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ION CONDUCTION AND SELECTIVITY IN THE RYANODINE RECEPTOR CHANNEL

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1. ABSTRACT

The ryanodine receptor channel is an intracellular membrane Ca^{2+}-release channel. The investigation of ion translocation and discrimination in individual channels under voltage-clamp conditions has revealed that the channel can sustain very high rates of cation translocation, has high affinity for divalent cations and displays relatively poor discrimination between physiologically relevant cations. In this article I will discuss the mechanisms underlying these characteristic properties, the regions of the channel molecule likely to be involved in ion handling and speculate on the structure of the conduction pathway of Ca^{2+}-release channels.

2. INTRODUCTION

Ion channels are multimeric protein molecules that provide pathways for ion translocation through otherwise impermeable membranes. In all cases ions move through a membrane ion channel in response to an electrochemical driving force. A defining feature of channel mediated translocation is that rates of ion movement are very high and for the most part, very specific. The ability of channel proteins to distinguish between closely related ions is reflected in their generic classification as, for example, K^+, Na^+ and Ca^{2+} channels. Our understanding of the mechanisms that make it possible for ion channels to perform the intuitively contradictory tasks of maintaining very high rates of flux whilst discriminating between physically related ions has been greatly enhanced by the determination of the structure of a bacterial K^+ channel at a resolution of 3.2 Å (1).

In this article I will review our current understanding of the structures and mechanisms involved in ion translocation and discrimination in a nominally Ca^{2+}-selective ion channel, the ryanodine receptor (RyR) and discuss how these might contribute to the function of this protein complex as a Ca^{2+}-release channel.

3. THE PHYSIOLOGICAL ROLE OF THE RYANODINE RECEPTOR CHANNEL

Ryanodine receptor channels are expressed in intracellular membrane networks such as the sarcoplasmic reticulum of striated muscle cells and provide pathways for the regulated release of stored Ca^{2+} in response to appropriate stimuli. In striated muscle the ryanodine receptor plays a pivotal role in the initiation of cell contraction; RyR channel open probability increases in response to the depolarisation of the sarcolemma and Ca^{2+} ions flow from the sarcoplasmic reticulum down the concentration gradient, established by the ATP-driven Ca^{2+} pump, to initiate contraction (2).

RyR is clearly a very efficient Ca^{2+}-release channel. RyR gating responds to appropriate physiological stimuli and is modulated by a variety of physiologically relevant processes (3-5). Equally important, when open, RyR provides a conduction pathway that allows for the release of enough Ca^{2+} from the sarcoplasmic reticulum to raise the free concentration of the highly buffered cytosol from its resting level of 100 nM to 1μM in a matter of milliseconds (2). It does this under prevailing conditions that include a relatively low concentration of the relevant cation (the concentration of free Ca^{2+} within the sarcoplasmic reticulum is likely to be in the region of 1 mM) and in the presence of a number of potentially competing ions such as K^+ and Mg^{2+}. To achieve its physiological role it appears that RyR must have structural features and employ mechanisms that result in high rates of Ca^{2+} translocation, high affinity for Ca^{2+} and the ability to discriminate between Ca^{2+}, K^+ and Mg^{2+}.

4. THE ION HANDLING PROPERTIES OF RyR

The sarcoplasmic reticulum membrane system contains a variety of ion transporting systems including ion channels permeable to monovalent cations (6) and anions (7;8). Detailed investigations of ion translocation and discrimination in RyR require the separation of this channel from the other cation transporting systems of the sarcoplasmic reticulum membrane network (9;10) and the incorporation of individual RyR channels into planar phospholipid bilayers. RyR channels incorporate into planar phospholipid bilayers in a fixed orientation so that the cytosolic and luminal faces of the channel protein can be defined (11). A rigorous investigation of ion translocation and discrimination can then be carried out under voltage clamp conditions. Using this approach we have characterised ion handling in the sheep cardiac isoform of the receptor channel (RyR2) by monitoring relative permeability, relative conductance and relative affinity of permeant ions and blocking parameters of impermeant ions. Details of these properties can be found in earlier publications (12;13) and I will summarise only the most important features here.

4.1. Physiologically relevant cations

The first striking feature to emerge from these studies is that whilst within the cell Ca^{2+} is undoubtedly the physiological charge carrier in RyR, the channel is in fact permeable to a very wide range of divalent and monovalent cations. Measurements of RyR channel activity in solutions of the group 1a monovalent cations in the absence of divalent cations demonstrate that K^+, Na^+, Cs^+, Rb^+ and Li^+ are all translocated at very substantial rates through RyR. With symmetrical 210 mM solutions single channel current-voltage relationships are linear with single channel conductances ranging from approximately 210 pS in Li^+ up to approximately 720 pS in K^+. However these rates of monovalent cation translocation can be increased by raising the activity of the permeant ion. Determinations of the dependence of single RyR channel conductance on group 1a monovalent cation activity indicate that in all cases conductance saturates, with values of maximal conductance ranging from approximately 250 pS with Li^+ to 900 pS with K^+. Activities at which single channel conductance reach 50% of the maximal value range from 9.1 mM for Li^+ to 34 mM for Cs^+. Despite the almost four fold range in maximal conductance, the permeability of the group 1a monovalent cations differs only slightly. Calculations of permeability relative to K^+ from reversal potentials monitored with K^+ at the cytosolic face of the channel and another group 1a monovalent cation at the luminal face of the channel indicate that, with the exception of Cs^+ ($pCs^+/pK^+ = 0.61$), this group of cations are essentially equally permeant in RyR.

Despite the high single channel conductance of the group 1a monovalent cations, RyR does show some discrimination between this class of cations and Ca^{2+}. Calculation of the relative permeability of Ca^{2+} to K^+ from reversal potentials monitored with K^+ at the cytosolic face of the channel and Ca^{2+} at the luminal face of the channel demonstrates that Ca^{2+} is 6.5 times more permeant in RyR than K^+. However, equivalent determinations of relative permeabilities of Ba^{2+}, Sr^{2+} and Mg^{2+} relative to K^+ indicate that these alkaline earth divalents are effectively as permeant as Ca^{2+} in RyR. These observations are confirmed by measurements of the relative permeability of Ca^{2+}, Sr^{2+} and Mg^{2+} against Ba^{2+} which yield values in the range 1.0 to 1.1.

Single channel conductance with the alkaline earth divalents as charge carriers is very high, ranging from approximately 90 pS with 210 mM Mg^{2+} to approximately 200 pS with 210 mM Ba^{2+}. The observed unitary conductance of Ca^{2+} in RyR is approximately 10 fold higher than that monitored for the dihydropyridine-sensitive L-type Ca^{2+} channel under comparable ionic conditions (14). As is the case with monovalent inorganic cations, single RyR channel conductance increases and saturates as divalent cation activity is raised. The relationship between unitary conductance and Ba^{2+} activity demonstrates that 50% maximal conductance is seen at approximately 400 μM suggesting that the affinity of RyR for divalent cations is very significantly higher than that for the monovalent inorganic cations.

The experiments summarised above demonstrate that the majority of the features of ion handling monitored in individual RyR channels are consistent with our expectations for an efficient Ca^{2+}-release channel. Under appropriate conditions rates of translocation of Ca^{2+} through RyR can be extremely high. In addition, the conduction pathway of the RyR channel has a high affinity for divalent cations and is therefore likely to maintain near maximal rates of Ca^{2+} translocation at the relatively low Ca^{2+} activities found within the lumen of the sarcoplasmic reticulum. One feature of ion handling in RyR that appears to be at odds with our initial image of a Ca^{2+}-release channel is that RyR shows only limited powers of discrimination between physiologically relevant cations. As a result, Ca^{2+} flux through RyR is likely to be sensitive to variations in the intracellular activities of potentially competing cations such as Mg^{2+} that may occur in pathophysiological conditions including myocardial stunning (15).

4.2. Permeation and block by organic cations

Physiologically relevant inorganic cations are not the only ionic species that interact with the conduction

pathway of RyR. Organic monovalent cations have proved to be very useful tools in studying the mechanisms and structures underlying ion translocation and discrimination in many species of ion channel including RyR. We have investigated the relative permeability of a large number of monovalent organic cations in RyR by determining reversal potentials with the organic cation at the cytosolic face of the channel and K^+ at the luminal face of the channel (16). These experiments have demonstrated that the relative permeability of this class of cations in RyR is related to the size of the cation; or more precisely to the minimum circular radius of the cation. Relative permeability of these ions appears to be dependent upon a simple sieving mechanism; permeability decreases as minimum circular radius is increased. This proposal is supported by the application of excluded area theory to the data in which the relative permeability of an ion is related to the squared difference between the radius of the narrowest region of the conduction pathway of the channel and the radius of the ion. These analyses indicate that the minimum radius of the RyR conduction pathway is likely to be in the region of 3.3 to 3.5 Å.

Impermeant organic cations are not without effect in RyR. Many are effective blockers of permeant ion translocation. Tetraalkylammonium (TAA) ions are well established blockers of permeant ion translocation in K^+ channels (17). Short chain TAAs such as tetramethylammonium (TMA), tetraethylammonium (TEA) and tetrapropylammonium (TPrA) block K^+ flux in RyR. These cations are effective in millimolar concentrations acting as concentration- and voltage-dependent blockers when added to the cytosolic face of the RyR channel. Quantitative analysis of block of RyR by these small TAAs using the protocol devised by Woodhull for analysis of Na^+ channel block by H^+ (18) has revealed the presence of two sites of interaction for these cations within the conduction pathway of RyR. The smallest TAA, TMA, blocks by interacting with a site positioned approximately 50% into the voltage drop across the channel. Larger TAAs do not have access to this site. TEA, TPrA and derivatives of TMA in which one methyl group is replaced by either an ethyl or propyl group are blockers with a greater dependence on trans-membrane voltage than TMA. These cations (19;20), the local anaesthetics QX222 and procaine (21) and cocaine (22) interact with a site located approximately 90% into the voltage drop across the channel. The interaction of blocking cations with this site has proved to be very useful in the estimation of the physical distance over which the voltage drop across the RyR channel occurs. In this approach, which was originally used to monitor the length of the voltage drop in the sarcoplasmic reticulum K^+ channel (23), blocking parameters of monovalent (CH_3-$(CH_2)_{n-1}$-$N^+(CH_3)_3$) and divalent ($((CH_3)_3N^+$-$(CH_2)_n$-$N^+(CH_3)_3$) derivatives of trimethylammonium of varying chain length were determined. Both sets of cation were found to be voltage-dependent blockers of K^+ flux when added to the cytosolic face of RyR (24). Analysis of the voltage dependence of block by the monovalent cations revealed that this parameter was unaffected by chain length; all produced values of effective valence of block of approximately 0.9, consistent with interaction of the cation with a site located 90% into the voltage drop across the channel from its cytosolic origin. This was not the case for the divalent cations; increasing chain length from n=2 to n=7 resulted in a linear decrease in the dependence of block on trans-membrane voltage. Effective valence decreased from 1.5 to a value approaching that obtained with the monovalent derivatives at n=7. Further increases in chain length (n=8 or 9) produced no further alteration in effective valence. It is assumed that one cationic group of all the divalent derivatives interacts with the 90% site and that the high values of effective valence obtained with short chain derivatives reflects the presence of the second trimethylammonium cationic group within the voltage drop. The point at which the effective valence of the mono and divalent derivatives coincide indicates the point at which the second charged group of the divalent derivative "drops out" of the voltage drop. Knowing the N^+-N^+ distance for the divalent derivatives it is possible to estimate the physical length of the voltage drop across RyR and this calculation yields a value of 10.4 Å (24).

5. SUMMARY OF THE PROPERTIES AND DIMENSIONS OF THE CONDUCTION PATHWAY IN RyR.

Measurements of relative conductance, relative affinity, relative permeability and block obtained from a range of permeant and impermeant cations have provided useful information on the properties of RyR as a Ca^{2+}-release channel and estimates for the dimensions of the essential components of its conduction pathway. RyR is a high conductance, poorly selective, cation channel. The voltage drop across the channel occurs over a distance of approximately 10 Å and the narrowest region of its conduction pathway has a radius of approximately 3.4 Å. The observation that blocking cations such as TEA interact with a site located 90% into the voltage drop from its cytosolic origin indicates that the narrowest region of the conduction pathway must be located towards the luminal end of the voltage drop and that the constriction formed by this region is unlikely to be more than 1 Å in length (13). Does this information tell us anything about the mechanisms underlying ion translocation and discrimination in RyR? How do these features compare with those of other ion channels?

6. MECHANISMS INVOLVED IN CATION TRANSLOCATION IN RyR

Undoubtedly the most striking features of ion handling by RyR are the monumental values of single channel conductance obtained with both divalent and monovalent inorganic cations and the massive unitary currents seen at high holding potentials. Maximal conductance values in the range of 1 nS for K^+ and 200 pS for Ba^{2+} far exceed those monitored in other native membrane channels and for that matter non-selective channels such as porin (13). We have monitored unitary current amplitudes of 70 pA at 100 mV with K^+ (25) and 20 pA at 100 mV with Ba^{2+} (26). Under these conditions, current-voltage relationships are linear with no indication of saturation of single channel current amplitude. High unitary conductance and current amplitude are clearly advantageous for a Ca^{2+}-release channel but how are these rates of ion translocation achieved?

Information on the mechanisms responsible for high rates of ion translocation (although considerably lower than those observed in RyR) is available for K^+ channels. A considerable body of experimental evidence indicates that high rates of ion flux in K^+ channels are achieved as the result of electrostatic repulsion between cations in a multiply occupied selectivity filter. This proposal has been confirmed by the visualisation of this phenomenon in KcsA (1). The selectivity filter of the KcsA channel is a tunnel 12 Å in length with a radius of 1.5 Å. The structure is formed by the apposition of four identical loops (one from each monomer of a homotetramer) composed of residues (GYG) that are essential for K^+ selectivity. At physiological K^+ concentrations two cations are located within the selectivity filter separated by a distance of approximately 7.5 Å (1). Under these conditions the electrostatic repulsion between the two cations is sufficient to overcome the interactions between the ions and the residues in the selectivity filter so that the affinity of the selectivity filter for K^+ is reduced and rates of ion exit from the channel are maximised (27).

The suggestion that such a mechanism could underlie the phenomenal rates of cation translocation achieved by RyR is appealing; particularly as there are potential similarities between the K^+ channel signature selectivity sequence and the putative selectivity sequence of RyR (see later). Unfortunately, investigations of ion occupancy in RyR, whilst not entirely excluding the possibility, are not consistent with multiple ion occupancy; RyR appears to be a single-ion channel. Briefly the evidence supporting this proposal is as follows (17): RyR shows simple saturation of conductance with increasing ion activity (19;26); bi-ionic reversal potentials are not dependent on concentration (16;26); we observe no anomalous mole-fraction effects (19;26); the effective valence of small monovalent cation blockers is less than 1.0 (19-21) and ion handling can be described by a simple single occupancy rate theory model (28).

What mechanisms could produce extremely high rates of cation translocation in a single-ion channel? In such a system, conductance will be limited by the rate at which ions leave the channel and this will in turn depend upon the physical dimensions of the conduction pathway. Using a simple cylindrical pore as an analogous structure it can be shown that in a single-ion channel conductance will be maximised if the conduction pathway of the channel is short and wide (17;29). Is the conduction pathway of RyR short and wide? A radius of 3.3 – 3.5 Å is comparable with equivalent measurements made in relatively non-selective channels such as cGMP-activated channels (30) and nicotinic Ach receptors (31) and is more than twice the minimum pore radii estimated in K^+-selective channels (17) and seen in KcsA (1). It is much more difficult to compare the relative lengths of channel pores. Different approaches provide information on different entities. The method described in an earlier section of this article yielded an estimate of the length of the voltage drop across the RyR channel; the selectivity filter visualised in KcsA is a physical structure but the relationship between this and the voltage drop across the channel is not established.

The huge unitary current amplitudes seen at high holding potentials in RyR with both monovalent and divalent inorganic cations indicate that rates of ion entry into the channel are not limited by diffusion (13;28;32). As is the case with rates of ion exit from RyR, rates of ion entry into RyR could be optimised by specific structural characteristics of the channel. Adaptations that provide the channel with a large capture radius will aid rates of ion entry. The capture radius of a channel will be maximised by increasing the area via which ions can leave the bulk solution and enter the area of influence of the trans-membrane voltage. A large capture radius would be provided by a structure in which access to a short selectivity filter is via wide mouths or vestibules, with the voltage drop across the channel extending into the vestibules (13;29;33). The capture radius of a cation-selective single-ion channel could also be increased by the presence of fixed negative charge at the entrances of the channel (33;34). The possible involvement of such a mechanism in RyR is supported by experiments which demonstrate a reduction in channel conductance following chemical modification of luminal carboxyl groups (35) and by the observation that the rate of association of K^+ channel N-type inactivation peptides with sites at the cytosolic face of the RyR channel are increased approximately 500 fold when the net charge of the peptide is increased from +3 to +7 (36).

The picture that emerges for the region of RyR responsible for ion conduction and discrimination is of a structure comprising a short pore (or voltage drop) in which is located an even shorter, relatively wide, constriction or selectivity filter. The combination of these features is likely to maximise rates at which ions leave the channel and provide a large capture radius to maximise rates of ion entry (13).

7. IDENTIFICATION OF COMPONENTS OF THE RyR CONDUCTION PATHWAY

By analogy with other species of ion channel, the conduction pathway of RyR is expected to be formed by components of membrane spanning helices. Putative membrane spanning domains of the RyR monomer are located in the carboxyl-terminal region of the molecule although the identity and number of these regions is still an area of debate. Based on evidence from hydropathy predictions Takeshima et al (37) proposed that RyR1 possessed four trans-membrane (T-M) helices. In contrast Zorzato et al (38) identified 12 hydrophobic sequences considered long enough to form T-M helices. T-M 5, 6, 8 and 10 of this model correspond to T-M 1, 2, 3 and 4 of the Takeshima model. A third model was proposed by Tunwell et al (39) in which the RyR monomer contains 6 T-M helices. Strong support for a carboxyl-terminal location of the RyR conduction pathway comes from experiments in which channel activity has been observed following the incorporation of trypsinised (40) or truncated (41;42) RyR channels into planar phospholipid bilayers.

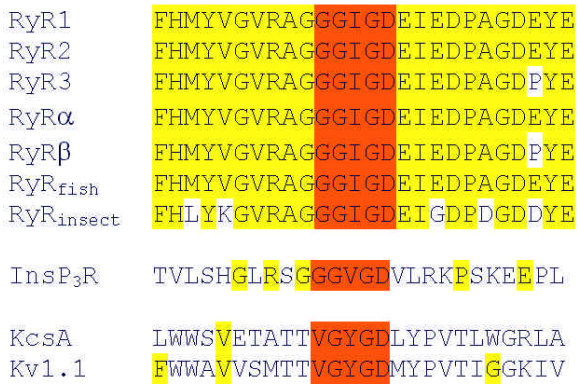

Figure 1. Sequence alignments for the putative pore-forming loops of RyR isoforms, $InsP_3R$, a voltage-gated K^+ channel (Kv1.1) and KcsA. The residues essential for K^+ selectivity in K^+ channels and the proposed "selectivity filter" of the Ca^{2+}-release channels are highlighted in red. Regions of identity are highlighted in yellow. Modified from (13).

The involvement of specific residues in the formation of the ion handling region of RyR was proposed by Balshaw et al (43) who noted a marked similarity between a sequence of residues present in luminal loops of both RyR and the related inositol 1,4,5-trisphosphate receptor ($InsP_3R$) Ca^{2+}-release channel (loop linking T-M 3 and T-M 4 in the RyR model of Takeshima et al (37); loop linking T-M8 and T-M10 in the Zorzato et al (38) model of RyR and loop linking T-M5 and T-M6 in $InsP_3R$ (44;45)) and the signature selectivity sequence in K^+ channels (46) (Figure 1). By analogy with known K^+ channel structure it was proposed that this loop, or more precisely one from each monomer of the homotetramer, might fold back into the membrane to form the selectivity filter or a component of the conduction pathway of the RyR channel.

Further support for this proposal comes from experiments in which residues in and around the RyR luminal loop have been mutated (13;47-49). Substitutions of amino acids in the GIGD sequence and contiguous regions of the luminal loop produce significant changes in the unitary conductance of RyR channels under voltage clamp conditions. Altered conductance is seen with either K^+ or Ca^{2+} as the charge carrying species. It should be noted that, in addition to ion translocation, mutations in this region of RyR modify properties such as the ability of the receptor to bind [^{3}H]-ryanodine and its ability to release Ca^{2+} when exposed, *in situ*, to caffeine (47-49).

8. CAN WE DRAW FURTHER ANALOGIES BETWEEN RyR AND K^+ CHANNELS?

It is established that the apparatus for pore formation, ion translocation and ion discrimination in both K^+ and RyR channels is located in a loop (extracellular in K^+ channels, luminal in RyR) that folds back into the membrane. In addition there is a similarity between the amino acid residues of the K^+ channel selectivity filter and residues known to be involved in ion translocation in RyR. Do these observations suggest that the structures and mechanisms involved ion handling in RyR might resemble those in K^+ channels?

The determination of the three-dimensional structure of the KcsA bacterial K^+ channel at a resolution of 3.2 Å (1) has led to a revolution in the understanding of the relationship between structure and function of membrane ion channels. On entering the KcsA channel from the intracellular solution, a permeant ion would encounter, in order, a water filled tunnel 18 Å in length, a water filled cavity approximately 10 Å in diameter and a selectivity filter 1.5 Å in radius and 12 Å in length. The functional channel is a homotetramer that spans the membrane with its pore formed by the apposition of the monomers. Each monomer consists of two trans-membrane helices linked by an extracellular loop that folds into the membrane to contribute to the pore. A portion of the loop forms a short helical region termed the pore helix (1). The four pore helices are positioned in such a way that their partial negative charge is orientated towards the 10 Å aqueous cavity and provide a mechanism for overcoming the dielectric barrier associated with the movement of an ion into the low dielectric environment of the membrane (50). The aqueous cavity is lined with hydrophobic residues of T-M2 (the inner helix) and the selectivity filter of KcsA is lined with oxygen atoms of the backbone carbonyls of residues of the signature selectivity sequence (GYG).

Given the superficial similarities between K^+ channels and RyR set out above it is interesting to explore the possibility that KcsA might be used, at least qualitatively, as a structural template for the pore region of RyR. Towards this end we have made predictions of the secondary structure of the last two T-M regions of RyR and $InsP_3R$ (T-M3 and T-M4 in the model of Takeshima (37) and T-M5 and T-M6 in $InsP_3R$ (45)) together with their linking, pore-forming, loops and have compared these predictions with the known structure of KcsA (13). These predictions indicate a striking correspondence. Not only do the pore-forming loops of RyR and $InsP_3R$ contain sequences of amino acids that are predicted to form helices analogous to the pore helix of KcsA but the sequence of predicted structural elements in the loops is the same as that found in the bacterial K^+ channel. That is trans-membrane helix (T-M3/ T-M5), pore helix, selectivity filter, trans-membrane helix (T-M4/ T-M6), carboxyl terminus (Figure 2).

If we were feeling particularly foolhardy we might suggest that in the light of these observations it would be possible to stretch the analogy and propose a completely hypothetical tertiary structure for the pore-forming region of the RyR and $InsP_3R$ monomers by folding the identified elements on the basis of the arrangement of the corresponding components in KcsA (13)(Figure 3). A hypothetical homotetramer formed from such monomers would provide the Ca^{2+}-release channels with the wherewithal to fulfil the basic requirements of an ion channel. As in KcsA, the homotetramer would contain a single conduction pathway. Helix dipoles arising from the putative pore helices and focused on a water-filled cavity opening to the cytosol would provide a mechanism for surmounting the electrostatic barrier faced by a cation in the low dielectric environment of the

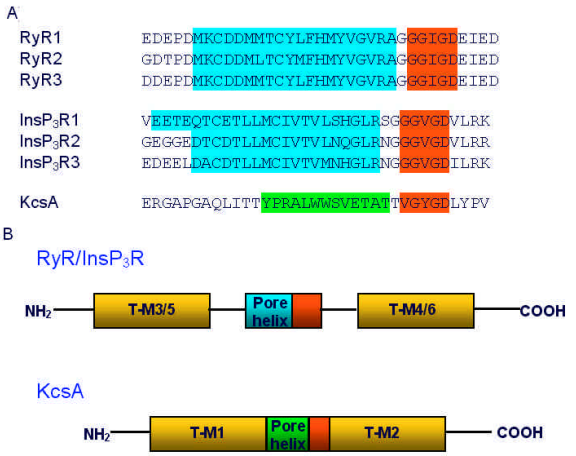

Figure 2. Secondary structure predictions of a putative pore helix in the pore-forming loops of isoforms of RyR and $InsP_3R$. A) Sequence alignments for RyR and $InsP_3R$ (blue) together with KcsA (green). Selectivity sequences are shown in red. Secondary structure was predicted using *PSI*pred: http://insulin.brunel.ac.uk/psipred. B) Diagram showing the similarity in sequence of structural elements in the pore-forming loops of RyR, $InsP_3R$ and KcsA. Modified from (13).

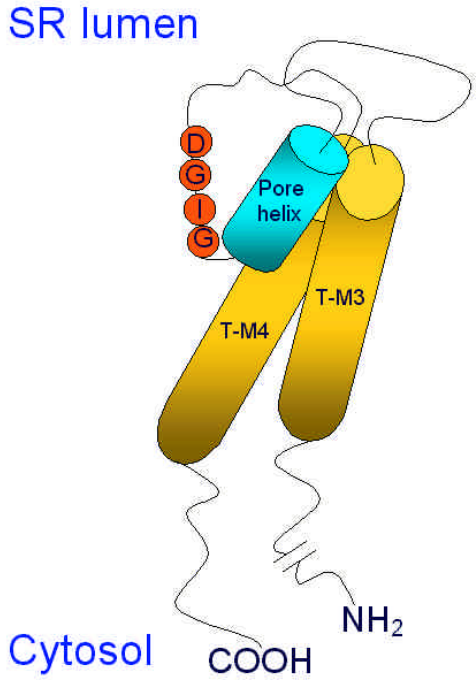

Figure 3. Cartoon depicting a hypothetical tertiary structure for the pore-forming loop of an RyR monomer. The relative location of the identified elements is based upon the established locations of corresponding elements in KcsA.

membrane. A constricted region in the putative conduction pathway of the channel, formed by the apposition of loops containing the GIGD motif, could provide the short, wide, region of the channel over which trans-membrane voltage falls and limited discrimination between cations takes place.

The proposal that the pore-forming loops of the RyR and $InsP_3R$ Ca^{2+}-release channels might share a range of general structural traits with equivalent regions of other membrane ion channels does not require that the detailed architecture and hence the mechanisms governing ion translocation and discrimination in the Ca^{2+}-release channels be similar to those resolved in K^+-selective channels such as KcsA. RyR and $InsP_3R$ are not K^+ channels and the relationship between the structure of the conduction pathways of these channels and their unusual ion handling properties remains an area of great interest and investigation.

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