Ryanodine receptors, voltage-gated calcium channels and their relationship with protein kinase A in myocardium

A mini-review

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Short title: Ryanodine receptors, voltage-gated calcium channels and PKA

Abstract

We present the review of the data from the literature about relationship between ryanodine receptors and voltage-gated calcium channels in myocardium, and also how both of them are related to protein kinase A. Ryanodine receptors, which have three subtypes (RyR1-3), are located on the membrane of sarcoplasmic reticulum. Different subtypes of voltage gated calcium channels interact with ryanodine receptors in skeletal and cardiac muscle tissue. The mechanism of excitation-contraction coupling is therefore different in skeletal and cardiac muscle. However, in both tissues ryanodine receptors and voltage-gated calcium channels seem to be physically connected. FK-506 binding proteins (FKBPs) are bound to ryanodine receptors, thus allowing for their concerted activity, called coupled gating. Activity

of both ryanodine receptors and voltage gated calcium channels is positively regulated by protein kinase A. These effects are, therefore, components of the mechanism of sympathetic stimulation of myocytes. The specificity of this enzyme's targeting is achieved by using different A kinase adapting proteins. Different diseases are related to inborn or acquired changes in ryanodine receptor activity in cardiac myocytes. Mutations in cardiac ryanodine receptor gene can cause catecholamine provoked ventricular tachycardia. Changes in phosphorylation state of ryanodine receptors can provide a credible explanation for development of heart failure. The restoration of their normal level of phosphorylation could explain the positive effect of beta-blockers in treatment of this disease. In conclusion, molecular interactions of ryanodine receptors and voltage-gated calcium channels with PKA have significant physiological role. However, their defects and alterations can result in serious disturbances.

Introduction

In general, Ca^{2+} ions can flow into the cell from the outside, through different types of channels, or they can be released from internal stores. This local increase in cytosolic $[Ca^{2+}]$ that creates a microdomain is used by various systems to start separate events in the cell. (Fill *et al.* 2002). Ryanodine receptors (RyRs) are a particular type of channels located on the membranes of sarcoplasmic reticulum. They are widely expressed (of particular importance is their distribution in CNS (Verkhratsky 2005)), but here we will describe their role in myocardium only. RyRs have a critical role in excitation-contraction coupling in striated muscles (both skeletal and cardiac). They are inextricably related to voltage-gated calcium channels (VGCC). Recently, however, several different concepts emerged that try to relate changes of phosphorylation state of both voltage-gated calcium channels and RyRs to physiological and pathological events in the myocardial cells. We will primarily describe

these recent advances in ryanodine receptor research, their interaction with VGCCs and then the influence that protein kinase A has on them.

List of abbreviations: VGCC - voltage-gated calcium channel, RyR – ryanodine receptor, PKA – protein kinase A, DHPR – dihydropyridine receptor, AKAP – A kinase binding protein, FKBP – FK-506 binding protein

Ryanodine receptors - Genes and Expression

So far, three types of ryanodine receptors have been isolated, RyR1- RyR3 (Zorzato *et al.* 1990; Hakamata *et al.* 1992). They are coded by three different genes, giving rise to enormous polypeptides (4872-5037 amino acids). The distribution of RyRs in the organism is generally tissue-specific: RyR1 predominates in skeletal muscle, RyR2 in cardiac muscle, whereas, RyR3 has a wide and, at a first glance, irregular, distribution.

Structure of ryanodine receptors

Structurally, there are larger receptors and larger proteins than RyRs, but RyRs are, without any doubt, the largest ion channels known to exist. Each molecule has more than 5000 amino acids, with the molecular mass of ~600 kDa. Both ends of the molecule (amino- and carboxy-) are on the cytosolic side of the membrane, i.e. on the outside from the sarcoplasmic reticulum lumen (Grunwald *et al.* 1995), which implies an even number of transmembrane segments. The protein itself is disproportionally divided into cytosolic (~4000 aa) and transmembrane segment (~1000 aa)(Rossi *et al.* 2002).

Since the complete receptor is homotetramer, composed of subunits attached at their angles, its total mass is ~2.5 MDa! (Serysheva *et al.* 2005) Due to such enormous size, RyRs are clearly visible under electron microscope (the foot processes at the junction of sarcoplasmic reticulum and t-tubules).

Voltage-gated Calcium Channels

There are several existing nomenclature systems related to VGCC and this produces a lot of confusion. Throughout this paper, we shall use the newest one (Catterall 2000). The voltage-gated calcium channels in the heart are still usually designated as L-type calcium channels because their current, after activation, is **L**ong-lasting. Due to their enormous importance in cardiovascular pathology, large groups of drugs acting on them (blocking them, actually) were developed. Structurally, these calcium blockers can be divided into phenylalkylamines, dihydropyridines and benzothiazepines. This explains still other name used for this same type of channels – dihydropyridine receptors (DHPR). According to this system, there are other types of voltage-gated calcium channels, such as P/Q, R, N and T-type channels. Comparative list of these three nomenclature systems is presented in Table 1.

IUPHAR nomenclature	alpha subunit nomenclature	Electrophysiological nomenclature
$Ca_V 1.1$	$\alpha 1_S$	L
$Ca_V 1.2$	$\alpha 1_C$	L
$Ca_V 1.3$	$\alpha 1_D$	L
$Ca_V 1.4$	$\alpha 1_F$	L
$Ca_V 2.1$	$\alpha 1_A$	P/Q
$Ca_V 2.2$	$\alpha 1_B$	N
$Ca_V 2.3$	$\alpha 1_E$	R
$Ca_V 3.1$	$\alpha 1_G$	T
$Ca_V 3.2$	$\alpha 1_H$	T
$Ca_V 3.3$	$\alpha 1_I$	T

Table 1. shows the nomenclature(s) used to describe the voltage-gated calcium channels. Presented are: the current system, the system according to the alpha subunit distribution and the electrophysiological system.

Regulation of ryanodine receptor activity

Ryanodine is a substance originally derived from the plant *Ryania sp*. Initially, it was used as the insecticide, but it was soon noticed that it is also toxic for people and animals, because of its paralytic action on skeletal and cardiac muscle. However, in the beginning, it was not known why cardiac paralysis caused by ryanodine was flaccid, whereas skeletal

muscle paralysis was spastic. The difference is not trivial, because it indicates essential difference in the mechanism of calcium handling in these two tissues: ryanodine blocks RyRs in the open state, thus practically emptying the sarcoplasmatic reticulum, because the calcium ions leak out. Opposite to cardiac muscle cells, the skeletal myocytes do not possess the strong membrane transport systems for elimination of Ca^{2+} (Fill *et al.* 2002), so free cytosolic $[Ca^{2+}]$ rises, causing muscle contraction.

Function or ryanodine receptors in striated muscles

As in other types of ion channels, we can describe selectivity, conductivity and activation properties of RyR receptors. Compared to other ion channels, their conductivity is enormous, the highest known, at the order of nS, when the charge carriers are monovalent cations. RyRs are moderately selective cation channels (only 6 times more permeable to calcium than to potassium). For comparison, $Ca_V1.x$ channels are 20 times more selective for calcium over potassium (Chen *et al.* 1997).

RyRs should not be considered isolated structures: instead, they are connected to a number of proteins from both cytosolic and luminal side (Zhang *et al.* 1997). Here, we will primarily deal with their interaction with $Ca_V1.x$ channels. There is a functional selectivity in interaction between subtypes of these channels, in other words *in vivo* they don't mix (Nakai *et al.* 1997). More precisely, RyR1 in skeletal muscles interacts with $Ca_V1.1$, whereas in myocardium RyR2 interacts with $Ca_V1.2$ (Sun *et al.* 1995; Ertel *et al.* 2000).

Structurally, this is evidenced by the organization of RyRs and $Ca_V1.x$, which is much more regular in the skeletal muscles: RyR1 and $Ca_V1.1$ are organized in tetrads, such that one $Ca_V1.1$ is connected to each of the four RyR1 subunits (Franzini-Armstrong 2004). In cardiac muscle, the organization is irregular, *i.e.* the distribution of $Ca_V1.2$ above adjacent RyR2s is haphazard. In addition, their ratio is significantly lower: there are 4-10 times more $Ca_V1.2$ than RyR2s, *i.e.* only 10-25% of calcium channels are in contact with RyRs (Bers *et al.* 1993).

Due to these structural differences, a different mechanism of excitation-contraction coupling exists in these two types of striated muscles. It is a well-known fact that calcium ions in extracellular solution bathing the tissue are essential for cardiac, but not for skeletal muscles (Armstrong *et al.* 1972; Dirksen *et al.* 1999). In other words, membrane potential change *per se* is not sufficient for cardiac contraction: calcium influx is required, as well (Franzini-Armstrong *et al.* 1997).

Except this anterograde interaction between $Ca_V1.1$ and RyR1 (a synonym for excitation-contraction coupling), a retrograde interaction between them exists, as well: after stimulation, RyR1 can increase the activity of its corresponding $Ca_V1.1$. It has been shown that the effect is RyR1 specific, because RyR2 cannot replace it neither in orthograde (cannot be activated by depolarization only) nor in retrograde coupling (Nakai *et al.* 1997).

However, as much as it is simplistic to assume that $Ca_V1.2$ and cardiac RyR2 are not physically connected, the recent literature data overthrow this dogma. It has been shown that cross-reactions do occur, both for peptide A (Dulhunty *et al.* 2004) and peptide C (Dulhunty *et al.* 2005) (these peptides are the fragments of voltage-gated calcium channels). The interaction of RyRs and $Ca_V1.x$ is shown in Figure 1.
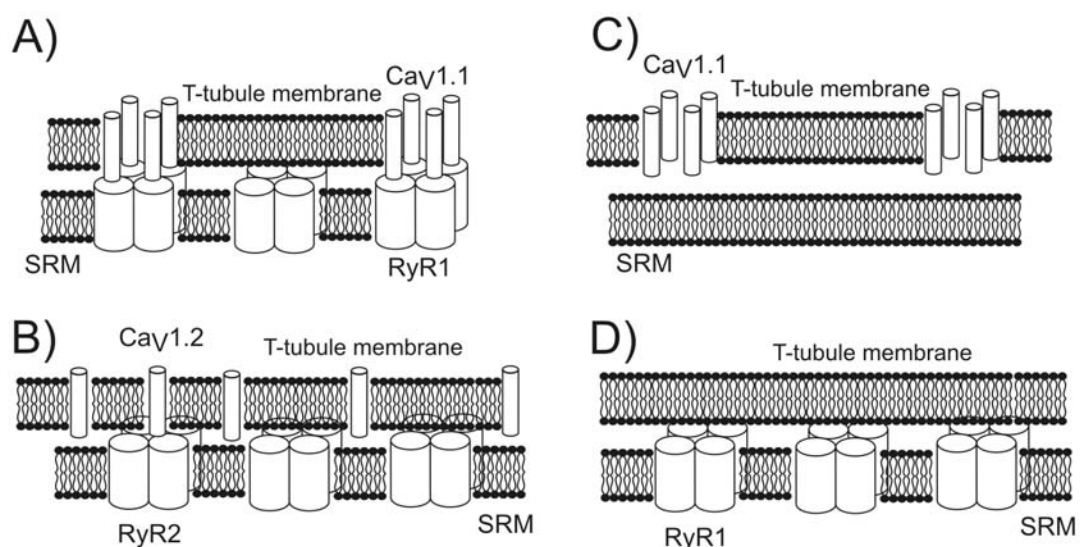
Figure 1. Interaction and positioning of RyRs and voltage-gated calcium channels in skeletal in cardiac muscle **A)** In the skeletal muscle, there is an orderly association between RyR1 and $Ca_V1.1$: every other complete RyR1 channel is in contact with 4 $Ca_V1.1$ channels, each calcium channel interacting with a single RyR1 subunit. **B)** In cardiac muscle, there is a functional interaction of RyR2 with $Ca_V1.2$. However, their positioning is less regular, although physical interaction between them may exist (see text). **C)** Mutant animals without RyR1 genes are called dyspedic. The lack of RyR1 can not be compensated for by RyR2 to reestablish excitation-contraction coupling **D)** Mutant animals without $Ca_V1.1$ are called dysgenic. **SRM** – sarcoplasmic reticulum membrane

VGCC and PKA

For their relationship to RyR, it is important to describe the modulation of $Ca_V1.x$ by sympathetic, β-adrenergic receptor mediated stimulation. The stimulation of β-adrenergic receptors and PKA produces significant increase in calcium current through $Ca_V1.2$ (Sculptoreanu *et al.* 1993). This could be a part of a mechanism that explains the positive inotropic (increase in the contraction strength) and lusitropic effects (increase in the rate of heart relaxation) of sympathetic system on working musculature of the heart. On a single channel level, this is manifested as increase in open channel probability (Yue *et al.* 1990).

In both skeletal and cardiac muscles, it was found that the moment of initiation of PKA effect was too rapid if PKA were simply floating free in the cytosol. It is believed that PKA is anchored to the cellular membrane by A-kinase anchoring proteins (AKAPs), which both raises the local concentration of the enzyme and localizes it in the close proximity of its targets (Johnson *et al.* 1994), therefore acting as efficient means to control the spatiotemporal resolution of cAMP-responsive phosphorylation events (Gao *et al.* 1997). AKAPs are used to bring PKA in the close vicinity of RyR2, as well. The specificity of the same enzyme for acting on two different molecules is conferred by different adaptor proteins. So, in skeletal

muscles, AKAP15/18 is the one that attaches PKA to $Ca_V1.1$ (Gray *et al.* 1997). Both

AKAP79 (Gao *et al.* 1997) and AKAP15/18 (Fraser *et al.* 1998) are candidates for targeting

PKA to $Ca_V1.2$.

Ryanodine Receptors and PKA

The targets of PKA in cardiac muscle cells are many (phospholamban, troponin I,

myosin protein C): here, we deal only with $Ca_V1.2$ and RyR2. ß-agonist-induced

phosphorylation of RyR2 was observed in intact myocytes (Shen 2006), as well as in isolated

myocytes, in which RyR2 activity was stimulated by isoproterenol and blocked by

propranolol (Yoshida *et al.* 1992). Various enzymes are involved in this, the most important

being protein kinase A (PKA). It is brought to a close proximity of its target on RyR2, Ser-

2809 (Witcher *et al.* 1991), by an A kinase adaptor protein, mAKAP, (aka AKAP100), which

colocalizes with RyR2 (Yang *et al.* 1998). The compartmentalization of PKA effect on
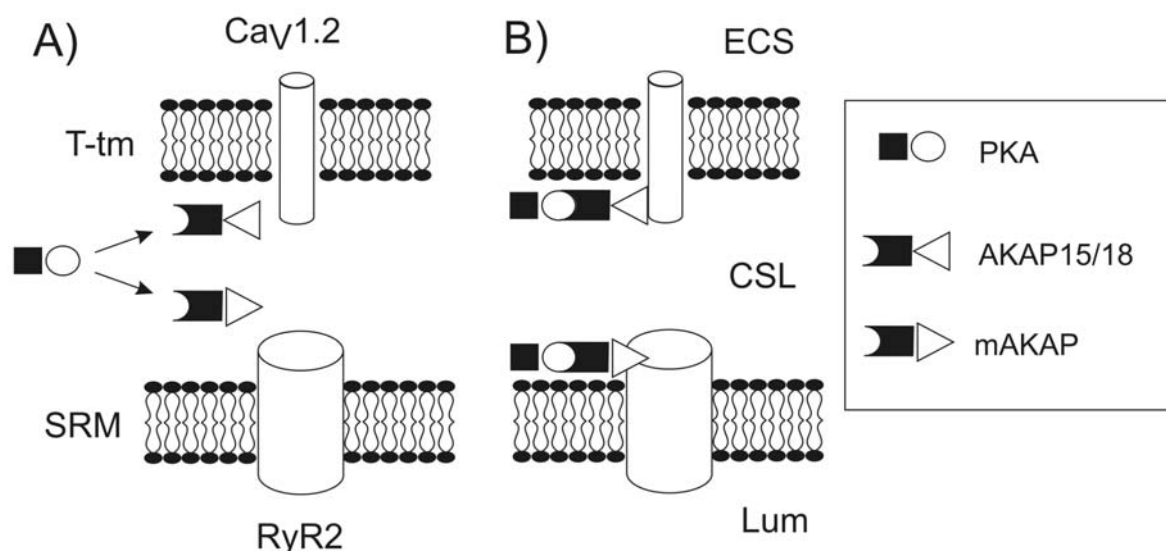
VGCCs and RyRs is shown in Figure 2.

Figure 2. A) The same enzyme, PKA, can be differentially targeted to RyR2 or $Ca_V1.2$.

B) To achieve this, different adaptor proteins are used to localize the effect of PKA to the

desired protein. It is still a matter of debate whether dissociation of catalytic from regulatory

subunit of PKA is necessary. **T-tm** – t-tubule membrane, **ECS** – extracellular space, **CSL** – cytosol, **SRM** – sarcoplasmic reticulum membrane, **Lum** – sarcoplasmic reticulum lumen

Thus, we have seen how PKA comes into proximity of RyRs. What remains to be seen is how its effect is converted into higher RyR activity. In order to do so, a new player must be introduced in the game.

Various cells in the human body contain a group of proteins designated as FKBPx (FK-506 binding proteins with molecular weight x), named so because they bind a drug called FK-506. The smaller, archetypal members are FKBP12 and FKBP12.6. In skeletal muscles, both FKBP12 and FKBP12.6 bind to RyR (Timerman *et al.* 1993), whereas in the myocardium only a slightly heavier isoform, FKBP12.6, binds to RyR2 (Timerman *et al.* 1996).

This is important for the explanation of the β1-adrenergic agonists on the heart: acting via cAMP, they activate PKA, which phosphorylates RyR2. While it is bound, FKBP12.6 inhibits RyR2. Phosphorylation of RyR2 by PKA causes FKBP12.6 to unbind from RyR2 (Marx *et al.* 2000), which then become more sensitive to the cytosolic $[Ca^{2+}]$. The effect is rapidly reversed by phosphatases, making it a component of a physiological mechanism for inotropic sympathetic effect. Fortunately, the effect of β1-agonists still does not enable RyR2 to be activated at the normal diastolic $[Ca^{2+}]$ of 10^{-7} M, otherwise, each one of us would be susceptible to life-threatening arrhythmia even at moderate physical exertion (Marks 2003).

The unusual phenomenon related to RyRs is that, normally, they don't operate as isolated units. Instead, there's a physical and functional connection between them, producing a two-dimensional network in the membrane. This has been shown in both skeletal (Marx *et al.* 1998) and cardiac muscle (Marx *et al.* 2001). This means that RyRs create a higher-order entity, opening and closing at the same time, which is called coupled gating. This system of

coupled gating of RyR2s works while FKBP12.6 is bound to RyR2. However, phosphorylation of RyR2 by PKA unbinds FKBP12.6 and channels start operating individually. In certain individuals, this effect, combined with the higher sensitivity of phosphorylated RyR2 to calcium, can have serious detrimental consequences.

Ryanodine receptors in cardiac pathology

At least two conditions can be related to a change (inborn or acquired) in activity of RyR2 in the heart. These are catecholamine provoked ventricular tachycardia (CPVT) and the heart failure (HF).

Ryanodine receptors and catecholamine provoked ventricular tachycardia

A sudden cardiac death is a term that indicates "…natural death due to cardiac causes, heralded by abrupt loss of consciousness within 1 h of the onset of acute symptoms, in an individual who may have known preexisting heart disease but in whom the time and mode of death are unexpected" (Myerburg RJ 2001). This can occur in people with chronic heart failure, but also in children and healthy adults without previously proven cardiac disease, cardiac arrest being the first (and the last) clinical manifestation of underlying disease. In a certain percentage of cases, no visible cause of death can be found in autopsy (Chugh *et al.* 2004). Therefore, it should be sought for at the molecular level (Swan *et al.* 1999). The results of post mortem analysis show that 1 in 7 (14%) of sudden cardiac deaths can be ascribed to RyR2 mutations (Tester *et al.* 2004).

These persons (even at early age) become susceptible to bouts of ventricular arrhythmias during physical exertion (Lehnart *et al.* 2004). Structurally, there is nothing wrong with their hearts, but increased sympathetic stimulation during exercise can cause sudden cardiac death. Some of the mutations decrease the binding affinity of mutated RyR channels to FKBP12.6. In non-stimulated state (no PKA activity), these channels behave

normally. Phosphorylation, however, causes a 10 times increase in probability of opening which could account for diastolic calcium leakage and triggering of lethal arrythmias.

Ryanodine receptors and the heart failure

In the heart failure, several changes occur in the heart cells: prolonged action potential, slower $[Ca^{2+}]$ systolic rise (longer time to peak), lower maximal amplitude of the calcium systolic pulse, as well as higher resting $[Ca^{2+}]$ in diastole, the latter effect being even more pronounced by inotropic drugs (Jiang *et al.* 2002). These events have logical consequences to cardiac contraction and relaxation: the systole is slower and weaker, whereas the diastolic relaxation is incomplete. We will try to focus on the events in diastole and indicate a possible role of RyR2 in it.

People with heart failure have an elevated level of circulating catecholamines (Kinugawa *et al.* 1996). The prolonged activation of β1 receptors produces their desensitization and down-regulation (Bristow *et al.* 1982). Still, beta-blockers are used in the treatment of heart failure (Gottlieb *et al.* 2002), although their beneficial effect is contrary to the common sense (Marks *et al.* 2002): why would the drugs that weaken cardiac contraction be useful for the heart that is already weakened by disease?

To provide an explanation for this, we shall focus on the previously mentioned higher diastolic $[Ca^{2+}]$. In theory, it could be explained either by lower activity of the systems that remove cytosolic calcium released during diastole (Yano *et al.* 2005), primarily by Ca^{2+}-ATPase, or by persistent leakage of Ca^{2+} through RyR2 that should be closed during diastole (Shannon *et al.* 2003). It has been shown that, in heart failure, activity of PKA is high. This is combined with lower phosphatase activity (Marx *et al.* 2000), leading to hyperphosphorylation of RyR2s. This seems to be a critical point that accounts for beneficial effect of beta-blockers: blockade of chronic catecholaminergic activity and reduction of PKA activity allow for dephosphorylation of RyR2s. When reverted into normal state, they rebind

FKBP12.6 which has two consequences: it reduces the calcium sensitivity and re-enables RyR2 to operate as a unit, *i.e.* restores the coupled gating. Both of these effects reduce the possibility for stochastic activation of RyR2 during diastole. This prevents the diastolic calcium leakage (leaving more calcium in the SR for the stronger systole) and reduces the diastolic $[Ca^{2+}]$, thus preventing life-threatening ventricular arrhythmias.

This explanation sounds reasonable and well-supported by experimental data. However, many studies have challenged it. It was found that FKBP12.6 binds to RyR2 equally well regardless of the level of phosphorylation and that it even binds to RyR2 mutated in the way to imitate constitutive hyperphosphorylation (Jiang *et al.* 2002). Also, no difference was found in the level of phosphorylation of RyR2 in the healthy and diseased hearts. The role of RyR2 phosphorylation in diastolic Ca^{2+} leakage was denied and instead ascribed to increased $[Ca^{2+}]$ in SR by increased uptake (Li *et al.* 2002). The effect of PKA on RyR phosphorylation and its interaction with FKBP12.6 is shown in Figure 3.
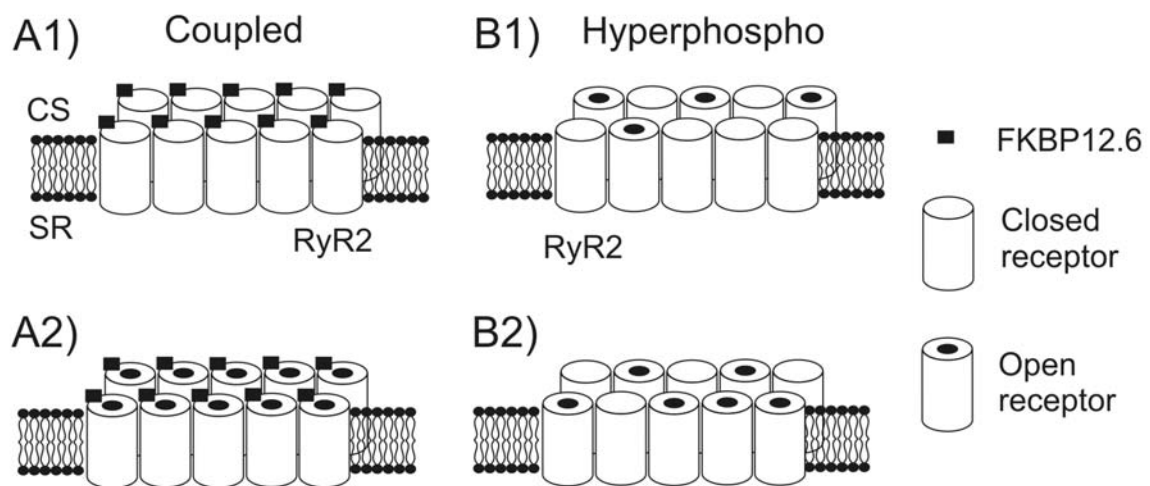

Figure 3. **A1)** In the healthy individual, with normal level of RyR2 phosphorylation, FKBP12.6 is bound to RyR2 (for simplicity reasons, only one FKBP12.6 per RyR2 is shown, although actually there are four, each bound to one RyR2 subunit). **A2)** When activated, RyR2 then operate in concert, opening and closing simultaneously (during systole and

diastole, respectively). **B1)** In hyperphosporylated state, FKBP12.6 dissociates from RyR2 and the coupling is lost. For this reason, some RyR2 channels can open even without stimulation, during diastole, giving rise to a tendency to lethal arrhythmias **B2)** In addition, not all channels open when they should, producing changes in calcium handling during heart failure. **CS** – cytosolic side, **SR** – sarcoplasmic reticulum lumen

Conclusion

The data presented in this paper indicate that investigating a single molecule, however well its role may be defined, is often misleading. Many solid truths have been questioned and overturned, once again showing that nature's mechanisms are not limited to only one type of cells, but are universally applicable in different cells of an organism, to the horror of those that try to systemize them. Ryanodine receptors are a good example of this. A large body of data accumulates about their interactions with many different proteins, both from cytosolic (as some of them are described here) and luminal side. More and more mutations in the constituents of this complex system are related to human diseases. Converted into clinical setting, given the high risk of sudden death and the efficacy of beta-blockers or implantable cardioverter defibrillators in therapy of CPVT, it should become routine to perform genetic screening, early diagnosis, and subsequent preventive strategies in treatment of this condition (Postma *et al.* 2005). The idea that aberrant Ca^{2+} release from RyR2 can lead to arrhythmogenic activity was shown in hearts of people with atrial fibrillation, with hyperphosphorylation and decreased FKBP12.6 binding to RyR2 in atrial myocytes (Vest *et al.* 2005). Recently, it has been unequivocally shown in patients with heart failure that well-known beneficial effect of beta-blockers occurs through stabilization of RyR2 function in failing hearts (Reiken *et al.* 2003). This finding is particularly distressing in the light of clinical evidence that many of such patients do not receive this therapy, even when they have no contraindications. In addition, recent pharmacological advances present the opportunity for

practical clinical use of these findings. The benzothiazepine derivatives that induce binding of FKBP12.6 to RyR2 had beneficial effects in experimental models of heart failure, manifested as significant increase in fractional shortening and ejection fraction, increase in peak dP/dt and decrease in end-diastolic ventricular pressure (Kohno *et al.* 2003; Wehrens *et al.* 2005), as well as in models of triggered arrythmias in heart failure and CPVT (Wehrens *et al.* 2004). Therefore, these drugs may constitute a novel class that is directed to gene-defect specific treatment of arrythmias. Therefore, better understanding of the role of ryanodine receptors in pathophysiology of these conditions could lead us to the correct path in the search for improved therapy. Many of the new concepts work well at the molecular level. The time will show to what extent the initial simplistic assumptions become complicated in its ultimate setting, a diseased patient.

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