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Biochemical changes and their relationship with morphological and functional findings in pig heart subjected to lasting volume overload: a possible role of acylphosphatase in the regulation of sarcoplasmic reticulum calcium pump

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■ **Abstract** We evaluated the changes in sarcoplasmic reticulum (SR) function and the parallel hemodynamic and morphological modifications in a heart subjected to volume overload. We also determined the levels of acylphosphatase, a cytosolic enzyme, that could play a regulatory effect on SR Ca^{2+} pump by hydrolyzing the phosphorylated intermediate of this transport system. For this, swine hearts were subjected to volume overload by aorta-cava shunt for 1, 2, or 3 months. Changes in heart contractility reflected modifications of SR function, whose reduction after 1 month of overload was followed by a gradual recovery. A decrease in SERCA2a protein and mRNA content was shown from 1 month and remained for the following 2 months. Phospholamban content and its phosphorylation status were not modified. Acylphosphatase was unchanged at 1 month, but at 2 months this enzyme exhibited an increased activity, protein and mRNA expression. Morphological alterations consisting of the cytoskeletal architectures, intermyofibrillar oedema, swollen mithochondria and abnormality of the membrane system (T-tubule and SR cisternae) were particularly evident after 1 month but almost disappeared after 3 months. These results suggest that our overloaded hearts underwent a substantial recovery of their structural and biochemical properties at 3 months after surgery. A possible involvement of acylphosphatase in the modification of SR function is discussed.

■ **Key words** Cardiac volume-overload – sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) – acylphosphatase – phospholamban – ultrastructure

Introduction

As an adaptive response to biomechanical stress myocardial cells develop a compensatory hypertrophy through the activation of several parallel and/or converging signalling pathways (9). Subsequently, there can be a transition to cardiac chamber dilatation that is associated with a loss of viable myocytes, a decrease in contractile elements, myofilament disarray and interstitial fibrosis. In addition to these modifications, other signalling pathways can induce an impairment of cardiac excitation-contraction coupling and severe defects in cardiac contractile performance that are the clinical hallmarks of the

progression of heart failure (12, 22). Our understanding of the biochemical mechanisms leading to heart failure is still largely incomplete, but it is generally accepted that altered intracellular calcium handling can play an important role in the development of the systolic and diastolic dysfunction which characterizes this disease (14). In this regard, the sarcoplasmic reticulum (SR) function is crucial for the movements of cytosolic Ca^{2+} during each cycle of cardiac contraction and relaxation. It is well known, that Ca^{2+} is released from SR to activate the contraction of heart muscle, while the subsequent re-accumulation achieves relaxation. Cardiac SR Ca^{2+} ATPase (SERCA2a) is a calcium pump whose activity is the rate-determining factor of Ca^{2+} uptake into SR and, indirectly, also of its release, which depends on the amount of calcium stored in this structure: in this way SERCA2a can regulate both cardiac relaxation and contractility. In the action mechanism of SERCA2a Ca^{2+} uptake is tightly coupled with ATP hydrolysis through a series of elementary steps involving the formation and the decomposition of an acylphosphorylated phosphoenzyme (EP) intermediate (33). SERCA2a activity is itself regulated by phospholamban (PLB), a membrane protein whose phosphorylation leads to an enhancement of active Ca^{2+} transport (30). An additional regulatory effect on the SR calcium pump might be exerted by acylphosphatase (ACPase, EC 3.6.1.7), a cytosolic enzyme, well represented in cardiac muscle, that catalyzes the hydrolysis of acylphosphates (6, 26, 28). In previous studies we have shown, in fact, that acylphosphatase can interact with SERCA2a and actively hydrolyze the EP intermediate of this active transport system, an effect that results in enhanced activity of the SR Ca^{2+} pump (23, 24).

Given this background, the goal of the present study was to explore the changes in heart SR function and the role of acylphosphatase related to the parallel hemodynamic and morphological modifications arising in the course of volume overload, a condition that can trigger the above mentioned alterations and lead, ultimately, to heart failure (21, 25). To this aim volume overload was induced by an aorta-inferior cava shunt (31) for 1, 2 and 3 months in swine heart, chosen as a model for our studies since its structural and functional characteristics are similar to those of the human heart.

Methods

■ Experimental model

The experimental protocol described in the study followed the recommendations of the National Research Council's *Guide for the Care and Use of Laboratory Animals.* Farm pigs (n = 16; four for each group), fasted overnight, weighing 30 – 45 kg, were used. Animals were premedicated with intramuscular azaperone (1 mg/kg), ketamine (15 mg/kg) and diazepam (1 mg/kg). Anesthesia was induced with ketamine (0.5 mg/kg) and atropine (0.5 mg/kg) and the pigs were subsequently intubated and ventilated with oxygen supplemented by with 50 % N_2O and fluothane at 1 – 1.5 % Pancuronium bromure (0.1 mg/kg) was given to achieve muscle relaxation at the beginning of the surgical procedure. The abdomen was opened via a midline incision, and the inferior part of the vena cava and abdominal aorta distal to the renal arteries were cleaned of fat and adventitia. The shunt was performed using a Dacron prosthesis (8-mm diameter) which was sutured latero-laterally to the abdominal aorta and to the inferior vena cava using partial occluding clamps. When the anastomosis was performed, the clamps were released, hemostasis was obtained and the abdomen was closed. The animals were kept on antibiotic therapy for the first 5 days. At the time of sacrifice, which was at 1, 2, or 3 months after surgery, the animals were again anesthetized, subjected to hemodynamic and echocardiographic measurements and then killed using humanitarian methods. After sacrifice specimens of myocardial tissue of left ventricle (LV) were taken for biochemical and histological evaluation. Control animals were simply anesthetized for hemodynamic and echocardiographic measurements and immediately sacrificed.

■ Hemodynamic and echocardiographic measurements

Two 6F pigtail catheters were introduced into the left femoral artery and advanced to monitor left ventricular and descending aortic pressure. A Swan-Ganz catheter was advanced from an external jugular vein to the pulmonary artery to measure pulmonary capillary wedge pressure and cardiac output (thermodilution). Two-dimensional and M-mode echocardiographic studies (2.25/3.5-Mhz transducer, SIM 5000) and relative measurements were performed from the right parasternal area and recorded on videotape (20); wall thickness and left ventricular internal dimensions were measured according to the recommendations of the American Society for Echocardiography (29). Left ventricular mass (LVM) and left ventricular volumes were calculated using validated formulas (11, 34). End-systolic stress (ESS) was calculated from echocardiographic recordings in combination with invasive left ventricular pressure (13). The contractile state was evaluated by measuring the ESS/end-systolic volume (ESV) ratio, which has proved useful in previous studies (8). Measurements were analyzed independently by two experienced echocardiographers. Inter-observer and intra-observer variabilities were 4.1 ± 0.5 % and 2.5 ± 0.3 % for cavity size and 3.7 ± 0.4 % and 2.1 ± 0.3 % for wall thickness, respectively.

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■ Ca²⁺-ATPase Activity activity and Ca²⁺ uptake measurements

Sarcoplasmic reticulum vesicles (SRVs), purified from cardiac LV specimens according to Nediani et al. (25), were measured for protein (4) and used to assay Ca^{2+}-ATPase activity and Ca^{2+} uptake.

Ca^{2+}-ATPase total activity was assayed in a standard reaction mixture containing 50 mmol/l Tris-HCl, pH 7.4, 3 mmol/l $MgCl_2$, 100 mmol/l KCl, 5 mmol/l NaN_3, 50 μmol/l $CaCl_2$, 3 mmol/l ATP, 1 mmol/l ouabain and 50 μg/ml vesicle protein. Ouabain and sodium azide were added to inhibit respectively sarcolemmal Na^+,K^+-ATPase and mithochondrial ATPase, possibly present as contaminants in SRV preparations. In these conditions a free Ca^{2+} concentration of approximately 10 μmol/l was calculated using the equations of Katz et al. (16). To determine basal ATPase activity, the assays were carried out in the presence of 1 mmol/l Tris-EGTA instead of $CaCl_2$. Reactions were started by the addition of ATP and stopped after 10 min with 1 volume of ice-cold 20 % trichloroacetic acid. After centrifugation (12,000xg for 5 min), the amount of Pi released was measured by malachite green procedure (3). Ca^{2+}-dependent ATPase activity was calculated by subtracting the basal ATPase activity from the total Ca^{2+}ATPase.

For Ca^{2+} uptake, the reaction mixture was the same as for ATPase assays except that it included $^{45}CaCl_2$ and 5 mmol/l oxalate. After 30 s of incubation at 37 °C, the vesicles were separated from the medium by filtration through a Millipore filter (0.45-μm pore size), and then the filter was immediately washed twice with 4 ml of ice-cold 20 mmol/l Tris-HCl, pH 7.4, 1 mmol/l EGTA, and 100 mmol/l KCl. Oxalate-facilitated ^{45}Ca uptake was measured as the difference between ^{45}Ca influx into vesicle at 0 time and at the end of incubation. Radioactivity trapped on the filter was determined by liquid scintillation spectroscopy.

Table 1 Acylphosphatase, SERCA 2a, phospholamban and GAPDH primers for RT-PCR

Primers	Sequences 5'-3'	cDNA Sizes bp
Acylphosphatase	5' ATGTATACAGAAGATGAAGC 3' ATGGTTTTTTCATTAGAAAAGTT	113
SERCA2a	5' ACAATGGCGCTGTCTGTTCTA 3' AAGCGGTTACTCCAGTATTGC	311
Phospholamban	5' CCAGCTAAACACCGA 3' TATTGGACTTTATCCATG	302
GAPDH	5' ATGGTTTACATGTTCCAATATGAT 3' GTTGTCATACTTCTCATGGTT	311

SERCA2a sarcoplasmic reticulum calcium ATPase class 2a; *GAPDH* glyceraldehyde-3-phosphate dehydrogenase

■ ACPase activity measurement

Cardiac LVs specimens were homogenized with 4 volumes of 0.1 mol/l HCl and centrifuged at 15000xg for 20 min. The supernatants were collected, adjusted to pH 7 and centrifuged at 15000xg for 20 min. The supernatants of this centrifugation were assayed for protein and for activity. ACPase activity was measured by a continuous optical test at 283 nm, using benzoyl phosphate as substrate (27). Benzoyl phosphate was synthesized according to Camici et al. (7).

■ RNA purification, reverse transcriptase (RT)-PCR and quantitative analysis

For RT-PCR studies, total RNA was extracted from tissues using TRIreagent (Sigma-Aldrich, Milan, Italy) and purified according to the manufacturer's instructions. Primers were designed using the published sequence information from GenBank and corresponding to the coding regions of the genes; the sequences of primers and the cDNA sizes (bp) are summarized in Table 1. Reverse transcriptase and PCR reactions were performed with a commercial RT-PCR system (SuperScript One Step, BRL-Life Technologies) on a Hybaid Thermocycler (Teddington, Middlesex, UK) using 125 ng of total RNA. To ensure that an equivalent cDNA template was used in each reaction, amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. The number of cycles was optimized for each message to ensure linearity between PCR amplified product and message and were 30 cycles for ACPase, SERCA2a and PLB and 25 for GAPDH, respectively.

RT-PCR products were resolved on 1.8 % agarose gel and digital images were captured from the stained gel with a videocamera. Quantitative analyses were performed via a Scion Image-Capture 2 board software and density of PCR products at different time points were was normalized over GAPDH expression.

■ Western blot analysis of SERCA 2a, PLB and ACPase

Ten microgram of SRV protein mixtures, boiled for SERCA2a and not boiled for PLB, were separated respectively on 12 % and 15 % SDS-PAGE gels. ACPase separation was performed on the same 15 % gel using 50 μg of boiled supernatant protein mixtures. Then the gels were electroblotted to nitrocellulose membranes and nonspecific binding sites were blocked for 2 h in 5 % BSA T-TBS solution (50 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl, 0.1 % Tween 20) with agitation. For immunoreaction a monoclonal (mouse) anti-SERCA 2a ATPase antibody (Affinity Bioreagents, Inc., Golden, CO), a polyclonal (rabbit) anti-ACPase antibody, purified as previously

described (5, 19) and a monoclonal (mouse) anti-PLB antibody (Upstate biotechnology, Lake Placid, NY) were used. For detection, a second antibody, a peroxidase-coniugated antirabbit IgG or antimouse IgG (Amersham Pharmacia Biotech, Italy) and enchanced chemiluminescence assay (Amersham Pharmacia Biotech, Milan, Italy) were used. After exposure to BIOMAX Light-1 film (Eastman Kodak Company, Rochester, NY), signals were quantified using the program for image analysis and densitometry Quantiscan (Biosoft, Cambridge, UK). The same nitrocellulose used to reveal PLB protein expression was stripped and reprobed with 10 μg/ml monoclonal (mouse)anti-P-serine-16 antibody (Sigma-Aldrich, Milan, Italy) to detect PLB-phosphorylation status.

Before quantification of immunoreactive bands of SERCA2a, PLB and ACPase immunodetection of the separated proteins on western blots was ensured to be in the linear range and electrophoretic transfer was complete in the relevant molecular weight range. The data from densitometric analysis were related to the amount of protein used on the PAGE and were not significantly altered when these values were related to β-MHC proteins levels. Quantitative data are expressed as percentage of control value.

■ Morphological analysis

Myocardial biopsies were taken from the left ventricle wall of each animal, immediately fixed by immersion in cold 2.5 % glutaraldehyde in 0.1 mol/l cacodylate buffer, pH 7.4 at room temperature and postfixed in 1 % osmium tetroxide in 0.1 mol/l phosphate buffer, pH 7.4 at room temperature for ultrastructural analysis. The specimens

were then dehydrated in a graded acetone series, passed through propylene oxide and embedded in Epon 812. For each specimens two sets of semithin sections (2 μm thick) were cut at different levels, stained with toluidine blue sodium tetraborate and observed under light microscope. Ultrathin sections obtained from the same specimens were placed on 200 mesh copper grids and stained with uranyl acetate and alkaline bismuth subnitrate. Approximately a number of 200 tissue sections from control and volume overloaded myocardium were examined under a transmission electron microscope (T.E.M) (JEOL 1010). In particular three independent ultrathin sets (grids) were observed for each biopsies.

■ Statistics

All data are presented as mean ± standard deviation (S.D.). Comparisons between the different groups were performed by ANOVA followed by the Bonferroni t-test. A value of $p < 0.05$ was accepted as statistically significant.

Results

■ Echocardiographic and hemodynamic data

During the whole study period the aorta-cava fistula remained pervious in all the operated pigs and no animal showed signs of heart failure. The data in Table 2 reveal a significant hypertrophy in the overloaded hearts since

Table 2 Hemodynamic and echocardiographic changes after aorta-cava shunt[a,c]

Months	0	1	2	3
Heart rate (beats/min)	90 ± 5	105 ± 3*	98 ± 8	95 ± 3
Cardiac index (mL/kg min)	78 ± 20	143 ± 27*	152 ± 42*	196 ± 10*
Systolic pressure (mmHg)	107 ± 4	108 ± 3	107 ± 2	109 ± 3
LVMi (g/kg)	2.69 ± 0.54	4.54 ± 0.59*	4.97 ± 0.46*	6.08 ± 0.18*º†
ESS/ESVi (kdyne/cm²) (mL/kg)	124 ± 22	87 ± 14*	103 ± 14	106 ± 9
PWP (mmHg)	12 ± 1	20 ± 3*	14 ± 2†	13 ± 1†
RWT	0.42 ± 0.02	0.41 ± 0.03	0.39 ± 0.03	0.36 ± 0.02
Body weight (kg)	35 ± 5	38 ± 4	40 ± 3	43 ± 4

[a] Values are means ± S.D.
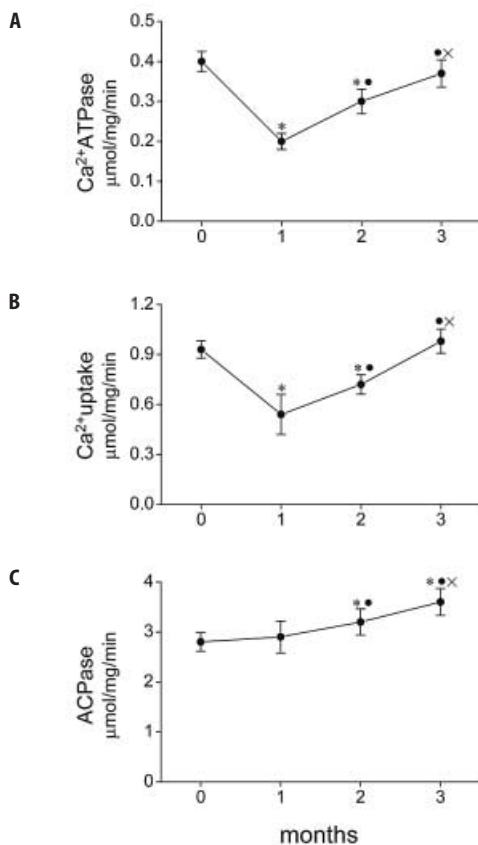[b] *$p < 0.05$ vs 0 (control); † $p < 0.05$ vs 1 months; º $p < 0.05$ vs 2 months.
[c] *LVMi* left ventricular mass, normalized for body weight; *ESS/ESVi* end-systolic stress/end-systolic volume normalized for body weight; *PWP* pulmonary wedge pressure; *RWT* relative wall thickness

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1 month after surgery; at 3 months of overload the mean left ventricular mass, normalized for body weight (LVMi), taken as an index of hypertrophy, was about doubled with respect to the control value. A similar effect was observed for the cardiac index. The values calculated for the ratio end-systolic stress/end-systolic volume (8) normalized for body weight (ESS/ESVi) indicate a loss of contractility at 1 month after surgery and a subsequent recovery of this property. These modifications of left ventricle contractility corresponded to changes of opposite sign observed for pulmonary wedge pressure.
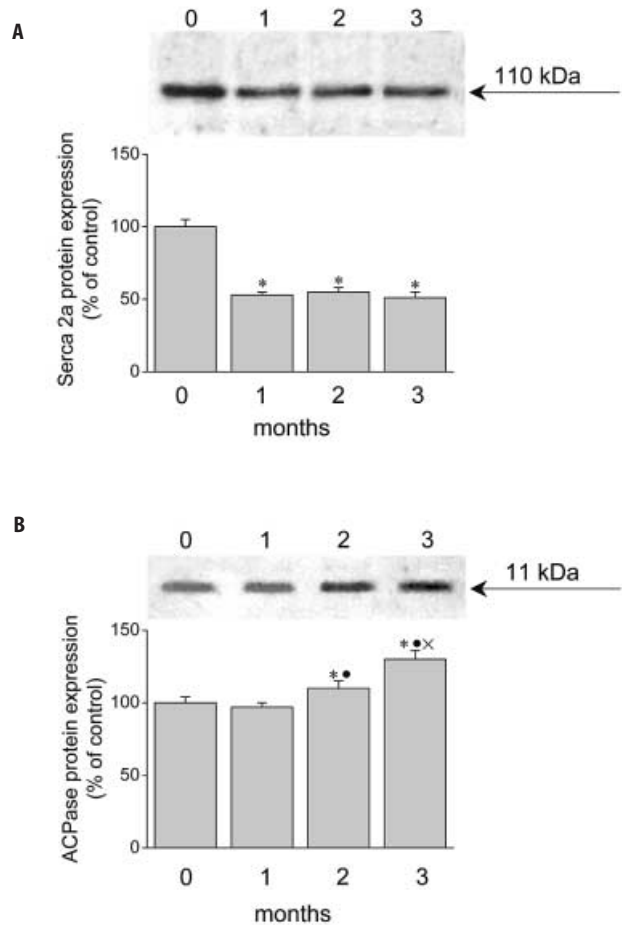
■ Biochemical data

To investigate the changes induced by our volume-overload conditions in SR Ca^{2+} pump function we measured the rates of Ca^{2+}-dependent ATP hydrolysis and of active Ca^{2+} uptake in cardiac SRVs isolated from LV at 1, 2 and 3 months from surgery. As shown in Fig. 1A and 1B,

1 month after the establishment of the arterial-venous shunt the overloaded hearts exhibited a significant decrease in both these activities whose mean levels were about 45 % lower those observed in the hearts of control animals. Subsequent measurements showed a progressive recovery of SR Ca^{2+} ATPase activity and Ca^{2+} uptake in the overloaded hearts since the mean differences from the control values were reduced at by about 25 % after 2 months and became statistically not significant 3 months after surgery. The time courses of Ca^{2+}-ATPase activity and Ca^{2+} uptake were very similar, so that the Ca^{2+}/ATP ratio appeared to be substantially unchanged at all the considered times (data not shown). ACPase activity did not show significant changes after 1 month of overload but 2 months it was significantly increased reaching at 3 months a mean level that was about 30 % higher than the control value (Fig. 1C).

Fig. 1 Time-course of SR Ca^{2+}-ATPase activity (**A**), Ca^{2+} uptake (**B**), and ACPase activity (**C**) in control heart (0) and in hearts subjected to 1, 2, 3 months of volume overload. All the activities are expressed as μmol/min/mg SR vesicle protein. Each value represents the mean value of four experiments $\pm$ S.D. * p < 0.05 vs control; ● p < 0.05 vs 1 month; × p< 0.05 vs 2 months.

Fig. 2 Top: Representative Western blotting of SERCA2a (**A**), and ACPase (**B**) from control heart (0) and hearts subjected to 1, 2, 3 months of volume overload. Bottom: Quantitative data. Each bar represents the mean value $\pm$ S.D. of four different blots. Signals were quantified by densitometric analysis and are expressed as percentage of control value. * p < 0.05 vs control; ● p < 0.05 vs 1 month; × p < 0.05 vs 2 months.

To assess whether the observed alterations of active Ca^{2+} transport in SR were due to changes in the expressions of the proteins involved in this process, we measured the mass concentrations and the mRNA levels of the SERCA2a, PLB and ACPase. Immunochemical detection of SERCA2a (Fig. 2A), obtained with highly specific monoclonal antibody, revealed a single band at the expected position of 110 kDa. Densitometric analysis indicated that the concentration of this protein was significantly decreased in the overloaded hearts compared to the control 1 month after surgery and no further changes were evident at the following times. Immunochemical detection of PLB in unboiled protein samples using monoclonal antibody revealed a single band at the expected position of 27 – 29 kDa (Fig. 3A), corresponding to the pentameric form of this protein, and no significant difference was detected in PLB protein levels between control and volume-overloaded hearts, at all the consid-
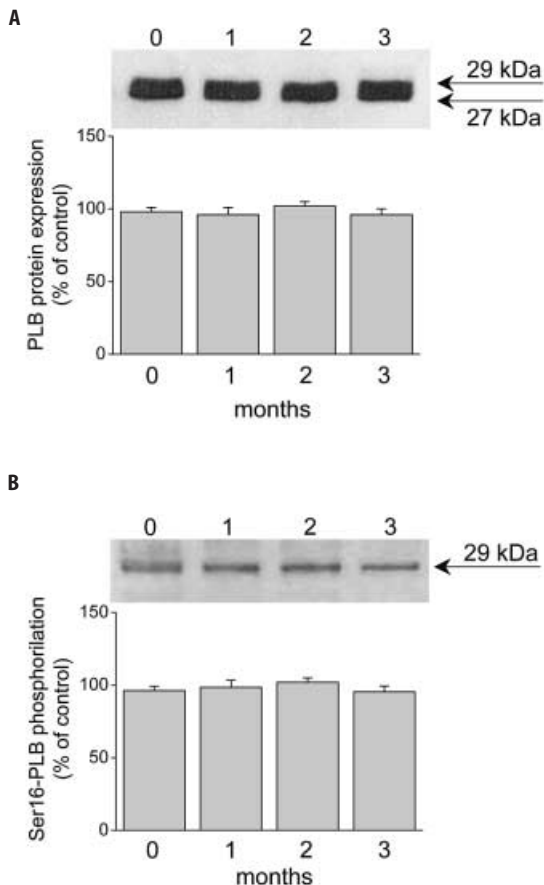
Fig. 3 Top: Representative Western blotting of PLB (**A**), and of PLB phosphorylation at Ser16 residue (**B**) from control heart (0) and hearts subjected to 1, 2, 3 months of volume overload. Bottom: quantitative data. Each bar represents the mean value ± S.D. of four different blots. Signals were quantified by densitometric analysis and are expressed as percentage of control value. No significant changes were observed for PLB protein expression and for its Ser16 phosphorylation.
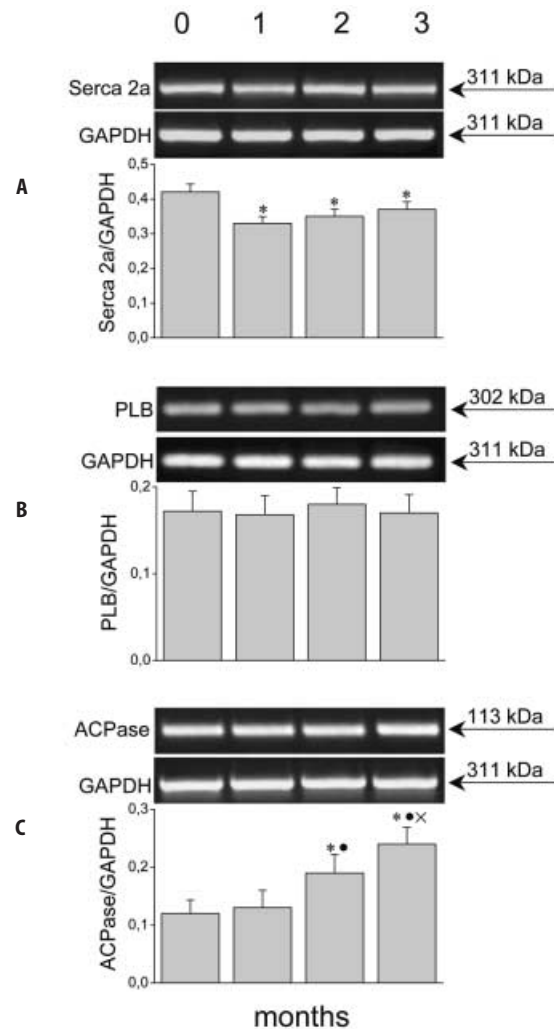
Fig. 4 Top: RT-PCR analysis for SERCA2a (**A**), PLB (**B**), ACPase (**C**) and GAPDH mRNAs, from control heart (0) and hearts subjected to volume overload (1,2,3). Bottom: Quantitative data. Each bar represent the mean ± S.D. of 3-4 values of amplification products normalized to GAPDH. * $p < 0.05$ vs control; • $p < 0.05$ vs 1 month; × $p < 0.05$ vs 2 months.

ered times. Equally, PLB-phosphorylation status was not modified, as judged from the immunodetectable signals revealed using a specific antibody that exclusively detects PLB at its serine-16 phosphorylated site (Fig. 3B) (10). Western blot analysis of ACPase, using specific polyclonal antibodies obtained as previously described (5, 19) revealed a well separated band at 11 kDa (Fig. 2B) whose quantification indicated that the mass concentration of this enzyme significantly increased in comparison with the control value as from at 2 months of volume overload.

As shown in Fig. 4A and 4C, the myocardial levels of both SERCA2a- and ACPase-mRNAs, normalized to GAPDH, showed modifications that paralleled the above reported changes in the expression of the corresponding

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proteins, while the PLB-mRNA remained unchanged as well as its protein concentration (Fig. 4B).

■ Morphological analysis

The morphological analyses of the volume overloaded myocardium revealed marked disruptions in the cytoskeletal architecture of cardiomyocytes subjected to 1 month of increased workload. These modifications, in sharp contrast with the organized assembly of the contractile apparatus in control hearts (Fig. 5A), consisted of focal clumps of contracted myofibrils interspersed with areas of overstretched myofilaments and/or of rarefaction of sarcomeres (Figs. 5B, C, D). However, other organelles in these fibers showed abnormalities. Indeed, in association with pronounced intermyofibrillar edema, mitochondria appeared swollen with loss of cristae and the tubular system with the adjacent cisternae of sarcoplasmic reticulum were often dilated (Fig. 5D). These modifications could also be identified, although to a lesser extent, in cardiomyocytes subjected to 2 months of volume overload, and surprisingly they further attenuated 3 months after surgery. At this time, in fact, the cytoskeletal organization seemed almost completely recovered in most of the cardiomyocytes (Figs. 6A-D) except for the persistence of focal areas of sarcomere hypercontractions, mainly located beneath the sarcolemma (Fig. 6C). Moreover, the 3-month-overloaded cardiomyocytes showed mitochondria with tightly
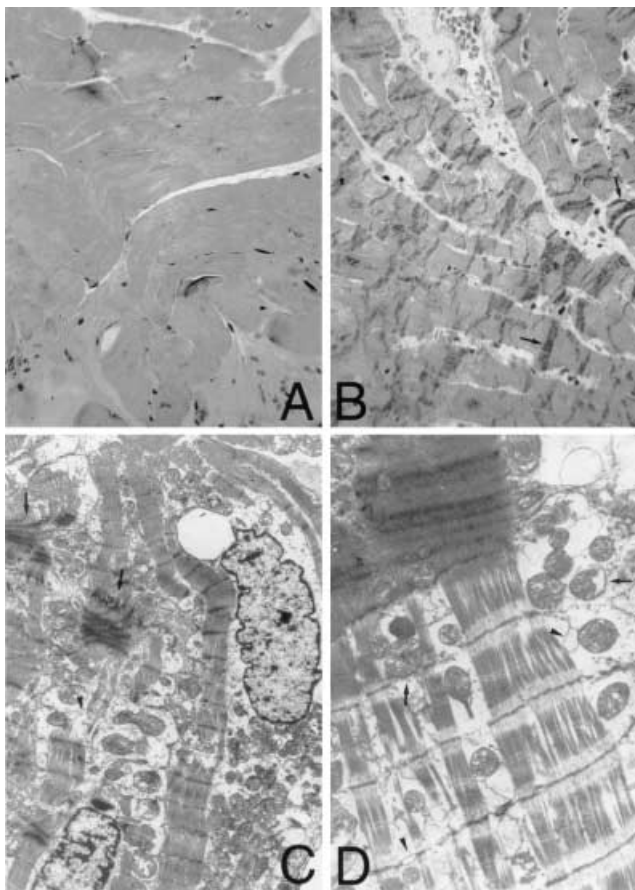
Fig. 5 **A** Myocardium from control swine heart. Cardiomyocytes show a normal myofibrillar structure. Semithin sections stained with Toluidine Blue. L.M. X750. (**B-D**) Myocardium from pig hearts subjected to 1 month of volume overload. **B** Virtually all the fibers show grossly disorganized myofibrillar network (arrows). Semithin sections stained with Toluidine blue L.M.X750. **C** Extensive hypercontractions through cardiomyocytes. Clumps of dark myofibrillar materials (arrows) are associated with disrupted sarcomeres (arrowhead)(TEMX7,000) and **D** with overstretched myofilaments. Intermyofibrillar edema, swollen mitochondria (arrows) with separated and disrupted cristae and dilated elements of T tubules and sarcoplasmic reticulum (arrowhead) are also evident. (TEMX13,000). Each figure is representative at least 3 independent observations from randomly selected mesh areas.
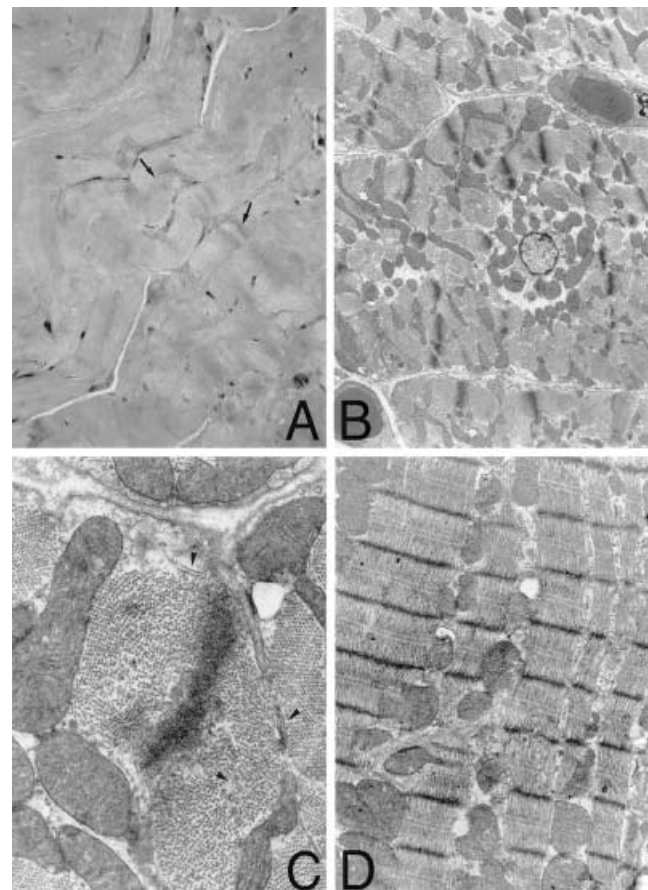
Fig. 6 Myocardium from swine hearts subjected to 3 months of volume overload. **A** Most of the fibers display a normal myofibrillar organization, apart from the persistence of occasional focal areas of hypercontraction (arrows) (LMX750). **B** Several cardiomyocytes in cross section show normal mitochondria interspersed with regularly arranged myofilaments and absence of oedema (TEMX7,000). **C** Higher magnification of **B** showing a dark area of myofilament hypercontraction. Mitochondria contain regularly packed cristae and the cisternae of sarcoplasmic reticulum (arrowheads) display a quite normal appearance (TEMX30,000). **D** Longitudinal section through a cardiomyocyte showing sarcomeres in register (TEMX10,000).

packed cristae, normal triads and no signs of intracellular edema (Figs. 6C, D).

Discussion

The results of hemodynamic and echocardiographic measurements indicate that our experimental conditions induce a marked hypertrophy, as shown by the LVMi data, that, owing to the RWT values, was eccentric. This, however, did not result in left ventricle pump dysfunction, since the cardiac index progressively increased during the study period. In our model, the ESS/ESVi ratio, assumed as an index of contractility (8), showed a reduction after 1 month of overload, subsequently increased, so that no significant differences from control values were evident 3 months after surgery. These findings suggest the development of adaptive process(es) and, in connection with this, it is noteworthy that a similar time course was observed for the changes in SR function, notably for Ca^{2+} uptake and Ca^{2+}–ATPase activity.

In order to elucidate the mechanism(s) responsible for the modifications of SR activity, we evaluated the expression, as protein and as mRNA, of SERCA2a and of its best-known modulator phospholamban. Similar measurements, besides the determination of catalytic activity, were performed for acylphosphatase, which we propose as a candidate for an additional regulatory role in Ca^{2+} transport through SR membrane.

As stated in the Results, although Ca^{2+} pump activity was significantly decreased at 1 month after surgery, it was partially restored at 2 months and after 3 months of overload it was not significantly different from the control value. These modifications, however, were not justified by the changes in SERCA2a protein expression because this decreased at 1 month of volume overload, reaching a level which was not modified both at 2 and or 3 months after surgery. The changes in SERCA2a protein concentrations paralleled those in the content of the corresponding mRNA, in agreement with other reports (1, 18) showing that hemodynamic overload can trigger regulatory mechanisms acting at a transcriptional level on the genes codifying for proteins involved in cardiac intracellular calcium handling. Since these findings suggested the involvement of mechanisms acting on the functional properties rather than on the mass concentration of SERCA2a, we examined the behavior of phospholamban, the best-known modulator of the SR Ca^{2+} pump. In this connection it is accepted that an enhanced Ca^{2+} pump activity may result either from a decrease in the relative PLB/SERCA ratio or from an increase in PLB phosphorylation status (17). Our measurements, however, indicated that phospholamban, both at the early and the late phase of overload, does not undergo significant changes in its expression (as protein and as mRNA) nor in its

phosphorylation status at the serine-16 residue, which is the main target in physiological myocardial regulation and the prerequisite for other residue phosphorylations (15). These results allow us to exclude an involvement of PLB in the changes of SR function observed in our overloaded hearts, notably in the modifications of Ca^{2+} pump activity at different times following surgery. In our opinion this is not surprising since, despite its importance to normal cardiac function, the role of PLB in heart pathophysiology remains largely unknown and the reports on this subject are often controversial (30). Conversely, acylphosphatase levels were unchanged with respect to control values at 1 month of overload: at 2 months, however, this enzyme exhibited a significant increase not only in its activity but also in its protein and mRNA expression. As mentioned in the Introduction, we showed that acylphosphatase can affect the functional properties of SERCA2a stimulating Ca^{2+} pump activity. Our studies (23, 24) also suggest that the acylphosphatase effect was mainly due to its hydrolytic activity on the phosphoenzyme intermediate of SR Ca^{2+} pump; however, because of a structural analogy with phospholamban, acylphosphatase may also interact with SERCA2a, taking the place of unphosphorylated phospholamban and removing the inhibitory effect of this regulatory protein. In any case, given this stimulatory effect – in whichever manner it is exerted – and the time course of acylphosphatase levels during the study period, it seems reasonable to propose that our enzyme may be responsible, at least in part, for the recovery of Ca^{2+} pump activity observed after the first month of overload and resulting in the restoration of control levels at 3 months. Also the morphological findings were consistent with the activation of cellular adaptive processes in cardiomyocytes subjected to a prolonged period of volume overload. The marked alterations observed after 1 month of volume overload, cytoskeletal changes and abnormalities of the membrane system (T tubule system and SR cisternae), are probably a consequence of the abnormal mechanical forces acting on cardiomyocytes because of the new hemodynamic condition. Another factor contributing to cytoskeletal disorganization may be a limited proteolysis caused by calcium activated proteases (2, 32). Indeed the reduced activity of the SR Ca^{2+} pump that we found, especially after 1 month of overload, may result in an upregulation of sarcoplasmic Ca^{2+} levels and in the consequent activation of these enzymes. In any case, the morphological alterations, as well as the biochemical changes, were markedly attenuated at 2 months and almost disappeared at 3 months from after surgery, thus, suggesting a a marked morphofunctional recovery of our overloaded hearts.

In conclusion, the present study provides some original findings about the hemodynamic, biochemical and morphological changes occurring in swine heart subject to a lasting volume overload. In particular, our results

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suggest a novel mechanism, based on acylphosphatase, that could play a role in the modifications of cardiac SR function associated with an increased working. In our opinion, further studies focusing on the relevance of this mechanism in other experimental conditions of cardiac overload and in spontaneous cardiomyopathies would be of interest.

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