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RYANODINE RECEPTOR TYPE 3: WHY ANOTHER RYANODINE RECEPTOR ISOFORM?

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1. ABSTRACT

The family of ryanodine receptor (RyR) genes encodes three highly related Ca^{2+} release channels: RyR1, RyR2 and RyR3. Until about 10 years ago, RyRs were essentially known only for being the Ca^{2+} release channels of the sarcoplasmic reticulum of striated muscles, because of the high levels of expression of the RyR1 and RyR2 isoforms in skeletal and cardiac muscles, respectively. In contrast with the above picture, the RyR3 gene has been found not to be preferentially expressed in one specific tissue, but rather to be widely expressed in various cells. This wide expression pattern has been subsequently observed also for the RyR1 and RyR2 genes, which in addition to their preferential expression in striated muscles, have been found expressed also in several other cell types, although at lower levels than in striated muscles. Thus a closer look reveals that in several cells of vertebrates two or even three RyR isoforms can be co-expressed. In this chapter we will review published work on the RyR3 gene and discuss a model where co-expression of different RyR channel isoforms is interpreted as an evolutionary solution to provide, by functional interactions of distinct isoforms of Ca^{2+} release channels, the several types of vertebrate cells with the cell-specific Ca^{2+} release machinery required for generating the sophisticated intracellular Ca^{2+} signals needed for optimal regulation of their functions.

2. INTRODUCTION

2.1. The ryanodine receptor gene family of Ca^{2+} release channels and their pattern of expression

The family of ryanodine receptor (RyR) genes encodes three highly related Ca^{2+} release channels (1-3).

The three RyR genes, named RyR1, RyR2 and RyR3 are located in humans on chromosomes 19q12-13.2, 1q42.1-43, 15q13-14 (1-3). Until about 10 years ago RyRs were only known as the Ca^{2+} release channels of the sarcoplasmic reticulum of striated muscles, as RyR1 and RyR2 were known to be expressed at high levels in skeletal and cardiac muscles, where they play a central role in skeletal and cardiac excitation-contraction (e-c) coupling, respectively (4). The preferential expression of RyRs in muscle tissues can be traced back to Caenorhabditis elegans, whose genome contains only one RyR gene (5). In Caenorhabditis elegans, that is the most primitive organism where a RyR gene has been studied, we can already observe a preferential expression and a functional role of the RyR gene in muscle contraction. This is at variance with the $InsP_3R$ gene that in Caenorhabditis elegans is expressed in a more wide range of tissues (6). With the appearance of vertebrates, three RyR genes evolved. These genes present distinct tissue-specific patterns of expression and encode proteins that display distinctive regulatory properties. From this point of view, evolution and association of RyR1 and RyR2 with skeletal and cardiac tissue may reflect the increasing sophistication in the mechanisms of excitation-contraction coupling of these tissues in vertebrates. Through processes of gene duplication and diversification, the RyR1 and RyR2 isoforms emerged as the molecular tools necessary for the more sophisticated muscle tissues of vertebrate compared to the more primitive ones of invertebrates (7).

In contrast with the above picture of RyR1 and RyR2, which may suggest that these genes were selected to encode specialized Ca^{2+} release channels for striated muscles, the RyR3 gene lacks specific localization and is found expressed in a wide variety of tissues (8). It should be noted however that, similar to RyR3, the RyR1 and RyR2 genes are also expressed in many other tissues and cells in addition to their preferential expression in striated muscles, although at lower levels (9,10). Actually, there are currently many examples of tissues or cells where two or three RyR isoforms are being co-expressed.

So the question could be presented in the following terms: if RyR3 is not preferentially associated with one tissue, what is its physiological role? And what is the functional significance of RyR1 and RyR2 gene expression outside of muscle cells? While at the moment little is known about tissues that express only RyR3, available evidence suggests that this isoform might contribute to form complex systems of Ca2+ release where more isoforms of RyR are required. Therefore, in addition to tissue-specific isoforms, co-expression of different RyR genes in a tissue may represent a developmentally regulated mechanism evolved to meet the signaling requirements of highly specialized cell types that are necessarily associated with the evolution of complex organisms (11). In the next paragraphs we shall review some examples in support of this hypothesis.

3. RYR3 IS DEVELOPMENTALLY REGULATED IN SKELETAL MUSCLES OF MAMMALS

Expression of RyR3 in mammalian skeletal muscle tissue is now well established. Initial studies performed shortly after the identification of the RyR3 gene revealed that mammalian skeletal muscles, in addition to expressing high levels of RyR1, also express detectable levels of RyR3. Studies in vitro using the C2C12 skeletal muscle cell line revealed that RyR3 channels were preferentially expressed in differentiated rather than in undifferentiated muscle cells and that RyR3 appeared simultaneously with RyR1 early during myotube differentiation (12). This was confirmed by the demonstration that RyR3 was expressed only in a few muscles of adult rodents (mainly diaphragm), but could not be detected in the majority of adult skeletal muscles (13). This picture has some analogies with what is known for skeletal muscles of non-mammalian vertebrates, where two RyR isoforms are often found at approximately equimolar ratio. These two isoforms (called ? and ?) have been demonstrated to be homologous to mammalian RyR1 and RyR3 respectively (14). The pattern of expression of RyR3 in mammals became clearer when a thorough analysis of RyR3 expression established that RyR3 was developmentally regulated in embryonic and neonatal skeletal muscles of mice (15,16). Accordingly, skeletal muscles of embryo contain the RyR3 isoform in all muscles in addition to RyR1. RyR3 expression increases after birth with a peak around 15 days after birth and a gradual decline after peak so that in adult musculature the RyR3 isoform is almost exclusively confined to the diaphragm and few other muscles. In general, however, RyR3 appears to contribute no more than 2-5 % of total ryanodine receptors of mammalian skeletal muscles (17,18).

3.1. Muscle contraction and Ca^{2+} signaling is altered in skeletal muscle of neonatal RyR3 KO mice

In order to identify a functional correlate of RyR3 expression in neonatal skeletal muscle, contraction analysis was performed in skeletal muscle of normal and RyR3 KO mice (15). These experiments revealed an impairment of muscle contraction in skeletal muscles of RyR3 KO mice only when skeletal muscles preparations were isolated from mice in the first weeks after birth. Under these conditions, tension developed following electrical stimulation was significantly lower in RyR3 KO than in control mice, and an even stronger difference was observed when neonatal muscles of RyR3 KO mice were exposed to high caffeine concentration. No significant difference between normal and RyR3 KO mice was observed when the analysis was extended to preparation of skeletal muscles of adult mice. The reduced contractility observed following electrical and caffeine stimulation in RyR3 KO mice is suggestive of a qualitative contribution of RyR3-mediated Ca^{2+} release to regulation of contraction in neonatal skeletal muscles (19) (Figure 1A).

In RyR1 KO myotubes in culture, Ca^{2+} release from the sarcoplasmic reticulum in response to increases in cytosolic Ca^{2+} concentration or caffeine was strongly reduced (20). A similar decrease in caffeine sensitivity was also observed in RyR3-null neonatal myocytes, suggesting a possible co-contribution of each RyR subtype to Ca^{2+} signaling, at least at embryonic / neonatal stages of myogenesis (21). In contrast, experiments on the rate of Ca^{2+} release in normal and RyR3 KO mice skeletal

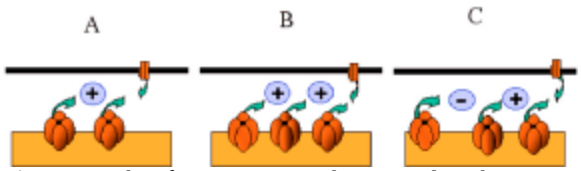

Figure 1. This figure presents three simple schemes on possible modalities of functional interactions that could form when more isoforms of RyR / Ca^{2+} release channels are co-expressed. Positive co-operation among two or three co-expressed channels are presented in Fig.1A and Fig.1B. The example in Fig.1C, on the other hand, indicates a different possibility where one isoform has an inhibitory effect on the activity of the other two.

myotubes did not indicate a modulatory effect of RyR3 on Ca^{2+} release following electrical stimulation (22). Both the voltage dependence of the Ca^{2+} release process and the time course of the release rate during strong depolarization were identical, indicating that if RyR3 were contributing to this process the contribution would be small and definitely not a decisive factor for E-C coupling in cultured myotubes. Investigation of localized spontaneous Ca^{2+} release events in muscle cells lacking the RyR3 gene indicated however that RyR3 channels contributed to generate localised spontaneous Ca^{2+} release events in normal skeletal neonatal muscle cells (23,24). Further investigation in skeletal muscle fibers from neonatal mice KO for either RyR1 or RyR3 genes revealed that Ca^{2+} sparks produced independently by either RyR1 or RyR3 channels had similar spatio-temporal parameters (25). However, since sparks observed in RyR1 or RyR3 KO mice were smaller than those of wild type muscles, these data indicated that co-expression of the RyR1 and RyR3 isoforms is required to generate the localized Ca^{2+} release events that are observed in embryonal and neonatal wild type cells that express both isoforms. Interestingly, sparks observed following addition of sub-optimal caffeine concentrations were larger in cells expressing RyR3 than in cells expressing only RyR1 (25).

Cheng and colleagues have more recently contributed a significant advancement on the differential contribution of RyR1 and RyR3 to Ca^{2+} signaling in skeletal myotubes (26). The use of an imaging system able to obtain a high spatially and temporally resolved variation of Ca^{2+} levels enabled this group to observe different patterns of Ca^{2+} release in neonatal skeletal muscle cells lacking RyR3 compared with normal neonatal myotubes (i.e. expressing both RyR1 and RyR3). These results indicate the existence of a functional interplay between RyR1 and RyR3, which may serve important roles in the regulation of Ca^{2+} movements in neonatal muscle cells, where RyR3 channels appear to be capable of amplifying the Ca^{2+} induced Ca^{2+} release promoted by voltage activated RyR1 channels. The principal observation reported indicated that the time required for diffusion of a Ca^{2+} signal following depolarization from the membrane to the central region of a muscle fiber is higher in RyR3 KO mice, where speed of this signal in wild type cells is 2200µ/sec and in the RyR3 KO fibers is of 190 µ/sec. These results indicate that co-expression of RyR3 with RyR1 contributes to build a system of amplification which results in a more uniform and synchronous activation of Ca^{2+} release across the whole cell body in neonatal skeletal muscle, which is about 10 fold higher in wild type compared with RyR3 KO cells. Given the absence or poor development of T-tubules in embryonal and neonatal skeletal muscle fibers, co-expression of RyR3 may be important to facilitate Ca^{2+} signaling in muscle fibers at this stage of development. This elegant analysis thus provides a significant explanation at the level of Ca^{2+} signals for RyR1 and RyR3 co-expression in neonatal muscle cells that directly support the initial model proposed following the identification of defective contraction of neonatal skeletal muscles of RyR3 KO mice (15,19).

4. RYR3 EXPRESSION IN THE CENTRAL NERVOUS SYSTEM

A considerable amount of evidence supports the notion that neuronal functions, like long-lasting changes of synaptic plasticity and long-term memory, require an initial elevation of the intracellular Ca^{2+} concentration (27). Such Ca^{2+} signals can result from an influx of Ca^{2+} via Ca^{2+} channels on the plasma membrane and/ or Ca^{2+} release from intracellular stores (28). All three isoforms of RyRs are expressed in the Central Nervous System (CNS) (8,9,10). In the brain the predominant isoform is RyR2; RyR1 and RyR3 contribute less than 5% of total RyR channels. Why multiple RyR isoforms exist in neurons and how these isoforms participate and interact in neuronal function is currently not understood. A role of RyRs in the induction of long-term potentiation (LTP) and/or depression (LTD) has been supported by a number of experiments based on the use of pharmacological agents (29); however these studies did not elucidate the functional contribution of each single isoform and the significance of the expression of multiple RyR isoforms within a single neuron.

On this basis, Balshun and colleagues adopted the RyR3 KO mice to study the eventual role of this RyR isoform in LTP, which is thought to mediate processes of learning and memory formation at the cellular level. RyR3 KO mice presented no obvious morphological alteration in the hippocampus, and LTP generated by strong tetanizations were not different between mutant and control animals. In contrast, LTP induced by a weak tetanization protocol and depotentiation was markedly changed by RyR3 deletion. In experiments performed with LTP generated by a weaker tetanization it was observed that RyR3 KO mice tended to have a lower initial amplitude of potentiation. In addition, LTP in RyR3 KO mice decayed within 30 min, whereas wild type mice still displayed a significant potentiation after 2 hours (30). Thus, RyR3 channels seem to play a distinct role in certain types of hippocampal synaptic plasticity.

A number of further behavioral tests were carried on RyR3 KO mice. Whereas the general behavior in the open field was not different between mutant and control mice, RyR3 KO mice displayed a higher speed of locomotion as also observed by Takeshima and co-workers

(21). The RyR3 KO mice also presented a mild tendency to circular running. The caudate/putamen area represents one of the preferential expression sites of RyR3 in CNS but whether these motor alterations result from a dysfunction of this region is not known. The specific effects of RyR3 gene deletion on synaptic plasticity agree with a role of these channels in hippocampus-dependent learning and behavior. Hippocampus-dependent spatial learning of RyR3 KO mice was examined in the Morris water maze test. In this type of experiments, during an acquisition phase the animals learn to use distant cues on the walls to locate an escape platform that is hidden at a constant location. In the acquisition phase and probe trial of the water maze task, RyR3 mutants were able to locate and to navigate to a hidden platform similarly to wild type mice. An interesting difference emerged after changing the platform position. Wild type animals learned the new position very rapidly, taking only 2-3 trials to reach the performance level they had shown at the end of acquisition. RyR3 KO mice, by contrast, needed the full 12 trials to learn the new position, apparently having no advantage with respect to the acquisition phase (30). An almost identical behavior in the water maze test has been reported by Futatsugi and colleagues (31). However these authors interpreted the persistent habit of the RyR3 KO mice in the reversal phase of the test as a sign of an increased capability to perform. By contrast, we think that RyR3 KO mice have a reduced flexibility to reuse and modify an acquired map once the goal position is changed in an otherwise unaltered environment. During the relearning part of the Morris test, in fact, the learning curve of RyR3 KO mice resembles that usually observed in a completely new setup indicating that hippocampal function may not be entirely normal in these mice. The response in the Morris test is generally considered as expression of the associative not-implicit learning which may find its cellular basis in hippocampal LTP, therefore the data discussed above indicate that RyR3 channels appear to participate of special forms of hippocampal synaptic plasticity, which might be required for the normal adaptation and modification of spatial maps. In a more general sense, RyR3 seems to be involved in a process that adapts the acquired memory flexibility to external /environmental changes or stimuli.

As of today, no evidence is available on defective Ca^{2+} release in neurons from RyR3 KO mice. Yet it is still reasonable to propose that, as in neonatal skeletal muscle cells, RyR3 channels may provide a qualitative contribution to Ca^{2+} release in neurons which if absent may result in altered regulation of neuronal activities.

5. RYR3 EXPRESSION AND FUNCTION IN SMOOTH MUSCLE CELLS

In smooth muscle cells the generation of global or localized changes in intracellular Ca^{2+} levels has been associated with the specific control of opposite functions such as contraction and relaxation (28,32). Increased global intracellular Ca^{2+} levels, although reaching modestly high concentrations, spread throughout the entire cell and regulate contraction by activating myosin light chain kinase, By contrast, Ca^{2+} -dependent relaxation in smooth muscle cells is mediated by discretely localized Ca^{2+} release events resulting from the activation of RyRs located near the plasma membrane. These events provoke local increases in Ca^{2+} concentration that stimulate adjacent Ca^{2+} -activated K^+ channels (BK), causing BK-type currents named "spontaneous transient outward currents" or STOCs. STOCs inhibit voltage-dependent Ca^{2+} channel activity, decrease global Ca^{2+} and diminish arterial contraction leading to decreased blood pressure.

5.1. studies on RyR3 channels in rat portal vein and mouse myometrial smooth muscle cells

All smooth muscle cells tested so far have been proven to express RyR channels, although smooth muscle cells from different organs have been found to express different combinations of the three RyR isoforms. In rat portal vein myocytes, spatial and temporal recruitment of Ca^{2+} sparks result in propagating Ca^{2+} waves that trigger cell contraction. All three RyR isoforms are co-expressed in these cells, but the specific contribution of a given isoform has only recently been studied. Mironneau and colleagues determined the RyR subtypes responsible for Ca^{2+} sparks and global Ca^{2+} responses in rat portal vein myocytes by antisense oligo-nucleotides that specifically targeted each one of the RyR subtypes (33). They found that inhibition of either RyR1 or RyR2 by treatment with antisense oligonucleotides strongly reduced the number of vascular myocytes with spontaneous Ca^{2+} sparks, whereas inhibition of RyR3 was ineffective, indicating that both RyR1 and RyR2, but not RyR3, were required for Ca^{2+} release during Ca^{2+} sparks and Ca^{2+} waves induced by activation of L-type Ca^{2+} currents. In the same cells, activation of RyR3 channel activity was obtained under conditions of increased Ca^{2+} loading of the sarcoplasmic reticulum (34). A similar requirement for increased Ca^{2+} loading of the sarcoplasmic reticulum in order to sensitize RyR3 channels to stimulation by Ca^{2+} and caffeine has been reported by Mironneau and colleagues in mouse myometrial smooth muscle cells that only express RyR3 channels (35).

5.2. RyR3 dictates stocs frequency in brain arterial smooth muscle cells: effects on blood pressure

Smooth muscle cells from cerebral arteries have been found to express the three RyR isoforms (36). To understand the contribution of the RyR3 isoforms to arterial contraction, Gollasch and colleagues tested the molecular role of RyR3 in Ca^{2+} spark regulation and STOCs generation in arterial smooth muscle cells of RyR3 KO mice (36). The spatial-temporal characteristics of individual Ca^{2+} sparks in arterial smooth muscle cells of RyR3 KO mice did not differ from those of control animals. Interestingly, analysis of BK current activation in arterial smooth muscle cells from RyR3 KO mice revealed a significant increase in the STOCs frequency compared to control mice. These results suggest that RyR3 channels specifically set the frequency of Ca^{2+} spark/STOCs in arterial smooth muscle cells. Thus, RyR3 channels seem to contribute to Ca^{2+} sparks by controlling the basal Ca^{2+} spark frequency while the remaining RyRs (i.e., RyR1 and RyR2) mainly contribute to the Ca^{2+} release underlying a single spark. To understand the physiological consequences

of the increased Ca^{2+} sparks/STOCs frequency in arterial smooth muscle cells, the effect of pressure on arterial diameter of intact, isolated cerebral arteries was tested. Arteries from RyR3 KO mice were less constricted at a given pressure than were control arteries, indicating that increased activity of BK channels in RyR3 KO arteries contributes to the regulation of arterial tone and that RyR3 limits arterial tone by an inhibition of basal BK channel/STOCs activity. On these bases Gollash and colleagues proposed a model in which BK channels are activated by Ca^{2+} released from a compartment containing multiple RyR channels (36). In this model, activation of RyR1 and RyR2 channels results in Ca^{2+} sparks formation while RyR3 channels contributes an inhibitory component that sets the basal Ca^{2+} spark frequency (see Figure 1C).

6. PERSPECTIVE

Experiments in both neonatal skeletal muscle and hippocampal neurons of RyR3 KO mice have revealed that ablation of RyR3 results in alterations of cellular activities, in spite of the presence in both systems of robust levels of other RyR isoforms. This effect can be rationalized considering that in neonatal skeletal muscles and hippocampal neurons RyR1 and RyR2 constitute the main RyR-mediate Ca^{2+} release signal transduction pathway. In both cases RyR3 channels may provide a positive feedback, probably via a Ca^{2+} induced Ca^{2+} release mechanism, which can contribute to build specific aspects of Ca^{2+} signaling of these cells. In such a model, deletion of RyR3 channels would not block the main pathways for activation of RyR-mediated Ca^{2+} release in skeletal muscle cells and neurons, but would result in non-optimal signaling, as evidenced by the altered performance of these cells in RyR3 KO mice. It should be reminded here that several laboratories have reported that RyR3 channels have properties that make these channels more adaptive than other RyR isoforms to Ca^{2+} induced Ca^{2+} release (17,18,37,38,39). Such properties would make the RyR3 channels particularly flexible for providing a more sustained Ca^{2+} release efflux from the endoplasmic reticulum following an initial increase in the intracellular concentration of Ca^{2+} mediated by other Ca^{2+} release channels on the endoplasmic reticulum or by Ca^{2+} channels on the plasma membrane. Depending on the system these channels are working in, positive or negative effects may be generated (Figure 1). Indeed, while in neonatal skeletal muscle cells (Fig 1A) and in neurons (Fig 1B) RyR3 channels appear to represent a positive contribution to the mechanism of Ca^{2+} release, work in smooth muscle cells from cerebral arteries suggests that RyR3 channels may have a negative role in regulating the frequency of Ca^{2+} sparks generated by RyR1 and RyR2 isoformsand hence STOCs frequency (Fig 1C).

An interesting and yet unanswered question is linked to the evidence reported by Mironneau and colleagues in smooth muscle cells from portal vein and mouse myometrium, where RyR3 channels do not seem to respond to activating stimuli unless the Ca^{2+} loading of the sarcoplasmic reticulum is increased (34,35). Why are not these channels activated by conditions that usually stimulate RyR1 and RyR2 isoforms? Interestingly, a similar finding was observed in mink lung epithelial cells expressing RyR3 (8). Further studies are needed to to answer this and other outstanding questions. Certainly, the idea of a cell-specific selective assembling of the molecular machinery that mediates Ca^{2+} release from the endoplasmic reticulum represents only a basic scheme in the complex world of Ca^{2+} signaling. However, it may provide a starting point to decipher the molecular players that generate the precise spatial and temporal aspects of the sophisticated Ca^{2+} signals required for controlling cellular functions. Although we have reviewed here initial evidence in support of a model on the functional significance of co-expression of RyR channels that represent our working hypothesis, more work in the future is certainly required to further support and eventually extend it.

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