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The Pyrrole Locus Is the Major Orienting Factor in Ryanodine Binding[†]

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ABSTRACT: Ryanodine, a natural product, is a complex modulator of a class of intracellular Ca^{2+} release channels commonly called the ryanodine receptors. Ryanodine analogs can cause the channel to persist in long-lived, subconductance states or, at high ligand concentrations, in closed, nonconducting states. In this paper, we further explore the relationship between structure and ryanodine binding to striated muscle. Ryanodine, 3-epiryanodine, and 10-ryanodine are three structural isomers of ryanodine. The dissociation constants of these compounds were measured using rabbit skeletal muscle ryanodine receptors. Placing the pyrrole carbonyl group at the 3-epi- and 10-positions of ryanodol largely restores the large loss of binding energy observed when ryanodine is hydrolyzed to ryanodol. Comparative molecular field analysis successfully predicts the enhanced binding and indicates that the pyrrole group controls the orientation of ligand binding. We propose that the ryanoids are reorientated in the binding site of the ryanodine receptors such that the pyrrole always occupies the same subsite. By applying this model, the binding constants of other ryanoids are predicted.

Ryanodine is a plant natural product (Rogers *et al.*, 1948) possessing powerful pharmacological (Jenden & Fairhurst, 1969) and insecticidal actions (DeVault, 1983). Contraction of both skeletal and cardiac muscle is profoundly influenced by the presence of ryanodine (Jenden & Fairhurst, 1969; Ogawa, 1994). Much evidence indicates that the site of action is an intracellular calcium channel located in the endoplasmic (sarcoplasmic) reticulum (Fairhurst & Hasselbach, 1970; Fairhurst, 1974; Pessah *et al.*, 1986; Campbell *et al.*, 1987; Inui *et al.*, 1987; Lai *et al.*, 1988; Hillyard & Procita, 1959; Nayler *et al.*, 1970; Penefsky & Kahn, 1970; Penefsky, 1974; Frank & Sleator, 1975; Sutko *et al.*, 1979; Jones *et al.*, 1979; Jones & Cala, 1981). This unusually large protein is generally termed the ryanodine receptor (RyR).[1] Binding of ryanodine alters the channel conductance by inducing a long-lived, partially open (or partially conducting) conformation of the RyR channel (Rousseau *et al.*, 1987; Smith *et al.*, 1988). At high (micromolar) concentrations, ryanodine induces a closed state of the channel (Rousseau *et al.*, 1987; Smith *et al.*, 1988). Under other experimental conditions, the RyR has been observed to have additional conductance states (Liu *et al.*, 1989).

Intracellular Ca^{2+} is a regulator of both excitable and inexcitable cells (Putney, 1993). There is much interest in the role of the RyR in the modulation of intracellular Ca^{2+}. The presence of RyR has been detected in a variety of cell types (McPherson & Campbell, 1993). The number of RyR isoforms and the presence of RyR in multiple tissue types (Airey *et al.*, 1990, 1993; Ellisman *et al.*, 1990; Ledbetter *et al.*, 1994; Ogawa, 1994; Sorrentino & Volpe, 1993) suggest involvement of this intracellular Ca^{2+}-release channel in multiple physiological processes. For that reason, it is increasingly important to map the receptor−ligand interactions both to understand intrinsic differences between the isoforms and to develop specific pharmacological reagents to dissect the function of the multiple isoforms.

Identification of the main elements of receptor recognition of ligand is most convenient when the structures of both are known. Identification of these elements in the case of the RyR is hampered by lack of a crystal structure for the binding site. Detailed structural information about the binding site will probably not be available in the near future. We therefore have approached the problem of modeling the RyR−ryanoid interaction using the comparative molecular field analysis (CoMFA) technique (Cramer *et al.*, 1988). This technique is described in the context of the family of three-dimensional quantitative structure−activity relation (3D-QSAR) analyses in a recent brief review (Green & Marshall, 1995). Several successful applications of CoMFA have been reported since introduction of CoMFA in 1988. Recent applications include the mapping of the steric, lipophilic, and polar interactions in a series of monoamine oxidase inhibitors (Thull *et al.*, 1995) and the prediction of ecotoxicity of chemicals (Briens *et al.*, 1995). Brandt *et al.* (1995) used CoMFA to engineer the most active known inhibitor of the serine protease thermitase.

In an earlier paper (Welch *et al.*, 1994), we presented a CoMFA of the structural factors correlated with tight binding to the ryanodine recognition site of the ryanodine receptor (RyR) of chicken and rabbit skeletal muscle. We presented evidence that the pyrrole and isopropyl loci are important structural components of the specificity of ligand binding to the ryanodine binding site of the ryanodine receptor. If the pyrrole group is the dominant factor in the molecular recognition of the receptor for the ligand, then one would surmise that relocation of the pyrrole should alter binding

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[1] Abbreviations: CBZ, benzyloxycarbonyl; CoMFA, comparative molecular field analysis; RyR, ryanodine receptor; SR, sarcoplasmic reticulum.

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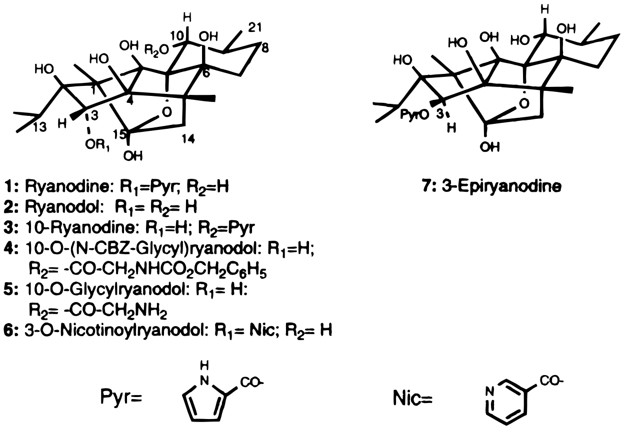
Scheme 1



1: Ryanodine: R_1=Pyr; R_2=H
2: Ryanodol: R_1= R_2= H
3: 10-Ryanodine: R_1=H; R_2=Pyr
4: 10-O-(N-CBZ-Glycyl)ryanodol: R_1=H;
R_2= $-CO-CH_2NHCO_2CH_2C_6H_5$
5: 10-O-Glycylryanodol: R_1= H:
R_2= $-CO-CH_2NH_2$
6: 3-O-Nicotinoylryanodol: R_1= Nic; R_2= H

7: 3-Epiryanodine

in a predictable manner. To analyze the importance of the pyrrole locus, we utilized two structural isomers of ryanodine. A ryanodol derivative (10-pyrrolecarbonylryanodine, called 10-ryanodine hereafter) produces the net effect of translocation of the pyrrolecarbonyl group from the 3-position to the 10-position. Compared with substituents at the 2- and 3-positions, substituents at the 9- and 10-positions were shown to be unimportant for the binding of ryanoids to the ryanodine receptor [compare Welch *et al*. (1994), Gerzon *et al*. (1993), Humerickhouse *et al*. (1994), and Bowling *et al*. (1994)]. As a control for a simple, fortuitous correlation of CoMFA prediction and experiment, the 3-epimer of ryanodine was synthesized. In our previous work, we had demonstrated that the strength of binding was sensitive to structural changes at the 3-position. Therefore, a major alteration at this position should be a sensitive test for the predictive ability of the CoMFA and the importance of the pyrrole locus. It is unlikely that two such radically different structural isomers would have the same unrecognized biases that might lead to erroneous conclusions. In this paper, evidence is presented that the pyrrole nucleus is the dominant or key factor in orienting ligands for binding into the ryanodine receptor.

EXPERIMENTAL PROCEDURES

Preparation of Ryanoids. Two isomers of ryanodine (**3** and **7**, Scheme 1) were synthesized from ryanodine (**1**) following a procedure previously described (Ruest & Deslongchamps, 1993). One of the isomers, 3-epiryanodine (**7**), is the epimer of ryanodine obtained formally by inversion of configuration at the 3-position, thus reorienting the pyrrole nucleus in relationship to the polycyclic ring system. The second compound, 10-*O*-pyrrolecarbonylryanodol (**3**) (hereafter called 10-ryanodine), is the positional isomer of ryanodine where the pyrrolecarbonyl group has been relocated from the 3-position to the 10-position. For the purpose of comparison, we also include two additional 10-O-derivatives of ryanodol in this analysis: 10-*O*-(*N*-CBZ-glycyl)ryanodol (**4**) and 10-*O*-glycylryanodol (**5**). We report now the syntheses of these last two compounds. Ryanodol (**2**), obtained as described in the literature (Wiesner *et al*., 1972), was acylated following the methodology developed by Neises and Steglich (1978), and the CBZ-protecting group of the resulting compound **4** was removed by catalytic hydrogenation.

The H NMR spectra were recorded on a Bruker AC-300 instrument (with CHD_2OD as the internal standard centered at 3.30 ppm). The following abbreviations have been used: s, singlet; d, doublet; m, multiplet; br, broad. Mass spectra and peak matching (exact mass) were recorded on a VG Micromass ZAB-2F spectrometer. Tetrahydrofuran was dried by distilling over potassium benzophenone ketyl. Reactions were performed under an argon atmosphere. Analytical chromatography and preparative thin-layer chromatography were done on glass plates precoated with silica gel 60F-250 (Merck). Column chromatography was performed with Merck silica gel (200−400 mesh).

*10-O-(N-Benzyloxycarbonyl)glycylryanodol [10-O-(N-CBZ-Glycyl)ryanodol] (**4**)*. To a solution of ryanodol (**2**) (52 mg, 0.13 mmol) in dry tetrahydrofuran (THF, 5 mL) were added successively (*N*-benzyloxycarbonyl)glycine (54 mg, 0.258 mmol), dicyclohexylcarbodiimide (77 mg, 0.373 mmol), and 4-(dimethylamino)pyridine (1 mg). The mixture was stirred under argon at room temperature for 3 h, and then it was poured onto dilute aqueous sodium bicarbonate solution and extracted with five portions of ethyl acetate. The combined extracts were dried over anhydrous sodium sulfate and filtered, and the solvent was evaporated. Column chromatography of the crude product furnished unreacted starting material (22 mg, 42%) and 10-*O*-(*N*-CBZ-glycyl)-ryanodol (**4**) (30 mg, 40%): mp 140−142 °C; H NMR (CD_3OD) δ 7.35 (m, 5 H, aromatic hydrogens), 5.29 (d, 10.6 Hz, 1 H, HC10), 5.11 (d, 1 H), and 5.06 (d, 1 H) (12.5 Hz, $ArCH_AH_B-O-$); 4.06 (s, 1 H, HC3), 3.92 (d, 1 H), and 3.85 (d, 1 H) (17.7 Hz, $N-CH_AH_B-CO_2-$); 2.52 (d, 1 H) and 1.74 (d, 1 H) (13.4 Hz, H_AH_BC14); 2.10 (m, 3 H, HC13, HC9, $H_{ax}C7$), 1.57 (m, 2 H, $H_{eq}C7$, $H_{ax}C8$), 1.11 (s, 3 H, CH_3C5), 1.07 (d, 6.8 Hz, 3 H, CH_3C13), 0.97 (d, 6.4 Hz, 3 H, CH_3C9), 0.85 (d, 6.6 Hz, 3 H, CH_3C13); MS (*m/e*) 591 (M^+, weak), 573 (M^+-H_2O); exact mass calcd for $C_{30}H_{39}NO_{10}$ (M^+-H_2O), 573.2574; found, 573.2586.

*10-O-(2-Aminoacetyl)ryanodol (**5**) (10-O-Glycylryanodol)*. A solution of 10-*O*-(*N*-CBZ-glycyl)ryanodol (**4**) (23 mg, 0.039 mmol) in dry THF (2 mL) was transferred to a prehydrogenated mixture of palladium catalyst (10% Pd/C, 5 mg) in dry THF (3 mL). The mixture was stirred under hydrogen for 2 h; then it was filtered, and the solvent was evaporated. The crude product was chromatographed on a silica plate (Merck) (solvent: $CHCl_3CH_3OH$, 3:1) to give 10-*O*-glycylryanodol (**5**) (18 mg, 99%): mp 218−220 °C, dec; H NMR (CD_3OD) δ 5.30 (d, 10.6 Hz, 1 H, HC10), 4.06 (s, 1 H, HC3), 3.40 (br s, 2 H, $N-CH_2CO_2-$), 2.52 (d, 1 H), and 1.74 (d, 1 H) (13.4 Hz, H_AH_BC14); 2.19−1.95 (m, 2 H, HC13, $H_{ax}C7$, HC9), 1.57 (m, 2 H, $H_{eq}C7$, $H_{ax}C8$), 1.31 (m, 1 H, $H_{eq}C8$), 1.30 (s, 3 H, CH_3C1), 1.11 (s, 3 H, CH_3C1), 1.06 (d, 6.7 Hz, 3 H, CH_3C13), 0.97 (d, 6.5 Hz, 3 H, CH_3C9), 0.84 (d, 6.6 Hz, CH_3C13); MS (*m/e*) 457 (M^+, weak); exact mass calcd for $C_{22}H_{34}NO_8$ (M^+-OH), 440.2284; found, 440.2280.

Preparation of Ryanodine Receptors. Ryanodine receptors were prepared as previously described (Airey *et al*., 1990; Welch *et al*., 1994). Briefly, chicken and rabbit skeletal muscles were dissected, frozen in liquid nitrogen, and stored at −80 °C. The frozen tissue was homogenized and the heavy sarcoplasmic reticulum fraction isolated by differential centrifugation. The vesicle preparation was frozen with liquid nitrogen and stored at −80 °C. Routinely, the specific binding was 3−5 pmol of ryanodine binding sites per milligram of protein as assayed by direct binding of radioactive ryanodine under the conditions described below.

This level of binding is similar to that obtained by others [for example, pig skeletal muscle (Fruen *et al*., 1994)].

[^{3}H]Ryanodine Binding Assay. The dissociation constants of the ryanoids were determined by a variation of the competitive binding assay used previously (Welch *et al*., 1994). Ligand binding was measured at 37 °C in a 200 μL final volume containing 0.5 M KCl, 20 mM Tris, pH 7.4, 200 μM $CaCl_2$, and 2.5 mM AMP. The concentration of [^{3}H]ryanodine (Sutko *et al*., 1986) was varied for direct binding assays. Binding to the ryanodine receptor was taken as the Ca^{2+}-dependent binding and quantitated as the difference in observed [^{3}H]ryanodine binding in the absence and presence of 5 mM EGTA (calculated $[Ca^{2+}] = 2$ nM). In other cases, the RyR binding was measured by using computer algorithms to fit the data to theoretical binding isotherms (see below). The apparent dissociation constants of the other ligands were measured in competitive binding assays where the concentration of tritiated ryanodine was held constant at 7 nM and the concentration of the ryanoid of interest was varied from 0.1 nM to 30 μM.

The binding reaction was initiated by the addition of 40 μg of microsomal protein and terminated 2 h later by collection of the microsomal membranes by filtration through Whatman GF/B glass fiber filters and washing 4 times with 4 mL aliquots of an ice-cold solution containing 0.5 M KCl, 20 mM Tris, pH 7.4, and 200 μM $CaCl_2$. The radioactivity retained on the filters was assayed by placing the filters in scintillation vials containing 9 mL of scintillation fluid (EcoLite, ICN, Costa Mesa, CA). Filters and scintillation fluid were vortexed for 20 s and incubated at room temperature for 24 h before final determination of the radioactivity in a scintillation counter. Results represent averages of at least three different determinations. The apparent dissociation constant of ryanodine was estimated from the Ca^{2+}-dependent binding measured as above. The net binding was analyzed according to the relation $Y = B[R]/(K_D + [R])$ where Y is the binding in picomoles per milligram of protein, B is the total (maximal) binding in picomoles per milligram of protein, [R] is the ryanodine concentration, and K_D is the apparent dissociation constant. Binding to the RyR was also analyzed by the relation $Y' = [B[R]/(K_D + [R]) + K'[R]$ where Y' is the total binding (binding in the absence of EGTA) in picomoles per milligram of protein and $K'[R]$ represents the contribution of the low-affinity binding sites to the total binding isotherm. Both equations were evaluated by direct fits using ORIGIN (MicroCal, Northampton, MA) and SCIENTIST (Micromath, Salt Lake City, UT). The two equations gave excellent agreement for the values of B and K_D. The displacement assays were analyzed by the relation $Y = Y_{max}/(1 + [C]/IC_{50})$ where Y is the Ca^{2+}-dependent binding, Y_{max} is Y when $[C] = 0$, and [C] is the concentration of the displacing ryanoid. Alternatively, the data were analyzed by the relation $Y' = [Y_{max}/(1 + [C]/IC_{50})] + K''$ where Y' is the total binding in picomoles per milligram of protein and K'' represents the contribution of the low-affinity ryanoid binding sites. Both equations yielded values of IC_{50} in good agreement. Apparent dissociation constants of the displacing ryanoids were calculated using the relation $K_C = K_D IC_{50}/(K_D + [R])$ where K_C is the apparent dissociation constant of the ryanoid under consideration and IC_{50} is the concentration of displacing ryanoid required to produce half-maximal binding (see previous two equations). Although chicken skeletal muscle contains approximately equal amounts of two RyR isoforms (Airey *et al*., 1990), no binding isotherm revealed any systematic deviation from the single hyperbolic relationships described above. Note the assay conditions used here promote ryanodine binding. Apparently both isoforms have quite similar properties, at least under these conditions.

CoMFA. The SYBYL 6.0 (Tripos Associates, St. Louis, MO) suite of programs was used to compute the global energy minima and perform the comparative molecular field analysis (CoMFA). An Evans and Sutherland ESV50 and PS390 and also a PhaseX X-terminal were used for the calculations and visualizations of the results. The basis data set previously described (Welch *et al*., 1994) was used for these studies. Employing the Tripos force field (Clark *et al*., 1989), the global minima of the molecules were determined by a combination of molecular mechanics [Powell (1977) minimizer], molecular dynamics (including simulated annealing), and also systematic and Monte Carlo searching of the torsional angles of the rotatable bonds of the molecules. As before, the method of Gasteiger and Marsili (Gasteiger & Marsili, 1980, 1981; Marsili & Gasteiger, 1980) was used to compute the charges on the molecules. Some small improvements were made, principally in the precision of the location of the conformations corresponding to the global energy minima and some consequent alterations in the field-fit alignments of the molecules. There were only slight improvements in the predictive ability of the model; therefore, no details are presented here.

Field-Fit Alignments. The CoMFA option within SYBYL allows rigid-body alignments between molecules matching the steric and electrostatic fields of a template molecule to a target molecule. The internal coordinates of neither molecule are altered in the process. In the present case, the template molecule was ryanodine; all of the other molecules (the target molecules) were fit to this single template. The underlying rationale is that ligands will orient to be as complementary to the binding site as possible. We assume that ryanodine is complementary to the binding site because of the high affinity of the binding site for this ligand, and therefore, that ryanodine is the best model structure. Two possible sources of error are evident in this approach: (1) The ryanodine molecule may not be entirely complementary to the binding site; therefore, fitting to ryanodine may miss other potential ligand−receptor interactions. (2) All points in the target and template fields receive equal weight. From our previous work, it is clear that some ligand−receptor interactions are more important than others. The proper weighting of the fields is a complicated issue. The focus of this paper is to demonstrate the critical role of the pyrrole locus in binding. This role is so evident that unweighted fits are more than sufficient. Furthermore, unweighted fits may be the best choice in this paper as it will avoid unconscious biases in the data.

RESULTS

The major changes in structure of the ryanodine isomers 3-epiryanodine and 10-ryanodine produce surprisingly small alterations in the dissociation constant of the ligand from the receptor (Table 1). The differences in the dissociation constants listed in Table 1 were converted to differences ($\Delta\Delta G$) in the free energy (ΔG) of binding. The $\Delta\Delta G$ (37 °C, chicken) for 10-ryanodine, 3-epiryanodine, and ryanodol

Table 1: Comparison of Measured and Predicted Dissociation Constants[a]

compound	source	dissociation constant (nM) experimental	dissociation constant (nM) predicted[b]
ryanodine	avian skeletal muscle	7	N/A
ryanodol	avian skeletal muscle	3600	N/A
10-ryanodine	avian skeletal muscle	83	210
3-epiryanodine	avian skeletal muscle	700	870
ryanodine	rabbit skeletal muscle	7	N/A
ryanodol	rabbit skeletal muscle	12000	N/A
10-ryanodine	rabbit skeletal muscle	52	203
3-epiryanodine	rabbit skeletal muscle	350	315
CBZ-glycylryanodine	rabbit skeletal muscle	38	75
CBZ-glycylryanodol	rabbit skeletal muscle	2000	1200
glycylryanodol	rabbit skeletal muscle	1300	1600

[a] The preparation of the ryanodine analogs and the method used to measure the ligand dissociation constants are described in the text. [b] The predicted values shown in this table are those of the optimum alignment of analog and ryanodine, based on the similarity of the predicted and measured values of the dissociation constants. The predictions are based on a refined version of the basis set described by Welch *et al.* (1994).

binding is 1.5, 2.8 , and 3.8 kcal/mol, respectively, compared with a total binding energy (ΔG) of −11.6 kcal/mol for ryanodine. Both the 3-epi and the 10-equatorial isomers are good ligands for both the rabbit and chicken RyR. Therefore, they must be quite complementary to the binding sites. Ryanodine, 3-epiryanodine, and 10-ryanodine have similar values for hydropathy as calculated by the computer program HINT [Kellogg *et al.*, 1991; see Welch *et al.* (1994) for a previous application of HINT to ryanoids]. The Hansch partition coefficients calculated by this program are −1.232, −1.232, and −1.150, respectively, using the bond method. Using the space method, the values are −6.018, −5.823, and −6.018, respectively. Therefore, little difference in the overall hydrophobicity of the ryanoids is expected.

CoMFA Predict Function. The CoMFA model derived earlier (Welch *et al*., 1994) was used to predict the dissociation constants of 10-ryanodine and 3-epiryanodine. Predictions require that the molecule under consideration be oriented to the members of the basis set. In all of the work discussed in this paper, ryanodine was taken as the template molecule (see Experimental Procedure).

Molecular Superimpositions. Using the prediction function of CoMFA (as implemented in SYBYL), one can gain insight into the orientations of the two compounds most consistent with the previously derived CoMFA model of ryanoid binding. The "suggested overlap" or "superimpose" function in SYBYL was used to suggest alignments of the 3-epi and the 10-equatorial isomers of ryanodine (the target molecules) with ryanodine (the template molecule). The algorithm was adjusted to look at carbon atoms only and to suggest orientations whereby the steric bulk of the template molecule (ryanodine) would be satisfied by the target molecule. In our implementation, no matching of particular atoms was required; therefore, the widest number of possible orientations would be accepted. These general orientations were then field-fit to ryanodine using a rigid-body algorithm. The field-fit algorithm tends to fall into local minima readily; thus, in most instances the orientation of the field-fit molecule is not much different from the starting orientation.

FIGURE 1: Three isomers of ryanodine are shown as aligned by positions 1−3, giving a common alignment of the polycyclic ring system. Middle: Ryanodine is shown with the pyrrole group in the upper left and the isopropyl group in the lower left of the molecule. Left: 3-Epiryanodine. Right: 10-Pyrrolecarbonylryanodine (10-ryanodine). Note that the pyrrole group is now in the lower right of the molecule.

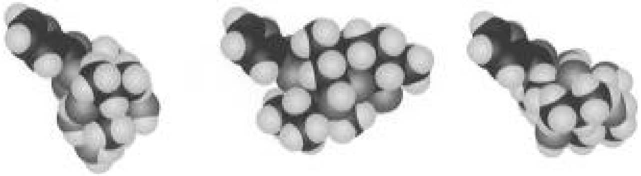


FIGURE 2: Three isomers of ryanodine are shown as aligned by the pyrrole locus (the alignment assumes the pyrrole group binds in the same subsite in all cases). The molecules have not been field-fit. Middle: Ryanodine shown in the same orientation as in Figure 1. Left: 3-Epiryanodine. Right: 10-Pyrrolecarbonylryanodine (10-ryanodine).

The alignments of the isomers can be divided into two general groups, those aligned by the atoms of the polycyclic ring system and those aligned by the pyrrole carbonyl groups. Orientation of the ryanoids (ryanodine, the 3-epi- and 10-isomers) by the common atoms in the polycyclic ring system (Figure 1) makes it difficult to understand how all three molecules could fit into the same binding site. Indeed, the CoMFA predicts dissociation constants far different from those actually observed (Table 1). Using chicken muscle as an example, such an alignment (least-squares fit of atoms 1−3) produces predicted dissociation constants of 2.3 μM for the 10-isomer and 16 μM for the 3-epi-isomer (experimental values 83 and 700 nM, respectively). Similar results were obtained for rabbit RyR. Field-fitting improved the predicted value only slightly. In comparison, the molecules aligned by the pyrrole yield much more satisfying predictions of 210 nM for the 10-isomer and 870 nM for the 3-epi-isomer (Table 1, chicken RyR). Note in this case the general similarity of the molecular shapes is evident (Figure 2). Several other orientations suggested by the superimpose algorithm were tested. The predicted values of the dissociation constants ranged from 300 to 2000 nM for the 10-ryanodine isomer. Only orientations based on alignment of the pyrrole locus produced the close agreement between predicted and experimentally measured dissociation constants. A similar result was obtained for the 3-epi series: all alignments obtained from the superimpose algorithm led to predicted dissociation constants larger than the experimental value.

10-Ryanodine. It had been our experience that in all cases the predicted value of the dissociation constant is significantly improved by field-fitting. However, that was not the case for 10-ryanodine. When the ryanoid was aligned by the pyrrole ring without any subsequent field-fitting, the predicted dissociation constant was 210 nM. Field-fitting increased the predicted value to 1.5 μM. The discrepancy most probably arises because the field-fit is performed without weighting, but our previous work has shown that the orientation of the pyrrole is a particularly critical element in the tight binding of the ryanoids. The present results simply affirm the major role of complementary interactions at the pyrrole locus.

In our previous paper, we reported that there were two, almost isoenergetic, minima in the torsional angle connecting the pyrrole ring to the carbonyl carbon. Both rotamers were predicted to have essentially the same dissociation constant. This suggested that the 10-isomer could be aligned by the pyrrole ring in either of two orientations, the first with all of the atoms (including the nitrogen) exactly aligned. The data for this orientation are given above. The second orientation has the fused ring system rotated 180° from the first (with all of the other internal coordinates of the molecules unchanged). The second rotamer was aligned as described above but produced a poorer predicted value of the dissociation constant both before or after field-fitting (710 and 430 nM, respectively). This suggests that although the pyrrole is an important determinant of binding, the remaining portions of the molecule make complementary interactions and the ligands bind in only one orientation.

3-Epiryanodine. As happened for the 10-ryanodine above, field-fitting after alignment of the pyrroles decreased the agreement between predicted and experimental (700 nM) values of the dissociation constant for the chicken RyR. For the 3-epi isomer, the predicted K_D fell from 870 to 325 nM. Rabbit RyR behaved differently. Field-fitting of 3-epiryanodine after alignment of the pyrroles improved the agreement between experiment (350 nM) and prediction. The initial predicted value of 960 nM fell to 230 nM.

The superimpose utility in SYBYL was used to find alternate alignments between ryanodine and the 3-epiryanodine. Ten alignments were found; each was field-fit to ryanodine. As noted above, the rigid-body field-fit quickly falls into local minima; in general, the final alignments were little changed from the initial alignment. The predicted values of the K_D of 3-epiryanodine ranged from 1.3 to 2.4 μM (chicken and rabbit RyR).

For the reasons outlined earlier, the pyrroles of 3-epiryanodine and ryanodine were aligned in the reverse orientation (the pyrrole group of 3-epiryanodine was rotated 180° on the axis formed by the bond between the pyrrole and the carbonyl carbon). For the chicken RyR, the predicted K_D values were 210 nM before and 550 nM after field-fitting. In this case, field-fitting improved the agreement between experiment and prediction. The results suggest that there may be two binding orientations for 3-epiryanodine. A similar situation was observed for rabbit RyR where the final predicted value was 315 nM (rabbit, Table 1). There is no experimental evidence for two different binding modes. When fitting synthetic data based on the two predicted dissociation constants with a single hyperbolic function (see Experimental Procedures), we find a maximum error of 0.3% (equal amounts of the two modes, data not shown). The dissociation constants would need to be more widely separated to be detectable by our present methods. Molecular dynamics simulations at 37 °C indicate that the two conformers modeled do not interconvert under physiological conditions (data not shown). This is similar to ryanodine (Welch *et al*., 1994). Only one conformer has been found for ryanodine [see Welch *et al*. (1994) for more discussion of experimental and predicted conformations]. Based on similar calculated rotational barriers for 3-epiryanodine (data not shown), one would expect the same situation for this epimer. Crystal structures are consistent with the modeled conformers used to predict the values in Table 1 (Marc Drouin, personal communication). The closeness of the two predicted dissociation constants of the two conformers indicates that the unlikely selection of the wrong conformer as the global minimum of 3-epiryanodine will not affect the major conclusion of this report.

The data in the section above make it clear that the 10-isomer binds with the pyrrole in the same general orientation as does ryanodine. However, it can be argued that the configuration at the 3-position of 3-epiryanodine prevents the pyrrole from fitting into the receptor in the same orientation as does ryanodine. Conceivably, the 3-epiryanodine could bind to the receptor in an orientation with the pyrrole in a position equivalent to the 10-position of ryanodine. This is an orientation analogous to the 10-equatorial ryanodine derivatives described by Gerzon *et al*. (1993). Such an orientation would leave a vacancy at the pyrrole site on the receptor but would avoid the possibility of steric clashes because the 10-position has been shown to accommodate bulky groups with little perturbation of binding (Gerzon *et al*., 1993; Welch *et al*., unpublished results). (For example, Gerzon *et al*. obtained IC_{50}'s of 6, 6, and 2 nM for ryanodine, 10-*O*-CBZ-β-alanylryanodine, and 10-O-guanidinoacetylryanodine respectively.) To test this possibility, the 3-epi isomer was aligned with ryanodine with the 3-epi position superimposed over the 10-position of ryanodine in two orientations 180° apart. The predicted values calculated for chicken and rabbit RyR after field-fitting ranged from 2.3 to 8.3 μM, far from the experimentally determined values. The result indicates that 3-epiryanodine binds in the same general orientation as ryanodine and that the pyrrole is a major determinant of ligand binding to the receptor.

Inspection of the electrostatic fields surrounding the three ryanodine isomers showed both similarities and decided differences (data not shown). Neither orientation of the 3-epi nor the 10-isomers had an obviously better fit of the electrostatic field to that of ryanodine. Therefore, electrostatics alone are not sufficient to explain the results.

Comparison of Rabbit and Chicken RyR. Realignment results obtained with a single skeletal muscle RyR may have been a fortuitous result. Therefore, binding measurements were conducted on two skeletal muscle RyR and on additional ryanoids. Although the dissociation constants are different, the observations made above about the orientation of the ryanodine isomers apply to both rabbit and chicken RyR. Further, although the chicken preparation contains two RyR isoforms (Airey *et al*., 1990), all experimental data fit a single hyperbolic binding isotherm (see Experimental Procedures). Therefore, the pyrrole group is a major determinant of ryanoid binding to at least two isoforms.

Flexible Field-Fitting. Because rigid-body field-fitting decreased the agreement between prediction and experiment, flexible field-fitting was applied. In the flexible field-fit algorithm, the target molecule is allowed more degrees of freedom in matching the force-field of the template molecule.

In addition to allowing the target molecule to move in space, the internal coordinates of the target molecule are allowed to move in order to obtain a better fit to the steric and electrostatic force-fields of the template molecule. The flexible field-fit of 10-ryanodine to ryanodine was accomplished by a combination of molecular mechanics and molecular dynamics. Several alignments were made after field-fitting. No improvement in agreement between prediction and experiment was obtained. As noted in the preceding results, the alignment giving the best agreement was that obtained by the alignment of the pyrrole groups of 10-ryanodine and ryanodine.

N-CBZ-Glycylryanodine. The derivative 10-*O*-*N*-CBZ-glycylryanodine was synthesized by Gerzon *et al*. (1993). These authors found introduction of this bulky group had only a minimal perturbation of the dissociation constant (rabbit skeletal muscle, ryanodine = 6.2 nM, *N*-CBZ = 4.8 nM). At Reno, we find that the CBZ group reduces binding affinity (7 nM for ryanodine *vs* 38 nM for the CBZ derivative). In any event, addition of the *N*-CBZ-glycyl group does not enhance binding to rabbit skeletal muscle. For the present study, we synthesized 10-*O*-*N*-CBZ-glycylryanodol. The *N*-CBZ-glycyl group enhanced binding (12 μM [ryanodol] *vs* 2 μM [*N*-CBZ derivative]; see Table 1).

The long flexible chain makes modeling this compound difficult because of the large amount of conformational entropy of this group. Using a conformation that we believe corresponds to the global energy minimum, we obtain a predicted dissociation constant of 8.3 μM if the *N*-CBZ-glycylryanodol is aligned to ryanodine by the ring positions 1−3 (that is, the *N*-CBZ derivative binds to the protein in the ryanodine orientation). If the benzene ring is aligned to the pyrrole of the template ryanodine (that is, the *N*-CBZ derivative binds to the RyR with the benzene ring of the derivative in the pyrrole binding site), the predicted dissociation constant is 1.2 μM, in good agreement with the experimentally measured value. This suggests that the *N*-CBZ group is binding in the pyrrole subsite of the ryanodine binding site. Previously we found that ryanodyl nicotinate had a dissociation constant of 1.2 μM (Welch *et al*., 1994). The slight increase in steric bulk caused by the addition of a single ring carbon at the pyrrole locus had a dramatic effect on binding. Apparently, ligand−receptor interactions are quite strict in this region. Based on analogy with the nicotinyl group, one might expect the *N*-CBZ-glycyl group to be too large to replace the pyrrole carbonyl group in promoting tight binding.

10-O-Glycylryanodol. The incorporation of a glycyl adduct at the 10-position of ryanodol increases the binding to rabbit skeletal muscle (the dissociation constant of the glycyl derivative is approximately one-ninth that of ryanodol, Table 1). The glycyl side chain is quite flexible, making it difficult to choose a proper alignment of molecules. Because of the positive charge on the side chain, movement of the glycyl chain has decided effects on the electric field. The first alignments of 10-glycylryanodol were performed with the glycyl chain in the conformation we believe corresponds to the global minimum. Alignment of 10-glycylryanodol to ryanodine by positions 1−3 (that is, assuming that 10-glycylryanodol binds in the same orientation as ryanodine) yields a predicted dissociation constant of 27.4 μM, considerably higher than the experimentally determined value of 1.3 μM. Field-fitting only slightly changes the predicted value (28 μM). The other tested orientation aligned the glycyl atoms with the corresponding atoms of the pyrrole carbonyl atoms of ryanodine. That is, the nitrogen of the glycyl group was aligned with the nitrogen of the pyrrole, the ester oxygens of the glycyl adduct were aligned with the ester oxygens of the pyrrole adduct, position 10 of glycylryanodol was aligned with position 3 of ryanodine, and the carbons of the glycyl side chain were aligned with those of the pyrrole carbonyl group of ryanodine. This alignment assumes that 10-glycylryanodol binds with the glycyl group partially occupying the pyrrole binding site. The predicted dissociation constant for this orientation was 1.9 μM, somewhat higher than the measured value (1.3 μM). Field-fitting altered the predicted value to 1.4 μM. The result suggests that both these ryanodol derivatives bind with the 10-position occupying the location normally occupied by the 3-position of ryanodine.

The proper control for 10-glycylryanodol is 10-glycylryanodine. Gerzon *et al*. (1993) have measured this binding constant. Based on the reported difference between the dissociation constants of ryanodine and 10-glycylryanodol, the addition of the glycyl group adds about 760 cal/mol to the free energy of binding. This value is consistent with the values reported for aqueous-faced salt-links (ionic bonds) in proteins [see Dao-pin *et al*. (1991) and references cited therein]. The energy is considerably less than reported for known salt links (ionic bonds). Experimentally measured energies for ionic links with ammonium groups include (A) 2.9 kcal/mol in chymotrypsin (Fersht, 1972) and (B) 4.3 kcal/mol in tyrosyl-tRNA synthetase (Santi & Pena, 1973). An ammonium ion was found to contribute 2.2 kcal/mol to the substrate−thrombin interaction energy (Stone *et al*., 1991). We find (see Table 1) that addition of the glycyl group to the 10-position of ryanodol adds about 1.5 kcal/mol to the free energy of binding. This result indicates that the ammonium group of the glycine of the ryanodol derivative is in a more restricted, less solvated environment than the case of the ryanodine derivative. This result is consistent with the glycyl group serving as a poor surrogate pyrrole group for the ryanodol derivative.

Dipole Moment. The dipole moments were measured for the three ryanodine isomers (data not shown). There was no correlation between dipole orientation and either predicted or experimentally measured dissociation constants.

DISCUSSION

The importance of the pyrrole locus to ligand binding to the ryanodine receptor was illustrated in our previous paper (Welch *et al*., 1994). The pyrrole (3-position) is absolutely required for tight binding. Any alterations in the conformation of the pyrrole caused major losses in binding strength. The data in this paper support and extend this observation. A small enlargement in steric bulk at this position, for example, 3-nicotinylryanodol where the pyrrole is replaced by a pyridine ring, also caused a drastic loss in the ligand−receptor interaction energy (Welch *et al*., 1994). We also demonstrated that large modifications at the 9- and 10-positions had only minor effects on binding (*p*-nitrophenylryanodine, β-alanylryanodine, *etc*.). An initial CoMFA of the binding of 19 ryanoids to chicken and rabbit RyR inferred that the isopropyl group promoted strong binding (Welch *et al*., 1994). These data taken together suggest a binding mode

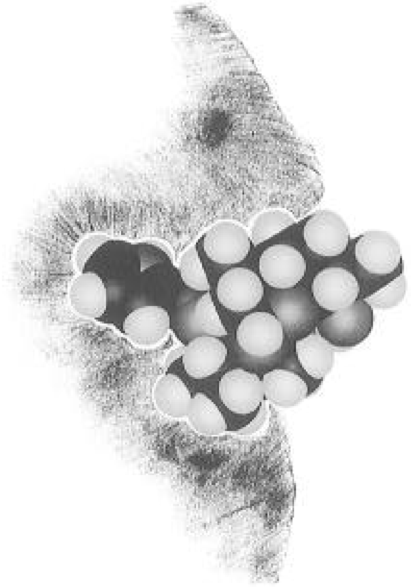


FIGURE 3: Cartoon illustrating the current hypothesis of the binding orientation between the RyR and ryanodine.

as illustrated in Figure 3 where the pyrrole and isopropyl groups are buried within a crevice on the receptor. Important interactions with polar groups are also contained within the crevice. The 9- and 10-positions are at the mouth of the binding site and extend into the solvent. However, in the previous work, the structural variety was limited. The tested molecules were quite homologous with similar polycyclic backbones. A pyrrole group was located at the 3-position in all but two cases. In one case, nicotinic acid replaced the pyrrole carbonyl (again at the 3-position). In the other case, the pyrrole carbonyl was simply absent (ryanodol). In the present series of ryanoids, the pyrrole carbonyl group has been moved. These isomers greatly enhance our understanding of the principle loci in the molecular recognition of ligands by the RyR. We note that in all cases, the best agreement between prediction and experiment occurs only when the template ryanodine and the structural isomer are aligned by the pyrrole groups. An interesting sidelight is that rigid-body field-fitting is required for best agreement between prediction for the 3-epi isomer in contrast to the 10-isomer where rigid-body field-fitting lessens the agreement. At a superficial level, the results seem to indicate the relative importance of the other portions of the molecule to binding. In the 3-epi isomer, the atoms surrounding the pyrrole locus have significant effects on the complimentarity between receptor and ligand whereas for the 10-isomer the surrounding atoms have lesser importance.

In many ligands, compared with the active configuration, the corresponding epimer has little affinity for the receptor. However, in the case of ryanodine, inversion of configuration at the 3-position has much less effect on binding than either removal of the pyrrole group or enlargement of the ring (Welch *et al.*, 1994). The best explanation for the result is that the pyrrole causes the ligand to bind in an alternate orientation. This orientation has many of the characteristic features of ryanodine, and, therefore, the affinity between ligand and receptor remains high.

In the case of the positional isomer, the importance of the pyrrole as a rudder for the orientation of the ligand is also clear. If the 10-pyrrolecarbonylryanodol were to bind in the same orientation as ryanodine, one would expect from previous results a dissociation constant on the order of 2.3 μM. The large dissociation constant is expected because, as with ryanodol, there is no pyrrole at the 3-position. Therefore, only weak binding is expected. Addition of a pyrrole to the 10-position is expected to have little-to-no effect based on the experimental measurements with the guanidino, β-alanyl, and *N*-CBZ-glycyl derivatives where these groups have been added to the 10-position of ryanodine (Gerzon *et al.*, 1993). In each case, these groups caused only a slight perturbation of the dissociation constant. We submit that in these ryanodine isomers the presence of the pyrrole at the 3-position orients the substituents outward into the solvent. In the case where the pyrrole has been removed from the 3-position, a major factor in ligand orientation (molecular recognition) has been lost, thus accounting for the poor binding of ryanodol. If the pyrrole is relocated to the 10-position, the orientation factor guides the ryanoid into a productive orientation in the binding site. The interactions between ligand and receptor are not as complementary as in the case of ryanodine, and therefore a small increase in the ligand dissociation constant is observed.

Note that there is specificity of the effect of 10-*O*-substituents on the enhancement of ryanodol binding. While both the bulky *N*-CBZ derivative and the small, charged glycyl derivative enhanced binding, neither was nearly as effective as the pyrrole carbonyl group. Several other 10-esters of ryanodol (Mitchell *et al.*, unpublished data) were found to bind with much lower affinity than ryanodol. Therefore, it is not the case that any substituent at the 10-position will restore strong binding.

Previously we had divided the interactions responsible for ryanoid binding to the RyR into two broad categories: (1) direct interactions with the functional groups altered and (2) indirect effects such as conformational changes in the ryanoid induced by the covalent modification (Welch *et al.*, 1994). We found that the indirect effect can be greater than the direct interactions. The results reported herein are consistent with this finding. The pyrrole is clearly an important orientating group, but the fused ring system also has important interactions with the site. These interactions are responsible for the difference in 3-epiryanodine and 10-ryanodine binding. Although we previously mapped the areas of the ryanodine molecule strongly correlated with binding strength, we have not yet reported a quantitative examination of the location of indirect conformational effects (as opposed to direct effects) to see if some areas were more critical than others. Using the present results, a preliminary analysis is presented in the following paragraph.

Ryanodine, 3-epiryanodine, and 10-ryanodine aligned in the orientation which gave the best agreement between predicted and experimental values were used to compare common molecular volumes and common areas of polar and hydrophobic interactions. In making the comparisons, the locations will be made in reference to ryanodine. The union of the molecular volumes of the three isomers is shown in Figure 4. The molecular volume common to all three isomers includes the pyrrolecarbonyl group and most of the fused ring system. Positions 9 and 10 are not included in the common volume. The picture is in general agreement for the minimum volume required for tight binding of ryanoids reported earlier (Welch *et al.*, 1994). A major

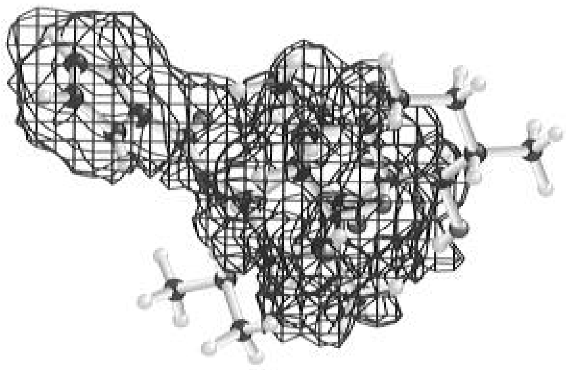


FIGURE 4: Molecular volumes common to the three ryanodine isomers after alignment of the pyrrole groups. Ryanodine is shown for orientation.

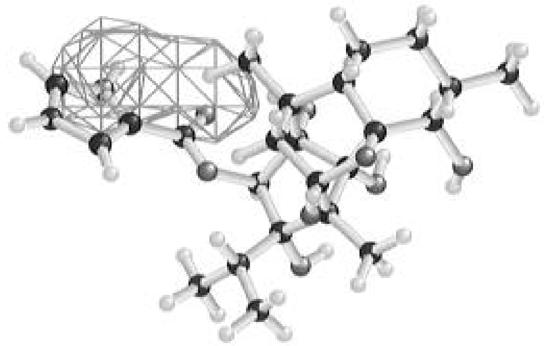


FIGURE 5: Polar area (contoured at −8) common to the three ryanodine isomers after alignment of the pyrrole groups. Ryanodine is shown for orientation.

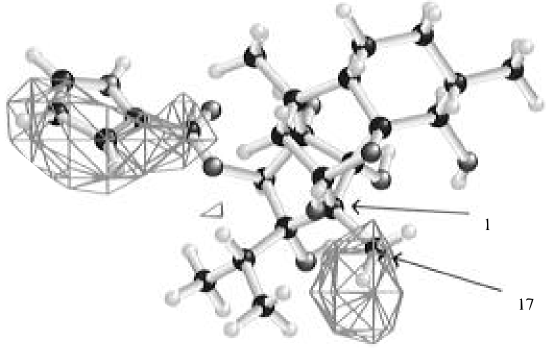


FIGURE 6: Hydrophobic area (contoured at 4) common to the three ryanodine isomers after alignment of the pyrrole groups. Ryanodine is shown for orientation.

difference is noted, however. The isopropyl locus is not included in the common volume whereas the isopropyl locus was clearly identified previously as extremely important for binding. This may be a reason why neither ryanodine isomer binds as well as ryanodine.

Using the same alignment as used in the paragraph above, the hydrophobic and polar fields were calculated and contoured using HINT (Kellogg *et al*., 1991). The union of these two fields was contoured at values of 4 (hydrophobic) and −8 (electrostatic). At this level of contour, one common polar area was noted along the nitrogen edge of the pyrrole to the bridging carbonyl (see Figure 5). The common hydrophobic areas are the hydrocarbon edge of the pyrrole and regions around positions 1 and 17 (Figure 6). These areas correlate well with the region where increased steric bulk enhances binding (Welch *et al*., 1994). The preceding initial analysis extends our earlier CoMFA (Welch *et al*., 1994). From Figures 5 and 6, we surmise that the essential interactions between ligand and receptor are regions around the pyrrole and positions 1 and 17. An isopropyl group *per se* may not be essential for strong ligand−receptor binding: hydrophobic bulk near the isopropyl locus may be sufficient. This information can be used to engineer ligands for the RyR with minimum structure for binding.

Understanding of the interactions between receptor and ryanoid is important to engineer pharmacologically useful reagents with high specificity. A more immediate application is to develop alignment rules so that ligands with widely different structure can be aligned to study functional correlations. An understanding of the proper orientation was essential to interpret the results of studies correlating ryanodine structure to RyR calcium ion channel function (Welch *et al*., unpublished data).

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