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# The role of molecular biology in medicine
## New horizons in the 21st century

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Ο ρόλος της Μοριακής Βιολογίας στην Ιατρική. Νέοι ορίζοντες στον 21ο αιώνα

*Περίληψη στο τέλος του άρθρου*

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### 1. INTRODUCTION

One of the most important and exciting scientific achievements at the beginning of the 21st century was the completion of the human genome project. The human genome consists of ~3 billion bases, but only ~1.5% of these bases code for the 31,000 known genes, corresponding to ~1,000,000 proteins. Thus, there is a vast amount of information based on the human genome, and it is imperative for the scientific world to efficiently and cautiously examine the association or link between specific human genes and diseases. To accomplish this, it is now evident that scientists need to associate variations in specific genes to corresponding alterations in proteins and to link these to functional and physiological alterations in cells, tissues and intact organs.

We are now in the 21st century well equipped to elucidate the function of each gene and its variants. The complete genomic sequence for over 60 species has been catalogued, providing an immense amount of information about gene coding regions, gene promoters, introns and regulatory regions. When the characterization of each gene and its variant is complete, there will be a «Periodic Table of Life», which may revolutionize the way medicine is practised and provide a huge knowledge base for deriving new treatments. Molecular dissection of human disease has proceeded at a remarkable pace, with more than 1500 genetic mutations already established as causes of diseases. In a similarly revolutionary way this information is now shared and disseminated. At OMIM, or the Online Mendelian Inheritance of Man (www.ncbi.nlm.nih.gov/omim), one can easily acquire information on the genetic basis of disease. Additionally, scientists worldwide have contributed to the on-line reporting of SNPs, or single nucleotide polymorphisms, uncovered by sequencing of genes. This information can also be easily accessed at www.ncbi.nlm.nih.gov/SNP. Clearly, scientists of the 21st century have the daunting task of characterizing each gene, its variant and the effects of non-coding regions at the single cell and *in vivo* levels. Genetic information obtained by screening for diseases and disease predispositions will lead to preventative therapy, as in the case of phenylketonuria (PKU). Furthermore, direct and tissue-specific gene therapy may allow manipulation of genomic DNA at the bedside. In addition, scientists have now recognized that there is a genetic basis for the varying action of drugs in different individuals. Customizing drug therapy for individual patients may soon become a reality in the era of «pharmacogenomics». Whether at the level of prevention, direct gene therapy or targeted pharmaceutical treatment, the era of «molecular medicine» has begun and is likely to proceed very rapidly in the 21st century. An example of the shift toward this era, based on the work of our laboratory, is provided below. We will discuss how the function of an important protein, phospholamban, was elucidated in the heart and indicate the approach towards clinical application of this knowledge in heart failure.

Congestive heart failure is a leading cause of morbidity and mortality in the United States, with more than 4

million Americans affected at any one time and with more than 500,000 new cases occurring each year. Furthermore, congestive heart failure is a major cause of hospitalization and is a disease that has a dire prognosis, with a predicted mortality at 5 years after diagnosis. Despite half a century of pharmacological research and drug development targeting congestive heart failure, little progress has been made in improving the prognosis of these patients. Uncovering the molecular basis of heart failure, however, promises to open up new avenues in the treatment of the disease.

Studies from several laboratories, including ours, have suggested that the major characteristic of the failing heart is the disturbed $Ca^{2+}$ homeostasis in the cardiomyocyte. Specifically, $Ca^{2+}$ uptake and release by the sarcoplasmic reticulum have been shown to be impaired. This calcium cycling or the sequential rise and fall of cytosolic $Ca^{2+}$ concentration is responsible for the contraction-relaxation cycle in the cardiomyocyte. Physiological studies at the cellular and subcellular levels have helped to characterize the molecular machinery involved in coupling of electrical excitation of the cell membrane to cardiac contraction and subsequent relaxation of the myofilaments. The endogenous pacemaker cells of the heart (SA and AV nodal cells) induce depolarization of the cardiac tissue; this depolarization at the sarcolemma, the outer cell membrane, alters the conductivity of the dihydropyridine sensitive calcium channels, a rapid calcium influx occurs and this triggers the release of a much larger amount of calcium from the sarcoplasmic reticulum calcium release channels (ryanodine receptors) (fig. 1). The cytosolic $Ca^{2+}$ then acts as a signaling molecule in the cardiomyocyte; it binds to troponin C inducing conformational change of tropomodulin on the thin (actin) filament, making the myosin binding site of actin accessible, and facilitating contraction. Intracellular $Ca^{2+}$ concentration changes greatly over systole (1–10 mM) and diastole (0.1 mM); this rise and fall of $Ca^{2+}$ concentration regulates cardiomyocyte contraction. The sarcoplasmic reticulum, an intracellular membranous network, plays an integral role in regulating intracellular calcium levels over systole and diastole, and thus helps mediate the contraction and relaxation cycle. During contraction, the sarcoplasmic reticulum serves as a reservoir for $Ca^{2+}$, and releases $Ca^{2+}$ through the ryanodine sensitive $Ca^{2+}$-channels. However, during relaxation, the sarcoplasmic reticulum functions as a $Ca^{2+}$ sink, sequestering $Ca^{2+}$ from the cytosol by the sarcoplasmic reticulum $Ca^{2+}$-ATPase, which is under regulatory control by the phosphoprotein phospholamban.

## 2. PHOSPHOLAMBAN

Phospholamban, named from the Greek root words meaning "to receive phosphate", was discovered in the early 1970s.[1] Phospholamban is a small protein, comprising 52 amino acid residues, and it is present in cardiac, smooth, and slow-twitch skeletal muscles. However, its regulatory effects have been mainly studied in cardiac muscle. *In vitro* studies indicated that phospholamban can be phosphorylated at three distinct sites by various protein kinases: serine 10, by protein kinase C; serine 16, by cAMP- or cGMP-dependent protein kinase; and threonine 17, by $Ca^{2+}$-calmodulin-dependent protein kinase. Each phosphorylation is associated with stimulation of the initial rates of cardiac sarcoplasmic reticulum $Ca^{2+}$ uptake, which is pronounced at low $Ca^{2+}$, resulting in an overall increase in the affinity of the $Ca^{2+}$ pump for $Ca^{2+}$.[2,3] On the basis of these observations, it was initially hypothesized that phosphorylated phospholamban functions as a stimulator of the cardiac sarcoplasmic reticulum $Ca^{2+}$-ATPase enzyme. However, in the late 1980s, a significant breakthrough occurred with the demonstration that dephosphorylated phospholamban is actually an inhibitor of cardiac sarcoplasmic reticulum transport of $Ca^{2+}$ and that phosphorylation relieves this inhibitory effect, giving the appearance of phosphorylation-induced stimulation.[4] This finding, together with the identification of a cardiac sarcoplasmic reticulum-associated protein phosphatase, which can dephosphorylate phospholamban,[5] has led to current understanding of phospholamban as a reversible inhibitor of the cardiac sarcoplasmic reticulum $Ca^{2+}$-ATPase activity.

The mechanism of action and functional significance of phospholamban have been well characterized in cardiac muscle, because of its abundant expression in cardiac sarcoplasmic reticulum. In SDS polyacrylamide gels, phospholamban migrates predominantly as a pentamer (>25–28 kDa), but it dissociates into monomers (>6 kDa) upon boiling. The phospholamban monomer consists of 52 amino acids and is proposed to contain three domains: (a) cytosolic domain IA (amino acid residues 1–20); (b) cytosolic domain IB (amino acid residues 21–30); and (c) transmembrane domain II (amino acid residues 31–52).[6] Structure/function investigations on phospholamban suggest that there are two and possibly three sites at which phospholamban interacts with the sarcoplasmic reticulum $Ca^{2+}$-ATPase. Electrostatic and hydrophobic interactions between domain IA of phospholamban and sarcoplasmic reticulum $Ca^{2+}$-ATPase have been proposed to

play an important role in the functional association between these two proteins, and may modulate the transmembrane inhibitory actions. Phospholamban domain II functions as an inhibitory domain that regulates the apparent affinity of sarcoplasmic reticulum $Ca^{2+}$-ATPase for $Ca^{2+}$.[7] Domain IB has been suggested to regulate the long-range coupling between domains IA and II of phospholamban, but recent studies have shown that this domain may also be involved in regulatory interactions between phospholamban and sarcoplasmic reticulum $Ca^{2+}$-ATPase. Based on these findings, phospholamban and sarcoplasmic reticulum $Ca^{2+}$-ATPase interactions have been proposed to occur via a four (or possibly six) base circuit, through which long-range inhibitory interactions are propagated among a series of cytoplasmic and transmembrane interaction sites.

Phospholamban was first shown to be phosphorylated *in vivo* in response to β-adrenergic stimulation of the heart by Kranias and Solaro.[8] *In vivo* phosphorylation occurs at two sites, Ser16 and Thr17. Phosphorylation of phospholamban in intact beating hearts was demonstrated to be associated with increases in the affinity of sarcoplasmic reticulum $Ca^{2+}$-ATPase for $Ca^{2+}$ and enhanced rates of relaxation.[9,10] In addition to phospholamban, other key cardiac phosphoproteins have been shown to be phosphorylated by β-agonists, and they may also play a role in mediating the responses of the heart to β-adrenergic stimulation. These substrates are phospholemman, L-type $Ca^{2+}$ channel, ryanodine receptor, protein phosphatase 1 inhibitor-1, troponin I and C-protein (fig. 2). However, in 32P perfused hearts, it was demonstrated that the rates of phosphorylation-dephosphorylation of phospholamban were faster than those of troponin I, C-protein or phospholemman,[10,11] and paralleled the changes in contractile parameters, suggesting that phospholamban is a prominent mediator of the response of the heart to β-adrenergic stimulation.

With the advent of mouse transgenic and gene targeting (ablation) technology in the early 1990s, the role of phospholamban in the regulation of basal cardiac contractility has been elucidated, through the generation and characterization of mouse models with increased,[12] reduced[13] or ablated[14] phospholamban expression levels. Ablation of phospholamban was associated with enhanced rates of contraction and relaxation in work-performing hearts.[14] The hypercontractile function in these phospholamban-deficient hearts was further confirmed at the cellular level and in intact animals.[15–17] These enhanced contractile parameters reflected subcellular alterations which had occurred at the level of the cardiac sarcoplasmic reticulum. Biochemical assessment of sarcoplasmic reticulum function revealed that phospholamban deficiency was associated with increases in the apparent affinity of sarcoplasmic reticulum $Ca^{2+}$-ATPase for $Ca^{2+}$ and this correlated with the increases observed in the intraluminal sarcoplasmic reticulum $Ca^{2+}$ content of phospholamban-deficient hearts.[14,18] To determine further whether cardiac contractility would be affected by a partial reduction in phospholamban levels, phospholamban-heterozygous hearts expressing reduced protein levels (40%), were studied in parallel with wild-type and phospholamban-deficient hearts.[13] A close linear correlation was observed between the relative levels of phospholamban expression in the mouse heart and (a) the apparent affinity of sarcoplasmic reticulum $Ca^{2+}$-ATPase for $Ca^{2+}$; (b) isolated cardiomyocyte contractile parameters; and (c) rates of contraction and relaxation in intact beating hearts.[13] These findings indicate that the relative ratio of phospholamban to sarcoplasmic reticulum $Ca^{2+}$-ATPase plays a prominent role in regulating sarcoplasmic reticulum function and basal cardiac contractility. As it was not clear from these studies whether all the $Ca^{2+}$-pumps in cardiac sarcoplasmic reticulum membranes were regulated by phospholamban, transgenic mice overexpressing wild-type phospholamban, specifically in the heart, were generated and characterized at the molecular, biochemical and physiological levels. Two-fold overexpression of phospholamban was associated with depressed cardiac contractile parameters in isolated ventricular myocytes and intact animals, which was consistent with the apparent decreased affinity of cardiac sarcoplasmic reticulum $Ca^{2+}$-ATPase for $Ca^{2+}$.[12] These findings, together with observations in rat cardiomyocytes overexpressing phospholamban through adenoviral gene transfer, suggested that a fraction of the sarcoplasmic reticulum $Ca^{2+}$-pumps is not regulated by phospholamban and the functional stoichiometric ratio of phospholamban to the sarcoplasmic reticulum $Ca^{2+}$-ATPase in the native sarcoplasmic reticulum is less than 1:1. Moreover, a close linear correlation was observed between the levels of phospholamban expression and the EC50 or $Ca^{2+}$ concentration at which half-maximal sarcoplasmic reticulum ATPase activity was achieved.[19] This indicated that spare pumps in the sarcoplasmic reticulum are inhibited by the overexpressed phospholamban in transgenic hearts and the functional stoichiometry of phospholamban/sarcoplasmic reticulum $Ca^{2+}$-ATPase is less than 0.5/1.0. An accurate assessment of the stoichiometric ratio of phospholamban/sarcoplasmic reticulum $Ca^{2+}$-ATPase *in vivo*

was obtained through the generation of several lines of transgenic mice, which overexpressed increased levels of a mutant form of phospholamban which could not become phosphorylated (S16A, T17A)[20] and thus alleviate its inhibitory effects on sarcoplasmic reticulum $Ca^{2+}$-ATPase. Examination of sarcoplasmic reticulum transport rates in these transgenic hearts revealed increased inhibition of the apparent affinity of sarcoplasmic reticulum $Ca^{2+}$-ATPase for $Ca^{2+}$ with increased levels of phospholamban expression up to 2.6 phospholamban/1.0 sarcoplasmic reticulum $Ca^{2+}$-ATPase. These findings suggest that approximately 40% of the sarcoplasmic reticulum $Ca^{2+}$ pumps are functionally regulated by phospholamban *in vivo*.[20]

To investigate further the contribution of phospholamban in the β-agonist response of the heart, phospholamban-deficient and wild-type hearts were stimulated with isoproterenol and studied in parallel. Phospholamban-deficient hearts exhibited attenuated responses to isoproterenol at the cellular, organ and intact animal levels, which were not due to any alterations in the β-adrenergic signaling pathway or the degree of phosphorylation of other cardiac regulatory phosphoproteins in myofibrils and sarcolemma.[14–17,21,22] Thus, phospholamban is a major regulator of the β-adrenergic stimulatory effects in the heart. Since phospholamban is phosphorylated by both cAMP-dependent and $Ca^{2+}$/calmodulin-dependent protein kinases during isoproterenol stimulation, several studies have focused on elucidating the physiological significance of dual site phosphorylation of phospholamban. Studies in perfused hearts demonstrated that phosphorylation of Ser16 by cAMP-dependent protein kinase precedes phosphorylation of Thr17 by $Ca^{2+}$/calmodulin-dependent protein kinase, and that Ser16 phosphorylation is largely responsible for the β-adrenergic increases in cardiac relaxation.[11]

In addition, it has been shown that hearts perfused with high extracellular concentrations of $Ca^{2+}$, to activate the $Ca^{2+}$/calmodulin-dependent protein kinase, did not exhibit phospholamban phosphorylation, suggesting that Thr17 phosphorylation could not occur independent of Ser16 phosphorylation *in vivo*. These findings were further substantiated by the generation of a transgenic mouse model, in which the Ser16 phosphorylation site was mutated to Ala16 in phospholamban and this phospholamban mutant was then inserted into the phospholamban null background.[23] Ser16 mutant hearts exhibited a depressed response to isoproterenol, which was postulated to be due to lack of phospholamban phosphorylation, since Thr17 in addition to Ser16 phosphorylation was not detected. However, phosphorylation of Thr17 in the mutant hearts could be achieved *in vitro* by activating the $Ca^{2+}$/calmodulin dependent protein kinase. Thus, these findings suggest that Ser16 phosphorylation is a prerequisite for Thr17 phosphorylation of phospholamban in the heart, and that Ser16 phosphorylation appears sufficient in mediating the contractile responses of the heart to β-agonist stimulation.

Alterations in the phospholamban/sarcoplasmic reticulum $Ca^{2+}$-ATPase ratio and/or phospholamban phosphorylation state have become a current subject of investigation in several cardiac pathophysiological conditions. Phospholamban and the phospholamban/sarcoplasmic reticulum $Ca^{2+}$-ATPase ratio have also been implicated as important determinants of the depressed sarcoplasmic reticulum function and altered $Ca^{2+}$-cycling in the failing human myocardium. Specifically, decreases in the apparent affinity of sarcoplasmic reticulum $Ca^{2+}$-ATPase for $Ca^{2+}$ in failing hearts have been associated with alterations in (a) the relative phospholamban/sarcoplasmic reticulum $Ca^{2+}$-ATPase ratio; and/or (b) the phosphorylation status of phospholamban. Investigations in our laboratory have revealed that the levels of sarcoplasmic reticulum $Ca^{2+}$-ATPase are decreased in human heart failure.[24] In addition, the phosphorylation state of phospholamban has also been implicated as a factor that may contribute to the impaired diastolic function of failing hearts. Recent studies in failing human hearts revealed (a) reduced levels of phospholamban phosphorylation;[24] and (b) increased mRNA expression and activity of type 1 protein phosphatase,[25] suggesting that a higher fraction of phospholamban is in the dephosphorylated state and contributes to greater inhibition of sarcoplasmic reticulum $Ca^{2+}$-ATPase.

Heart failure is a disease associated with distinct changes in intracellular $Ca^{2+}$-handling. Cardiac relaxation and the rapid removal of cytosolic $Ca^{2+}$ by the sarcoplasmic reticulum $Ca^{2+}$-ATPase are impaired, leading to increased diastolic $Ca^{2+}$ levels. As a result, the load of the cardiac sarcoplasmic reticulum is decreased and less $Ca^{2+}$ is available for release in subsequent muscle contractions. Thus, a potential therapeutic strategy for heart failure is the enhancement of the reuptake of $Ca^{2+}$ into the cardiac sarcoplasmic reticulum. Along these lines, two unique approaches have been employed to (a) alter phospholamban or sarcoplasmic reticulum $Ca^{2+}$-ATPase protein expression levels; or (b) disrupt the phospholamban-sarcoplasmic reticulum $Ca^{2+}$-ATPase complex. Studies on decreases of the phos-

pholamban/sarcoplasmic reticulum $Ca^{2+}$-ATPase ratio through increased expression of sarcoplasmic reticulum $Ca^{2+}$-ATPase, utilized recombinant adenoviral-mediated gene transfer in hearts of aortic-banded rats[26] or in myocytes from failing human hearts[27] and indicated that impaired intracellular $Ca^{2+}$-handling could be restored. Moreover, introduction of antisense phospholamban in to failing human cardiomyocytes resulted in decreased phospholamban expression at 48 hours, increasing the velocity of both contraction and relaxation.[28] Strategies to alter the phospholamban/sarcoplasmic reticulum $Ca^{2+}$-ATPase ratio or the interaction of these two proteins hold therapeutic promise the treatment of heart failure. Alteration of the phosphorylation status of phospholamban may provide an additional therapeutic avenue by which SERCA function can be enhanced; adenoviral transfections of failing human cardiomyocytes with a constitutively active form of protein phosphatase 1 inhibitor (I-T35D), enhanced cardiomyocyte contractility and calcium transients,[29] presumably because the levels of phosphorylated phospholamban are increased due to attenuated protein phosphatase 1 activity. Alteration of phospholamban levels, phospholamban phosphorylation status and the interaction of phospholamban with sarcoplasmic reticulum $Ca^{2+}$-ATPase all provide promising targets for the treatment of heart failure.

Support of these strategies has also been provided by other genetic studies, in which phospholamban ablation was shown to rescue the phenotype of the muscle-specific LIM protein knockout mouse which exhibits characteristics similar to human heart failure.[30] The double knockout mice (muscle-specific LIM protein -/-. Phospholamban -/-) exhibited a long-term "suprarescue" of systolic and diastolic cardiac function and reversal of the pathological alterations associated with heart failure. Since the defects in $Ca^{2+}$-cycling of muscle-specific LIM protein knockout hearts appeared to be due to functional impairment of excitation-contraction coupling and not to alterations in the expression levels of sarcoplasmic reticulum $Ca^{2+}$-handling proteins, this genetic complementation strategy demonstrated that inhibition of phospholamban expression or phospholamban-sarcoplasmic reticulum $Ca^{2+}$-ATPase interaction may prevent the onset of heart failure. Similarly, ablation of phospholamban resulted in the rescue of the depressed function in calsequestrin overexpression hearts.[31] Thus, interventions designed to decrease phospholamban expression or inhibit phospholamban activity may be applied selectively to certain forms of heart failure, characterized by depressed $Ca^{2+}$-cycling.

## 3. SUMMARY AND FUTURE DIRECTIONS

Phospholamban is a major regulator of cardiac contractile kinetics through its ability to modulate the sarcoplasmic reticulum $Ca^{2+}$-ATPase function, and thus the sarcoplasmic reticulum $Ca^{2+}$ load. Significant advancements have been made in understanding the dynamics of the interaction of phospholamban with sarcoplasmic reticulum $Ca^{2+}$-ATPase, which affect cardiac calcium homeostasis and contractility. The ability of molecular biologist to manipulate the genomic DNA has thrust us into an era of genetically altered models, which in the most recent decade has yielded copious information on the function of individual genes. The challenge now is the individual characterization of the physiological and pathological effect of the more than 30,000 genes in the human genome and their variants. The translation of knowledge from the genetic code to functional understanding at the cellular, organ and intact animal levels will be tremendously rewarding, since new pathways of physiology and pathophysiology will be uncovered and these will lead to new, innovative, specific and efficacious therapies.

Such progress has paved the way for the development of therapeutic strategies, involving inhibition of phospholamban expression or activity, which may be valuable in the treatment of defective $Ca^{2+}$ homeostasis in various cardiomyopathies. The elucidation of phospholamban function may serve as a «case-study» of how protein function will be elucidated in the future.

Based on the achievements of basic research in molecular biology and the link between these findings and clinical therapy, there is hope for better therapeutic approaches at the sunrise of the 21st century. The recent quantum leaps in molecular biology and genetics will clearly enable us to practise preventative medicine at the molecular level, to rescue deficits via gene therapy and to customize drug therapy based on the specialized «genetic material» of each patient. The era of molecular medicine is before us. The «Periodic Table of Life» will be established with time and given the honest work and assiduous collaboration of basic scientists and pioneering clinicians, our knowledge and arsenal will undoubtedly grow vast. Perhaps Michelangelo's metaphor of Adam himself touching the hand of God, which beautifully adorns the Sistine Chapel and which represents man's infinite potential and divine origins, will be achieved in this era of molecular medicine.

## ΠΕΡΙΛΗΨΗ

### Ο ρόλος της Μοριακής Βιολογίας στην Ιατρική. Νέοι ορίζοντες στον 21ο αιώνα

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Η ολοκλήρωση του προγράμματος του ανθρώπινου γονιδιώματος και η τεράστια αύξηση των γνώσεων σχετικά με τις γονιδιακές αλληλουχίες δημιούργησαν την υποδομή για πολλές μελλοντικές ανακαλύψεις. Στο άρθρο αυτό, χρησιμοποιώντας την περίπτωση μιας μικρής φωσφορυλιωμένης πρωτεΐνης, της φωσφολαμπάνης, παρουσιάζεται η σημασία της εκπληκτικής αύξησης της γενετικής πληροφορίας και περιγράφεται ο τρόπος με τον οποίο αυτή η γενετική πληροφορία "μεταφράζεται" σε λειτουργική γνώση. Ειδικότερα, ανασκοπείται η διαδικασία, μέσω της οποίας έγινε κατανοητή η λειτουργία της φωσφολαμπάνης και ο τρόπος με τον οποίο η συγκεκριμένη γνώση οδήγησε σε κλινικές εφαρμογές.

**Λέξεις ευρετηρίου:** Ανθρώπινο γονιδίωμα, Γονιδιακή θεραπεία, Καρδιακή ανεπάρκεια, Μοντέλα πειραματοζώων, Φωσφολαμπάνη

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