Structure-Function Relationships among Ryanodine Derivatives

PYRIDYL RYANODINE DEFINITELY SEPARATES ACTIVATION POTENCY FROM HIGH AFFINITY*

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Ryanodine derivatives are differentially effective on the two limbs of the ryanodine concentration-effect curve. This study comparing ryanodine, ryanodol, and pyridyl ryanodine and nine C₁₀-Oₐ esters of them focuses on structure-function relations underlying their differential effectiveness. Ryanodol and pyridyl ryanodine had significantly lower affinities than ryanodine, but their EC₅₀ values (concentration of ryanoid that induces one-half of full efficacy), potencies, and efficacies were not diminished in like fashion. Ryanodine and ryanodol were partial agonists, whereas pyridyl ryanodine was a full agonist, having a diminished deactivation potency. C₁₀-Oₐ esterification enhanced affinities and efficacies of the base ryanoids. The C₁₀-Oₐ esters of ryanodine and pyridyl ryanodine, but not those of ryanodol, lost their capacity to deactivate RyR1s. Thus, affinity differences among ryanoids clearly do not predicate functional differences as regards activation of Ca²⁺ release channels. The pyrrole-carboxylate on the C₁₀ of ryanodine is dispensable to ryanoid activation of Ca²⁺ release channels. Ryanodine lacks this ring, but it nevertheless effects substantial activation. Moreover, its C₁₀-Oₐ esters display full efficacy. The increased ability of all the C₁₀-Oₐ derivatives to release Ca²⁺ from the vesicles strengthens their role in directly impeding deactivation of RyR1, perhaps by interaction with some component within the transmembrane ionic flux pathway.

Significant progress has been made recently in delineating the role of the sarcoplasmic reticulum (SR) in the excitation-contraction (E-C) coupling processes of muscles (1–6). In major part, pharmacologic dissection of the pivotal role of the Ca²⁺ pump of SR in E-C coupling has been achieved with aid of the microbial metabolites cyclopiazonic acid (7) and thapsigargin (8), which are potent inhibitors of Ca²⁺, Mg²⁺ ATPases (sarcoplasmic reticulum Ca²⁺-ATPase) (9). Furthermore, knock-out genetic mutants (10) have solidified the importance of the regulator protein phospholamban (2) on physiologic function of the sarcoplasmic reticulum. Especially contributory to our current understanding of SR function has been studies with the complex plant alkaloid ryanodine (C₁₇-O-(pyrrole-2-carbonyl) ryanodol), a potent and specific modulator of a class of intracellular Ca²⁺ release channels (11–15).

For over a decade it has been appreciated that Ca²⁺ release channels (both on sarcoplasmic reticulum and endoplasmic reticulum) exhibit a complex response to ryanodine. At the single channel level, the effects of ryanodine are now appreciated to be quantal, occurring in a minimum of two discrete steps. Submicromolar concentrations induce an abrupt change from a flickery, predominantly closed (zero conductance) state to a sustained, higher (fractional, i.e. less than full) conductance state. “Locked” in this fractional conductance state and hovering about half-way between closed and fully open, the Ca²⁺ release channel modified by ryanodine is less restive, displaying fewer opening events than in the absence of ryanodine (16, 17). Rarely at submicromolar but more readily at micromolar concentrations, ryanodine may induce a second step change in the ion conductance of the Ca²⁺ release channel to a persistently closed state displaying opening events only rarely. For convenience of discussion, we adopt the convention describing the locked, fractional conductance state as “modified” (18) and the persistently closed, zero conductance state produced at high ryanodine concentrations as “shut.”

Assays at the multi-channel level with muscle microsomes reveal graded responses to ryanodine, reflecting the ensemble of the many individual quantal events. Passive Ca²⁺ efflux assays evaluating ensemble effects typically employ sarcoplasmic reticulum membrane vesicles prepared from fast skeletal muscles (19–21). Low concentrations of ryanodine (≤60 μM) activate or open the RyR1s of such preparations, whereas higher concentrations deactivate, that is they close them. The resultant, biphasic response curves reflect the aggregate of the step quantal changes occurring in the multitude of individual Ca²⁺ release channels of such microsomes. We have recently shown that the behavior of single channels incorporated into lipid bilayers (18) is consistent with their aggregate behavior judged from flux studies with sarcoplasmic reticulum vesicles. Calcium sparks seem to demonstrate a similar phenomenon (22).

Details of the molecular processes that trigger RyRs to respond to ryanodine in a biphasic manner remain obscure. A robust strategy to approach understanding of the modulation of RyR1 could be assembled from structure-activity relationships that would identify chemical functionalities on the molecule that prove to be integral for high affinity interactions and comparison of these with topological features found essential for ryanoid channel modulating characteristics. The present study is a step in the latter direction.

It is generally recognized that the C₃ substituent on the A-ring of ryanodine is important to its affinity for ryanodine-
binding sites on RyR1 (23–25). However, few studies have been conducted to evaluate whether the chemical nature of C3 substituents are especially important for the efficacy of ryanoids at opening, on the one hand, or closing, on the other, the Ca2+ release channel to ionic flux. This paucity results from chemical synthetic limitations. Although de-esterification of the C3 ester is facile, re-esterification with original stereochemistry is not. In fact, it is presently unattainable. Thus, comparisons of efficacy of C3-modified ryanoids have been limited for the most part to comparisons between ryanodine and ryanodol, the latter having no C3 ester. The present study adds pyridyl ryanodine to the mix. Also termed ryanodyl-3-nicotinate in the literature, the affinity but not the functional consequences of pyridyl ryanodine have been investigated. Jefferies et al. (23) first identified this ryanodine as a naturally occurring minor secondary metabolite from Ryania wood. They found pyridyl ryanodine to be “essentially inactive compared with ryanodine for insecticidal activity, for toxicity to mice, and for competition with [3H]ryanodine at the Ca2+-ryanodine receptor complex of skeletal muscle.” For use in the present study, we purified to >98% a sufficient quantity of pyridyl ryanodine to permit a more extensive functional evaluation of this natural ryanoid.

Among the numerous derivatives of ryanodine thus far reported, only a few C10-Oeq esters increased RyR1 affinity (26–28). Such effective ester substituents were incorporated into pyridyl ryanodine and compared in the present study with ryanodine and ryanodol and certain of their esters. The results show clearly that affinity consequences of C3 ester substituents do not predict their channel activating character. Furthermore, anchoring substituents at the C10-Oeq position effect no greater changes in activation potency of weak ryanoids than they do of highly effective ryanodines. Apparently the topologic features determinant for RyR1 activation and deactivation are not congruent with those responsible for apparent affinity for binding to RyR1.

**EXPERIMENTAL PROCEDURES**

**Materials**

Ryanodine (C3-O-(pyrrole-2-carbonyl) ryanodol) and pyridyl ryanodine (C3-O-(pyridyl-3-carbonyl) ryanodol) used in this study were obtained from Integrated Biotechnology Corp. (Carmel, IN) or were isolated and purified to >98% single compounds from chipped Ryania speciosa wood supplied by them, using procedures previously described (21, 29). [3H]Ryanodine (specific activity 87 Ci/mmol) and 45CaCl2 (specific activity 2.7 Ci/mmol) were purchased from NEN Life Science Products. Brevital® sodium was obtained from Lilly. All other reagents and solvents used were of the highest purity available commercially.

**Skeletal Muscle Junctional Sarcoplasmic Reticular Membrane Vesicles (JSRV)**

Crude (mixtures of junctional and longitudinal) sarcoplasmic reticular membrane vesicles (CVs) were prepared from fast twitch skeletal muscles of rabbits. The CVs were then subfractionated using a discontinuous sucrose gradient as described previously (21, 26–28, 30). The interface between 1.2 and 1.5 M sucrose which typically contains the highest percentage of ryanodine receptors was collected by aspiration and resuspended in freshly prepared isolation buffer at concentrations of 5–10 mg/ml, quickly frozen, and stored at –70 °C.

**Chemical Methods**

**Preparation of Ryanodol**

The C3 hydroxyl substituent was removed from ryanodine using the alkaline de-esterification procedure described by Kelley et al. (31) and ryanodol the product of interest, was further purified by column chromatography giving a yield of 85%. Detection of ryanodol on thin layer chromatographic (silica gel) plates was achieved by spraying with 10% ceric ammonium nitrate in 85% phosphoric acid and heating for 5 min. Ryanodol was identified as a reddish-brown spot. The product was characterized by mass spectroscopy and 1H NMR.
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12-15% MeOH in CH₂Cl₂ and on rotary evaporation and freeze-drying from dioxane afforded yields of at least 85%. The structures of the C₁₀-Oeq β-alanyl ryanoids were characterized by ¹H NMR and mass spectroscopy.

Preparation of C₁₀-Oeq Guanidino-propionyl Ester Derivatives of the Base Ryanoids—C₁₀-Oeq guanidino acyl ester derivatives of these ryanoids were prepared from their C₁₀-Oeq aminoacyl esters as described previously (26–28) or by the procedure of Bernatowicz et al. (32). The structures of the derived compounds were confirmed by ¹H NMR and mass spectroscopy.

Biochemical and Pharmacological Properties of the Base Ryanoids and Selected C₁₀-Oeq Ester Derivatives

Relative Binding Affinities of the Base Ryanoids for RyR1s

Relative binding affinities of ryanodol, ryanodine, and pyridyl ryanodine, as well as their C₁₀-Oeq Cbz-β-alanyl, β-alanyl, and guanidino-propionyl ester derivatives were assessed using equilibrium displacement binding affinity assays based on rabbit skeletal muscle JSRV as described (18, 21, 24–30, 33). Briefly, JSRV (0.1 mg/ml) were incubated...
in binding buffer (500 mM KCl, 20 mM Tris-HCl, 0.2 mM CaCl₂, pH 7.4 at 37 °C) containing 6.7 nM [³H]ryanodine and varying concentrations of the ryanoids (up to 50 μM) for 2 h at 37 °C. At the end of the incubation, the vesicles were filtered through Whatman GF/C filters (0.45 μm) using a cell harvester (Brandel model M-24R), washed 3 times with 3 ml of ice-cold binding buffer (pH 7.4 at 0 °C), quantitated by liquid scintillation counting. Nonspecific binding was determined simultaneously by incubating JSRV with a concentration of the respective unlabeled ryanoid 10 x higher than the highest concentration used in the binding assay. Displacement curves and IC₅₀ and Kᵢ values were calculated.

FIG. 2. Relative binding affinities of the base ryanoids (a) and their derivatives (b–d). The relative binding affinities of ryanodol, ryanodine, pyridyl ryanodine, and their C₁₀-Oeq Cbz-β-alanyl, β-alanyl, and guanidino-propionyl ester derivatives for rabbit skeletal muscle RyR1 were assessed using displacement binding affinity assays. Briefly, skeletal JSRV (0.1 mg of protein/ml) were incubated for 2 h at 37 °C in the presence of 6.7 nM [³H]ryanodine and varying concentrations of the designated unlabeled ryanoids, in a buffer consisting of 500 mM KCl, 20 mM Tris-HCl, and 0.2 mM CaCl₂ (pH 7.4 at 37 °C) as described under “Experimental Procedures.” At the end of the incubation, the vesicles were filtered and washed, and the [³H]ryanodine bound to the receptors was determined by liquid scintillation counting. The data for each compound represent the means from several separate experiments (n = 3) using at least two different JSRV preparations. Standard deviations were <10% of the mean values. To facilitate visual comparisons, the displacement binding affinity isotherm for the parent molecule and for ryanodine, taken from a, are reproduced in each of the other panels. Ryanodine data are shown in dashed lines. Note the expanded abscissa of c.
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### Table I

| Ryanoid                  | IC\(_{50}\) (nM) | Fold shift (lost in affinity) | Fold shift (gain in affinity) | EC\(_{50}\)act (nM) | Fold shift (lost in potency for activation) | EC\(_{50}\)deact (nM) | Fold shift (lost in potency for deactivation) |
|-------------------------|------------------|------------------------------|------------------------------|---------------------|-------------------------------------------|----------------------|-------------------------------------------|
| Base ryanoids           |                  |                              |                              |                     |                                           |                      |                                           |
| Ryanodine               | 6.2              | 1                            | 1                            | 3.4                 | 1                                         | 260                  | 1                                         |
| Pyridyl ryanodine       | 700              | 113                          | 1                            | 2.5                 | 0.74                                      | 1700                 | 6.54                                      |
| Ryanodol                | 8000             | 1290                         | 1                            | 1400                | 412                                       | 950                  | 3.65                                      |
| C\(_{10}\)-O\(_{eq}\) derived ryanodol | 4000             | 645                          | 2.0                          | 330                 | 97                                        | 8900                 | 32.2                                      |
| C\(_{10}\)-O\(_{eq}\) derived ryanodol | 1100             | 177                          | 7.3                          | 330                 | 97                                        | 5800                 | 22.3                                      |
| GP ryanodine            | 415              | 63                           | 19                           | ND\(^{d}\)          |                                           |                      |                                           |
| C\(_{10}\)-O\(_{eq}\) derived pyridyl ryanodine | 5.9              | 0.95                         | 1.1                          | 6.9                 | 2.03                                      | AS\(^{e}\)           |                                           |
| C\(_{10}\)-O\(_{eq}\) derived pyridyl ryanodine | 2.2              | 0.35                         | 2.8                          | 2.3                 | 0.68                                      | AS\(^{e}\)           |                                           |
| C\(_{10}\)-O\(_{eq}\) derived pyridyl ryanodine | 1.1              | 0.18                         | 5.6                          | 2.9                 | 0.85                                      | AS\(^{e}\)           |                                           |
| C\(_{10}\)-O\(_{eq}\) derived pyridyl ryanodine | 225              | 36                           | 3.1                          | ND\(^{d}\)          |                                           |                      |                                           |
| C\(_{10}\)-O\(_{eq}\) derived pyridyl ryanodine | 35               | 5.6                          | 20                           | 3.9                 | 1.15                                      | AS\(^{e}\)           |                                           |
| C\(_{10}\)-O\(_{eq}\) derived pyridyl ryanodine | 20               | 3.2                          | 35                           | ND\(^{d}\)          |                                           |                      |                                           |

\(^{a}\) Compared to ryanodine.  
\(^{b}\) Computed for base ryanoid.  
\(^{c}\) GP, guanidino-propionyl.  
\(^{d}\) ND, not done.  
\(^{e}\) AS, activator selective ryanoids; deactivation values not available.

using Microsoft Excel®, CA-Cricket Graph (version 1.1), the coupled binding analysis program EBDALigand (34), and GraphPad Prism 2.0 (San Diego, CA).

Channel Modulating Characteristics of the Ryanoïds

Ryanoid-induced changes in Ca\(^{2+}\) efflux were assessed by measuring their abilities to alter passive Ca\(^{2+}\) efflux from JSRV previously loaded with \(^{45}\)Ca\(^{2+}\) as described (19–21, 26–30). In this assay, JSRV (3.5 mg/ml) were incubated in Ca\(^{2+}\) loading buffer (140 mM NaCl, 20 mM HEPES, 1.1 mM Ca\(^{2+}\) (spiked with 0.25 \(\mu\)M \(^{45}\)Ca\(^{2+}\)), 0.1 mM EGTA, and 1 mM MgCl\(_{2}\) (pH 7.0 at 22 °C)), in the presence of varying concentrations of the ryanoids (up to 5 mM) for 2 h at 22 °C. The Ca\(^{2+}\) load achieved was 20–30 nmol/mg JSRV protein. Passive Ca\(^{2+}\) efflux was determined by diluting the vesicles 5-fold into an ice-cold stop solution (140 mM NaCl, 20 mM HEPES, 0.2 mM Ca\(^{2+}\), and 1 mM EGTA, pH 7.0 at 22 °C). After 3 s, efflux was stopped by further diluting the vesicles 6-fold into an ice-cold stop solution (140 mM NaCl, 20 mM HEPES, 0.1 mM EGTA, 5 mM MgCl\(_{2}\), and 0.01 mM ruthenium red) and rapidly filtering. The vesicles on the filters were then washed 3 times with 3 ml of rinse solution (identical to stop solution except without ruthenium red), and the \(^{45}\)Ca\(^{2+}\) remaining inside the vesicles was determined by liquid scintillation counting.

Since Ca\(^{2+}\) efflux may be different from different vesicle populations (reflecting, for example, different densities of RyRs), the measurements can be taken to represent only an average over all vesicles in the preparation. We use herein the term ensemble functional potency to describe the effect of a given ryanoid-induced opening toward net Ca\(^{2+}\) efflux. The lower the amount of \(^{45}\)Ca\(^{2+}\) remaining in its presence. Concentration-effect curves with several dozen ryanoid derivatives never revealed efflux any greater than this combination (data not shown). Thus we used as an operational definition of full efficacy that which occurs with 1 \(\mu\)M guanidino-propionyl ryanodine in the presence of 1 mM AMP-PCP. It was 88 ± 5% (mean ± S.D.) for all nine preparations used in the present studies. The residual 12% could be released within 2 s by 1 \(\mu\)M A23187 (data not shown).

RESULTS

Relative Binding Affinities of the Base Ryanoïds for Rabbit Skeletal Muscle RyR1

Binding affinities were determined from the ability of the compounds to compete with 6.7 nM \(^{3}\)Hryanodine for binding to receptor sites on RyR1, using displacement binding affinity assays. As shown in Fig. 2a, the model ryanoids exhibit substantial differences in apparent receptor affinity. Ryanodol (\(\Delta\)) was weakest in this regard, exhibiting an IC\(_{50}\) value of 8000 ± 50.9 nM (\(K_{d} = 2970 ± 49.6\) nM) for RyR1. Derivatives varying at the C\(_{3}\) secondary hydroxyl of ryanodol, containing either pyridyl-3-carbonate (pyridyl ryanodine) or pyrrole-2-carbonate (ryanodine), showed significantly enhanced affinity for RyR1 (IC\(_{50}\) value of 700 ± 10.2 nM (\(K_{d} = 260 ± 12.2\) nM), for pyridyl ryanodine and IC\(_{50}\) value of 6.2 ± 0.2 nM (\(K_{d} = 2.4 ± 0.2\) nM) for ryanodine, respectively (Fig. 2a, ● and △). All three displacement binding affinity isotherms display apparent saturation binding kinetics and are parallel to each other, as anticipated from their similarities in chemical structure.

These results are compatible with those from previous studies showing that an ester substituent on the C\(_{3}\) carbon enhances high affinity ryanodine interactions with RyR1 from rabbit skeletal muscles. The present studies suggest that, in addition, the chemical nature of the C\(_{3}\) ester plays a substantial role in the high affinity interaction of the ryanoids with RyR1 (see also Table I, data column 2). High affinity interactions of ryanoids with RyR1 apparently depend to some degree on an appropriately sized substituent on the C\(_{3}\) carbon. These results confirm and extend reports from prior investigations (23–25, 35).

It should be noted, however, that there is a substantial potential for distortion of affinity assays by minor variations in purity of the applied ryanoids. To minimize this confounding variable in the present study, we re-assessed ryanoids’ affinities using exactly the same ryanoid preparations subsequently

Full Efficacy of Ryanoid Actions

To compare the ability of the various ryanoids to open the Ca\(^{2+}\) release channels fully, it was necessary to institute a protocol that would establish it for each JSRV population. In previously published studies with activation selective ryanodine derivatives (26–38), we found guanidino-propionyl ryanodine to consistently produce maximal Ca\(^{2+}\) efflux at 30 \(\mu\)M (in the absence of 1 mM AMP-PCP) and at 300 nM in its presence. Concentration-effect curves with several dozen ryanodine derivatives never revealed efflux any greater than this combination (data not shown). Thus we used as an operational definition of full
Relative Binding Affinities of C10-Oeq Derivatives of the Base Ryanoids with Cbz-β-Alanyl, β-Alanyl, and Guanidino-propionyl Esters

Ryanodol—The relative binding affinities of ryanodol derivatives, including C10-Oeq Cbz-β-alanyl ryanodol, C10-Oeq β-alanyl ryanodol, and C10-Oeq guanidino-propionyl ryanodol were compared with ryanodol using displacement binding affinity assays as described above. As shown in Fig. 2b, increasing the basicity of the C10-Oeq ester substituent terminus, from Cbz-β-alanyl to β-alanyl, and then to guanidino-propionyl progressively enhanced the affinity of these ryanoids for binding sites on RyR1 (see also Table I, data column 3). These values demonstrate that C10-Oeq anchoring by the esterifications restored affinity toward that of ryanodol by 2-, 7-, and 19-fold, with Cbz-β-alanyl, β-alanyl, and guanidino-propionyl side chains, respectively (compared with parent ryanodol).

Although esterification of the C10 secondary hydroxyl with anchoring substituents significantly enhances the affinity of ryanodol for the ryanodine-binding sites, the affinities of these semi-synthetic ryanoids for RyR1 nevertheless remain significantly lower than that of ryanodine (Fig. 2 (A)). Even when a ryanoid experiences anchoring by basic C10-Oeq esters, maximal affinity apparently depends substantially on the C3 substituent, pyrrole-2-carbonate.

Ryanodine—Esterification of the C10 equatorial secondary hydroxyl of ryanodine to produce the Cbz-β-alanyl, β-alanyl, and guanidino-propionyl derivatives also progressively enhances their affinities for RyR1 (Fig. 2c). Notably, the degree of leftward shift by each of the ryanodine esters in the series was not as large as that of the corresponding ryanodol ester in the same series (Table I, data column 3). These displacement binding isotherms are all parallel, consistent with the notion that all four ryanoids are interacting with the same site(s) on RyR1. The higher affinities of these semi-synthetic ester derivatives of ryanodine are apparently due to the anchoring influence of the C10 ester substituents on RyR1, but they are able to add only a lesser stabilizing influence to the predominant binding strength afforded by the C3 pyrrole-2-carbonyl (and the remaining polycyclic, polyhydroxylic backbone of the parent ryanodine molecule).

Pyridyl Ryanodine—As for ryanodol and ryanodine, esterification of the C10 secondary hydroxyl group of pyridyl ryanodine with basic Cbz-β-alanine, β-alanine, and guanidino-propionate side chains produced derivatives that exhibited enhanced affinities for RyR1 (Fig. 2d; Table I, data column 3). Notably, the affinity enhancements by anchoring esterifications of pyridyl ryanodine were greater than those for either ryanodol or ryanodine. The guanidino-propionyl derivative of pyridyl ryanodine demonstrated an apparent affinity of about 20 nM, a value not substantially different from that of ryanodine.

It was unexpected that the binding affinity of C10-Oeq guanidino-propionyl pyridyl ryanodine (Fig. 2d, ◆) would be only 3-fold less than that of ryanodine (▲) because a recent study proposed overwhelming dominance of the pyrrole for binding affinity (36). The present data (especially those on potency and efficacy, see below) suggest that the six-membered C4 substituent of pyridyl ryanodine is accommodated almost equally well as the five-membered substituent of ryanodine. Its affinity enhancement apparently is reinforced when it is held in the vicinity by anchoring the pyridyl ryanodine molecule via C10-Oeq substitutions having basic termini.
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Ca\(^{2+}\) loading buffer an additional 10-fold, to 300 \(\mu\text{M}\), resulted in no further change in Ca\(^{2+}\) efflux. Only when presented with concentrations of pyridyl ryanodine greater than 300 \(\mu\text{M}\) did ensemble Ca\(^{2+}\) efflux begin to diminish (EC\(_{50}\text{deact} = 1.7 \text{ mM}\)). What had been peaks for concentration-effect curves of ryanodol and ryanodine became instead a plateau for pyridyl ryanodine. The plateau was entirely due to a 7-fold rightward shift of the deactivation curve compared with that of ryanodine. Pyridyl ryanodine activated as potently as ryanodine but deactivated only as impotently as ryanodol.

Normalizing the Ca\(^{2+}\) efflux profiles of ryanodol and pyridyl ryanodine based on their affinities relative to that of ryanodine for RyR1 (Fig. 3a, inset) suggests that at calculated equivalent receptor occupancies, pyridyl ryanodine is the most potent among the base ryanoids in ability to activate RyR1, followed by ryanodol and then ryanodine. Ryanodol apparently is able to induce channel deactivation at a receptor occupancy lower than that of either ryanodine or pyridyl ryanodine. This might be due to ryanodol's greater intrinsic activity on binding to some lower affinity ryanodine-binding site(s) on RyR1.

Collectively, these data show clearly that the pattern of affinity values among the base ryanoids (Fig. 2a) does not predicate their channel modulation profiles (Fig. 3a; Table I). A hydrogen for pyrrole-carbonate substitution (ryanodine to pyridyl ryanodine) diminished affinity 1290-fold and simultaneously decreased the activating efficacy of this compound to about one-third of that for ryanodine and one-half that of pyridyl ryanodine. Nevertheless, all three base ryanoids effectively deactivated RyR1. A pyridyl-carbonate for a pyrrole-carbonate substitution on the C\(_3\) carbon (ryanodine versus pyridyl ryanodine) diminished ryanoid affinity for the receptor by 113-fold but produced no decrease in its activating potency. In fact, pyridyl ryanodine was slightly more potent and substantially more efficacious than ryanodine in its ability to activate RyR1. At the same time, its deactivation profile was shifted rightward to that of ryanodine, by a full log unit. The intrinsic activity of pyridyl ryanodine was therefore less than that of ryanodine on binding to the putative lower affinity ryanodine-binding site(s) on the receptor.

Relative Efficacies of C\(_{10}\)-O\(_{eq}\) Derivatives of Ryanodol—Cbz-\(\beta\)-alanyl Ryanodol and \(\beta\)-Alanyl Ryanodol—C\(_{10}\)-O\(_{eq}\) ester derivatives of ryanodol, like ryanodol itself, also activated and at least partially deactivated RyR1, with only small changes in potencies (Fig. 3b; Table I). However, these semi-synthetic derivatives demonstrated enhanced activating efficacies. Up to concentrations of 30 \(\mu\text{M}\), these ryanoids were unable to alter significantly the ensemble Ca\(^{2+}\) efflux. Thereafter, progressively increasing the concentrations of C\(_{10}\)-O\(_{eq}\) Cbz-\(\beta\)-alanyl ryanodol and C\(_{10}\)-O\(_{eq}\) \(\beta\)-alanyl ryanodol up to 3 \(\text{mM}\) resulted in activating efficacies greater than that of ryanodol and perhaps even of ryanodine (Fig. 3b [] and []). Whereas C\(_{10}\)-O\(_{eq}\) Cbz-\(\beta\)-alanyl ryanodol (3 \(\text{mM}\)) approached full agonist activity, C\(_{10}\)-O\(_{eq}\) \(\beta\)-alanyl ryanodol (3 \(\text{mM}\)) released a maximum of only 84.5\%. Their EC\(_{50}\text{act}\) values were similar, at about 30 \(\mu\text{M}\). Thus, the latter compounds increased the ensemble functional potency of RyR1 to Ca\(^{2+}\) efflux well beyond the maximum affordable by parent ryanodol.

At a concentration of 5 \(\mu\text{M}\), C\(_{10}\)-O\(_{eq}\) Cbz-\(\beta\)-alanyl ryanodol and C\(_{10}\)-O\(_{eq}\) \(\beta\)-alanyl ryanodol appeared to deactivate the channels, to at least some extent. However, it was not possible to characterize fully the deactivating effects of either compound because of their limited aqueous solubilities. Nonetheless, if higher aqueous concentrations of C\(_{10}\)-O\(_{eq}\) Cbz-\(\beta\)-alanyl ryanodol and C\(_{10}\)-O\(_{eq}\) \(\beta\)-alanyl ryanodol could have been achieved, they would be expected to further deactivate the channels, giving extrapolated EC\(_{50}\text{deact}\) values of 8.9 and 5.8 \(\mu\text{M}\), respectively. In a single passive Ca\(^{2+}\) efflux assay, C\(_{10}\)-O\(_{eq}\) guanidinopropionyloxy ryanodol exhibited a channel modulating profile similar to that of its congener, C\(_{10}\)-O\(_{eq}\) Cbz-\(\beta\)-alanyl ryanodol (data not shown). However, we were unable to confirm these values due to the limited availability of this compound.

Normalizing the data based on the affinities of ryanodol and its derivatives relative to that of ryanodine for RyR1 (Fig. 3b, inset) suggests that at calculated equivalent receptor occupancies, ryanodol and C\(_{10}\)-O\(_{eq}\) Cbz-\(\beta\)-alanyl ryanodol are approximately equally potent in ability to trigger Ca\(^{2+}\) loss from the vesicles, whereas C\(_{10}\)-O\(_{eq}\) \(\beta\)-alanyl ryanodol was slightly less so. C\(_{10}\)-O\(_{eq}\) Cbz-\(\beta\)-alanyl ryanodol was the most efficacious. In addition, ryanodol appears to a more potent deactivator of RyR1 (at a calculated equivalent receptor occupancy) than either C\(_{10}\)-O\(_{eq}\) Cbz-\(\beta\)-alanyl ryanodol or C\(_{10}\)-O\(_{eq}\) \(\beta\)-alanyl ryanodol.

Relative Efficacies of C\(_{10}\)-O\(_{eq}\) Derivatives of Ryanodine—As previously reported, C\(_{10}\)-O\(_{eq}\) derivatives of ryanodine (Fig. 3c) induced Ca\(^{2+}\) release from vesicles, with greater efficiencies than ryanodine. All three became full agonists or nearly so. C\(_{10}\)-O\(_{eq}\) Cbz-\(\beta\)-alanyl ryanodine demonstrated an EC\(_{50}\text{act}\) of 6.9 \(\mu\text{M}\), releasing a maximum of 87.5\% of the intravesicular Ca\(^{2+}\) at a concentration of 60 \(\mu\text{M}\), and C\(_{10}\)-O\(_{eq}\) \(\beta\)-alanyl ryanodine exhibited an EC\(_{50}\text{act}\) of 2.3 \(\mu\text{M}\), releasing a maximum of 98.5\% of the Ca\(^{2+}\) from JSRV at a concentration of 30 \(\mu\text{M}\), and C\(_{10}\)-O\(_{eq}\) guanidino-propionyl ryanodine released a maximum of 94.5\% at a concentration of 30 \(\mu\text{M}\) (EC\(_{50}\text{act} = 2.9 \mu\text{M}\)). Clearly, the channel activating profiles of C\(_{10}\)-O\(_{eq}\) \(\beta\)-alanyl ryanodine and C\(_{10}\)-O\(_{eq}\) guanidino-propionyl ryanodine did not mimic that of ryanodine. At the low end of the concentration-effect curve (concentrations less than about 1 \(\mu\text{M}\)), their channel activating profiles are approximately superimposable on that of ryanodine. Higher concentrations of these derivatives resulted in greater Ca\(^{2+}\) efflux than ryanodine. C\(_{10}\)-O\(_{eq}\) Cbz-\(\beta\)-alanyl ryanodine appears to be less potent than ryanodine, or either of the two other derivatives in ability to activate RyR1, i.e., its concentration-effect curve was shifted slightly to the right.

Up to the maximally available concentration of 1 \(\text{mM}\) C\(_{10}\)-O\(_{eq}\) Cbz-\(\beta\)-alanyl ryanodine, C\(_{10}\)-O\(_{eq}\) \(\beta\)-alanyl ryanodine (as well as C\(_{10}\)-O\(_{eq}\) guanidino-propionyl ryanodine) entirely lacked ability to deactivate RyR1 and diminish Ca\(^{2+}\) efflux from the vesicles. These data suggest that although esterification of the C\(_{10}\) secondary hydroxyl of ryanodine with a basic substituent enhances its affinity for the receptor, this modification does not predicate a leftward shift of the channel activation profiles. Instead, these ester functionalities on the C\(_3\) carbon produced ryanoids unable to produce channel deactivation. This could be due either to their antagonistic effect when they bind to the low affinity ryanodine site(s) or their inability to bind to the low affinity site(s) and thereby prohibit channel deactivation. Our reported prior studies support the former.

Normalizing these data based on the binding affinities of the compounds relative to that of ryanodine for RyR1 (Fig. 3c, inset) suggests that at calculated equivalent receptor occupancies, the channel activating potency of C\(_{10}\)-O\(_{eq}\) derivatives of ryanodine are approximately equivalent to each other and greater than that of ryanodine (Fig. 3c, inset).

Relative Efficacy of C\(_{10}\)-O\(_{eq}\) \(\beta\)-Alanyl Pyridyl Ryanodine—Like the other ryanoid derivatives, C\(_{10}\)-O\(_{eq}\) \(\beta\)-alanyl pyridyl ryanodine was assessed for its channel modulating characteristics using passive Ca\(^{2+}\) efflux assays. As is shown in Fig. 3d (\(\%\)), it too activated the RyR1 in a concentration-dependent manner (EC\(_{50}\text{act} = 3.9 \mu\text{M}\)) and exhibited full agonist action. At a concentration of 300 \(\mu\text{M}\), 98\% of the intravesicular Ca\(^{2+}\) was released. In fact, up to a concentration of 30 \(\mu\text{M}\) the channel activating effects of pyridyl ryanodine and its C\(_{10}\)-O\(_{eq}\) \(\beta\)-alanyl derivative were essentially superimposable. Concentrations of
Pyridyl ryanodine above these appeared to be slightly more efficacious than C_{10}-Oeq β-alanyl pyridyl ryanodine in ability to induce Ca^{2+} loss from the vesicles. In preliminary passive Ca^{2+} efflux assays, we found that C_{10}-Oeq Cbz-β-alanyl pyridyl ryanodine and C_{10}-Oeq guanidino-propionyl pyridyl ryanodine were also activator-selective ryanoids and that their channel activating profiles were superimposable on that of parent pyridyl ryanodine. Further experimentation is currently ongoing to confirm or refute these latter observations.

Unlike pyridyl ryanodine which could deactivate RyR1s, C_{10}-Oeq β-alanyl pyridyl ryanodine, up to the maximally available concentration of 5 mM in the Ca^{2+} loading buffer, could not. As mentioned above, this lack of deactivator actions of C_{10}-Oeq esters of pyridyl ryanodine could be due either to their inability to bind to the lower affinity site(s) on the receptors or their antagonism at the lower affinity site(s). As shown in competition studies with JSRV, at least one of the C_{10}-Oeq ester derivatives of 9,21-dehydroyanodine, namely C_{10}-Oeq N-methyl-9,21-didehydroyanodine succinimide appears to be a pure antagonist on RyR1 (27). Others prevent channel closure in vesicles and at the single channel level (18).

Normalizing the present data based on the affinities of pyridyl ryanodine and C_{10}-Oeq β-alanyl pyridyl ryanodine for RyR1 relative to that of ryanodine (Fig. 3d, inset) suggests that at calculated equivalent receptor occupancies pyridyl ryanodine is more potent than either its β-alanyl derivative or parent ryanodine itself.

**DISCUSSION**

A principal result of the present study is that pyridyl ryanodine, a natural secondary metabolite from *R. speciosa*, shows for the first time a definitive separation of activation potency from high affinity for the ryanodine receptor. Pyridyl ryanodine had only 1% of the affinity of ryanodine for RyR1-binding sites but showed 100% of ryanodine’s activation potency and even greater efficacy than ryanodine. The 100-fold loss in apparent affinity was associated with a 10-fold loss in deactivation potency.

We previously suggested that the effects of ryanodines on Ca^{2+} efflux might usefully be rationalized as the algebraic sum of individual activation and deactivation concentration-effect curves for the ryanodines (21). A straightforward means to characterize the degree of overlap of the deactivation limb on the activation limb of ryanodine effects is to compare the mean values for their midpoints. For convenience of discussion, we have termed the ratio of EC_{50,deact} to EC_{50,act} the concentration coupling ratio, abbreviated as the CCR (21). Recently Meissner et al. (37) coined the term “Ca^{2+} window of receptor activation” to describe a similar phenomenon, namely anionic influences that separate the limbs of the Ca^{2+}-ryanodine binding curve.

The CCR for ryanodine in the present study was approximately 76, but it increased >8-fold to 680 for pyridyl ryanodine. The CCR for ryanodol, on the other hand, was only 0.68. Strong overlap of the deactivation curve on the activation curve rationalizes the poor efficacy of ryanodol, and the lack of overlap rationalizes the full channel opening character of pyridyl ryanodine. The wide berth between the EC_{50,act} and the EC_{50,deact} for pyridyl ryanodine was caused entirely by a loss of potency for deactivation; activation potency for pyridyl ryanodine was equal to that of ryanodine. Pyridyl ryanodine thus activated the channel equally as well as ryanodine but deactivated it only as poorly as ryanodol.

Taken together, these results strongly imply that sites at which ryanodines enlist functionally differential effects on RyR1 are physically separate as well. Both activation and deactivation appear to arise from initially high affinity interactions. If such suggested sites actually exist, they are kinetically indistinguishable, so far, by binding competition studies using 6.7 nM [3H]ryanodine.

Displacement binding affinity assays are most discriminant of subsites when the concentration of the radiolabeled ligand used is less than the lowest concentration of unlabeled ligand against which it is allowed to compete. To approach this ideal, in an earlier study we prepared a radioiodinated derivative of ryanodine, namely C_{10}-Oeq N-(4-azido-5-125I-iodosalicyloyl) glycy ryano- dine, having a specific activity (1400 Ci/mmol) 12-fold higher than that of [3H]ryanodine (120 Ci/mmol). By using 0.5 nM of this radioligand in displacement binding affinity assays, we reported a pseudo-sigmoidal displacement curve that had a distinct shoulder at about 6 nM ryanodine (28). This could represent two sequential ryanodine binding isotherms within the nanomolar concentration range. Applied to the present results, the higher affinity site would have an IC_{50} of 0.5 nM (K_{d} = 0.26 ± 0.02 nM) while the lower (high) affinity site would demonstrate an IC_{50} of about 15 nM (K_{d} = 11.7 ± 1.2 nM).

An alternate explanation for those results is that the intense radiation from the 125I label produced an autodegradation product as a minor contaminant in the binding displacement medium. Although this seems unlikely, we are currently investigating this possibility. If such an artifact can be ruled out, those findings would complement the present data, suggesting that the biphasic effects of ryanodines on RyR1 may arise from sequential interactions at functionally antagonistic binding sites (38) that both apparently have high affinity constants, at least initially (39). If one of these (the deactivating sites?) were multicomponent and displayed negative cooperativity, Scatchard plots based on [3H]ryanodine could be misconstrued to represent only a single high and one (or a few) low affinity sites (40, 41). This possibility is under current investigation using a more selective derivative, aryldiazirine ryanodine (42).

Prior studies with two other minor secondary *Rymania* metabolites, namely ester E (C_{8,5}-hydroxy-ryanodine) and ester F (C_{8,5}-hydroxy-C_{10}-epi-dehydroyanodine) provided evidence that the topologic features responsible for activator actions of ryanodines are not coincident with those inducing deactivation of the channels (21). However, those natural ryanodines lost activation potency by about as much as they lost deactivation potency. Furthermore, affinities of ester E and ester F were much greater than those of the present studies. Pyridyl ryanodine is the first (natural or semi-synthetic) ryanoid to possess full ability to activate the Ca^{2+} release channel while losing, by over 100-fold, the ability to compete for binding at the high affinity ryanodine-binding sites.

A second major conclusion from the present results is that the C_{8} ester substituent on ryanodines plays only a secondary role in their potency and efficacy for activation of Ca^{2+} release channels. This was an unexpected finding in light of the apparently primary role of the pyrrole substituent in affinity (36). As noted above in the discussion of our prior results with a radioiodinated ryanoid, the present results suggest that if receptor affinity may be correlated to potency of unidirectional effects on RyR1, then such a correlation appears more consistent with the deactivating than the activating limb of the biphasic concentration-effect curve.

In any case, the present results show that a C_{8} ester is dispensable for activation of the Ca^{2+} release channel. Ryanodol lacks an ester substituent at C_{8} but nevertheless is able to open the Ca^{2+} release channel to Ca^{2+} flux. The potency and efficacy of ryanodol, however, are limited. Its half-activation of Ca^{2+} efflux (EC_{50,act}) was 412-fold higher than that of ryanodine. This was not simply consequent to the diminished affinity of ryanodol for RyR1, as expressed in its IC_{50} value, because the latter was 1290-fold less than that of ryanodine. Thus,
while ryanodol binds weakly to RyR1, it nevertheless has fairly strong efficacy for opening the Ca\(^{2+}\) release channel.

The chemical nature of the C\(_9\) substituent appears determinant of potency for activation of RyR1 by the base ryanoids of the present study. Pyrrole- and pyridyl-carbonate derivatives of ryanodol were approximately equally potent at opening the channel. This was not further enhanced by substituent esters, despite the fact that such C\(_{10}\)-Oeq substituents anchored them to the receptor (as suggested by their up to 35-fold increase in affinity on esterification). The same was true for ryanodol. What C\(_{10}\)-Oeq esters with basic termini provide is an increased efficacy for channel opening. Anchoring substituent esters at the C\(_{10}\)-Oeq position of the base ryanoids enhanced affinity and especially efficacy of the derived ryanoids but produced little consequence as regards potency.

Two direct and an additional indirect experimental paradigms are generally used to evaluate structure-activity effects on ryanodine receptor/calcium release channels as follows: flux measurements, lipid bilayer measurements, and \(^{3}H\)

ryanodine binding studies. The first and third provide data averaged over many Ca\(^{2+}\) channels, with time courses over hours. The second reflects single (or a few) channels, with time courses over minutes and individual gating events in the millisecond range. In previously published studies we have used all three methods. The present results rely on direct flux for functional measurements because these permit a wide range of experimental manipulations. Furthermore, the present results confirm that, at least under some conditions, ryanoid bind affinity does not go hand-in-hand with activity.

Qualitatively, results with single channel measurements nevertheless are in general consistent with efficacy results with vesicle population measurements. Certain ryanoids possessing full efficacy in vesicles lack ability to produce the shut state in single channel studies (18). This translates as full efficacy in the alternate experimental paradigm. Interestingly, however, at the higher resolution possible with bilayer measurements, a new feature of ryanoid actions emerges (18). Each ryanoid in our series produced a characteristic modified partial conductance state of the channel. The resultant slope conductances were not related to efficacy as judged from ensemble Ca\(^{2+}\) efflux data.

Specifically, ryanodol induces the largest modified conductance state of any ryanoid examined thus far. Yet ryanodol produces the least efficacy. Furthermore, C\(_{10}\)-Oeq derived ryanodines produce even smaller modified conductance states, in direct proportion to their enhanced affinities, but exhibit full efficacy for ensemble Ca\(^{2+}\) efflux.

Apparently the determinant feature of full agonists is not the residual size of the intermediate subconductance state, but rather the probability of inducing the terminal shut state. Fully efficacious C\(_{10}\)-Oeq derivatives seem to lock the channel into a state where \(P_e\) approximates 1. Not only do they not close the channel, they prevent ryanodine from closing it, even when ryanodine is elevated to 1 mM (18, 27). Ryanodol, on the other hand, is able to produce the shut channel state at submillimolar concentrations. (We have not yet completed studies with pyridyl ryanodine at the single channel level. They may prove particularly instructive.)

These results are compatible with the notion that basic termini of C\(_{10}\)-Oeq side chains interact with the ryanodine receptor in such a manner as to interfere physically with ionic flux through the pore region of the transmembrane conduction pathway, regardless of the experimental paradigm. By extension, the C\(_{10}\)-hydroxyl of ryanodine may also interact within the transmembrane conduction pathway.

Almost a decade after the total synthesis of ryanodol (43), the complete synthesis of ryanodine per se has yet to be achieved. Ruest and Deslongchamps (44) reincorporated the pyrrole-2-carbonate onto the C\(_9\) secondary hydroxyl of anhydroryanodol using the mild reaction conditions of Neises and Steglich (45) that we adapted for C\(_{10}\)-Oeq esterification of ryanodins (26–30). However, owing to stereochemical hindrances, Ruest and Deslongchamps were able to produce only C\(_3\)-epi-ryanodine. It has so far proved impossible to vary chemically the ester substituent on the C\(_9\) carbon while maintaining the original stereochemical orientation found in ryanodine. The present studies with the naturally oriented C\(_3\)-pyrrole substituent of ryanodine were permitted by our isolation of sufficient quantities of the natural secondary metabolite pyridyl ryanodine from *Ryania* wood.

C\(_3\)-epi-ryanodine had an affinity 100-fold less than ryanodine. In collaboration with Ruest and Deslongchamps (44), Welch et al. (36) confirmed the low affinity of C\(_3\)-epi-ryanodine and further showed that esterification of the C\(_{10}\)-Oeq position of ryanodol with the basic side chain pyrrole-2-carbonate partially restored the affinity of ryanodol for RyR1, to within 7-fold that of parent ryanodine. We interpret their data to indicate that a C\(_{10}\)-Oeq pyrrole carbonate is able to anchor the ryanoid polycyclic backbone structure to the ryanodine receptor, much as does other basic termini such as the guanidino-propionyl moiety. They concluded differently. However, in their binding paradigm, both the C\(_9\) and C\(_{10}\)-Oeq positions were systematically excluded from their elegant comparative molecular field analysis relating topologic ryanoid features to ryanodine affinity. They modeled both to be in solvent space. Whereas this exclusion may well be justified for C\(_9\) substituents, it seems less likely for C\(_{10}\)-Oeq esters, since the latter can considerably increase the binding affinity of all ryanoids, including ryanodine itself (24, 26–30, 33 and present results). If effective, basic C\(_{10}\)-Oeq ryanodines had been included in the Welch et al. (36) basis set, the interpretation that certain pyrrole derived ryanoids might be “reoriented in the binding site... such that the pyrrole [when present] always occupies the same subsite” likely could not have emerged as a viable hypothesis. In fact, if a substituent pyrrole-2-carbonate predicated the binding orientation of any ryanoid containing it, then pyrrole-2-carbonate itself logically should compete with \(^{3}H\)

ryanodine for high affinity binding sites. Having none of the steric hindrances associated with the bulk polycyclic backbone of ryanodine and none of its numerous polar groups, pyrrole-2-carbonate should be an especially effective competitor for \(^{3}H\)

ryanodine binding. It is not. Up to 1 mM, pyrrole-2-carbonate has absolutely no effect on \(^{3}H\)

ryanodine binding (data not shown).

Numerous other studies of function-activity relationships among ryanoids support the proposition that the polycyclic backbone of ryanodins and its multiple polar moieties figure prominently in affinity and effectiveness of ryanodine derivatives. For example, Casida and co-workers (46) showed that partial oxidation of the C\(_{10}\) secondary hydroxyl to a ketone reduced the affinity of this ryanodin for RyR1. These investigators also showed that conversion of the C\(_{10}\) secondary hydroxyl to its axial isomer or other axially oriented functionalities (e.g. amino) decreased the affinity of ryanodins for RyR1 (47). Systematic evaluation of ryanodin-structure activity relationships among many ryanoids have demonstrated affinity consequences of various polar regions of the ryanodine molecule (48). Jeffries et al. (49) examined 22 derived ryanodins prepared by nucleophilic additions to 4,12-seco- and 4,12-dioxoryanodines and extended such studies to two additional series of derivatives. Additionally, Welch and co-workers (24, 33, 36) sought to identify structural components of ryanodine responsible for modulation of sarcoplasmic reticulum Ca\(^{2+}\) channel function.
For the most part, however, prior studies have focused on changes in affinities of ryanoids consequent to imposed structural variations. In a recent review, Sutko and colleagues (50) provided an excellent summary of ryanoid structure-activity studies to date. Prior studies also have shown that the affinities of natural ryanoids for their receptor sites may be altered by modifications and derivatizations. We have recently shown that esterification of the C10 secondary hydroxyl of ryanodine and 9,21-didehydroryanodine with substituents having basic termini significantly enhances their affinities for the receptor sites by as much as 6–8-fold. The potencies and efficacies of these molecules were not correspondingly shifted.

Taken collectively with the present data, the composite results permit assignment of the ester on the C9 secondary hydroxyl to less than a central role for high affinity interactions of ryanoids with RyR1.

As regards the anchoring substituents, for each of the base ryanoids of the present study, C10-Oeq esterification with side chains bearing basic termini advanced their affinities approximatel actively. For each, the most basic terminus, guanidino-propionyl, produced the highest affinity interaction. Whereas chains bearing basic termini advanced their affinities approximately, it was more advantageous, C10-Oeq substituents anchoring the ryanoids to ryanoid receptor site(s) were found to affect primarily efficacy for channel opening, with little effect on potency. Thus increased binding affinity clearly desegregated from increased potency.

Unlike ryanoids that demonstrate a bifasic effect on RyRs, most of the semi-synthetic C10-Oeq derived ryanoid molecules showed only activator-selective actions. Assuming that the bifasic effects of ryanoids on RyRs are due to their interactions with (at least) two functionally antagonistic receptor sites, it still remained uncertain whether the activator selective actions of certain ryanoids resulted from their inability to interact with a putative lower affinity binding site or whether they might be antagonists at such a site. Our competitive function studies with JSRV favored the latter interpretation, and this conclusion has recently been strengthened by our single channel experiments. The present communication provides further evidence in support of our working hypothesis that the activator selective actions of these C10-Oeq ester derivatives of ryanoids result from their physical interference with deactivation.

In summary, the present results show that affinity consequences of ryanoid chemical modifications experimentally segregate from their consequences on potency and efficacy. Pyridyl ryanodine was poorly effective in its ability to compete with ryanodine for binding to high affinity sites on the ryanodine receptor but nevertheless was fully effective in its capacity to open the channels to Ca2+ influx. In fact, pyridyl ryanodine opened the Ca2+ release channels fully at a concentration some 100-fold lower than its minimal concentration for initiating channel closure. Ryanoid anchoring via C10-Oeq side chains with basic termini also desegregated affinity consequences from potency consequences; affinities were increased but potencies remained essentially unchanged.

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