole, Ca^{2+} dissociated from the TnC and bound to PV resulting in cardiac myocyte relaxation. At mid to late diastole, intracellular Ca^{2+} levels reduce and the metal binding sites on PV changed back from binding Ca^{2+} to binding Mg^{2+} again. Therefore, a decrease of PV level may lead to prolonging cardiac relaxation. Abnormal cardiac relaxation and diastolic dysfunction have been widely demonstrated in a rat model of

senescence (Weisfeldt 1998; 1980). In addition, in several mammalian species including humans, postmaturational aging is associated with a slower rate of relaxation. The cellular and molecular mechanisms underlying this age-related change have only been partially identified to date. The rat model has been used for a number of studies on age related changes. These have identified an age-related decrease in the Ca^{2+} - sequestering activity. In the aging human heart, a decline in the myocardial sarcoplasmic reticulum content was found to prolong the contraction period by slowing the removal of Ca^{2+} following contraction (Cain et al., 1998). In aged Wistar rat, it has been demonstrated that the time to peak tension and peak shortening is increased and the duration of contraction is prolonged. This is most likely due to a reduced rate of Ca^{2+} uptake by the sarcoplasmic reticulum generated by a decline in either the Ca ATPase content or a reduction in the Ca ATPase activity (Jiang and Narayanon 1990). Thus, a decrease in either the Ca ATPase content or reduction in the Ca ATPase activity and a decrease in PV level in the aging rat heart may explain a reduction in the rate of Ca^{2+} uptake by the sarcoplasmic reticulum and contributes to a prolonged cardiac myocyte relaxation. This may explain the diastolic dysfunction that increases with age and is a hallmark of the aging heart (Schmidt et al. 2004).

Expression of PV in exercised rat heart

From our data, long term swimming exercise could induce up-regulation of PV expression in the rat heart of all age groups. However, the effect of exercise on mediating an increase of PV expression was different among the different age groups. In the 9 and 12-month rat heart, the exercise induced percentage increase of PV expression was lower than that of the 18-month sample (9-month = 133 %, 12-month = 129 % and 18 month =167 %) This may be due to the basal PV expression in the 9- and 12-month samples being already high before exercise whereas in the 18-month sample, down regulation of PV expression was observed before exercise, therefore, exercise could induce a bigger increase of PV expression in the 18-month sample than in the 9-and12-month sample. These data indicate that the age group in which long term swimming exercise could induce an increase of PV expression was at 18-months. This is an important age group with clinical implications for preventing diastolic dysfunction in the elderly. From our data, we suggest that exercise in the young adult, adult and middle aged could induce PV expression better than exercise in the aging group. According to the work of Michele et al. (2004), it was reported that after the PV gene was transferred to the myocardium *in vivo*, the expression of PV is reduced in old rat heart compared to young rats. They explained that the reduced PV expression in the old rat heart compared to the young rat heart was due to morphological changes in the aged myocardium including cardiac hypertrophy. This may impact on the percentage of myocytes that can be targeted by a single injection of adenovirus and any alteration of protein synthesis in the aged myocardium may account for the lower levels of PV expression. It has been demonstrated that the rate of RNA and protein synthesis decreases in the aging group (Meerson et al. 1987). This may explain the low level of PV expression in the aging and

exercised aging groups. In contrast, from previous studies a high intensity running program could induce PV expression in both young and old skeletal muscles (Cai et al. 2001). This may be due to the different training regime of the exercise programs that might involve different tissues. It has been demonstrated that aging produces a decrease in cardiac function, such as cardiac myocytes contraction and relaxation and the risk of cardiovascular morbidity increases in the aged heart (Cappasso et al. 1983; Lakatta 1987). On the other hand, exercise training improves the aging-induced decrease in cardiac myocyte contraction and relaxation (Levy et al. 1993; Li et al. 1986). One explanation is that aging decreases the expression of SR Ca^{2+}-ATPase mRNA in the heart (Maciel et al. 1990). On the other hand, it has been shown that exercise training in aged rats improves the aging-induced decrease in expression of SR Ca^{2+}-ATPase mRNA in the heart (Take et al. 1996). In addition, exercise has been shown to enhance calcium uptake of the cardiac sarcoplasmic reticulum (Take et al. 1990) and could lead to an improvement of cardiac relaxation. Our data has demonstrated the down regulation of PV expression in the aging rat heart and long term swimming exercise induce an increase in PV expression in all age groups (adult, middle age and aging), this may also explain a prolongation of cardiac myocyte relaxation in the aging heart and exercise training improves the aging induced reduction in cardiac myocyte relaxation.

In conclusion, PV expression is down regulated in rat heart during aging and long term swimming exercise could reverse this aging-related change. Our results indicate that long term swimming exercise could be recommended to improve heart function during aging because PV plays an important role in regulating cardiac myocyte contraction and relaxation.

Acknowledgements

This study was financially supported by the Commission on Higher Education and Thailand Research Fund under MRG4880155. We would like to thank Dr. Brian Hodgson at PSU for assistance with the use of English.

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