SCIENCE CHINA
Life Sciences

• REVIEWS •

August 2011 Vol.54 No.8: 691–698
doi: 10.1007/s11427-011-4199-1

Ca^{2+} signalling in cardiovascular disease: the role of the plasma membrane calcium pumps

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Received May 17, 2011; accepted June 10, 2011

The plasma membrane calcium ATPases (PMCA) are a family of genes which extrude Ca^{2+} from the cell and are involved in the maintenance of intracellular free calcium levels and/or with Ca^{2+} signalling, depending on the cell type. In the cardiovascular system, Ca^{2+} is not only essential for contraction and relaxation but also has a vital role as a second messenger in signal transduction pathways. A complex array of mechanisms regulate intracellular free calcium levels in the heart and vasculature and a failure in these systems to maintain normal Ca^{2+} homeostasis has been linked to both heart failure and hypertension. This article focuses on the functions of PMCA, in particular isoform 4 (PMCA4), in the heart and vasculature and the reported links between PMCAs and contractile function, cardiac hypertrophy, cardiac rhythm and sudden cardiac death, and blood pressure control and hypertension. It is becoming clear that this family of calcium extrusion pumps have essential roles in both cardiovascular health and disease.

plasma membrane calcium/calmodulin-dependent ATPase, Ca^{2+} homeostasis, Ca^{2+} signalling, heart failure, hypertension

Citation: Cartwright E J, Oceandy D, Austin C, *et al*. Ca^{2+} signalling in cardiovascular disease: the role of the plasma membrane calcium pumps. Sci China Life Sci, 2011, 54: 691−698, doi: 10.1007/s11427-011-4199-1

1 Introduction: The need to develop new treatment strategies for heart failure

It is well established that diseases of the cardiovascular system are the world's largest killers. The World Health Organisation reports that currently 17.1 million deaths a year are attributed to these diseases, and the number is set to rise. This group of diseases which includes hypertension, stroke, coronary heart disease, heart failure, cardiomyopathies, rheumatic and congenital heart disease, and peripheral vascular disease are not only prevalent in western countries, with approximately one in three deaths in the USA resulting from cardiovascular disease (CVD), but are fast becoming a very major health concern in the developing world. Although deaths from several of these CVDs are reducing year on year, deaths as a result of heart failure (HF) are increasing largely because the prevalence of HF increases sharply with age. The proportion of the population classified as elderly (>60 years) is the fastest growing age group across the world; for example, in western countries it is estimated that by 2050 approximately a quarter of the population will be older than 60.

The life time risk of developing this chronic disease reaches one in five, and unlike a number of cardiovascular diseases the risk is equal in both men and women [1]. Heart failure is a syndrome associated with high rates of morbidity and mortality with only 35% of patients surviving five years post-diagnosis [2]; this outcome impacts not only on individual human health but causes an increasing financial burden on the health and welfare system. It is therefore essential that we continue to improve our understanding of the

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molecular mechanisms and intracellular signalling pathways underlying the progression of heart failure in order to inform our development of new, more effective treatment strategies.

1.1 Alterations in calcium homeostasis are associated with heart failure

Heart failure is a complex syndrome often associated with cardiac hypertrophy and subsequent chamber dilation, reduced contractile function, altered myocardial energetics, and changes to cellular calcium cycling. Hence, the mechanisms governing the Ca^{2+} transient have been investigated in detail [3]. Cardiac excitation-contraction (E-C) coupling involves a tightly regulated sequential series of events which results in the cyclical increase in free intracellular Ca^{2+} ($[Ca^{2+}]_i$) from 100 nmol L^{-1} to 1 μmol L^{-1}. Following depolarisation of the plasma membrane, contraction is initiated when Ca^{2+} enters the cell through the L-type Ca^{2+} channel triggering the release of Ca^{2+} from the sarcoplasmic reticulum (SR) via the ryanodine receptors. Both Ca^{2+} influx and release from the SR lead to an increase in $[Ca^{2+}]_i$, which subsequently activates Ca^{2+}-sensitive contractile proteins and elicits muscle contraction. Cardiac relaxation requires Ca^{2+} removal from the cytoplasm by four different molecules, the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA), Na^+/Ca^{2+} exchanger (NCX), plasma membrane Ca^{2+}/calmodulin dependent ATPase (PMCA) and to a lesser extent the mitochondrial Ca^{2+} uniporter.

Alterations in calcium homeostasis during excitation-contraction coupling have been recognised in many studies associated with heart failure [4–6], and numerous genes involved in calcium homeostasis have been reported to be altered in heart failure. For example, many studies show the downregulation of SERCA in both animal models [7] and human tissue samples from failing heart [8]. Borlak and Thum [9] have shown that in human end stage heart failure, left ventricular expression of PMCA1, PMCA4, and SERCA2 is reduced to 30%, 50%, and 30%, respectively, compared with the controls. Additionally, an alteration in ryanodine receptor (RyR) expression has been associated with heart failure [10], and increased expression of NCX has been observed in some heart failure models, which is thought to be a compensatory mechanism for SERCA downregulation [11].

Of this array of Ca^{2+} handling mechanisms this review will focus on the PMCA and its role in cardiovascular function.

2 The plasma membrane calcium ATPases (PMCAs)

The plasma membrane calcium ATPases are ATP-consuming calmodulin-dependent pumps which eject Ca^{2+} into the extracellular space [12,13]. There are four isoforms of PMCA (PMCA1–4; gene names *atp2b1–4*), each encoded by an independent gene, on different chromosomes [14,15]. PMCAs are 134 kD proteins which belong to the P-type ATPase family of proteins. All four proteins have a similar structure comprising 10 transmembrane (TM) domains and four major intracellular regions: The N-terminal region is a region of low sequence similarity between the four isoforms (56.6% homology at the protein level in human genes) and its function has not been fully elucidated; the loop between TM domains 2 and 3 is involved in calcium pore function; whilst the very large loop between TM domains 4 and 5 is the site of ATP binding and contains the aspartate residue which is phosphorylated during the calcium transport cycle; finally the C-terminal tail contains the calmodulin binding domain (CaM-BD) which is essential in the regulation of pump activity. Binding of the CaM-BD to regions on the two large intracellular loops leads to autoinhibition of the pump and this inhibitory effect is released upon binding of calmodulin to the CaM-BD [16–18].

Essentially, PMCA is ubiquitously expressed in that all cells express at least one isoform of PMCA and it is hypothesised that the presence of the different isoforms and their numerous splice variants [19] reflects the specific and differing Ca^{2+} requirements in different cell types. Thus, it is likely that each isoform serves different functions in different tissues with specificity being mediated by tissue-specific factors/interaction partners, as with other calcium transporters [18].

PMCA1 is widely regarded as the housekeeping isoform of this family of genes. A study of mouse embryogenesis has revealed that PMCA1 is the isoform expressed earliest in development [20]; its expression being ubiquitous in both the embryo and the adult mouse. Gene knockout studies corroborate the view that PMCA1 is a housekeeping gene as gene deletion *in vivo* leads to lethality during a very early stage of embryonic development [21]. The expression of PMCA2 is much more specific, being localised to inner ear cilia, Purkinje cells and the mammary gland [22–26]. PMCA3 is also expressed in the brain as well as skeletal muscle cells and pancreatic islet cells [22,27–29], whilst PMCA4 is expressed in a wide range of cell types including erythrocytes, platelets, spermatozoa, heart, vascular smooth muscle, kidney, skeletal muscle, stomach, intestine and brain [21,22,30–35].

In recent years this family of genes has been associated with a number of very important human diseases. Loss of PMCA2 has been associated with hereditary hearing loss [24,36] and its overexpression with poor prognosis of breast cancer [37]. PMCA4 has been implicated in Long QT syndrome which can lead to sudden cardiac death due to arrhythmia [38,39] and there is now an extensive body of genome-wide association study data indicating single nucleotide polymorphisms (SNP) in the PMCA1 gene as the single strongest association with blood pressure variance and hy-

pertension [40–43]. These findings provide compelling evidence of the essential roles of the PMCAs in health and disease.

Much of what is known about the physiological and pathophysiological functions of PMCAs comes from the study of genetically modified mice; for example, PMCA1 knockout mice die during very early embryonic development revealing the essential role of this calcium pump during development [21]. PMCA2 knockout mice, as well as spontaneous mouse mutants of PMCA2, exhibit both deafness and ataxia [44–46]. Deletion of PMCA4 leads to sperm immotility and subsequent infertility [21,35] and alterations in aspects of platelet function including aggregation [32] suggesting that although PMCA4 is essentially ubiquitously expressed in the adult mouse its function is highly specialised.

Traditionally the role of the PMCAs in the cardiovascular system has been understudied; PMCA has long been presumed to have a minor role in contraction/relaxation. However, over recent years, work from our own group and others, with a focus on gene knockout and transgenic studies, has revealed the critical importance of PMCAs in key cardiac and vascular functions.

3 PMCA in the heart

In the heart two isoforms of PMCA are expressed, PMCA1 and PMCA4; there is also some evidence that PMCA2 is expressed at a low level [28] although not in the cardiomyocytes. In non-excitable cells, the primary function of PMCA is to expel calcium from the cytosol, but it is clear from our work and that of others, that in the heart many of the actions of PMCA4 are not as a direct result of its role in the maintenance of intracellular calcium levels and its action as a bulk calcium extrusion pump [33,47,48]. It is as a signalling molecule that PMCA4 is involved in the modulation of physiological and pathophysiological cardiac functions including the β-adrenergic contractile response, cardiac rhythm/rhythm disorders, as well as chronic processes such as hypertrophy [33,38,39,47,48] (Figure 1).

3.1 PMCA4 and β-adrenergic responsiveness in the heart

In recent years, it has become clear that neuronal nitric oxide synthase (nNOS) is a key regulator of heart function which is needed to sustain the cardiac β-adrenergic response, prevent remodelling after myocardial infarction, and to regulate oxygen radical production [49–53]. As a consequence, the question as to which are the regulators of nNOS has become an area of major interest in HF research. In a series of publications it has been demonstrated that PMCA4 is such a regulator of nNOS. A number of years ago our own group identified that PMCA4 binds to nNOS, via a PDZ domain, and subsequently tightly regulates nNOS activity; this interaction and regulation occurs both *in vitro* and *in vivo* in the heart [33,47,54,55]. PMCA4 negatively regulates nNOS by extruding calcium and thus reducing the $[Ca^{2+}]_i$ in the vicinity of the PMCA4-nNOS complex and since nNOS

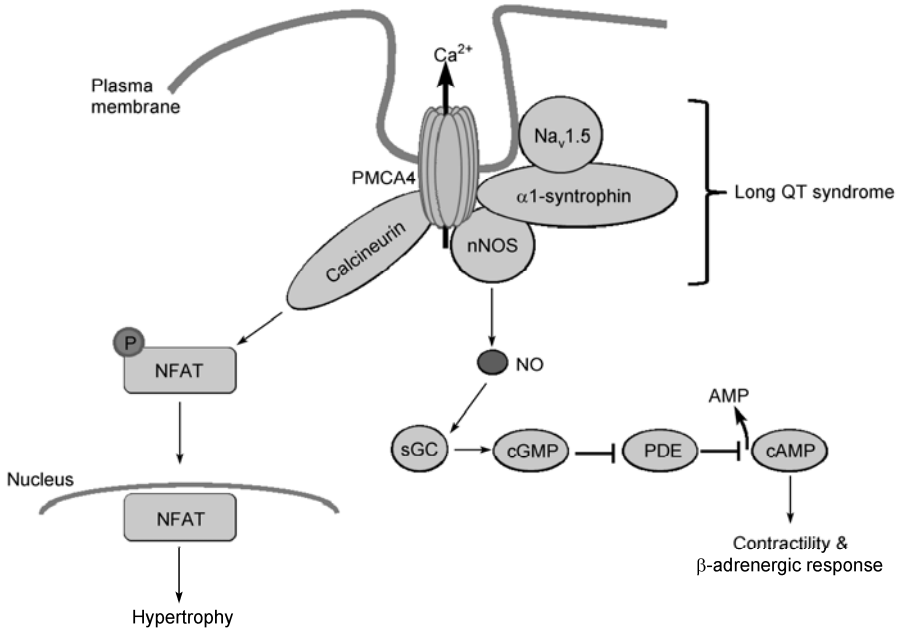

Figure 1 PMCA4 has a number of roles in cardiac health and disease. PMCA4, through its interaction with nNOS, regulates β-adrenergic signal transmission in the heart and influences β-adrenergic responsiveness and cardiac contractility [47]. PMCA4 modulates cardiac hypertrophy through regulation of the calcineurin/NFAT signalling pathway [48]. Disruption of a complex formed between PMCA4, nNOS, α1-syntrophin and the voltage gated sodium channel $Na_v1.5$ is associated with long QT syndrome [38].

activity is Ca^{2+}/calmodulin dependent this leads to a decrease in nNOS activity.

Mice which overexpress PMCA4 in the heart have recently been shown to display reduced β-adrenergic responsiveness *in vivo*, via an nNOS dependent mechanism [33,47]. The PMCA4-nNOS complex controls the cardiac β-adrenergic response through a novel signalling pathway. PMCA4 inhibits nNOS and thus leads to a reduction in nNOS activity. The complex in turn determines local cyclic nucleotide concentrations such that a reduction in nNOS activity leads to a reduction of cGMP production by soluble guanylyl cyclase (sGC). A decrease in the cGMP level results in the reduction of phosphodiesterase activity, which prevents cAMP degradation and hence increased protein kinase A (PKA) activity, which ultimately leads to an altered contractile response to β-adrenergic agonists through phosphorylation of a number of proteins involved in E-C coupling [47].

3.2 PMCA4 and cardiac hypertrophy

It is well established that many proteins that regulate $[Ca^{2+}]_i$ and Ca^{2+} signalling in the heart are involved in the cardiac hypertrophic response. It has been suggested that stimulators of hypertrophy, such as angiotensin II, noradrenaline and aldosterone, cause an increase in the frequency of the oscillation rate of the calcium transient. This leads to an increase in cell size through the mediation of the calcineurin-NFAT signalling pathway [56], a pathway with extremely well characterised associations with cardiac hypertrophy and heart failure [57].

PMCA4 has been found to be important in the regulation of calcineurin/NFAT signalling; the two proteins bind directly at the large intracellular loop between TM domains 4 and 5 of PMCA4 [58]. Calcineurin, which is a serine/threonine-specific phosphatase, is activated by a sustained increase in $[Ca^{2+}]_i$ and an increase in the calcium transient [59,60]. Upon activation calcineurin binds to and dephosphorylates NFAT (nuclear factor of activated T-cells) which translocates to the nucleus and subsequently activates a number of hypertrophic genes [61]. Both *in vitro* and *in vivo* studies have clearly demonstrated that overexpression of PMCA4 inhibits calcineurin which reduces the activation of NFAT which in the heart protects against the development of pathological hypertrophy [48,58].

3.3 PMCA4 and long QT syndrome

The vast majority of patients with heart failure will die either from circulatory insufficiency due to increasing left ventricular dysfunction or sudden cardiac death. It is reported that nearly half the deaths from heart failure are sudden and are likely caused by ventricular arrhythmia; however, the underlying mechanisms are not fully understood. It is also interesting to question why the other 50% do not develop lethal ventricular arrhythmias and instead die from pump failure despite exposure to similar unspecific pro-arrhythmic factors such as fibrosis. Similarly to the susceptibility of the myocardium to failure, there may be genetic determinants of rhythm disorders.

As has been described above, PMCA4 forms a complex at the plasma membrane with nNOS; in fact this complex can exist as part of a larger complex along with α1-syntrophin, which binds to PMCA4 via the large intracellular loop between TM domains 4 and 5 [62]. A mutation (A390V) in the α1-syntrophin gene, which lies within the region which binds to PMCA4, has been associated with long QT syndrome [38], a condition associated with sudden cardiac death due to arrhythmias. Analysis of the A390V mutant shows that it is unable to bind to PMCA4; this results in enhanced nNOS activity likely because PMCA4 is not available to negatively regulate the activity of the enzyme. The link between PMCA4 and cardiac rhythm was made stronger by the findings of a recent genome wide association study which showed that a mutation in CAPON, a protein which interacts with PMCA4 and nNOS [63], is associated with long QT syndrome and sudden cardiac death [39].

4 PMCA in the vasculature

In the vasculature both conduit and small resistance arteries have been reported to express PMCA1 and PMCA4 [34,64] and as such it is possible that both isoforms may modulate vascular contractility, vascular structure and thus resistance.

Recently very strong evidence has emerged that PMCA is involved in blood pressure variance and hypertension in humans. Hypertension affects around 15% of the general population and is strongly associated with heart failure, with 80% of HF patients having hypertension. Hence, a better understanding of the mechanisms regulating blood pressure and those underlying the development of hypertension is essential if we are to improve treatment strategies for controlling blood pressure and ultimately CVD risk.

Four independent genome wide association studies (GWAS) have established a strong link between the PMCA1 gene (*atp2b1*) and blood pressure/hypertension which appears to be independent of the population/lifestyle because the association is similar in White, Japanese and Korean populations [40–43]. However, it is evidence from studies of PMCA4 that have so far provided clues to the functional role the PMCA in regulating vascular tone and blood pressure control.

4.1 Ca^{2+} homeostasis in vascular smooth muscle

Hypertension is associated with a raised peripheral vascular resistance and increases in arterial tone as well as structural remodelling of resistance arteries [65,66]. The diameter of

small arteries, and thus the resistance to flow, is ultimately determined by the contractile state of the vascular smooth muscle. This in turn is modulated by changes in $[Ca^{2+}]_i$. It has long been known that alterations in $[Ca^{2+}]_i$ homeostasis occur in hypertension [67–69].

Vascular smooth muscle contractility is initiated by an elevation of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ may be elevated as a consequence of either influx of Ca^{2+} across the plasma membrane or release of Ca^{2+} from intracellular stores such as the sarcoplasmic reticulum (for reviews see [70,71]). The rise in $[Ca^{2+}]_i$ leads to binding to calmodulin which activates myosin light chain kinase (MLKC) resulting in phosphorylation of the regulatory chains of myosin (MLC_{20}) and ultimately the formation and cycling of actin-myosin cross-bridges to generate contraction. Relaxation generally occurs when $[Ca^{2+}]_i$ falls and MLC_{20} is dephosphorylated. Although certain stimuli may modulate contractility via effects on the Ca^{2+} sensitivity of the contractile apparatus [72,73] it is clear that changes in $[Ca^{2+}]_i$ play a direct role in the modulation of smooth muscle, and therefore resistance artery, tone. Advancements in cellular imaging, fluorescent probes and molecular biology have established the presence of subcellular Ca^{2+} microdomains, signalling compartmentalisation and subcellular fluxes within smooth muscle cells which can modulate numerous cellular functions including contractility in the absence of changes in global $[Ca^{2+}]_i$ [74–76]. Thus, any agent which modifies Ca^{2+} homeostasis has the potential to alter contractility.

The $[Ca^{2+}]_i$ content of a cell at any one time will be determined by a balance between the activity of the mechanisms which elevate $[Ca^{2+}]_i$ (i.e., by movement of Ca^{2+} into the cell or release of Ca^{2+} from intracellular stores) and those that remove it from the cytoplasm [70,71,77]. Whilst the mechanisms responsible for $[Ca^{2+}]_i$ elevation in vascular smooth muscle have been well characterised, those responsible for decreasing $[Ca^{2+}]_i$ to resting levels have received less attention.

Smooth muscle $[Ca^{2+}]_i$ is reduced by the action of calcium transporters which either extrude Ca^{2+} out of the cell or re-sequester it into intracellular stores [78,79]. These mechanisms are important for relaxation, the maintenance of low $[Ca^{2+}]_i$ under resting conditions and also to re-load intracellular stores with Ca^{2+}. SERCA and the mitochondrial uniporter transport Ca^{2+} into the SR and mitochondria respectively while Ca^{2+} can be transported out of the cell via the NCX and by PMCA. Although the NCX is known to be the major Ca^{2+} removal mechanism in other excitable tissues such as the heart [80], its involvement in the removal of Ca^{2+} in resistance artery smooth muscle cells has been questioned [81] thus lending weight to the probability that the PMCA is a major route of regulated Ca^{2+} removal from the cytosol. In uterine muscle it has been shown that 70% of Ca^{2+} is extruded from the cell by PMCA4 [82] while in bladder 25%–30% is carried out of the cell by PMCAs [83]. Taken together this suggests that PMCAs play an important role in Ca^{2+} extrusion in smooth muscles. Modulation of PMCA in vascular smooth muscle therefore has the potential to modulate smooth muscle $[Ca^{2+}]_i$ homeostasis and thus arterial tone. However, its effects appear complex and are not yet entirely understood.

4.2 PMCA4 and vascular contractility

Evidence for the role of PMCA4 in the vasculature has come largely from the study of transgenic mouse models which overexpress PMCA4 in the vascular smooth muscle under the control of the SM22α promoter. Contrary to expectations, these mice were hypertensive suggesting that alteration of PMCA4 expression and/or activity may modulate blood pressure [84,85]. It was shown that conduit arteries from these mice exhibit enhanced contractility to depolarisation and that resistance arteries exhibited heightened myogenic responsiveness and increased agonist sensitivity, all of which likely play a key role in the observed elevated blood pressure [84,85].

The increased contractility observed with overexpression of PMCA4 may at first be surprising. It is logical to hypothesise that overexpression of a calcium extrusion pump would reduce $[Ca^{2+}]_i$ and thus lower blood pressure due to relaxation of the vascular smooth muscle. However, the effects observed in the PMCA4 overexpressing mice do not appear to involve changes in global $[Ca^{2+}]_i$ [85], but levels of cGMP, a marker for nNOS activity, are significantly reduced [84]. As has been discussed above, in other cell types such as cardiomyocytes, PMCA4 plays a more significant role in signal transduction through its interaction with and regulation of nNOS than in the modulation of Ca^{2+} sub-serving direct excitation contraction coupling. As with cardiac myocytes, the effects of PMCA4 on nNOS activity may involve changes in sub-cellular Ca^{2+} homeostasis. It appears that such a mechanism may be responsible, in part at least, for the enhanced vascular contractility observed with overexpression of PMCA4.

In contrast to studies on transgenic mice it has been shown that the PMCA inhibitor caloxin 1c2, which has 10x greater affinity for PMCA4 when compared to PMCA1, 2 or 3, increased coronary artery smooth muscle contractility [86]. Studies in cultured vascular smooth muscle cells have shown that inhibition of PMCA4 with another PMCA4 inhibitor, caloxin 1b1, results in a rise in $[Ca^{2+}]_i$. Thus, both inhibition and over-expression of PMCA4 have been reported to increased arterial contractility.

Taken together this suggests that PMCA4 may modulate arterial contractility in different ways which may, or may not, involve changes in global $[Ca^{2+}]_i$. It is currently unknown whether PMCA1 modulates $[Ca^{2+}]_i$ and arterial contractility and indeed whether either PMCA1 or PMCA4 modulates arterial structure. These studies are particularly important given the strong association between hypertension and arterial structure and function. It is perhaps appo-

site that endothelial nitric oxide synthase, a known major determinant of vascular function/dysfunction, has recently been found to be negatively regulated by PMCA1 [87].

5 Conclusion

The field of PMCA research, particularly in the cardiovascular research field, has recently made a key transition from basic scientific discovery to immediate human relevance in key diseases. It is essential that this impetus is maintained and that we continue to investigate the functional role of this family of genes in order to determine the mechanisms underlying major diseases including hypertrophy and heart failure, lethal arrhythmias and hypertension. To this end it is essential that we identify and develop pharmacological agents to specifically inhibit each of the PMCA isoforms [88]; the use of such inhibitors as scientific tools would greatly enhance this area of research and would inform the development of future potential therapeutic reagents.

Abbreviations

$[Ca^{2+}]_i$	intracellular free Ca^{2+}
CaM-BD	calmodulin binding domain
CAPON	C-terminal PDZ domain ligand of neuronal nitric oxide synthase
CVD	cardiovascular disease
E-C	excitation-contraction
GWAS	genome wide association studies
HF	heart failure
MLKC	myosin light chain kinase
NCX	Na^+/Ca^{2+} exchanger
NFAT	nuclear factor of activated T-cells
nNOS	neuronal nitric oxide synthase
PDZ	PSD 95, Drosophila Discs large protein and Zona occludens-1
PKA	protein kinase A
PMCA	plasma membrane calcium ATPase or plasma membrane calcium/calmodulin-dependent ATPase
RyR	ryanodine receptor
SERCA	sarcoplasmic reticulum Ca^{2+} ATPase
sGC	soluble guanylyl cyclase
SNP	single nucleotide polymorphisms
SR	sarcoplasmic reticulum
TM	transmembrane

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