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QUANTITATIVE RELATIONSHIPS BETWEEN RYANOIDS, RECEPTOR AFFINITY AND CHANNEL CONDUCTANCE

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1. ABSTRACT

The review examines the relationship between the structure of several ryanodine analogs and (A) binding, (B) channel conductance, and (C) ligand binding kinetics. Comparative molecular field analysis (CoMFA) and comparative molecular similarity analysis (CoMSIA) are used to quantitatively assign structural correlations. Hydrogen bond donating (but not accepting) ability was found to be highly correlated with ligand affinity. Analysis of the correlation between hydrophobicity and ligand affinity indicates that, in general, deviation from the amphipathic nature of ryanodine weakens binding. Affinities and binding kinetics obtained *in vivo* are comparable to those obtained in the less-than-physiological *in vitro* conditions. Therefore, the structure-activity relationships surveyed are relevant to the living cell. The review presents arguments favoring the propositions that (A) the pyrrole is a major factor orienting the ligand in the receptor binding site and (B) that ryanoids alter ryanodine receptor function through allosteric mechanisms.

2. INTRODUCTION

The ryanodine receptor (RyR or calcium-induced, calcium-release channel) interacts with so many other cellular components sometimes it seems that, with little exaggeration, one could teach an entire biochemistry course based on the ryanodine receptor. Fortunately, in this paper, the focus will be on only one class of the myriad of ryanodine receptor ligands, the ryanoids. Many have taken on the task of examining other aspects of the ryanodine receptor: a Medline search lists more than 200 reviews dealing with one or more aspects of ryanodine receptor function. While only one of many modulators, ryanodine has served, and continues to serve, as an important tool in understanding the function of ryanodine receptors in the many cell types in which they are found.

3. PREVIOUS QSAR

Ryanodine binds to the ryanodine receptor with remarkable affinity and specificity. Compared to the free receptor, the ryanodine complexes have radically altered conductance and gating kinetics. For that reason, the relationship between structure and function of the ryanoids has attracted the interest of researchers. Suitable ryanoids could serve as effective insect control agents, they could serve as useful therapeutic agents, and they could be extraordinarily useful research tools allowing

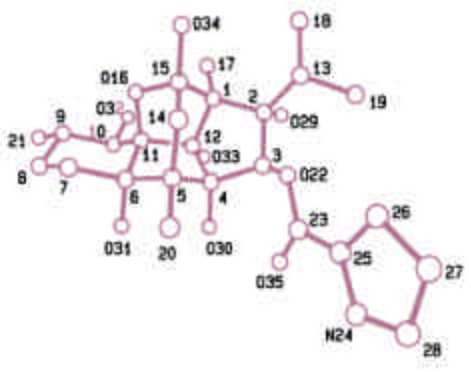

Figure 1. A perspective drawing to illustrate the numbering system used for the ryanodine and related compounds.

pharmacological manipulation of calcium channel function in single cells, perfused tissues and intact organisms. The relationship between structure and ryanoid function has been reviewed previously. Sutko *et al.* (1) reviewed the properties of the receptor and described several of the reactions leading to derivatives and analogs of ryanodine. Jefferies & Casida (2) provided an excellent overview of structure-activity relationships for vertebrate and non-vertebrate animals. In this review emphasis will be placed on some of the developments since the review by Welch (3). The focus will be on quantitative structure activity relationships (QSAR) at the high-affinity ryanodine binding site. The principal computational tool will be three-dimensional quantitative structure-activity relationships (3D-QSAR), in particular, comparative molecular field analysis (CoMFA, Reference 4) and comparative similarity index analysis (CoMSIA, Reference 5). Therefore, much elegant work measuring biological properties will not be mentioned. Much of that data should be mined in the future for QSAR studies.

In this review of ryanoid CoMFA, the desire is to focus on general trends between structure and function. Therefore, lists of compounds and structures will be absent. Please see Sutko *et al.* (1) and references therein for a comprehensive lists of structures. The figures here summarize QSAR that includes some compounds added since the previous review (3) and may therefore be somewhat different, reflecting refinement of the model.

The ryanodine receptor is generally considered to bear one high affinity site and one or more low affinity sites per tetramer. Binding of ryanodine to the high affinity site is thought to cause the channel to enter a long-lived, partially-conducting state whereas binding to the low affinity site causes the channel to enter a long-lived, non-conducting state (see Reference 1 for a discussion of these points). Unless otherwise indicated, the data refer to ryanoid interactions with the high affinity site.

Ryanodine is illustrated in Figure 1 along with the numbering system that will be used in this review. For convenience, the ryanodine structure will be divided into six areas: (I) pyrrole group or position 3, (II) isopropyl group or position 2, (III) polycyclic fused ring system or diterpene, (IV) 2-hydroxyl group, (V) 9-position, and (VI) 10-position.

4. CoMFA MAP OF DISSOCIATION CONSTANTS

The relationship between structure and dissociation constant has been examined for many ryanodine analogs. The QSAR in this review is based on 38 compounds. The dissociation constants were determined by the ability of the test compounds to displace 7 nM radiolabed ryanodine in a competitive binding assay. In all cases the competition follows a hyperbolic equation, completely displacing the ryanodine and giving no hint of multiple sites. At 7 nM ryanodine, the high affinity site is either ~50% (rabbit skeletal) or ~80% (rabbit cardiac) of saturation. These conditions were chosen to minimize contributions from the low affinity site. Therefore, all 3D-QSAR discussed here refer to characteristics of the high affinity site.

Figure 2 summarizes the correlation between changes in ryanoid structure and dissociation constant. The wire frames enclose those regions where the correlation is the strongest. These areas give clues about the nature of the interaction between ligand and receptor. Changes in steric bulk and electrostatic charge contribute about equally to the correlation between structure and dissociation constant (42% and 58% respectively). Interestingly, most of the steric correlation is located near the isopropyl and pyrrole groups (2- and 3-positions) in contrast to electrostatic contributions that are mostly located, near the 9- and 10-positions. Steric (or van der Waals) interactions in CoMFA are approximated by the Lennard-Jones potential and, therefore, are most strongly responsive to local changes. Electrostatic changes are approximated by a modified Coulombs Law relationship. In practical terms this means that addition of an amino group to the 21-position will produce only small changes in the steric field at the 8-position but will produce a large increase in the electrostatic potential at the 8-position. Therefore, correlations between electrostatic potential and dissociation constant may be the result of changes in charge at several remote locations. Changes in charge at the locus identified by the wire frame are important. There are just more ways of causing the change. Overall, Figure 2 is similar to, and a refinement of, previously reported QSAR.

Figure 3 summarizes the relationships between structure and function for the cardiac ryanodine receptor (RyR2). The contours are drawn at the same levels as Figure 2; therefore, the plots are directly comparable. There are numerous, but minor, differences between the 3D-QSAR of the two isoforms of the receptor. The largest differences are near the isopropyl and pyrrole groups.

5. CoMSIA MAP OF DISSOCIATION CONSTANTS

5.1. Hydrogen bonds

CoMSIA (comparative molecular similarity analysis) was conducted to find possible correlations between hydrogen bond acceptor sites on the ligand and

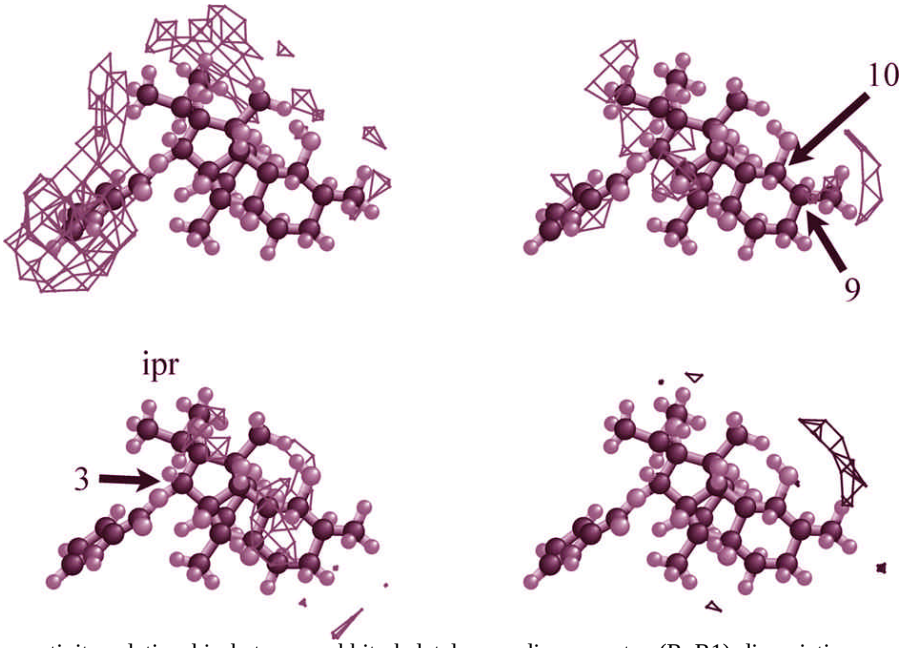

Figure 2. Structure activity relationship between rabbit skeletal ryanodine receptor (RyR1) dissociation constant and physical properties. The wire frames are contours at one-third of the absolute value of the maximum correlation between changes in physical property and dissociation constant. Numbered arrows locate specific atoms for orientation (See Fig 1 for numbering system; ipr = isopropyl). Ryanodine is used as a guide to the eye. Upper left: wire frames enclose volumes where increased steric bulk is strongly correlated with decreased dissociation constant. Upper right: wire frames enclose volumes where increased steric bulk is strongly correlated with increased dissociation constant. Lower left: wire frames enclose volumes where increased positive charge is highly correlated with decreased dissociation constant. Lower right: wire frames enclose volumes where increased negative charge is highly correlated with decreased dissociation constant.

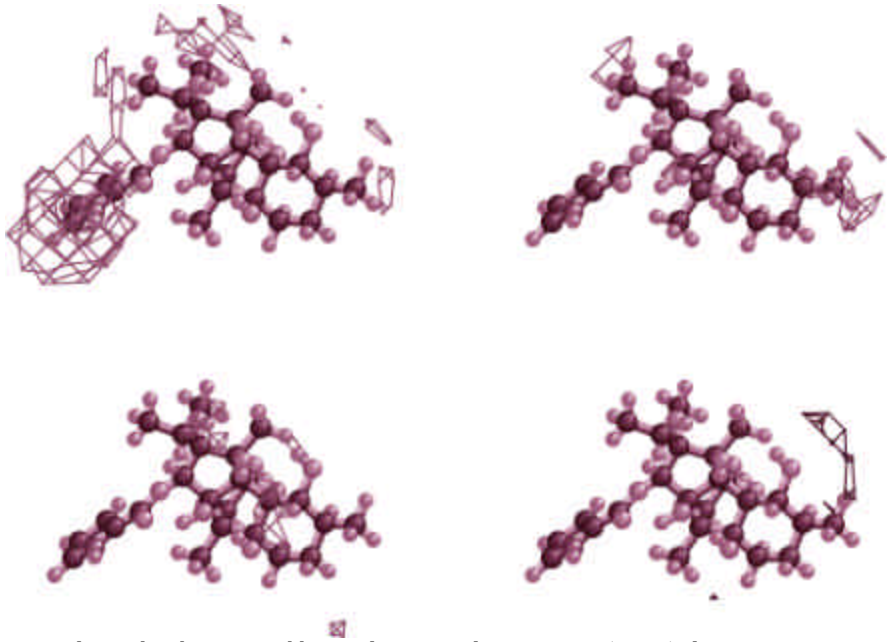

Figure 3. Structure activity relationship between rabbit cardiac ryanodine receptor (RyR2) dissociation constant and physical properties. The wireframes are contours at the same level as used in Figure 2. Ryanodine is used as a guide to the eye. Upper left: wire frames enclose volumes where increased steric bulk is strongly correlated with decreased dissociation constant. Upper right: wire frames enclose volumes where increased steric bulk is strongly correlated with increased dissociation constant. Lower left: wire frames enclose volumes where increased positive charge is highly correlated with decreased dissociation constant. Lower right: wire frames enclose volumes where increased negative charge is highly correlated with decreased dissociation constant.

binding. Only a random correlation between hydrogen bond accepting ability and binding was found. In contrast, changes in hydrogen bond donating ability are correlated with changes in dissociation constant. The positive correlations (the presence of hydrogen bonds is correlated with stronger binding) are focused at the pyrrole nitrogen and at the hydroxyl groups attached to the 2 and 12 positions on the fused ring system. Relative to the positive correlation, the negative correlation (the presence of hydrogen bond donors is correlated with weaker binding) is much more diffuse and falls below one-fourth the maximum significance (Figure 4). The correlations are essentially identical for both the skeletal and cardiac isoforms.

5.2. Hydrophobic interactions

Changes in hydrophobic interactions are highly correlated with changes in dissociation constant (Figure 5). As with the CoMFA maps (Figures 2 and 3), the hydrophobic CoMSIA contours are drawn at one third of the maximum correlation between changes in hydrophobic interaction and dissociation constant. In comparison to the correlation with hydrogen bond donating ability, which is confined to a small proportion of the molecular surface, the hydrophobic correlations cover a large portion of the surface of the molecule, indicating a more equitable participation in binding. The contour maps are almost identical for both cardiac (top) and skeletal (bottom) isoforms. The areas where increasing hydrophobicity is strongly correlated with decreasing dissociation constant are shown on the left; areas where increasing hydrophobicity is strongly correlated with increasing dissociation constant are shown on the right. In Figure 5, the polar face of ryanodine (with most of the hydroxyl groups and the nitrogen edge of the pyrrole) is facing the viewer. The take-home message is that, from the point of view of hydrophobicity, the amphipathic distribution of ryanodine is well designed for tight binding. The contours on the left cover hydrophobic regions of ryanodine whereas the contours on the right cover hydrophilic areas of ryanodine. The amphipathic nature of the pyrrole group (the protrusion on the right) is reflected in the contour maps. The hydrophobic edge of the pyrrole (the side facing away from the viewer) is an area where increases in hydrophobicity are highly correlated with decreased dissociation constant (tighter binding). The hydrophilic edge of the pyrrole (the nitrogen, facing the viewer) is an area where increases in hydrophobicity are strongly correlated with increasing dissociation constant. In the fused ring system (a dipterpene), strong correlations between increasing hydrophobicity and decreasing dissociation constant cover the 8-, 9- and 10-positions and extend toward the pyrrole carbonyl along the hydrophobic surface of ryanodine (away from the viewer). In contrast, the contours where increasing hydrophobicity is strongly correlated with decreased affinity cover most of the hydroxyl groups (on the polar face which is toward the viewer). In general, anything that makes the hydrophobic surface of ryanodine less hydrophobic is correlated with decreased binding, and any changes that make the hydrophilic surfaces of ryanodine less hydrophilic are correlated with decreased binding. The notable exception is the isopropyl group. Steric factors (Figure 2) are highly correlated with binding but not with hydrophobicity (Figure 5). This is in keeping with the observation that adding a hydroxyl group to the isopropyl group has little effect on dissociation constant (reference 6; unpublished results, and see below).

6. STRUCTURAL ANALYSIS

6.1. Pyrrole group

The pyrrole carbonyl group (attached to the 3-position) is the single most important locus related to binding. This is most dramatically seen in the 1200-fold increase in dissociation constant when the pyrrole carbonyl group is removed from ryanodine (K_D = 5±0.3 nM) to form ryanodol (K_D = 5800±900 nM; rabbit skeletal sarcoplasmic reticulum or SR). No other group is nearly as effective as the pyrrole. Slight changes in structure lead to large changes in affinity. Methylation of the pyrrole nitrogen produces an 8-fold increase in dissociation constant (7). Replacement of the pyrrole group with a pyridine, causes a 300-fold increase in dissociation constant (8). The complex nature of pyrrole interactions with the receptor is evident comparing Figures 2-5. The contours for antagonistic correlations of steric, electrostatic, and hydrophobic correlations surround the group. The presence of a hydrogen bond donor at the nitrogen edge of the pyrrole is highly correlated with binding to the high affinity site of both cardiac and skeletal RyR. Taken together, physical factors (steric, electrostatic, hydrogen bonding, and hydrophobicity) suggest a restrictive nature in the binding site in this region. Few pendant groups will have the necessary complimentary interactions.

A SAR of other substituents is limited because of synthetic difficulties at the 3-position in the ryanodine stereochemistry. The 3-hydroxyl is sterically hindered; so researchers have been limited to what nature has chosen to offer. However, Ruest and his colleagues have synthesized 3-epiryanodol and a series of analogs have been synthesized and tested. The affinity of ryanodol and 3-epiryanodol for the RyR is roughly the same (6000 nM vs. 4000 nM, unpublished results). Addition of pyrrole carbonyl to the 3-epi position increases affinity 10-fold (K_D of 3-epiryanodine = 400 nM, see also Reference 9). Therefore, the effectiveness of the pyrrole carbonyl is greatly reduced by the isomerization. However, the comparison is complicated. The fact that the 3-epimer of ryanodine binds at all suggests that 3-epiryanodine binds to the RyR in an alternate orientation (see References 9 and 10). Seemingly isosteric changes, for example replacing the pyrrole ring with a furan (K_D = 3000 nM) or a thiophene (K_D = 3000 nM) failed to produce even modest enhancements compared to pyrrole (unpublished results). As stated previously, interactions between ligand and receptor are complex at this locus (Figures 2 and 3).

The most dramatic demonstration of the importance of the pyrrole comes from studies of 10-O-pyrrolecarbonyl ryanodol (9, 10). In this compound (K_D = 200 nM) the pyrrole carbonyl group has moved from the 3-hydroxyl group to the 10-hydroxyl group with only a small

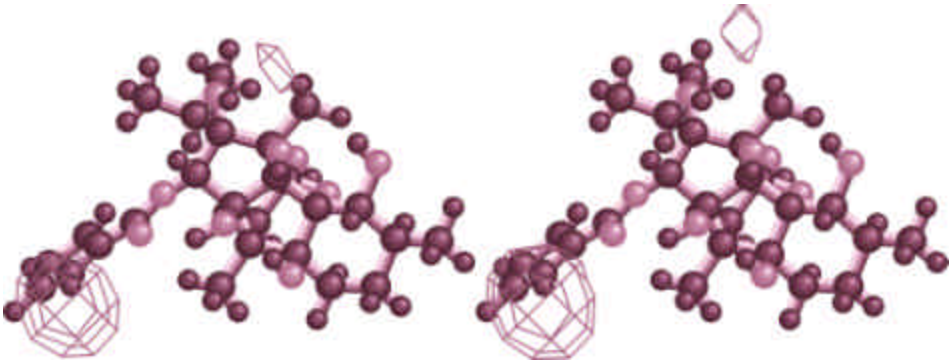

Figure 4. Structure activity relationship between rabbit skeletal (RyR1, left) and cardiac (RyR2, right) ryanodine receptor (RyR1 and 2) dissociation constants and the presence of hydrogen bond donors. The correlation between changes in hydrogen bond donor ability and dissociation constant are contoured at one-fourth of the maximum correlation. At this level the negative correlations are invisible. Therefore, the contours identify the regions where hydrogen bond donors strengthen binding. The contours are essentially identical for both RyR isoforms.

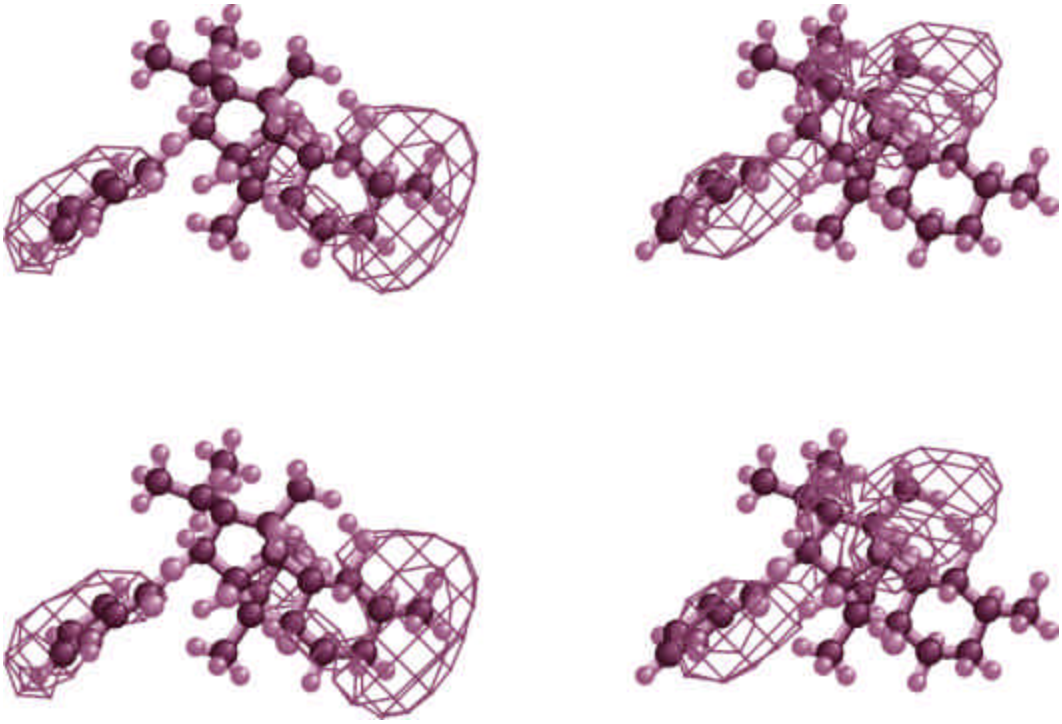

Figure 5. Structure activity relationship between rabbit cardiac (RyR2, top) and skeletal ryanodine receptor (RyR1, bottom) dissociation constant and hydrophobicity. The wire frames are contours at one-third of the absolute value of the maximum correlation between changes in hydrophobicity and dissociation constant. Ryanodine is used as a guide to the eye. Left side: wire frames enclose volumes where increased hydrophobicity is strongly correlated with decreased dissociation constant. Right side: wire frames enclose volumes where increased hydrophobicity is strongly correlated with increased dissociation constant.

penalty (25-fold) in dissociation constant. Although the 40-fold increase in affinity relative to ryanodol might be due to interactions with parts of the binding site near the 10-position, all other substituents at that position on either ryanodine or ryanodol at best produce smaller enhancements in binding (see below or Reference 1). At the 3-position, the pyridine-3-carbonyl (nicotinyl) group enhances the binding of ryanodol 10-fold (compound named ryanodyl nicotinate or 2-O- (pyridyl-3-carbonyl) ryanodol, References 8, 11); whereas this same group at the 10-position doubles the dissociation constant of ryanodol (unpublished results). In fact, the latter result was predicted by previous CoMFA and is consistent with the complex CoMFA at the 10-position (see Figures 2 and 3). Therefore, the effect is specific for the pyrrole. In addition, the relationship between structure and fractional

conductance (CoMFA) of the ryanoid-modified channel is markedly improved if the 10-O-pyrrolecarbonyl ryanodol and 3-epiryanodol are oriented so that the pyrrole carbonyl occupies the same subsite (10).

It has been argued that the pyrrole caused a reorientation of the ligand in the binding site in order to maximize the strong interactions between the receptor and the pyrrolecarbonyl group (9, 10). The most direct support for this hypothesis comes from the dissociation constant of 10-O-pyrrolecarbonylryanodine (K_D = 12 nM), essentially that of ryanodine. Therefore, if the pyrrole is to interact with the receptor, the ligand must bind in an alternate orientation.

The importance of the pyrrole has been disputed by Bidasee & Besch (11). They ask why, if the pyrrole group is so important, pyrrole is not an effective competitive inhibitor of ryanodine binding. They claim that "up to 1 mM pyrrole carbonate" produces no detectable inhibition of ryanodine binding. Using their numbers (11), in the context of ryanodol the pyrrole carbonyl group provides 4.4 kcal/mol binding energy. Using this energy as an intrinsic energy of binding, one calculates a dissociation constant of approximately 1 mM for the binding of the pyrrole carbonyl fragment alone. The corresponding EC_{50} for the displacement of the radiolabeled ryanodine would be approximately 2 mM. However, the experimental value is expected to be considerably higher due the entropic effect of binding a ligated pyrrole vs. a free pyrrole. A back-of-the-envelope calculation of the estimated loss of translational and rotational entropy is 9 kcal/mol (see References 12-14 for a discussion on estimation of ligand receptor interactions). The loss of entropy will likely overwhelm the intrinsic interaction energy of the pyrrole carbonyl fragment. In addition, the introduction of a negative charge (from the carboxyl in place of the carbonyl) is also likely to be deleterious to affinity (an ester or pyrrole itself would have been a better choice). In fact, the experimentally observed failure of 1 mM pyrrole to displace radiolabeled ryanodine is very much in keeping with the experimental binding data.

Referring to Figure 2, increasing steric bulk on the far edge (hydrophobic edge) of the pyrrole (upper right) increases the dissociation constant. In contrast, increasing steric bulk everywhere else around the pyrrole (including the bay between the pyrrole and the isopropyl group) enhances binding. Meanwhile, electronic changes at the 3-position are only weakly correlated with changes in affinity (Figure 2, bottom). This locus is the most highly correlated with hydrogen bond donating ability (Figure 4). While the presence of a hydrogen bond acceptor is not correlated with high affinity binding, the presence of a hydrogen bond donor is an important component of high affinity binding.

Compared to the skeletal isoform, increases in steric bulk around the pyrrole group are not as strongly correlated to increases in dissociation constant of the cardiac isoform (note the loss of contours comparing the upper right panel of Figures 2 and 3).

Casida's laboratory has produced large numbers of structure-activity studies on both vertebrate and invertebrate RyR. Much of this data has not yet been incorporated into quantitative structure-activity analyses. Although the pyrrole is critical to the binding of ryanoids to the receptor, loss of the pyrrole has relatively little effect on toxicity to insects (2,6). In insect bioassays, ryanodol has one-fourth the potency of ryanodine. In rabbit cardiac and skeletal SR, the dissociation constant of ryanodol is 700- to 1200-fold larger than that of ryanodine. Gonzalez-Coloma *et al.* (15) made similar observations while investigating a number of alkaloid (ryanodine-like) and non-alkaloid (ryanodol-like) compounds (15). The authors interpret their data as supporting a hypothesis of a ryanodol-specific mode of action in insects. Lehmborg & Casida (1994) made such a proposal based on the weak binding (relative to ryanodine) of ryanodol to insect RyR. Gonzalez-Coloma *et al.* (15) used compounds isolated from *Persea indica* (Lauraceae) and *Spigelia anthelmia* (Loganiaceae) rather than the more common source, *Ryania speciosa*. Other sources for ryanoids are welcome.

With successful isolation of insect RyR, a SAR for binding affinity and channel conductance will be helpful. Scott-Ward *et al.* (16) found that the insect RyR binds ryanodine with a dissociation constant of 4 nM, similar to vertebrate RyR. This is consistent with the earlier report of ryanodine dissociation constants of about 5 nM in various insect tissues (17). Ryanodine modified insect RyR have a fractional conductance of 36% compared to 38% for rabbit cardiac RyR (16). The difference in fractional conductance between rabbit cardiac and sheep cardiac (57%; Reference 18) is the use of Ca^{2+} as permeant ion in the former and K^+ in the latter case.

Ryanodine (at nanomolar levels), 9,21-didehydroryanodine and 9,21-didehydroryanodol can act on insect potassium channels (19, 20). The modification puts a double bond between the 9 and 21 carbons (see Figure 1) and causes little to no change in binding affinity. The channels are considerably less sensitive to the ryanodol than to the ryanodines. The results do not explain the similar toxicity of ryanodine and ryanodol on intact insects but does support multiple sites of ryanoid action.

6.2. Isopropyl group

Compared to the pyrrole group, the isopropyl group can tolerate quite large changes with little change in affinity. Steric interactions are as complex as those at the pyrrole locus. Some of these are indirect as the isopropyl (unlike the pyrrole) group changes conformation in response to modifications at nearby locations. In general, increases in steric bulk on the far side of the isopropyl group are correlated with increases in dissociation constant (upper left, Figure 2) whereas such increases on the near side of the isopropyl group decrease the dissociation constant (upper right, Figure 2). Changes in charge at the isopropyl group are strongly correlated with changes in binding (bottom, Figure 2). However, changes in hydrophobicity are only weakly correlated with changes in binding constants.

Changes in the isopropyl group lead to complex changes in binding. EsterC2 contains a double bond between the C1 and C2 atoms (positions 1 and 2), and contains a lactone ring (1). The double bond causes a reorientation of the isopropyl group. These changes cause a 400-fold increase in dissociation constant of both the cardiac isoform (from 2.5 nM to 500 nM) and skeletal isoform (from 5 nM to 2000 nM). Addition of a double bond between the isopropyl group and the fused ring (as in 2-deoxy-2(13)dehydroryanodine) causes the group to become planar and non-rotatable. This modification causes only a slight (2-fold, cardiac; 1.2-fold, skeletal) increase in dissociation constant. Apparently most of the loss of affinity of EsterC2 arises from changes in the fused ring system. Addition of a hydroxyl group to the isopropyl group causes a 9-fold increase in dissociation constant (inferred from Reference 6). We have observed a 20-fold (skeletal) to 30-fold increase (cardiac) in dissociation constant when hydroxyl groups are added to either methyl group of the isopropyl group (unpublished results).

Although not clear from Figures 2 and 3, the distribution of contours around the isopropyl group is remarkable. In both figures the contours depict the strong correlation between increased steric bulk and decreased dissociation constant. For the skeletal isoform (RyR1, Figure 2, upper left) the contours are behind the isopropyl group. In contrast, for the cardiac isoform (RyR2, Figure 3, upper left) the contours are in front of the isopropyl group.

6.3. Polycyclic fused ring system

Changes in the fused ring system (diterpene) are correlated with changes in binding albeit the contours of high electrostatic and steric correlations are less density packed than on pendant groups. The CoMSIA contours show strong correlations between changes in hydrophobicity and binding. In general, these match the hydrophobic and polar surfaces of ryanodine. While some modifications produce dramatic changes in dissociation constant (for example see EsterC2 above), many changes in hydroxyl groups produce relatively little change in dissociation constant (7, 21, 22). No modifications that increase binding have been found. The CoMFA model above has successfully predicted all molecules tested from these references. The results are consistent with a model that allows the fused ring system to bind in more than one orientation. Additional alterations in the ring structure may reveal more detailed information.

A few examples of modification of the fused ring system are offered. A fairly minor modification, the oxidation of the hemiacetal ring to the corresponding lactone (esterC2, Reference 8) results in a 370-fold increase in dissociation constant. Introduction of a positive change at the eight position strongly inhibits binding. An 8-equatorial amino group increases the dissociation constant 540- fold whereas the axial isomer increases dissociation constant 110-fold (8). These findings are noteworthy due to the different effects of introducing positive charge at the 9- and 10-positions (see below).

In summary, it appears that the fused ring system can bind to the receptor in at least two orientations (9, 10). One surmises that the requirement for tight binding does not require a large number of specific interactions with the fused ring system. Instead, a limited number of essential contacts are required and these interactions can be met in more than one way. For example, of the six hydroxyl groups on ryanodine, only two (at the 2- and 12-positions) are strongly correlated with hydrogen bonding. The map is consistent with large variation of the impact of modifications on the diterpene.

6.4. 2-hydroxyl group

As was noted previously (1) interactions at the hydroxyl group at the 2-position (the same attachment point as the isopropyl group) is context dependant. The most prominent of these interactions are reflected in the upper right of Figure 2. Increases in steric bulk at the 2-hydroxyl position are strongly correlated to increases in dissociation constant. There are no strong correlations with electrostatic changes at the 2-hydroxyl group. However, there is a strong correlation between the presence of a hydrogen bond donor at the two position and decreased dissociation constant. No such correlation has been found for hydrogen bond acceptors.

Compared to the skeletal isoform, the cardiac isoform is much less sensitive to increases in steric bulk at the 2-hydroxyl position (compare upper right panels of Figures 2 and 3).

6.5. 9-position

Because of its accessibility, this has been a site of many modifications. Fortunately, large groups can be introduced at this position with little loss of binding. Therefore, a number of experimentally useful groups can be introduced. Steric interactions with the receptor are complex at this locus with increased steric bulk both promoting and inhibiting binding (upper panels, Figure 2). However, the introduction of large bulky groups such as BODIPY (8) indicates that the 9-position is not sterically restricted. In general (lower right, Figure 2), introduction of ionic groups at this position decreases binding (for example, 21-amino-9-alpha-hydroxy ryanodine, 21-amino-9-beta-hydroxyryanodine).

6.6. 10-position

Again, this is a position that has enjoyed much attention because of the relative accessibility of this group to modification. In general, addition of steric bulk at the 10-position enhances binding (upper left, Figure 2). Strong electrostatic correlations at this position are more complex. Positive charge generally favors binding (see the following paragraph). The tightest binding ryanoid has a positively charged pendant group attached to the 10-position (10-O-guanidinopropylryanodine, References 10, 11, 23, 24). Hydrophobic interactions are also complex (Figure 5). The 10-position is at the junction between contours showing strong positive and negative correlations between changes in hydrophobicity and dissociation constant.

The binding site at the 10-position, like the 9-position, must be extremely commodious. The bulky 10-O-(NCBZ-aminoacyl)- and 10-O- (NCBZ-3-amino propanoyl)ryanodines bind with affinities equal to or better than ryanodine (9, 23, 25). We have modeled this part of bound ryanodine as facing into the solvent (1, 9). This feature is preserved in the virtual site model of Schleifer (26) although no ryanoids with bulky substituents at either the 9- or 10-positions were used to build the model. A Glu and a Leu are the only residues near this region of the ryanoids. 10-O-guanidinoacetylryanodine and the slightly longer 10-O-guanidinopropanoylryanodine are the tightest binding ryanodine analogs discovered to date (9, 23, 25). The 10-O-guanidinopropanoyl group is particularly effective at promoting ryanoid binding in multiple contexts (10, 11, 23, 24). Using the data of Bidasee and Besch (11), one calculates that this particular group enhances ryanodine binding by 1.1 kcal/mol, 3-O-nicotinyl ryanodol binding by 2.2 kcal/mol (Reference 27; also called pyridyl ryanodine, Reference 11), and ryanodol binding by 1.8 kcal/mol. In cardiac SR, the 10-O-guanidinopropanoyl group enhanced ryanodine binding 0.8 kcal/mol (10). These values are consistent with solvent-faced electrostatic (including hydrogen bonding) and hydrophobic interactions. The smaller, but cationic, 10-O-beta-alanyl group is also effective at reducing the dissociation constant of ryanodine (0.6 kcal/mol, skeletal SR, Reference 11; 0.8 kcal/mol, cardiac SR, Reference 10). As before, the cationic group is about twice as effective in the context of the weaker-binding ryanoids. 10-O-beta-alanyl group enhances binding of both 3-O-nicotinyl ryanodol and ryanodol by 1.8 kcal/mol (data from Reference 11). Ionic interactions are long range and it is difficult to assess what differences in binding might be sufficiently dramatic to account for the > 1 kcal/mol differences between compounds. Both the guanidino and beta-alanyl groups are capable of forming hydrogen bonds where one of the partners will be charged. Hydrogen bonds are short-range interactions. Changes in ligand binding orientation or induced conformational changes in the RyR may be sufficient to form stronger ligand-receptor hydrogen bonds. The bulkier, but non-ionic, CBZ-beta-alanyl group also promotes binding (ryanodine, 0.03 kcal/mol; ryanodol, 0.4 kcal/mol; 3-O-nictotinyl, 0.7 kcal/mol; data from Reference 11). At the 10-position the shorter CBZ-glycyl group actually inhibits ryanodine binding by 1.3 kcal/mol (10). In contrast, the same group in the same position slightly enhances binding of ryanodol to skeletal SR by 0.6 kcal/mol and to cardiac by 0.4 kcal/mol (unpublished results).

For contrast, an anion has replaced the cation. The 10-O-succinyl group enhances the binding of ryanodol by 0.1 kcal/mol (unpublished results). The fact that the succinyl group did not antagonize ryanodol binding suggests that enhanced binding of the cationic ryanoids is not due to formation of a salt link: the predominant interactions are more likely derived from steric and hydrogen bond interactions. However, the succinyl group antagonizes binding of other ryanoids: 10-O-succinyl-ryanodine binding was so weak as to be unmeasurable (23) and addition of a succinyl group 9,21-dehydroryanodine (a double bond between atoms 9 and 21) increased the dissociation constant from 5 nM to 244 nM (24).

In passing, recall from the previous section that positive charge in this region of the molecule does not automatically enhance binding. For example, converting the methyl group at the 9-position to the amino methane group (21-amino-9-alpha-hydroxyryanodine, 21-amino-9-beta-hydroxyryanodine) increases the dissociation constant of ryanodine from 8.5 nM to 3700 nM (alpha) and 2000 nM (beta). This is a robust change (as much as 3.7 kcal/mol) for a site that can accommodate such large derivatives as BODIPY with little to no penalty. Based on molecular dynamics simulations the average distance between the amino groups of 21-amino-9-alpha-hydroxyryanodine and 10-O-beta-alanyl ryanodine is approximately 5A (unpublished). All this indicates the complex relationship between receptor and ligand near the 9- and 10-positions.

In summary, the ability of the 10-position to accommodate large bulky substituents argues that this position is exposed to solvent in the receptor-ligand complex. The ability of the various substituents to deferentially enhance ryanoid binding argues for receptor-ligand interactions at this site. The most likely analogy is to affinity chromatography. The linker used to anchor the affinity ligand while not part of the binding site per se, none-the-less has profound effects on solute binding.

7. MAP OF THE BINDING SITE

The CoMFA and CoMSIA contours provide a map of the ryanodine binding site. Regions of CoMFA and CoMSIA that have high correlation between structural changes and dissociation constant identify corresponding regions in the receptor. Therefore, areas where increased steric bulk enhance binding correspond to cavities in the receptor that provide a good fit with protrusions on the ligand. Likewise, regions where increased positive electrical potential are highly correlated with lower dissociation constant are regions of negative potential in the receptor. One can quantify this relationship by investigating the effect of removing charge or atoms (or both) on virtual molecules and examining the effect on predicted dissociation constant (or free energy of binding). The relationship can be visualized using the model by drawing contours that show the areas of a ligand that contribute to the experimentally observed dissociation constant (e.g., Figure 25, Reference 1).

Schleifer (26) has carried mapping a step further by constructing a virtual ryanodine receptor binding site. Using the computer algorithm PrGen, amino acid residues were positioned around a collection of ryanodine analogs to obtain the strongest correlation between experimental and predicted free energies of binding. In the final pseudoreceptor model, six amino acid residues are positioned to make essential contacts with the bound ligands. It likely that the binding site of the ryanodine receptor includes more than six amino acids, the

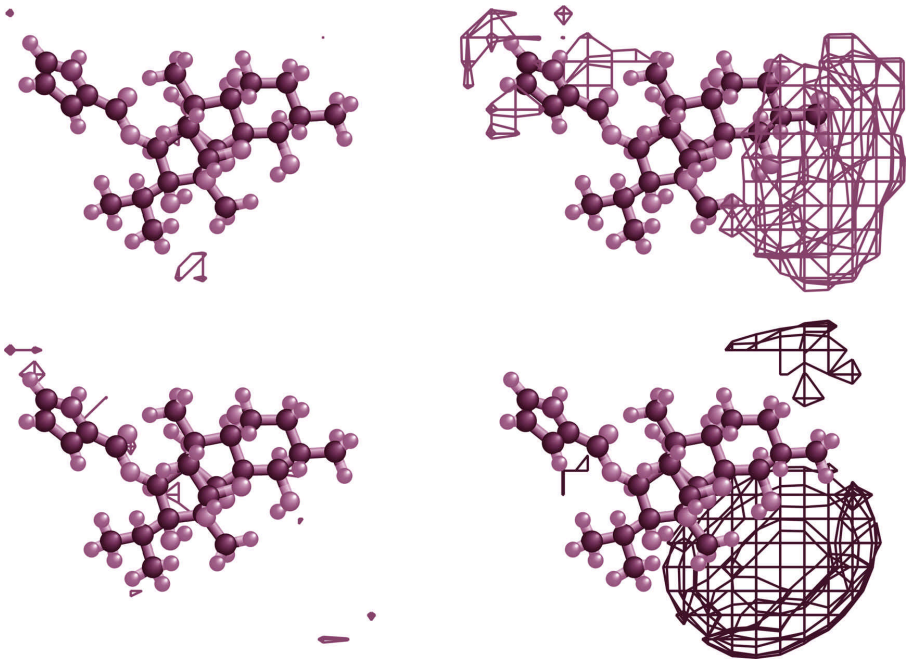

Figure 6. Relationship between structure and fractional conductance of the ryanoid-modified ovine cardiac RyR. To improve visibility of the contours, the ryanodine molecules as shown in Figure 2 have been rotated approximately 180 deg. along the X-axis. In Figure 6 the pyrrole is at the upper left and the isopropyl group at the lower left of each molecule. The wire frames are contours at one third of the absolute value of the correlation between steric bulk (top panels) and electrostatic bulk (lower panels). Upper left: regions where increasing steric bulk are highly correlated with increased fractional conductance; upper right: regions where increasing steric bulk are highly correlated with decreasing fractional conductance; lower left: regions where increasing negative electrostatic charge are highly correlated with decreasing fractional conductance; lower right, regions where increasing positive charge are highly correlated with decreasing fractional conductance.

pseudoreceptor model provides a concrete representation of interactions sufficient to explain experimental binding data. Although different methods are used from those that generated Figures 2 and 3, the same important interactions are determined. These interactions are with the pyrrole carbonyl, the isopropyl group and the 9- and 10-positions. The pyrrole is surrounded by an asparagine (to provide polar contacts) and a combination of three hydrophobic residues (phenylalanine, tyrosine, isoleucine) to provide strong, but sterically restrictive, interactions with the pyrrole. The isopropyl group interacts with the phenylalanine and isoleucine residues. The other end of the binding site is defined by a leucine (to make steric and hydrophobic interactions) and a glutamate (to make polar and hydrogen bonds with the 9- and 10-positions). Schleifer (26) proposes that when ryanodine is bound to the receptor, the hydrophilic hemisphere of the diterpene fused ring system remains exposed to the solvent. This is consistent with the weak correlations between hydrogen bond acceptors or donors in this part of the molecule. The concentration of residues in the vicinity of the pyrrole is consistent with the experimentally observed strong binding energy and specificity associated with the 3-position. It is also consistent with the CoMFA presented in Figures 2 and 3, which show complicated steric interactions but relatively weak correlations with changes in charge. The CoMSIA of hydrogen bonding is consistent with the presence of asparagine and tyrosine acting as hydrogen bond partners. It differs in that it would suggest these residues optimally would act as hydrogen bond acceptors for the pyrrole nitrogen and hydroxyl groups at positions 2 or 12. The glutamate and leucine are consistent with the strong correlations between charge and steric bulk at the 9- and 10-positions while retaining the ability to attach large ligands at either of these positions.

8. RYANOID-INDUCED CONDUCTANCE STATES

Ryanodine is a complex modulator of RyR channel function producing changes in both channel gating kinetics and channel conductance. At nanomolar concentrations ryanodine causes the channel to enter into a long-lived subconductance state. At micromolar concentrations ryanodine produces a long-lived, non-conducting state. These phenomena have been commonly observed in both calcium uptake and release studies and single channel recordings (28). Williams and his coworkers found that the magnitude of the subconductance state induced by binding to the high affinity site of sheep cardiac RyR was a function of the ryanoid structure. The structural determinants for channel modification map to loci different from the structural determinants of binding affinity (10).

8.1. CoMFA map of fractional conductance

Figure 6 is an update of earlier maps and includes compounds investigated since Tinker *et al.* (18) including 21-aminoryanodine, and 10-O-succinyl ryanodol. While ryanoids have been discovered that can almost completely block channel conductance while bound to the high affinity site, no ryanoids have been found that induce a near maximal conductance of the ryanoid-modified state. Ryanodol, with a fractional conductance of 69% (18), is about the largest fractional conductance observed in the ryanoid modified channels. Changes in structure are highly correlated with fractional conductance. In Figures 2, 3, and 6 the wire frames are contours at one third of the maximum absolute value of the respective physical property. Therefore, these figures are comparable in terms of the level of correlation. To improve the visibility of the wire frames in a two-dimensional drawing, the orientation of Figure 6 is approximately 180 deg. relative to Figures 2 and 3. High levels of correlation of increasing steric bulk (upper left) and increasing positive charge, (lower left) with increasing fractional conductance are confined to small loci. In comparison, high levels of correlation between increasing steric bulk (upper right; or increasing positive electrostatic field, lower right) and decreasing fractional conductance extend over a large region of space. Compared to binding, much more of the relationship between physical properties and fractional conductance is located in the vicinity of the 9- and 10-positions. Attachment of groups of any kind to these positions results in lowered fractional conductance. Interestingly, no matter low large the group attached, the ryanoid-modified channel still has an appreciable conductance. This observation argues against ryanoid binding near the ion conductance pathway. Should the ryanoid bind at or near the conduction pathway, one would expect that eventually the ryanoid would become so large as to completely obstruct the channel. Even bulky biotinylated ryanoids, while binding with about the same affinity as ryanodine, have fractional conductances of 20% (Williams, personal communication). To achieve nearly zero conductance, a combination of positive charge and bulk is required (guanidinoryanodine has a fractional conductance of 6% whereas beta alanyl has 14%). The dramatic effect of positive charge is shown in the lower right panel of Figure 6 (the correlation between increasing negative charge and increasing fractional conductance or, in other words, increasing positive charge and decreasing fractional conductance).

8.2. CoMSIA

Unlike the correlations observed for dissociation constant, CoMSIA reveals a random relationship between changes in hydrogen bonds and hydrophobicity and the fractional conductance of the ryanoid-modified channel. Note the CoMSIA does not say that these physicochemical factors are not important in determining fractional conductance. It does say that a model based on hydrogen bonds or hydrophobicity is insufficient to predict the fractional conductance of compounds, i.e., one could do as well by random guesses. From another point of view, none of these factors, by themselves, are essential for setting the magnitude of the fractional conductance. As pointed out previously, there is no correlation between the strength of binding and the induced fractional conductance (3). Together, these data support the hypothesis that receptor-ligand interactions that promote strong binding are both qualitatively and quantitatively different from those that modify channel conductance. At least in principle it is possible to modulate affinity independently of conductance.

8.3. Calcium release

Ryanoid structure independently alters binding to the high affinity site and the conductance of the resultant modified state of the RyR. One immediately asks questions about the low affinity site. For example, is the ratio of apparent free energy of binding to and high and low affinity sites the same for all ryanoids? Can all ryanoids induce a non-conducting state or an alternate subconducting state? If a ryanoid fails to induce a non-conducting state, does the failure result from the inability to bind to a low affinity site? Can a ryanoid bind to a low affinity site, but lack the structural components required to induce a non-conducting state? Some investigations along these lines have been reported (18, 24, 25). Intriguing observations have been made by Bidasee and Besch (11) based on enhancement and inhibition of passive calcium efflux from SR vesicles. Notable findings include the following, (A) the EC_{50} for stimulation of calcium efflux is orders of magnitude higher than the dissociation constant of the ryanoid, (B) no correlation is evident between the high affinity dissociation constant and EC_{50} for stimulation of calcium efflux, and (C) no correlation is evident between ryanoid-induced fractional conductance (18, 31, 32) and either the EC_{50} for stimulation of calcium efflux or the magnitude of the induced Ca^{2+} efflux. For example, 10-O-guanidinopropionyl ryanodine produced maximal calcium efflux (Ref. 11, EC_{50} = 2.9 uM) but induces the lowest fractional conductance in single channel measurements (Ref. 18, K_D = 1 nM). Dissociation constants of ryanoids are in the nanomolar range but the EC_{50} for stimulation of calcium efflux are in the micromolar range (11). The dissociation constants inferred from the kinetics of RyR modification of channel conductance are in agreement with dissociation constants from equilibrium binding experiments (32) suggesting the partially conducting, modified state is the same as that identified by binding isotherms in SR vesicles. Inhibition of Ca^{2+} efflux by ryanodol or 10-O-beta-alanyl ryanodine was not observed (11) whereas high concentrations of both of these compounds close the ryanodine-modified channel in single channel experiments (Tanna & Williams, personal communication). The conditions of the calcium efflux experiments (11) make it difficult to provide a mechanistic interpretation. Calcium loading occurred at 1 mM Ca^{2+} in the presence of the test ryanoid. Millimolar levels of calcium inhibit the open state of the channel and ryanoid binding (29, 30). The high levels of calcium ion may be responsible for some of the observed differences. Transmembrane electrical potential (32) may also be involved. Bidasee & Besch (11) suggest that magnitude of vesicular calcium efflux is determined by the difference in affinity of a high affinity and low affinity site. Fascinating differential structural determinants exist at two or more sites; however, additional mechanistic components are required to explain the dose-response curves and the magnitudes of the calcium efflux.

8.3. Modulation of RyR channel function

The binding of ryanoids to the receptor causes both a change in channel gating kinetics and channel conductance. These effects could result from the binding of the ryanodine at or near to the ion conduction path. The ligand would then be in a position to physically obscure both (A) the movement of amino acids that switch the channel from an open and a closed state and (B) the free flow of ions through the channel. However, the energetics of the relationship between structure and fractional conductance would appear to be at odds with this mechanism. The magnitude of the fractional conductance seems to bear no relationship to the size of the ryanoid. The effect of charge on anionic and cationic ryanoids appears to be too weak to have an intimate relationship between the ryanoid and the conduction pathway (see References 1 and 3 and the following section for more discussion).

Binding to allosteric sites could produce changes in fractional conductance and gating kinetics if one assumes that the ryanodine receptor exists in a number of rarely populated conformations. The binding of ryanoid then stabilizes one or more of these conformations. Support for the allosteric mechanism comes from the work of Fessenden *et al.* (33) who find that ryanodine binding restores calcium sensitivity to a calcium insensitive mutant while failing to block conductance.

9. APPLIED POTENTIAL VS. RYANOID BINDING (KINETICS OF RYANOID BINDING)

Typically, ryanodine and related compounds are found to be slow binding ligands. The slow dissociation rates make measurement of the kinetics of single channel modification by ryanoids unattainable within the lifetime (up to 1 hr) of a single-channel experiment (18, 28, 34). Kinetics have been confined to ensemble experiments such as radioligand binding assays and vesicular calcium uptake and release assays. However, four derivatives (ryanodol, 9-hydroxy-21-azidoryanodine, 10-O-pyrrolecarbonyl ryanodine, 3-epi-ryanodine) have dwell times in the substate lasting tens of seconds to minutes (18). Ryanodol modification is dependent on transmembrane voltage, with modification more likely to occur and lasting longer at +60 than at -60 mV holding potential (18). Tanna *et al.* (31, 32) have extended these observations using an uncharged ryanoid (ryanodol, Reference 32) a cationic ryanoid (21-amino-9alpha-hydroxyryanodine, Reference 31), and an anionic ryanoid (10-O-succinyl ryanodine, Tanna *et al.*, unpublished results). All three show similar dependencies on applied potential: therefore, interactions between the applied field and the ligand are minor if they exist at all. It is clear that the RyR is sensing the applied electrostatic field by undergoing a voltage-induced conformation change (31, 32). The isomerization of the RyR is not discerned by open probability of conductance but is detected by a change in the affinity of the RyR for ryanoids. This is manifested by voltage-induced changes in proportion of time the channel spends in the ryanoid-induced subconductance state, and in changes in the association and dissociation rate constants. Both the on and off rate constants are affected by applied potential. For the cationic, anionic and neutral ryanoids, increasing positive holding potential increases the association rate constant while the off rate constant decreases with increasing positive holding potential. At zero applied potential, the association and dissociation rate constants are ryanodol (0.035 uM^{-1} sec^{-1}, 0.095 sec^{-1}), 21-amino-9-alpha-hydroxyryanodine (0.365 uM^{-1} sec^{-1}, 0.99 sec^{-1}), and 10-O-succinyl ryanodol (0.0022 uM^{-1} sec^{-1}, 0.085 sec^{-1}). Although it is dangerous to do so (because neither binding nor dissociation of a ryanoid are elementary processes) one can estimate the dissociation constant from the ratio of the dissociation rate constant to the association rate constant. At zero applied potential the inferred dissociation constants for both ryanodol and 21-amino-9-alpha-hydroxyryanodine is 2.8 uM, while that of 10-O-succinyl ryanodol is 36 uM. The values obtained from binding isotherms to cardiac SR are 1.6 μM (ryanodol), 3.6 uM (21-amino-9-alpha-hydroxyryanodine) and 4.8 uM (10-O-succinyl ryanodol). The large discrepancy in the case of the anionic derivative is unresolved. It may point to a difference in rate limiting step in the binding of the anionic ryanoid and the inadequacy of the calculation to account for the difference. Note that the order of magnitude difference between the on and off rate constants between the cationic ryanoid and the neutral and anionic ryanoids is not paralleled in binding isotherms in SR preparations of the RyR.

10. CONFORMATION AND CONDUCTANCE

That change in structure should lead to changes in ligand-receptor affinity is useful but not surprising. That change in structure should lead to changes in fractional conductance and gating kinetics of ryanodine receptors is less expected. The fact that affinity and modulation of channel properties map to different structural loci indicates the potential for design of the ligands with useful properties. What was surprising is that the ryanodine receptor can sense the conformation of the bound ligand. Both 21-p-nitrobenzoylamino-9-alpha-hydroxylryanodine (32a) and succinyl ryanodol (Tanna *et al.* unpublished results) induce multiple conformational states. Three distinct subconductance states are induced by 21-p-nitrobenzoylamino-9-alpha-hydroxylryanodine. These subconductance states are correlated with three distinct conformations of the p-nitrobenzoyl group identified by extended, unrestrained molecular dynamics and family analysis. The fractional conductance states (and the percentage of total observations) are 0.27 (73%), 0.17 (20%) and 0.59 (7%). In Figure 7 the three major conformations are shown. They exist in the ratio of 73:24:1; the remaining 2% of the conformations correspond to several conformers that are rarely populated.

From examination of the single channel recordings, it appears that the receptor binds to one of the 21-p-nitrobenzoylamino-9-alpha-hydroxylryanodine conformers to enter the corresponding subconductance state. The ryanoid is released, and another or the same conformer is bound. There is no evidence for isomerization of the ligand within the binary receptor-ryanoid complex.

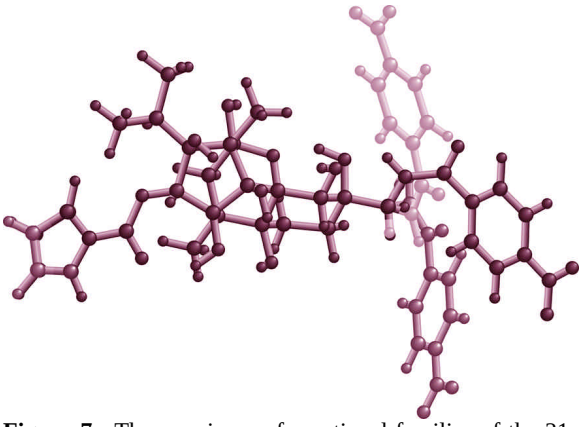

Figure 7. Three major conformational families of the 21-p-nitrobenzoylamino-9a-hydroxylryanodine. The compound was run in molecular dynamics and therefore includes entropic contributions to conformation. The high energy barrier to rotation about the bond connecting the 9- and 21-carbons divides the molecule into distinct conformational families. After dividing the conformers into families, the average conformation was computed and then minimized by molecular mechanics. The three major conformations are superimposed with the p-nitrobenzoyl group to the right.

Succinyl ryanodol produces a large number of subconductance states. Molecular dynamics demonstrates that unlike the 21-p-nitrobenzoylamino-9-alpha-hydroxylryanodine, the succinyl group exists in a large number of distinct conformers. Because of the complexity of the data, no analysis has been made. Conformational relationships of anionic ryanoids can be better addressed with pendant groups that are less conformationally mobile, for example a fumaric acid rather than a succinic acid.

11. MODULATION BY BINDING IN THE CHANNEL OR BY ALLOSTERIC INTERACTIONS

The location of the ryanodine binding site remains unknown. Covalent modification studies indicate that the ryanodine binding site includes, at the least, the carboxyl terminal part of the ryanodine receptor (which includes the transmembrane domain). In particular, a debate continues about how binding of ryanoids is translated into altered channel function. Any such model would have to explain both the altered channel gating kinetics and the altered conductance of the channel. In one model the ryanoids bind in the ion conduction pathway and partially block the ion conduction path. In this way ryanoids function in a manner analogous to a classical blocker. In another model, ryanoids bind at a site remote from the ion pore and alter channel function by stabilizing conformers rarely populated in unmodified RyR. The latter idea is attractive because of the many factors that modulate channel conductance. Cryoelectron microscopy shows conformational changes remote from the transmembrane domain suggesting that ligands may stabilize functional conformers by binding to sites remote from the conduction path. The ability of ryanodine to enhance the RyR calcium sensitivity is also consistent with an allosteric model where two or more ligands act to stabilize a conformation of a protein (35).

The original argument for allosteric binding came from analysis of binding isotherms. Pessah and Zimanyi (36) had proposed the binding isotherm of ryanodine could be interpreted as strong negative cooperativity between binding sites. This review will not discuss the many excellent papers that analyze the complex binding isotherms. An insufficient number of ryanoids have been used to conduct a QSAR. Instead this review will recite the arguments for allosteric modulation of channel function based on QSAR of fractional conductance (9, 31, 32). First, there is little, if any, correlation between the steric bulk of ryanodine analogs and fractional conductance. Extremely bulky substituents at the 9- and 10-positions (some more than doubling the molecular volume of ryanodine) have relatively minor effects of fractional conductance. Second, introduction of charge at either the 9- or 10-position produces far less change in conductance than would be expected from an intimate relationship between the ryanodine binding site and the ion conduction path through the RyR. These points are discussed elsewhere in this review and in Reference 3.

Fessenden *et al.* (33) created a RyR1 mutant (E4032A) that is unresponsive to calcium, nucleotides or caffeine. High concentrations of ryanodine (200-500 uM) convert the channel to a functioning state. Presumably this occurs by converting ryanodine binding energy to stabilization energy to compensate for protein interactions lost in the mutant. The authors provide a plausible mechanism to explain both the thermodynamics and kinetics of the observations. The fact that the restored channel activity will open to full conductance strongly suggests that ryanodine binds remotely from the ion conduction path.

The E4032A mutant provides a potential opportunity for a SAR to answer some fundamental questions about the mechanism of ryanoid-induced changes in channel function (3). Does the channel exist in only two states, open and closed, or does the channel exist as a manifold of conformers, many of which are rarely populated? Does the ryanoid alter channel function by binding at or near the cation conduction path, or does it alter channel function by binding to, and thereby stabilizing one or more of the rarely populated conformational states? Different ryanoids induce alternate fractional conductances. The E4032A mutant stabilizes a conformer that lacks channel function. In the presence of ryanodine, an alternate, functional conformation is stabilized. Would all ryanoids stabilize this same functional conformer? In other words, is occupancy of the site by any ligand sufficient to promote the functional conformer? Or does the ryanoid contain information recognized by the receptor (and decoded by stabilization of alternative isomers of the receptor)? For example, the pyrrole carbonyl appears to be a critical determinant of ryanoid binding but it plays only a minor role in setting the conductance of the ryanoid-modified channel (57% ryanodine vs. 69% ryanodol, Reference 18). What is needed to restore function to the

mutant? Does the pyrrole only provide interactions to increase the free energy of binding or does the pyrrole contain essential information required to induce a functional conformer of the mutant ryanodine receptor? In the absence of the pyrrole carbonyl, does the ryanoid bind differently or induce an alternate conformation of the RyR? Moving the pyrrole from the three position (ryanodine) to the 10-position alters the binding of ryanodol (see above). Would the positional isomer of ryanodine restore channel function? Would a ryanoid that induces a low conducting conformation (e.g., the guanidyl ryanoids) induce the same functional state as ryanodine? The mutant may serve as a lens to magnify these structural differences.

12. QSAR AND INTACT CELLS

A major purpose of 3D-QSAR is to develop useful probes of RyR function in cells, organs, and intact organisms. Proposed by John Sutko, fluorescent derivatives of ryanodine have been synthesized by conjugating BODIPY to ryanodine. Large fluorophores such as 4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3,4-diaza-s-indacene-3-propionic acid succinimidyl ester (or 6-((4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)amino) hexanoic acid, BODIPY) can be incorporated at the 9- or 10-position with only minor effects on dissociation constant. Welch *et al*. (8) find a 2-fold increase (7 nM vs. 13 nM) in the dissociation constant when BODIPY is attached to the 21-position. Cifuentes *et al.* (37) report that BODIPY at the 10-position of ryanodine (B-FL-X ryanodine) increases the dissociation constant about 20-fold. From Figure 8 of Reference 38 we obtain the same result. These data are consistent with the steric and electrostatic contours in Figures 2 and 3 and the model of ryanodine binding site presented in Reference 3.

While the function of the RyR in striated muscle is at least partially understood, the function of RyR in neurons or non-excitable tissues is far less clear. Localization of RyR in cells can provide insights into function. The BODIPY-ryanodines have been used to localize ryanodine receptors in microsomes of rat parotid acinar cells (38), porcine endothelial cells (39), pancreatic beta cells (42) and vascular myocytes (40, 41). Cifuentes *et al.* (37) used the fluorescent BODIPY conjugated ryanodine to identify ryanodine receptors on the Golgi of rat sympathetic neurons. Holz *et al.* (Reference 42, Figure 2) using fixed tissue found 4 nM ryanodine inhibited the binding of 1 nM BODIPY-ryanodine binding 50%. This finding is consistent with the data in the paragraph above: addition of the BODIPY group causes little perturbation of binding. The Texas red conjugate of ryanodine was used to localize RyR in transgenic insect cells (43).

Cifuentes *et al.* (37) have pioneered details of ryanoid-receptor interactions in living cells. Using a fluorescent ryanodine (BODIPY attached to the 10-position) they measured ryanoid binding kinetics in a living cell (rat neuron). Binding is complete within 60 min. This is typical of the time required for binding of ryanodine to SR *in vitro*. The apparent second order rate constant is $9X10^4\ M^{-1}\ min^{-1}$ (pseudo first order rate constant = 0.018 min^{-1}) compared to $3X10^6\ M^{-1}\ min^{-1}$ (ryanodine, unpublished results) or $5X10^6\ M^{-1}\ min^{-1}$ (44). As pointed out (37) the slower association rate constant may be due to the physical barriers not found in the SR binding assays. Dissociation of ryanoid from RyR in living cells consists of multiple kinetic components as seen by others *in vitro* (44; manuscript in preparation). The first order rate constants *in vivo* are 0.16 min^{-1} and 0.017 min^{-1}. In comparison, Wang *et al.* (44) observed three distinct dissociation rate constants under conditions of increasing concentrations of unlabeled ryanoid (k_{-1}= 0.0025 min^{-1}; k_{-2} = 0.00025 min^{-1}; k_{-3} = 0.012 min-1). Privette et al. observed rate constants of 0.025 min^{-1} and 0.001 min^{-1} (manuscript in preparation). The slower of the *in vivo* dissociation rate constants is comparable to those observed in SR.

The Williams group found certain ryanoids have dissociation and association rates sufficiently fast to measure with the artificial bilayer/single channel technique (see above). At zero applied potential, the association and dissociation rate constants are ryanodol ($2.1X10^6\ M^{-1}\ min^{-1}$, 5.7 min^{-1}), 21-amino-9-alpha-hydroxyryanodine ($2.19X10^7\ M^{-1}\ min^{-1}$, 59.4 min^{-1}; Reference 32), and 10-O-succinyl ryanodol ($1.32X10^5\ M^{-1}\ min^{-1}$, 5.1 min^{-1}; Tanna *et al.*, unpublished results). Although well below the diffusion-controlled limit, the association rate constants observed in the artificial bilayer are all faster than that estimated in the living cell. Again, this may be due to physical barriers in the cell not present in the artificial bilayer. Dissociation of weaker binding ryanoids in the bilayer experiments is faster than the BODIPY derivative *in vivo*, consistent with the considerably tighter binding of the fluorescent ryanodine adduct. *In vivo* direct binding of B-FL-X Ry had an EC_{50} of 160 nM, (essentially the same as observed in isolated SR, see Reference 38) and close to the dissociation constant of 21-BODIPY ryanodine (8). As mentioned earlier, the relationship between kinetic rate constants and thermodynamic dissociation constants is true only for elementary processes. In spite of this important caveat, we have often found good agreement between rate constants and thermodynamic constants for both radioligand binding to SR and from rates of modification of single channels in artificial membranes. Using the rate constants from Reference 37, the derived dissociation constants are 2 uM and 28 nM respectively. The relative magnitudes of the rate classes are consistent with the amount of binding to high and low affinity sites one would expect at a loading concentration of 200 nM BODIPY ryanodine. These values are in the range of dissociation constants (160 nM) estimated in living neurons (37) and acinar cells (38). The differences may simply reflect the inaccuracies of using kinetics of multistep processes to infer binding constants. It is not the differences that are remarkable: it is the similarities. This data is wonderful support for the notion that the highly artificial binding conditions used in the SR experiments and artificial bilayers reflect ryanoid interactions in living cells.

At 1 uM, ryanodine produces a use-dependent irreversible inhibition of the caffeine response and on Ca2+ transients resulting from Ca^{2+} influx through voltage-gated

channels. In comparison, application of 1 uM of BODIPY ryanodine produced a small elevation of the resting Ca2+ levels and an attenuated response to potassium-induced membrane depolarization and caffeine (37). The authors suggested that ryanodine binding may block interactions between RyR.

The BODIPY derivatives have been useful because the conjugation of the fluorophore to ryanodine extracts only a small price in binding energy and retains high specificity and slow binding kinetics of ryanodine. These physical properties facilitate the localization of functional RyR in living cells. The CoMFA suggests the possibility of attaching other useful groups to the ryanodine structure. For example, one can conceive of attaching a sensor or reporter group to monitor the environment near the RyR. Cifuentes *et al.* (37) suggest that binding of ryanodine derivatives may block interactions between RyR: none-the-less such derivatives may provide insights into the compartments formed by RyR. One such molecule might sense calcium kinetics at the SR junction.

13. CLOSING

Before ending one should note that two isoflavones, tectoridin and 3'-hydroxy tectoridin, were found to bind to and modulate skeletal and cardiac RyR (45). While the affinity is about a thousand-fold weaker than that of ryanodine, the isoflavones are able to fully displace ryanodine from the binding site. Removal of the sugar to form the aglycone tectorigenin resulted in a loss in affinity to RyR1 and could displace only 50% of the bound ryanodine. Remarkably, removal of the sugar apparently abolished binding to RyR2. While more experiments are required to prove that the isoflavones and ryanodine bind to the identical site on the RyR, these compounds are intriguing as they may provide clues into the synthesis of isoform-specific ryanomimetics.

While this brief review has mentioned only a few of the applications of ryanodine and related compounds, it is evident that ryanoids remain one of the most important tools in understanding ryanodine receptor function. The advantage of QSAR is one can make useful, quantitative predictions about ryanoid interactions without the necessity of synthesizing every possible variant. The application of three-dimensional QSAR to the ryanodine receptor is very much in the early stages. As computers and computational algorithms improve, three-dimensional structure-function relationships will be combined with experimental information and techniques to extend the insights provided by this important ligand and to guide synthesis of useful drugs and investigational tools.

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