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What Does Maurocalcine Tell Us About the Process of Excitation-Contraction Coupling?

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1. Introduction

Cardiac and skeletal muscle contraction is triggered by the arrival of an action potential that locally and transiently depolarizes the plasma membrane. The entire chain of molecular events that link the arrival of the action potential and muscle contraction is called excitation-contraction coupling. This chapter will focus on the way membrane depolarization is sensed and how it triggers a massive increase in the cytosolic Ca^{2+} concentration. Plasma membrane depolarization is detected by L-type voltage-gated calcium channels whose activation allows Ca^{2+} entry into muscle cells. Cardiac and skeletal muscle voltage-gated calcium channels differ by their Ca^{2+} permeability. While cardiac channels lead to an important and rapid Ca^{2+} influx, skeletal muscle channel activation allows a moderate and rather slow Ca^{2+} entry (Bean, 1989). In fact, the molecular identity of the pore-forming channel subunit differs in both tissues, cardiac fibers expressing the $\text{Ca}_v1.2$ isoform while skeletal muscles express the $\text{Ca}_v1.1$ isoform. Nevertheless, the overall subunit composition of both channels, illustrated in Figure 1, bears interesting similarities.

The opening of these ion channels in response to plasma membrane depolarization results from a complex series of conformational changes occurring mainly in the pore-forming subunits. In these subunits, positively charged S4 segments act as voltage-sensing mechanical transducers by altering their position within the plasma membrane as a function of voltage value (Nakai, Adams, Imoto, & Beam, 1994). How movements of other structural elements are related to these S4 repositioning is still an open but particularly important question with regard to the excitation-contraction coupling process as we will see later. In both tissues, the activation of L-type voltage-gated calcium channels produces the opening of another calcium channel located in the membrane of the sarcoplasmic reticulum, the main intracellular Ca^{2+} store in muscles. This process leads to a transient and important increase in cytoplasmic Ca^{2+} concentration that is at the basis of muscle contraction. This particular ion channel presents several interesting features: i) it is sensitive to the plant alkaloid ryanodine (and is therefore named “ryanodine receptor”), ii) it is positioned in such a way that it faces the L-type channel in order to maximize activation efficiency, and iii) in both tissues, its activation and inactivation are controlled by cytoplasmic Ca^{2+} and Mg^{2+} concentrations (Figure 2).

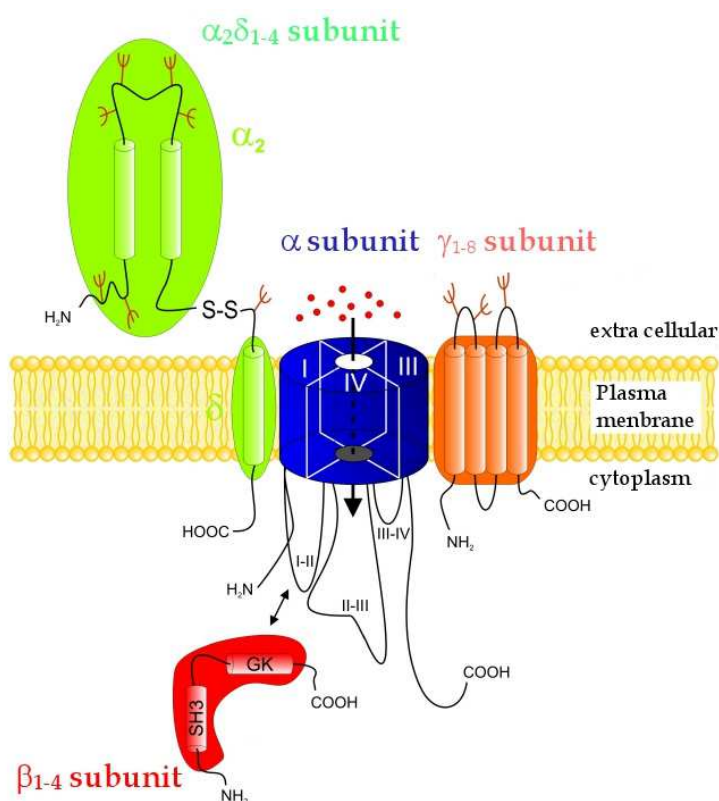


Fig. 1. Schematic representation of an L-type voltage-dependent calcium channel. $Ca_v\alpha$ is the voltage-sensing and pore-forming subunit, whereas $\alpha_2\delta$, β and γ are auxiliary subunits. $Ca_v\alpha$ is constituted of four homologous hydrophobic domains linked by cytoplasmic loops. The II-III loop, that links domains II and III, is the focus of this review. The β subunits is the only protein that is fully cytoplasmic.

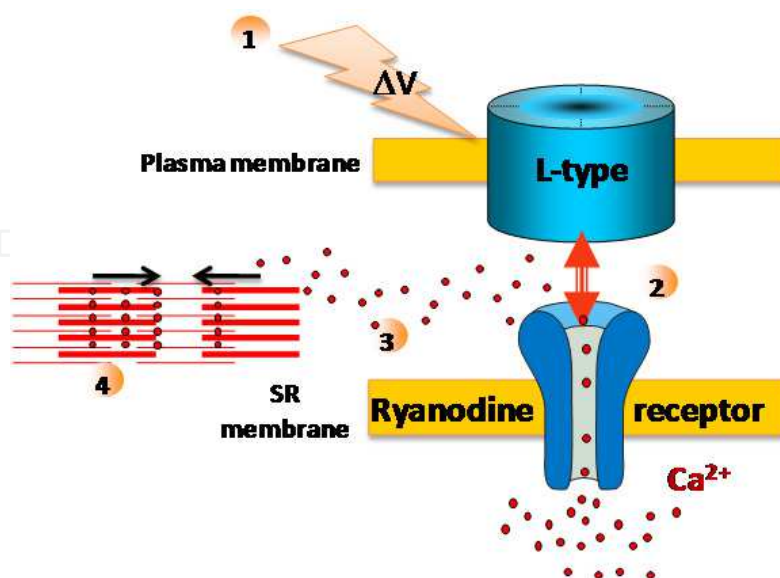


Fig. 2. Cartoon describing the general process of excitation-contraction coupling, starting by membrane depolarization (1), L-type channel activation and transfer of conformational change to the ryanodine receptor (2), ryanodine receptor opening and sarcoplasmic reticulum Ca^{2+} release (3), and muscle contraction (4).

As for the L-type calcium channels, two different types of ryanodine receptors are respectively expressed in skeletal (type 1 ryanodine receptor) and cardiac (type 2 ryanodine receptors) muscles. These two types of ryanodine receptors differ in their sensitivity to cytoplasmic Ca^{2+} concentration. Type 1 ryanodine receptor appears to be more sensitive to inhibition by high cytosolic Ca^{2+} concentration than type 2 ryanodine receptors. In contrast, there does not seem to be major differences with regard to activation by cytosolic Ca^{2+} (Chu, Fill, Stefani, & Entman, 1993; Fruen, Bardy, Byrem, Strasburg, & Louis, 2000; Michalak, Dupraz, & Shoshan-Barmatz, 1988). *In vivo*, cardiac type 2 ryanodine receptor appears to be less repressed by L-type calcium channel than type 1 ryanodine receptors as witnessed by the far greater likelihood to observe spontaneous channel openings (spark events) (H. Cheng, Lederer, & Cannell, 1993; Wier & Balke, 1999). The differences in biophysical properties between skeletal and cardiac L-type calcium channels on one hand, and skeletal and cardiac ryanodine receptors on the other hand, led to differences in excitation-contraction coupling mechanisms between these two tissue. In cardiac tissues, Ca^{2+} entering through activated L-type channels is sufficient to directly activate type 2 ryanodine receptors. This chain of events is called Ca^{2+} -induced Ca^{2+} -release. In this case, opening of the type 2 ryanodine receptor strictly depends on extracellular Ca^{2+} (Nabauer, Callewaert, Cleemann, & Morad, 1989). Indeed, the Ca^{2+} release process through type 2 ryanodine receptors is a U-shape function of voltage and follows the U-shape voltage-dependence of external Ca^{2+} entry. Moreover, blocking external Ca^{2+} entry precludes voltage-activated Ca^{2+} release through type 2 receptors. Type 1 ryanodine receptors in skeletal muscles differ from type 2 ryanodine receptors in their mode of activation. In spite of the capability of type 1 ryanodine receptors to perform Ca^{2+} -induced Ca^{2+} -release, the amplitude of Ca^{2+} entry through activated skeletal L-type channels is too low and its kinetic too slow to trigger a cardiac type Ca^{2+} -induced Ca^{2+} -release mechanism. Indeed, in these cells, the Ca^{2+} release process is no longer U-shaped in response to voltage increase and strictly follows the sigmoidal voltage-dependence of activation of L-type channels. Ca^{2+} permeability through skeletal L-type channels plays therefore a negligible role in the skeletal type excitation-contraction coupling. In skeletal muscles, the voltage-dependent trigger of type 1 ryanodine receptor activation is the changes in L-type channel conformation. This process has been termed voltage-induced Ca^{2+} release (Schneider & Chandler, 1973; Tanabe, Beam, Powell, & Numa, 1988). While it may seem comfortable for intellectual construction to oppose Ca^{2+} -induced Ca^{2+} release to voltage-induced Ca^{2+} release, one should keep in mind that such a drastic distinction between these two mechanisms also presents intrinsic restrictions. In skeletal muscles, the conformational transmission between L-type channel and type 1 ryanodine receptor requires a physical interaction between these channel types. While conceptually, the process of Ca^{2+} -induced Ca^{2+} release in cardiac tissues does not require a physical interaction between the two channels, it would be a mistake to eliminate the hypothesis that conformational changes in cardiac L-type channels can also be transmitted directly to type 2 ryanodine receptor. Conversely, while skeletal L-type conformational changes are essential to trigger Ca^{2+} release through type 1 ryanodine receptors in skeletal muscles, ruling out a contribution of Ca^{2+} (originating from internal sources) in the release of Ca^{2+} itself may also represent a shortcut attitude. In this review we will therefore not oppose the two modes of Ca^{2+} -release but simply take into account that in cardiac tissues external Ca^{2+} is an important factor, while in skeletal muscle conformational changes in L-type channels is a more important factor. We will assume that cardiac L-type channels might also be in interaction with the ryanodine type 2 receptor. The next paragraph will

describe what is known in terms of molecular interactions between L-type channels and ryanodine receptors, focusing mainly on the most important determinants for excitation-contraction coupling.

2. Molecular determinants of the interaction between L-type channels and ryanodine receptors

2.1 Contribution of two separate domains of the II-III loop of $\text{Ca}_v1.1$ in voltage-induced Ca^{2+} release

Because voltage-induced Ca^{2+} release clearly relies on a direct interaction between skeletal muscle L-type and type 1 ryanodine receptor, most of the researches have focused on the interaction between these two channels. In this context, the use of animal models has been decisive in identifying molecular determinants critical for the excitation-contraction coupling process. Three mice models have been widely used: i) the dysgenic mice *mdg* corresponding to a natural knockout of the pore-forming subunit of skeletal L-type channels (Tanabe et al., 1988), ii) the dyspedic mice, a knock-out of type 1 ryanodine receptor (Takeshima et al., 1995), and iii) the β -null mice, a knock-out of the β auxiliary subunit of the skeletal L-type channel. All these gene knock-outs produce lethality at birth by defective respiratory muscle function implying that studies are done with primary myotubes isolated from late embryos.

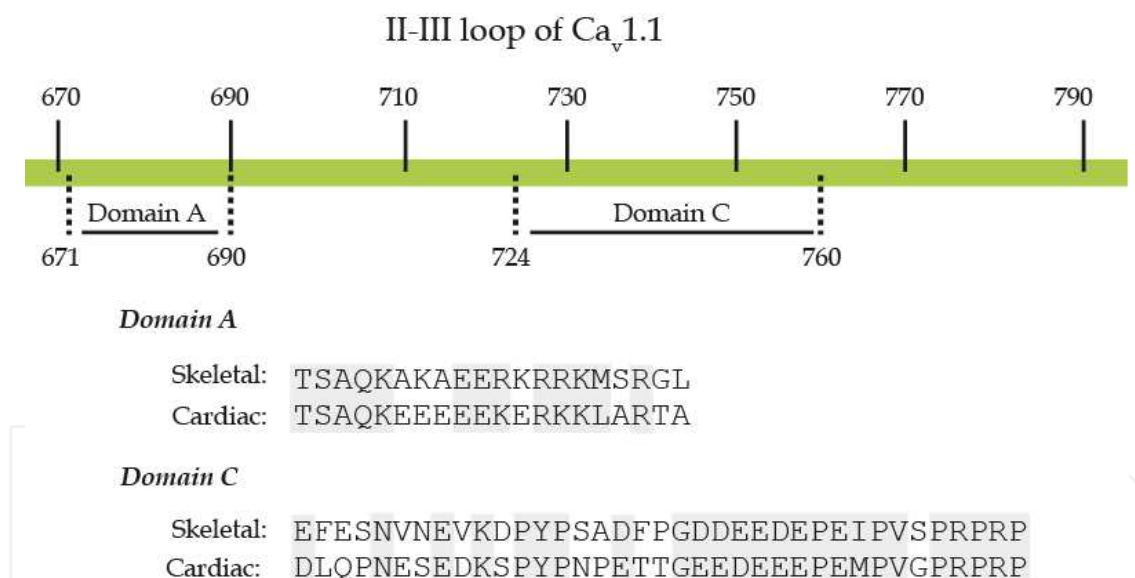


Fig. 3. Amino acid positions of skeletal muscle $\text{Ca}_v1.1$ Domains A and C. Amino acid sequences of these domains are compared to those of equivalent domains of the cardiac L-type $\text{Ca}_v1.2$ channel.

In a first series of precept experiments, *mdg* myotubes were used to express chimeras between the pore-forming $\text{Ca}_v\alpha$ subunits of skeletal and cardiac L-type channels in order to identify a skeletal muscle-specific L-type determinant responsible for voltage-induced Ca^{2+} release. This approach led to the identification of one particularly important determinant, the large cytoplasmic loop between the second and third hydrophobic domains (called II-III loop) of $\text{Ca}_v1.1$ (Nakai, Tanabe, Konno, Adams, & Beam, 1998) (see

Figure 3). According to structural predictions, this loop would be of 137 (Ca_v1.1) to 146 (Ca_v1.2) amino acids long. Further, restriction of the skeletal L-type domain responsible for voltage-induced Ca²⁺ release through type 1 ryanodine receptors locates a sequence of 36 amino acids, 62 amino acids downstream of the start of I-II loop sequence. This domain is called *Domain C*. While this region is undoubtedly important in Ca_v1.1, the sequence homology with the equivalent *Domain C* of Ca_v1.2 remains nevertheless quite high. The replacement of Ala⁷³⁹, Phe⁷⁴¹, Pro⁷⁴² and Asp⁷⁴⁴ of Ca_v1.1 by, respectively, Pro, Thr, Thr and Glu of Ca_v1.2 reduces voltage-induced Ca²⁺ release (Kugler, Grabner, Platzer, Striessnig, & Flucher, 2004; Kugler, Weiss, Flucher, & Grabner, 2004).

While these studies clearly demonstrated that *Domain C* is critical for allowing voltage-induced Ca²⁺-release by L-type channels, the exact mechanism whereby this domain contributes to the process has still not been resolved. There is no solid evidence for a direct interaction between *Domain C* and the ryanodine receptor, suggesting that its mode of action is indirect or that this interaction relies on a transient conformation of the Ca_vα subunit controlled by voltage. Following the identification of the II-III loop as an important determinant in voltage-induced Ca²⁺ release, synthetic peptides corresponding to fragments of this loop were screened for their ability to interact with purified ryanodine receptors and modify their gating activity. Curiously, this approach led to the identification of a ryanodine receptor-interacting domain of the II-III loop that is different of *Domain C*. This sequence has been termed *Domain A* and corresponds to the amino acid region Thr⁶⁷¹ to Leu⁶⁹⁰ of Ca_v1.1 (Figure 2). One particular feature of this domain is the presence of a stretch of basic residues (8 amino acids out of 20 are either Arg or Lys residues). *Domain A* of Ca_v1.2 bears sequence homology (10 residues are identical out of 20 and 3 have homology). In spite of this high sequence homology, cardiac *domain A* is considerably enriched in acidic residues suggesting that its function may have evolved differently than its skeletal muscle counterpart. Proof of functional differences between cardiac and skeletal *domain A* comes from the fact that skeletal *domain A* interacts with type 1 ryanodine receptor but not type 2 (O'Reilly et al., 2002). Conversely, cardiac *domain A* does not interact with type 1 ryanodine receptor (el-Hayek, Antoniu, Wang, Hamilton, & Ikemoto, 1995; O'Reilly et al., 2002). Curiously, there are no reports on the potential interaction between cardiac *domain A* and type 2 ryanodine receptor thereby illustrating the little credit the scientific community is giving to the possibility that cardiac L-type and type 2 ryanodine receptors physically interact. While direct interaction of skeletal *domain A* with type 1 ryanodine receptor, and evidence for functional effects (reviewed in paragraph 3), would ensure that this domain plays a critical role in excitation-contraction coupling, this possibility is hotly debated. In 2000, a study reported that the partial scrambling of skeletal *domain A* had little impact on excitation-contraction coupling (Proenza, Wilkens, & Beam, 2000). Careful examination of the scrambled sequence reveals that scrambling occurs on the ten last residues of *domain A* and that many of the residues remain in fact at the same position. Moreover a characteristic feature of *domain A*, a rich content in basic amino acid, is conserved in the scrambled sequence. At the functional level, while excitation-contraction coupling is preserved in principle, no quantification of the extent of this conservation is performed. While these data are interesting, it would have been preferable to perform a more extensive scrambling of the sequence (using the entire domain and avoiding conservation of position for some residues). Other evidences question the role of *domain A* in excitation-contraction coupling. Replacement of the skeletal Ca_v1.1 II-III loop by the II-III loop of *Musca domestica* Ca_vα

channel, which has a poor homology with $Ca_v1.1$, largely prevents excitation-contraction coupling, but not completely, while reintegration of skeletal type *domain C* in this construct is enough to restore voltage-induced Ca^{2+} -release (Wilkens, Kasielke, Flucher, Beam, & Grabner, 2001). It should be mentioned that the chimera construct in which the entire II-III loop is replaced displays very little Ca^{2+} permeability, possibly questioning the correct functioning of the chimera channel. The study lacks also a chimera construct with the integration of skeletal muscle *domain A* rather than *domain C*. Also, expression of two skeletal $Ca_v1.1$ hemi-channels, one lacking *domain A*, reconstitutes voltage-induced Ca^{2+} release in *mdg* myotubes (Ahern, Arikath et al., 2001). Similarly, complete deletion of *domain A* in $Ca_v1.1$ does not affect the amplitude of voltage-induced Ca^{2+} release, although it seems to speed the process (Ahern, Bhattacharya, Mortenson, & Coronado, 2001). Very curiously, in the later study, the same authors demonstrate that deletion of both *domains A* and *C* results in the reappearance of some voltage-induced Ca^{2+} release. While these studies indicate that the presence of *domain A* is not strictly necessary to observe voltage-induced Ca^{2+} release in skeletal muscles, it is hard to conclude that it has no function at all. Also, while *domain C* appears to be important to observe the excitation-contraction coupling process, the data tend to indicate that there might be some interdependence between II-III loop domains to exert their function. Indeed, it was observed *in vitro* that *domain C* exerts an inhibitory effect on the activating function of *domain A* on Ca^{2+} release from type 1 ryanodine receptor or on [3H]-ryanodine binding (el-Hayek, Antoniu et al., 1995; Ikemoto & el-Hayek, 1998).

2.2 The skeletal muscle L-type channel contains other important determinants for excitation-contraction coupling

While the II-III loop of $Ca_v1.1$ has focused much of the attention in the comprehension of the excitation-contraction coupling process, it is important to mention that other relevant L-type channel determinants have been proposed to play a role in the process. Two of these determinants are present on the $Ca_v1.1$ pore-forming subunit (the III-IV loop and the C-terminal domain), and the second is constituted by the auxiliary β_{1a} subunit. Their implication is summarized hereunder.

Suspicion of the involvement of the III-IV loop of $Ca_v1.1$ arose as a consequence of the identification of a point mutation (Arg¹⁰⁸⁶His) associated to malignant hyperthermia, a skeletal muscle pathology in which inhalation of volatile anesthetics provokes enhanced Ca^{2+} release from the sarcoplasmic reticulum, excessive ATP hydrolysis, heat production and therefore muscle damage (Monnier, Procaccio, Stieglitz, & Lunardi, 1997). A functional analysis of this mutation highlights a putative role of this loop in controlling the voltage-dependence of the Ca^{2+} release thru type 1 ryanodine receptor and its caffeine sensitivity (Weiss et al., 2004). Caffeine is a well characterized exogenous activator of all types of ryanodine receptors favors Ca^{2+} -induced Ca^{2+} release by sensitizing the ryanodine receptors to activating cytosolic Ca^{2+} . A potential interaction site of this loop on type 1 ryanodine receptor has been identified that encompasses amino acids 922 to 1112 (Figure 4) (Leong & MacLennan, 1998b).

It is noteworthy that this III-IV loop is extremely conserved among $Ca_v\alpha$ subunits (with 46 amino acids identical on 53 between $Ca_v1.1$ and $Ca_v1.2$). This suggests that the cardiac III-IV loop may also interact with type 2 ryanodine receptor.

The C-terminus of $Ca_v1.1$ has also been shown to be an interesting determinant. The amino acid region 1543 to 1647 of $Ca_v1.1$ plays a role in the transport and targeting of $Ca_v1.1$ to the triad (Flucher, Kasielke, & Grabner, 2000; Proenza, Wilkens, Lorenzon, & Beam, 2000). The 1393 to 1527 amino acid region of $Ca_v1.1$ binds Ca^{2+} and calmodulin. In the absence of calmodulin, this domain has been shown to bind type 1 ryanodine receptor at amino acid position 3609 to 3643. A synthetic peptide corresponding to the 1487 to 1506 region of $Ca_v1.1$ inhibits both ryanodine binding and ryanodine receptor channel gating in bilayers (Sencer et al., 2001; Slavik et al., 1997). The authors of these findings suggest that these domains could participate to Ca^{2+} and/or calmodulin regulation of voltage-induced Ca^{2+} release, but more studies are warranted to confirm their importance.

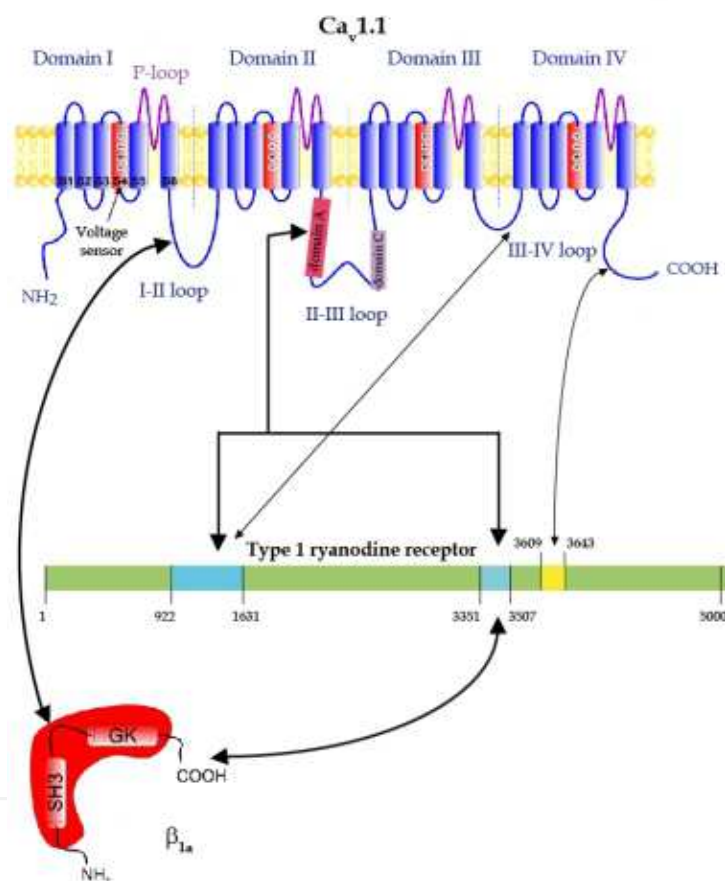


Fig. 4. Summary of known protein interactions between skeletal muscle L-type $Ca_v1.1$ and β subunits with type 1 ryanodine receptor. The amino acid positions of *Domains A* and *C* are defined in the text.

As mentioned above, absence of the β_{1a} subunit of the L-type calcium channel led to a complete loss of excitation-contraction coupling in skeletal muscle. Recently, this subunit has been shown to directly interact with the type 1 ryanodine receptor (W. Cheng, Altafaj, Ronjat, & Coronado, 2005). Binding experiments identified a cluster of positively charged residues of type 1 ryanodine receptor that control this interaction. Neutralization or deletion of these amino acids severely depress the amplitude of the depolarization induced Ca^{2+} release through type 1 ryanodine receptor, indicating that this interaction is important in the regulation of the excitation-contraction coupling in skeletal muscles.

3. Is *domain A* of relevance to excitation-contraction coupling?

Although the functional relevance of *domain A* is questioned by studies that have used chimera $\text{Ca}_v\alpha$ channels, we still believe that it is of interest to investigate the precise role of *domain A* in excitation-contraction coupling. Indeed, it is important to note the intrinsic limitations of several reports that rely on the use of chimera channels and *mdg* myotubes. For a chimera channel to integrate into the triad (the plasma membrane loci where skeletal muscle excitation-contraction takes place), it needs to preserve sufficient molecular determinants that guarantee normal cell trafficking and correct localization with regard to type 1 ryanodine receptor. The role of these determinants on excitation-contraction coupling, and specifically on voltage-induced Ca^{2+} -release, cannot be investigated. Also, these studies and conclusions are all based on the assumption (that is not yet proven) that Ca^{2+} -induced Ca^{2+} -release by cardiac L-type channels and type 2 ryanodine receptors can occur in the absence of conformational changes in $\text{Ca}_v1.2$ (invariably triggered by voltage changes). Testing this hypothesis would require to block voltage-dependent $\text{Ca}_v1.2$ charge movements without blocking Ca^{2+} permeability. While this is a provocative opinion, one could conclude that the chimera studies have demonstrated that skeletal muscle *domain C* governs the requirement on external Ca^{2+} to produce Ca^{2+} release: when skeletal *domain C* is present, external Ca^{2+} would not be required, while when absent, external Ca^{2+} is mandatory. *Domain C* is undoubtedly important in participating to voltage-induced Ca^{2+} release. However, how is *domain C* voltage-dependent and how does it transmit voltage-dependent conformational changes to type 1 ryanodine receptor without directly interacting with this channel?

In voltage-induced Ca^{2+} -release, which occurs in skeletal muscles, the initial trigger for Ca^{2+} release through type 1 ryanodine receptor is brought by the voltage change. However, voltage might be considered as the initial trigger for initiating the Ca^{2+} release process, and the released Ca^{2+} , once it has reached a critical concentration, may contribute to the process by promoting Ca^{2+} -induced Ca^{2+} release. This can be expected to occur as type 1 ryanodine receptor is perfectly capable to support Ca^{2+} -induced Ca^{2+} release instead of voltage-induced Ca^{2+} release such as when $\text{Ca}_v1.2$ replaces $\text{Ca}_v1.1$. While there is no doubt that type 1 ryanodine receptor can support both voltage- and Ca^{2+} -induced Ca^{2+} release, very little is known on the contribution of the different $\text{Ca}_v1.1$ *domains* (in particular *A* and *C*) to the balance between these two triggering processes. Among other arguments in favor of a contribution of *domain A* to excitation-contraction coupling is the fact that *domain A* is heavily charged. Obviously, voltage-induced Ca^{2+} release requires both the presence of $\text{Ca}_v1.1$ *domain C* in the context of an operational channel and a change in voltage, but it is expected that one type 1 ryanodine receptor-interacting $\text{Ca}_v1.1$ determinant is altering its conformation upon depolarization to influence excitation-contraction coupling. The basic nature of *domain A* is of interest in that respect since (i) it binds type 1 ryanodine receptor, (ii) influences the Ca^{2+} sensitivity of type 1 ryanodine receptor, and (iii) is susceptible to sense voltage changes owing to its close proximity with the plasma membrane. This latter point has never been investigated of course, but needless to say, *domain C* is not the most convincing candidate as voltage-sensor owing to its location within the channel and the lower net charge of the sequence. Earlier evidences that *domains A* and *C* may interact are also interesting since it could be envisioned that voltage-changes alter the interaction between these two domains thereby dynamically regulating excitation-contraction coupling. Finally, most of the studies on excitation-contraction coupling and on voltage-induced Ca^{2+} release in particular have focused their attention on the release process itself but not on the termination of the signal upon membrane

repolarisation. In that respect, it would be interesting to have a careful examination of the role of *domain A* on the ending of voltage-induced Ca²⁺ release.

4. Animal toxins presenting an intriguing sequence homology with *domain A*

In the course of ongoing studies on ryanodine receptor channel pharmacology, the group of Coronado identified imperatoxin A as an effector of type 1 ryanodine receptor (Valdivia, Kirby, Lederer, & Coronado, 1992). The toxin has high affinity for the channel (close to 10 nM), produces an increase in channel opening probability by reducing the closure times, induces Ca²⁺ release from purified sarcoplasmic reticulum and increases [³H]-ryanodine binding by conversion of the ryanodine binding site from a low to a high affinity state (el-Hayek, Lokuta, Arevalo, & Valdivia, 1995). Imperatoxin A was isolated from the venom of the African scorpion *Pandinus imperator*. It is a 33 amino acid peptide containing six cysteine residues and therefore three internal disulfide bridges. The pattern of connectivity results in a fold of the “inhibitor cysteine knot” type with three β-strands (Green et al., 2003). The peptide was first synthesized in 2007 (Zamudio et al., 1997). Since this initial discovery, other toxins have been identified, coming from the venom of other scorpions, that all share high sequence homology with imperatoxin A (Figure 5). These include maurocalcine in 2000 from *Scorpio maurus palmatus* (Mosbah et al., 2000), immediately synthesized in 2000 (Fajloun et al., 2000), two opicalcine variants in 2003 (S. Zhu, Darbon, Dyason, Verdonck, & Tytgat, 2003), hemicalcin in 2007 (Shahbazzadeh et al., 2007), and hadrucalcin in 2009 (Schwartz et al., 2009). All of these peptides have been tested on type 1 ryanodine receptor and shown to possess similar pharmacological properties as imperatoxin A. These toxins therefore define a family of functional homologous peptides.

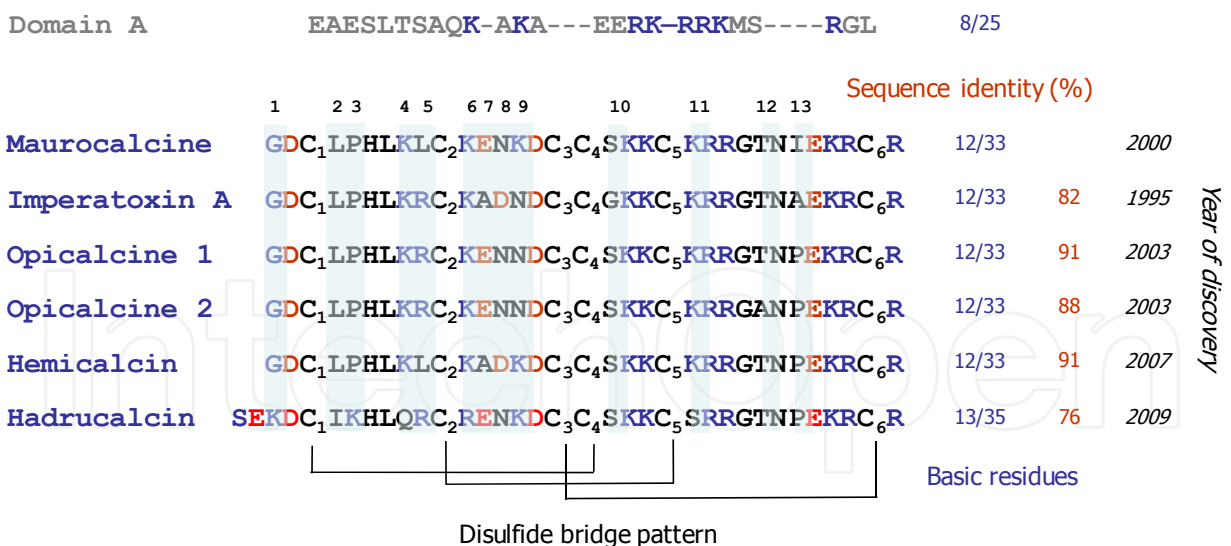


Fig. 5. Amino acid sequences and alignment of maurocalcine-like toxins acting on ryanodine receptors. Sequence homology with skeletal muscle *domain A* is also shown. Positively charged residues are shown in blue, sequence identities are given in red and the year of discovery of each toxin is also provided.

A close comparison of the peptide sequences reveals an important conservation. All peptides are heavily charged, containing mostly Lys residues and some Arg residues, and

the net charge of these peptides is high (+7 if one excludes the potential basic charge of the His residue that is present in all toxins). Most of these basic residues are located on one face of the molecule, creating an important dipole moment in these toxins (Boisseau et al., 2006). A closer examination of these sequences also reveals that a limited stretch of the primary structure of the toxins is highly homologous to the primary structure of skeletal muscle *domain A* (Figure 4). Moreover, modification of *domain A*, meant to further improve the structural similarity between *domain A* and imperatoxin A and maurocalcine, led to a mutant *domain A* with increased functional effects on type 1 ryanodine receptors (Green et al., 2003). These effects include stimulation of Ca^{2+} release from sarcoplasmic reticulum vesicles and increased channel opening probability of type 1 ryanodine receptor incorporated in lipid bilayers. Of note, following the action of this optimized *domain A* peptide, these toxins have no cumulative effect on channel activation, strongly suggesting that *domain A* and the toxins share the same binding site on the ryanodine receptors. These experiments highlight in any case the functional relevance of this limited structural homology between *domain A* and the toxins. Further evidence for the importance of this cluster of residues comes from an elegant study of the group of Valdivia. The cardiac *domain A*, which is poorly activator of type 1 ryanodine receptor, can be modified to become a strong type 1 ryanodine receptor activator by selected mutations within this cluster of residues (X. Zhu, Gurrola, Jiang, Walker, & Valdivia, 1999). The mutations were chosen in such a way that cardiac *domain A* integrates the cluster of basic residues of *domain A* that is homologous to the toxin sequences.

5. *In vitro* functional similarities between maurocalcine and *domain A*

Among the various toxin activators of ryanodine receptors, maurocalcine is one of the best characterized. Besides sequence similarities with *domain A*, and the emerging evidence that both skeletal muscle *domain A* and maurocalcine share the binding sites on type 1 ryanodine receptor, there is also cumulative indication for functional similarities. We rapidly summarize these similarities hereunder. First, both *domain A* and maurocalcine enhance channel activity by promoting an increase in channel opening probability and the appearance of a subconductance state (Chen et al., 2003; Fajloun et al., 2000; Lukacs et al., 2008; O'Reilly et al., 2002). Both peptides differ however with regard to the level of subconductance state and the duration of channel opening in this subconductance state. Toxins promote long-lasting openings in the subconductance state that are barely observed with *domain A*. Interestingly, the various toxins also differ among each other with regard to the level of subconductance states (Chen et al., 2003; el-Hayek, Lokuta et al., 1995; Tripathy, Resch, Xu, Valdivia, & Meissner, 1998), suggesting that sequence divergences may control this subconductance level. Indeed, we observed that single point mutations in maurocalcine modify this subconductance level (Lukacs et al., 2008). The absence of long-lasting events with *domain A* might also reflect its significantly lower affinity for type 1 ryanodine receptor compared to the toxin. Second, in agreement with their potentiating effects on channel activity, both maurocalcine and *domain A* induce Ca^{2+} release from purified sarcoplasmic reticulum vesicles (Dulhunty et al., 1999; el-Hayek, Antoniu et al., 1995; Esteve et al., 2003). These stimulating effects of *domain A* on Ca^{2+} release are observed at high concentrations (above 10 μM in general) and are not consistently reported. In one study, application of peptide A was reported to decrease the caffeine- and Ca^{2+} -induced Ca^{2+} release (Chen et al., 2003), indicating in any case a relationship between the binding site of *domain A* and the

Ca²⁺-sensitivity of the type 1 ryanodine receptors. In the same study, high concentrations of peptide A actually block the effect of maurocalcine, again suggesting that both peptides bind onto the same site on the type 1 ryanodine receptor. The variable response of type 1 ryanodine receptor to *domain A*, as can be concluded from the various reports, also suggest that peptide conformation or cofactors largely influence the response of type 1 ryanodine receptor to *domain A*. Third, both *domain A* and maurocalcine increase [³H]-ryanodine binding (Chen et al., 2003; el-Hayek, Antoniu et al., 1995; Gurrola et al., 1999; Lu, Xu, & Meissner, 1994; X. Zhu et al., 1999). This stimulation is expected since conditions that trigger ryanodine channel opening are reported to produce a conversion in the binding site for ryanodine from a low affinity state to a higher one. The amplitude of the increase in [³H]-ryanodine binding, triggered by *domain A*, maurocalcine or other toxins, appears quite variable. This stimulation depends on other factor, such as the concentrations of cytosolic Ca²⁺ (Esteve et al., 2003; Tripathy et al., 1998) and Mg²⁺ (unpublished observations). This dependence on cytosolic Ca²⁺ concentration deserves some important comments that may be of interest for the understanding of excitation-contraction coupling. Cytosolic Ca²⁺ concentration has a biphasic effect of [³H]-ryanodine binding and channel gating, being an activator at low concentrations (above 100 nM) and inhibitor at higher concentrations (between 10 μM and 1 mM). Interestingly, maurocalcine and imperatoxin A were reported to alter this Ca²⁺-dependence by sensitizing type 1 ryanodine receptors to both the stimulation and inhibitory effects of Ca²⁺ (Esteve et al., 2003; Tripathy et al., 1998). For instance, while 100 nM Ca²⁺ does not trigger Ca²⁺ release from type 1 ryanodine receptor, this same concentration will produce Ca²⁺-induced Ca²⁺-release in the presence of maurocalcine. This is an important observation because it means that binding of maurocalcine on type 1 ryanodine receptor makes it prone to perform Ca²⁺-induced Ca²⁺ release. The analogy between maurocalcine and peptide A questions the possibility that *domain A* controls the ability of type 1 ryanodine receptors to perform Ca²⁺-induced Ca²⁺ release. The possibility that this control is revealed during membrane depolarization opens interesting questions.

An important finding has been the identification of peptide A and toxin binding site on type 1 ryanodine receptors. A first report describes the location of biotinylated imperatoxin A binding sites on tetramers of type 1 ryanodine receptors (Samsó, Trujillo, Gurrola, Valdivia, & Wagenknecht, 1999). This study illustrates that four imperatoxin A can bind onto a cytoplasmic domain of the tetramer of type 1 ryanodine receptors between the clam and handle domains, 11 nm away from the transmembrane pore. The far distance of binding of imperatoxin A indicates that its effect on channel gating occurs through an allosteric mechanism. In a 3D reconstruction of type 1 ryanodine receptor, the imperatoxin A binding site is in close physical proximity to the Ca²⁺-calmodulin binding site (Wagenknecht & Samsó, 2002). More precise location of the binding sites of the toxins has been investigated using recombinant fusion protein of fragments of the type 1 ryanodine receptor. Using biotinylated maurocalcine and peptide A, Altafaj and coll. identified two type 1 ryanodine receptor sequences that bind either *domain A* or maurocalcine (Altafaj et al., 2005). Weak interaction was observed with a fragment encompassing amino acid region 1021 to 1631 that contains the 37 amino acid sequence that was previously shown to bind to the skeletal muscle Ca_v1.1 II-III loop (Leong & MacLennan, 1998a). Stronger interaction was shown to occur with amino acid region 3351 to 3507. These findings are in agreement with the predicted localization of imperatoxin A

binding site on the 3D structure of type 1 ryanodine receptor. They are also in agreement with the suggestion that these two regions are in close proximity in space, thereby suggesting that they constitute a single binding site. In any case, these data demonstrate for the first time that maurocalcine and *domain A* share the same binding site. Of importance, it has been observed that deletion of the 1272-1455 amino acid region within type 1 ryanodine receptor provokes the loss of depolarization induced Ca^{2+} release (Perez, Mukherjee, & Allen, 2003). Combined these observations reinforce the idea that *domain A* and its binding site are somehow involved in the control of voltage-induced Ca^{2+} release. While maurocalcine has limited functional effects on type 2 ryanodine receptors, it is interesting to note that it binds to this receptor through an homologous binding site (regions 1033 to 1622 and 3558 to 3609 of type 2 ryanodine receptors) (Altafaj et al., 2007). These results suggest that binding of the toxin can be dissociated from its functional effect. This possibility has lately been reinforced by unpublished observations showing that the effect of maurocalcine depends on the redox state of type 1 ryanodine receptor. Differences in redox states between type 1 and 2 ryanodine receptors may thus explain differences in maurocalcine effects on these two channels. Of note, the redox state of type 2 ryanodine receptor has been shown to control its activity under cardiomyocytes stretch conditions (Prosser, Ward, & Lederer, 2011).

6. Use of maurocalcine to understand excitation-contraction coupling in skeletal muscle cells

During the course of maurocalcine characterization, we have shown that external application of maurocalcine on cultured skeletal muscle myotubes produces a small, but detectable, transient cytoplasmic Ca^{2+} elevation (Esteve et al., 2003). This effect is also observed in the absence of external Ca^{2+} demonstrating the mobilization of intracellular stores. Finally, maurocalcine interferes with the action of 4-chloro-*m*-cresol indicating the involvement of type 1 ryanodine receptors. This was the first demonstration of a pharmacological effect of a maurocalcine-type toxin on intact muscle cells. Since then, similar observations have been made with imperatoxin A and hadrucalcin on cardiomyocytes (Gurrola, Capes, Zamudio, Possani, & Valdivia, 2010; Schwartz et al., 2009). These observations have prompted us to investigate the cell penetration properties of maurocalcine. A series of studies have demonstrated since 2005 that maurocalcine belongs to the cell penetrating peptide family with vector properties that can be derived for various diagnostic, imaging and therapeutic applications (Aroui et al., 2009; Boisseau et al., 2006; Esteve et al., 2005; Jayagopal et al., 2009; Mabrouk et al., 2007; Poillot et al., 2010). A more detailed investigation of the mode of action of maurocalcine in developing skeletal muscle cells and adult fibers reveals that the peptide acts preferentially on type 1 ryanodine receptors that are uncoupled from the L-type calcium channels (Szappanos et al., 2005). Indeed, maurocalcine increases the frequency of spontaneous Ca^{2+} release events followed by the appearance of ember-like long lasting Ca^{2+} release events in permeabilized adult muscle fibers. Muscle permeabilization is expected to favor the uncoupling between type 1 ryanodine receptors and L-type calcium channels. These observations tend to indicate that maurocalcine has difficulties to act on type 1 ryanodine receptors when *domain A* of L-type channels already occupies the binding site on type 1 ryanodine receptor. Besides this effect of maurocalcine on uncoupled type 1 ryanodine receptors, the effect of the peptide was also investigated on L-type coupled type 1 ryanodine receptors by using high concentrations of

the peptide to gain accessibility to its binding site on the ryanodine receptor. Maurocalcine was injected into adult skeletal muscle fibers and Ca^{2+} transients induced by membrane depolarization. In these experiments, incubation with maurocalcine does not induce *per se* a change in resting cytoplasmic Ca^{2+} concentrations or amplitude of the depolarization-induced Ca^{2+} release. Only minor effects of maurocalcine were observed on the onset kinetics and voltage-dependence of voltage-induced Ca^{2+} release. Although these effects are small, they tend to indicate that maurocalcine is able to occupy its binding site during depolarization. Interestingly, much stronger effects of maurocalcine were observed on the kinetics of the termination of Ca^{2+} release upon ending membrane depolarization (Pouvreau et al., 2006). These effects correlate with the duration and amplitude of membrane depolarization and confirm that depolarization progressively permits maurocalcine binding onto L-type coupled ryanodine receptors. Taken together, these experiments would suggest that *domain A* acts as a negative clamp on type 1 ryanodine receptor for Ca^{2+} release at resting membrane potential. In this configuration, its affinity for *domain A* is too high or the accessibility too restricted to allow maurocalcine binding to type 1 ryanodine receptor. Depolarization produces a change in conformation of *domain A* that reduces its affinity for the ryanodine receptor, thereby relieving the negative clamp for Ca^{2+} release. In this situation, maurocalcine gains access to the ryanodine receptor by competing with *domain A* for its binding site. Since maurocalcine does not trigger a gain of function with regard to Ca^{2+} release under membrane depolarization, this observation suggest that i) maurocalcine and *domain A* are functionally equivalent under depolarization, and ii) since maurocalcine is an activator of Ca^{2+} release, it is likely that *domain A* acquires activating functions under membrane depolarization. Upon membrane repolarization, *domain A* regains access to its binding site on the ryanodine receptor by increased affinity and competitive displacement of maurocalcine. The slow kinetics of maurocalcine displacement by *domain A* explains the slower termination of Ca^{2+} release. This sequence of events is depicted in Figure 6.

In the scheme of events presented in Figure 6, *domain A* acts as a functionally versatile ligand: a negative clamp under resting membrane potential and an activator under membrane depolarization. By analogy with maurocalcine action, we hypothesize that the activating function of *domain A* stems from an increase in sensitivity of type 1 ryanodine receptor to cytoplasmic Ca^{2+} . Two types of changes in Ca^{2+} sensitivity have been observed with maurocalcine: i) a shift in Ca^{2+} concentration required to activate Ca^{2+} -induced Ca^{2+} -release towards lowers concentrations (from a threshold of 100 to 10 nM Ca^{2+}), and ii) an enhanced amplitude of Ca^{2+} -induced Ca^{2+} release. With such a mechanism in hand, the initial trigger of Ca^{2+} elevation that sparks Ca^{2+} -induced Ca^{2+} release under these activating functions of *domain A* can be *domain C*. The Ca^{2+} sensitizing function of *domain A* under membrane depolarization highlights a new concept for excitation-contraction in skeletal muscles. Rather than opposing voltage-induced Ca^{2+} release and Ca^{2+} -induced Ca^{2+} release, the two phenomena might be closely linked. Voltage-induced Ca^{2+} release could be termed voltage-induced Ca^{2+} -induced Ca^{2+} release. The Ca^{2+} responsible for the induction of Ca^{2+} -induced Ca^{2+} release would originate from the initial opening of type 1 ryanodine receptor. In skeletal muscles, the presence of a negative clamp might be required to avoid spontaneous Ca^{2+} release events that otherwise would spark on Ca^{2+} release and contraction. In this scheme, the absence of *domain A* mainly alters the Ca^{2+} sensitivity of type 1 ryanodine receptors and does not prevent any activating function of *domain C*. When *domain A* is modified, change in Ca^{2+} dependence should be observed. However, this is

extremely difficult to address in classical experimental conditions where cytosolic Ca^{2+} concentration is not under control.

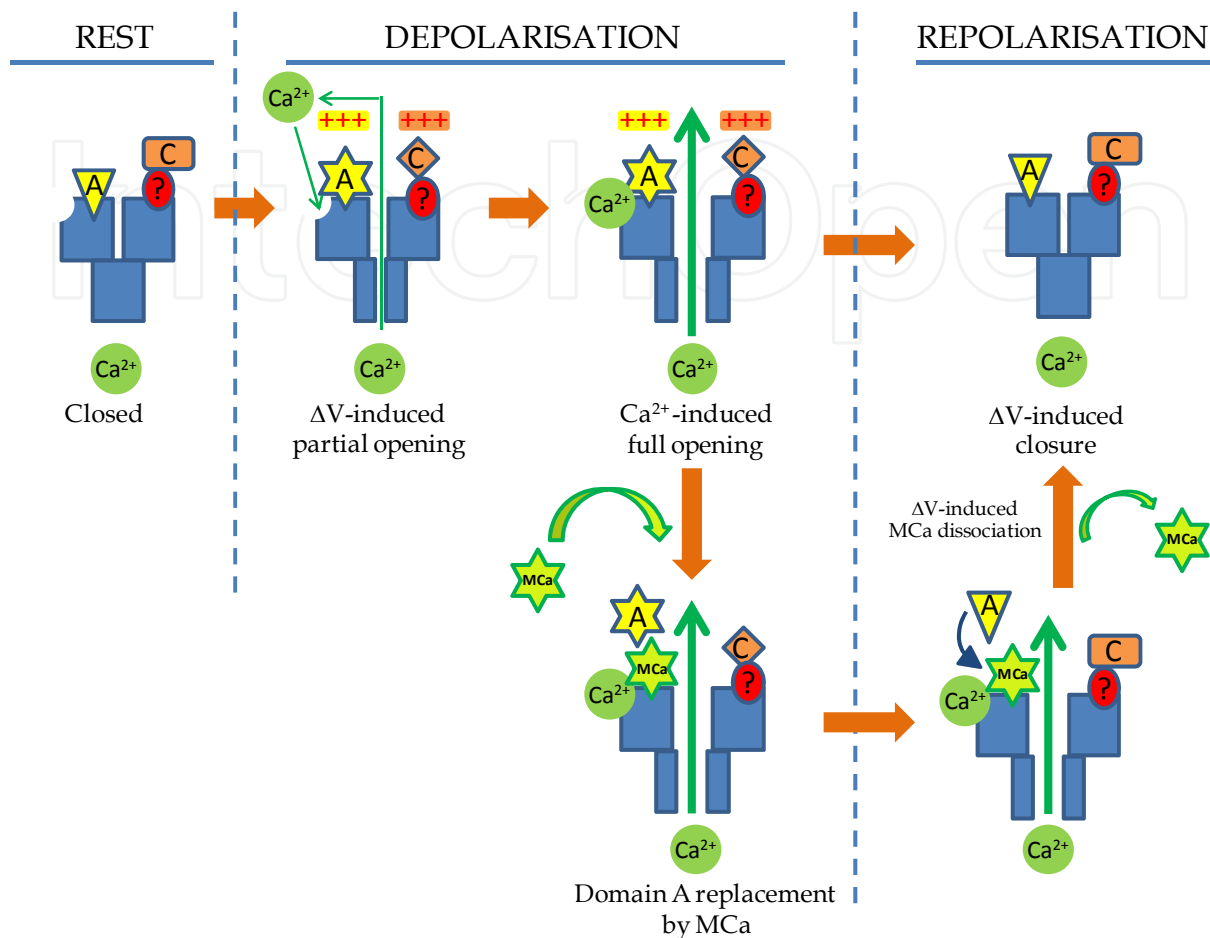


Fig. 6. Putative model of type 1 ryanodine receptor activation in skeletal muscles and role of domain A. A: domain A; C: domain C; ?: unknown putative domain C protein partner or receptor binding site. Membrane depolarization triggers domain C-mediated ryanodine receptor opening, while activating the Ca^{2+} -sensitizing function of domain A. Ca^{2+} -induced Ca^{2+} -release becomes the major sources of cytosolic Ca^{2+} elevation. Domain A interaction with the ryanodine receptor is weakened and maurocalcine can replace domain A with equivalent function. Upon membrane repolarization, the activating function of domain C is lost, domain A regains its affinity and the Ca^{2+} sensitivity is lost. If maurocalcine was occupying domain A binding site on the receptor, it is slowly displaced by the inhibitory conformation of domain A explaining the continuing Ca^{2+} release process in the interval, assuming that the activating function of maurocalcine is voltage-independent or at least less voltage-sensitive.

7. Conclusion

One common feature between domain A and maurocalcine is that both are positively charged. Interestingly, maurocalcine has been shown to possess a strong affinity for negatively charged membrane lipids (Boisseau et al., 2006; Ram et al., 2008), namely phosphatidylserine and phosphoinositides, but also gangliosides. Also, cell penetration of maurocalcine presents a voltage-sensitive component (Boisseau et al., 2006). These properties

introduce three interesting questions. First, does *domain A* interact with the plasma membrane, and in particular with negatively charged lipids? Second, owing to its close proximity with the plasma membrane and its charged nature, does *domain A* sense changes in membrane potential? Third, if yes, does *domain A* possess some of the plasma membrane translocation properties of maurocalcine by physically invading the plasma membrane during depolarization? While these questions remain unanswered, they may be of interest because these considerations could impact the possible voltage-dependent relationship existing between *domain A* and type 1 ryanodine receptor. Owing to its privileged interaction with type 1 ryanodine receptor through a discrete binding site, *domain A* may also confer voltage-sensitivity to the ryanodine receptor. Maurocalcine, as a tool, clearly demonstrated that the domain of type 1 ryanodine receptor on which maurocalcine or skeletal *domain A* binds plays an important role in the control of channel opening through pore-distant allosteric modifications. Therefore, it is conceivable that depolarization-induced movement of *domain A* within this site could impact the allosteric change that control type 1 ryanodine channel opening. Of note, while skeletal muscle *domain A* has a net positive charge of +6, cardiac *domain A* has a net charge of 0 making it far less suitable to sense changes in membrane potential. By extension, these considerations of voltage-dependence could apply to other domains of the II-III loop of voltage-gated calcium channels. One striking feature of *domain C* is that, contrary to *domain A*, it is negatively charged (12 residues out of 36 are negatively charged in skeletal and cardiac *domain C* - net negative charge -9). On the basis of these observations, one can therefore question the existence of a voltage-sensitivity in the conformation of *domain C*. Also, taking over an idea that has been postulated in the past, it wouldn't be unlikely, considering the electrostatic complementarities of *domains A* and *C*, that both domains interact with each other and that this interaction is under the control of voltage changes. This questions the specific relationship that might exist between *domain A* and *domain C* for the control of voltage-induced Ca^{2+} release.

Most studies that have been performed up to now in order to understand the roles of channel cytoplasmic domains in excitation-contraction coupling have suffered from a lack of structural knowledge. The identification of a restricted binding site of maurocalcine and *domain A* on ryanodine receptors opens the door to a better definition of the critical interacting amino acid residues through RMN studies. These structural studies are currently underway and indicate that some specific type 1 ryanodine receptor amino acids are involved in the interaction with both *domain A* and maurocalcine (unpublished results). At last, the identification of the essential amino acids will allow refined functional studies based on more precise $\text{Ca}_v1.1$ or ryanodine receptor channel mutagenesis. In the process of comparing the functional homologies between *domain A* and maurocalcine, it would be of interest to substitute *domain A* by maurocalcine sequence in $\text{Ca}_v1.1$.

The importance of maurocalcine as a tool to investigate excitation-contraction coupling is demonstrated by the fact that, for the first time, this peptide allows a perturbation of the excitation-contraction coupling process in native cells without requiring the modification of any of the proteins involved, by mutagenesis or chimeras. Swapping domains between various ion channel isoforms is indeed a particularly tricky approach considering that there must be a delicate voltage-dependent conformational relationship between numerous important structural determinants. The use of maurocalcine has highlighted two important matters in the study of excitation-contraction coupling. First, it illustrates that the nature of the relationship between *domain A* and type 1 ryanodine receptor changes during membrane

depolarization. Second, it uncovers the unexpected importance of *domain A* in the termination of Ca^{2+} release during membrane repolarization. The precise description of this voltage-induced type 1 ryanodine closure is lacking so far. Nevertheless, this result is the first evidence for the role of $\text{Ca}_v1.1$ *domain A* in this silencing process. The importance of this step in the control of Ca^{2+} homeostasis and contraction would justify that more studies be devoted to this issue.

The properties of maurocalcine to cross the plasma membrane suggest that it would be an interesting tool to modify excitation-contraction coupling in skeletal muscle *in vivo*. The fact that maurocalcine shares the same binding site than *domain A* on type 1 ryanodine receptor restricts its accessibility to L-type coupled ryanodine receptors. Obviously, it would thus be very interesting to investigate its effect on ryanodine receptors under conditions where its accessibility to the channel is not restricted, i.e. in the absence of $\text{Ca}_v1.1$. Effects of maurocalcine on *mdg* myotubes, lacking $\text{Ca}_v1.1$, are currently under investigation in our research team. These studies will provide important information on the effects of maurocalcine, and by inference on *domain A*, in a cellular context independent of the voltage-dependent channel environment and therefore also of the contribution of *domain C*.

The evidence for a voltage-modulated contribution of *domain A* to excitation-contraction coupling prompted us to emphasize the limits existing in the opposition between voltage-induced and Ca^{2+} -induced Ca^{2+} release mechanisms. Investigations focused on Ca^{2+} -induced Ca^{2+} release tend to take into account only the cell entry of Ca^{2+} from the extracellular space as trigger for Ca^{2+} release. The possible contribution of cytoplasmic or sarcoplasmic Ca^{2+} calcium in this process is not taken into account because of technical limitations. Indeed, it is quite difficult to maintain cytoplasmic Ca^{2+} at a certain concentration while at the same time measuring Ca^{2+} release from sarcoplasmic reticulum. Conversely, the possibility that type 2 ryanodine receptor Ca^{2+} release undergoes voltage-dependence might be masked by the voltage-dependence of external Ca^{2+} entry. If one takes into account these limitations, it is possible to draw a number of working hypotheses that do not necessarily oppose cardiac and skeletal muscle excitation-contraction coupling. Indeed, in light of the maurocalcine effects, we propose that the voltage-dependent modification of type 1 ryanodine receptor by *domain A* induces an increase of its sensitivity to cytoplasmic Ca^{2+} . Therefore, we propose to introduce the concept of voltage-induced- Ca^{2+} induced- Ca^{2+} release by which depolarization concomitantly promotes a change in Ca^{2+} sensitivity, through *domain A* of $\text{Ca}_v1.1$, and the opening, through *domain C*, of type 1 ryanodine receptor. In this model, the difference between cardiac and skeletal muscle excitation contraction coupling mechanism only resides on the absence of this change of Ca^{2+} sensitivity of the type 2 ryanodine receptor due to the absence of interaction of cardiac *domain A* with type 2 ryanodine receptor.

8. References

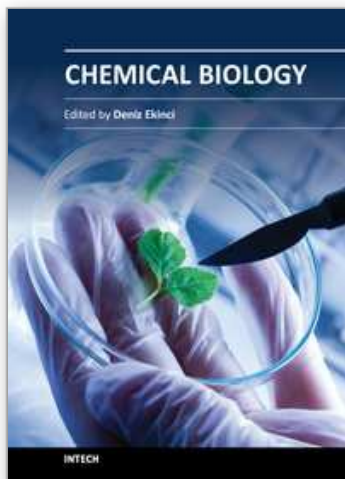
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