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Differential Activating and Deactivating Effects of Natural Ryanodine Congeners on the Calcium Release Channel of Sarcoplasmic Reticulum: Evidence for Separation of Effects at Functionally Distinct Sites

ROD A. HUMERICKHOUSE, HENRY R. BESCH, JR., KOERT GERZON, LUC RUEST, JOHN L. SUTKO, and JEFFREY T. EMMICK

Department of Pharmacology and Toxicology, Indiana University School of Medicine, Indianapolis, Indiana 46202 (R.A.H., H.R.B., K.G., J.T.E.), Department of Chemistry, University of Sherbrooke, Sherbrooke, Quebec, J1K 2R1, Canada (L.R.), and Department of Pharmacology, University of Nevada, Reno, Nevada 89559 (J.L.S.)

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SUMMARY

Two novel natural ryanoids from extracts of the wood of *Ryania speciosa* Vahl were evaluated with sarcoplasmic reticulum (SR) vesicles for their binding affinities and their activating and deactivating effects on Ca^{2+} release channels. The new ryanoids, which are more polar than the known *Ryania* constituents ryanodine and didehydro-(9,21)-ryanodine, were purified using silica gel column chromatography and reverse phase high performance liquid chromatography. The new ryanoids were designated ester E and ester F, in keeping with nomenclature previously used in the literature. These compounds were identified by NMR spectroscopy and mass spectroscopy as C_{9ax}-hydroxyryanodine and C_{9ax}-hydroxy-C_{10}-epi-dehydroryanodine, respectively. Binding of esters E and F to the high affinity (nanomolar K_d) site on SR Ca^{2+} release channels was determined from relative binding affinity assays using 6.7 nM [^{3}H]ryanodine. Apparent K_d values of ryanodine, ester E, and ester F for binding to this domain on the skeletal muscle ryanodine receptor/SR Ca^{2+} release channel were 4.4 ± 0.8, 65 ± 10, and 257 ± 53 nM, respectively (mean ± standard deviation, four or more experiments). Apparent K_d values for cardiac muscle receptors were 0.51 ± 0.01, 12 ± 0.4, and 57 nM, respectively. As a functional indication of the effects of the ryanoids, channel-opening (activator) and channel-closing (deactivator) actions were assessed from the ability of the ryanoids to alter the rate of Ca^{2+} efflux from passively loaded skeletal muscle junctional sarcoplasmic reticular vesicles (JSRV). Activator actions among the ryanoids were similar, in that they exhibited apparently parallel concentration-effect curves, having a slope of 40% Ca^{2+} loss/decade increment in ryanoid concentration. Half-maximal values for activation (EC_{50} values) were 2.5, 63, and 43 μM for ryanodine, ester E, and ester F, respectively. Maximal channel opening by ester E was significantly less than that produced by the other ryanoids. The deactivator actions of the compounds on skeletal JSRV were dissimilar, in that their concentration-effect curves appeared not to be parallel. The quotient of the EC_{50} for deactivation and that for activation was taken as the concentration-coupling ratio (CCR). The CCR for ryanodine was 114 and that for ester F was 72, but the CCR for ester E was only 21. ATP-dependent Ca^{2+} accumulation by cardiac JSRV provided a second means to evaluate deactivator actions of the ryanoids. Results from cardiac JSRV assays were in general similar to those from skeletal JSRV assays. CCRs among the natural ryanodine analogs were obviously disparate, providing strong evidence for separation of ryanoid effectiveness at functionally distinct domains on (or states of) the ryanodine receptor/SR Ca^{2+} release channel. In support of this conclusion, results of studies with the parent molecule ryanodine also revealed a variant CCR between half-maximally deactivating and half-maximally activating concentrations. In the presence of the allosteric channel activator adenosine-5′-β,γ-methylenetriphosphate, ryanodine exhibited an activation curve that lay >3 orders of magnitude to the left of its deactivation curve. The CCR of ryanodine in the absence of this adenine nucleotide was only 2% of that in its presence. The observation that activation is not invariantly concentration-coupled to deactivation suggests that the molecular features responsible for the activator actions of ryanoids on Ca^{2+} release channels are different from those inducing deactivation of the channels. The present results constitute the first evidence that the activator effects of ryanoids are functionally separable from their deactivator effects.

The plant *Ryania speciosa* Vahl produces the alkaloids ryanodine (1) and dehydroryanodine (2), which are specific effectors of Ca^{2+} release channels of both muscle (3–6) and nonmuscle tissues (7, 8). These ligand-gated channels are integral components of the junctional regions of the SR and mediate in striated muscle the release of Ca^{2+} from intracellular Ca^{2+} stores that triggers muscle contraction. The actions of ryanodine at the SR Ca^{2+} release channel disrupt intracellular Ca^{2+} regulation, producing tonic contracture in skeletal muscle (9) and prolonged contractions and negative inotropy in cardiac tissues

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(10). These characteristics have made ryanodine useful as a tool to investigate the role of intracellular Ca^{2+} release in excitation-contraction coupling.

Ryanodine exhibits two distinct opposing effects. Both actions can be observed in SR vesicles (11, 12), as well as in isolated SR Ca^{2+} release channels reconstituted into planar lipid bilayers (13–16). Lower ryanodine concentrations (nanomolar to low micromolar) activate (open) the channels, whereas concentrations exceeding those that produce maximal activation deactivate (functionally close) the SR Ca^{2+} release channels and thereby impede Ca^{2+} fluxes. Because activation always precedes deactivation, correlating the effects of ryanodine at the molecular and tissue levels remains problematic. Further confounding interpretation of the macroscopic physiologic effects of ryanodine, as well as its interactions with the Ca^{2+} release channel, is the fact that binding, like the functional effects of the alkaloid, displays complex concentration kinetics. Most analyses of binding suggest more than one class of binding sites. The results of several investigations are consistent with the concept of two distinct binding sites, one exhibiting a high affinity for ryanodine (K_d in the nanomolar range) and the other having a much lower affinity (K_d in the micromolar range). The reported K_d values for these sites vary substantially with the Mg^{2+} and Ca^{2+} concentrations, the ionic strength, and the presence of adenine nucleotides in the medium (17–20). From concentration-effect relationships it is generally considered that occupation of the high affinity site activates the channel, whereas deactivation results from ryanodine filling the low affinity site. However, substantial differences exist between the reported K_d values and the concentrations at which functional effects are observed.

At least some of the discrepancies between the concentrations at which ryanodine binds to its receptor and those purportedly responsible for its functional effects may be attributable to the slow association and dissociation kinetics of this alkaloid. As is the case for K_d values, rates of association and dissociation are influenced by the content of the assay medium. It is thought that the faster association and dissociation kinetics induced by optimal concentrations of Ca^{2+} and nucleotides result from allosteric effects on the channel (3, 11, 12, 21). Under optimal conditions ryanodine binding to its receptor domains appears to be facilitated. Recent studies suggest that the interaction of ryanodine with the SR Ca^{2+} release channel may involve positively (18, 22) or negatively (21, 23) cooperative interactions among multiple binding sites or states of the channel (18, 24).

Given the complexities inherent in the antithetical effects of ryanodine, attempts are warranted to identify and characterize ryanodine analogs that may exhibit greater, perhaps definitive, separation of activator from deactivator effects. Such separation of activators and antagonists has been possible for compounds affecting the L class of voltage-gated sarcolemmal Ca^{2+} channels (25, 26). Chemical alteration of ryanodine has yielded a variety of products, but in general these have markedly reduced affinities for the SR Ca^{2+} release channel (27). Recently, preliminary data on chemical derivatives of ryanodine prepared by modifying the hydroxyl function at C_{10} have been reported (28–30, 43). Several of these derivatives possess affinities greater than that of the parent ryanodine, enhancing the possibility that judicious chemical modification may yield high potency compounds with directionally unique actions.

Natural minor constituents of *Ryania* extracts have also been studied (31, 32). The functional effects of only one of these secondary metabolites, ester A, have so far been reported (33). In the present communication, we describe the identification and characterization of two ryanodine congeners isolated from crude extracts of *R. speciosa* Vahl. These compounds, designated ester E and ester F in keeping with the nomenclature previously used in the literature (31), bind to the SR Ca^{2+} release channel and differentially affect channel activation and deactivation. The relationship between relative binding affinity and activation or deactivation of the SR Ca^{2+} release channel was not constant among these ryanoids, nor was activation invariantly coupled to deactivation. Furthermore, even the parent molecule ryanodine exhibited variant coupling of its activation and deactivation effects in skeletal muscle JSRV, depending upon assay conditions. Addition of the adenine nucleotide AMP-PCP to the assay medium substantially increased the activator potency of ryanodine, whereas the deactivator effect remained virtually unchanged. These results show that activator binding sites on (or states of) the Ca^{2+} release channel/ryanodine receptor display functional independence from those effecting channel closure.

Experimental Procedures

Materials

[^{3}H]Ryanodine was obtained from DuPont-New England Nuclear Corp. (Boston, MA). Unlabeled mixtures of ryanodine and dehydroryanodine were obtained from S.B. Penick and Co. (Lindhurst, NJ) and from Agri-Systems International (lot LR171290; Wind Gap, PA). A crude *Ryania* extract (lot 8E09) was also obtained from Agri-Systems International. *Ryania* wood was supplied by Agri-Systems International and by Integrated Biotechnology Corp. (Carmel, IN). Individual ryanoids were purified by chromatography of organic solvent extracts of the crushed wood (see below). The purity of all ryanoids used in the binding and pharmacologic studies was verified to be at least 98% by analytical HPLC. Tris-ATP and AMP-PCP were from Sigma Chemical Co. (St. Louis, MO). All other chemicals and reagents were of the highest grade obtainable commercially.

Methods

Isolation of skeletal muscle SR membrane vesicles. Rabbit fast skeletal muscle SR vesicles were prepared by the following method modified from cardiac muscle procedures (34–36) (also see below). All preparative procedures were carried out on ice or at 4°. After induction of anesthesia with Brevital, New Zealand White rabbits were sacrificed by exsanguination. The white muscles of the back and hind legs were

ABBREVIATIONS: SR, sarcoplasmic reticulum; ester A, *Ryania* diterpene ester A (C_{4ax}-methoxy-C_{8ax}-hydroxy-C_{10}-epi-dehydroryanodine); ester E, *Ryania* diterpene ester E (C_{9ax}-hydroxyryanodine); ester F, *Ryania* diterpene ester F (C_{8ax}-hydroxy-C_{10}-epi-dehydroryanodine); dehydroryanodine, didehydro-(9,21)-ryanodine; EC_{50}, effective concentration inducing 50% of response; IC_{50}, concentration of competing ryanoid reducing specific [^{3}H] ryanodine binding by 50%; CCR, concentration-coupling ratio; JSRV, junctional sarcoplasmic reticular vesicles; AMP-PCP, adenosine-5′-β,γ-methylenetriphosphate; PMSF, phenylmethylsulfonyl fluoride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; PI, protease inhibitor; TLC, thin layer chromatography.

removed and ground in a Quisinart food processor in 200 ml of isolation buffer (0.3 M sucrose, 10 mM imidazole/HCl, pH 7.4) containing the PIs PMSF (230 μM) and leupeptin (1.1 μM). Approximately 50-g portions of ground muscle were placed in Beckman type JA-10 centrifuge tubes, buffer was added to reach a final volume of 300 ml, and the muscle was homogenized three times for 30 sec each time, using a Kinematica PT-600 Polytron at a setting of 4.5. The homogenates were centrifuged at 7500 × g_{av} for 20 min and the supernatants were discarded. The pellets were then rehomogenized (3 × 30 sec each time) in 300 ml of buffer, using the Polytron at a setting of 5.5. The homogenates were then centrifuged at 11,000 × g_{av} for 20 min. After filtering of the supernatants through four layers of cheesecloth, crude microsomal vesicles were obtained by sedimentation at 85,000 × g_{av} for 30 min in a Beckman type 45Ti rotor. The pellets were resuspended (using a 20-ml syringe and a blunt-tipped stainless steel needle) in 0.3 M sucrose, 10 mM imidazole/HCl, pH 7.4, containing freshly diluted PIs (see above). Crude microsomal vesicles were either immediately subfractionated on discontinuous sucrose gradients or divided into 100-μl aliquots, flash frozen in dry ice/acetone, and stored at −70° for later use.

To obtain subfractions of the skeletal SR vesicles, 20–25-mg portions of the crude microsomal vesicles were layered onto discontinuous sucrose gradients consisting of (from bottom to top) 5 ml of 1.5 M, 7 ml of 1.2 M, 7 ml of 1.0 M, and 7 ml of 0.8 M sucrose in isolation buffer. The gradients were then centrifuged in a Beckman SW-28 swinging bucket-type rotor at 110,000 × g_{av} for 2 hr. The membrane fractions sedimenting at the sucrose interfaces were collected by aspiration. After dilution by approximately 1/3 with 10 mM imidazole/HCl buffer, the vesicle subfractions were recovered by sedimentation at 100,000 × g_{av} for 30 min in a Beckman type 45Ti rotor. The pellets were then resuspended at a protein concentration of 4–8 mg/ml in isolation buffer containing freshly diluted PIs and were stored at −70°. The skeletal junctional SR membrane fraction sedimenting at the 1.2 M/1.5 M sucrose interface was identified as the fraction highest in [^{3}H]ryanodine-binding activity. The membrane vesicles sedimenting at the 1.0/1.2 M sucrose interface were also enriched in [^{3}H]ryanodine-binding activity and were used in some of the experiments described. The small differences in functional effects of the ryanoids on these two fractions were completely accounted for by the differences in JSRV content, as judged from maximal ryanodine binding. For the various preparations of skeletal muscle JSRV, maximal ryanodine binding was 6.5–12 pmol/mg of protein (subfractions 3 and 4) in the present studies.

Isolation of cardiac SR membrane vesicles. Cardiac SR membrane vesicles were prepared as described (33, 34). All buffers and solutions contained the PIs PMSF (230 μM) and leupeptin (1.1 μM). Canine ventricular tissue (250 g) was homogenized in 10 mM $NaHCO_3$ using a Quisinart food processor. Approximately 40 g of the homogenate were diluted into 200 ml of 10 mM $NaHCO_3$/PI solution and the membranes were disrupted (3 × 30 sec) using a Kinematica PT-600 Polytron generator at a setting of 5.5. The homogenate was centrifuged twice at low speeds (8500 × g_{av} and then 12,000 × g_{av}) for 20 min in a Beckman type JA-10 rotor, to remove contaminating cell particles, nuclei, and mitochondria from the SR-containing supernatant. Supernatant fractions were transferred to Beckman type 19 tubes, and crude SR vesicles were isolated by centrifugation at 27,500 × g_{av} for 30 min in a Beckman type 19 rotor. The isolated vesicles were washed twice with a buffer containing 0.6 M KCl and 30 mM histidine, pH 7.3, by sedimentation at 46,000 × g_{av} in a Beckman type 45Ti rotor. Finally, the vesicles were resuspended in 0.25 M sucrose, 10 mM histidine, pH 7.4, and stored in small aliquots at −70°.

To obtain subfractions of the crude cardiac SR, the vesicles described above were Ca^{2+}/oxalate loaded as described (35, 36). Loaded vesicles were resuspended in 1.5 M sucrose and layered at the bottom of discontinuous sucrose gradients consisting of (from bottom to top) 7 ml of 1.0 M, 7 ml of 0.8 M, 7 ml of 0.6 M, and 5 ml of 0.25 M sucrose in high ionic strength buffer. SR vesicle fractions were separated by floating the vesicles at 110,000 × g_{av} in a Beckman SW-28 rotor. Fractions were collected from the gradient interfaces, washed with ice-cold H_2O, resuspended in 0.25 M sucrose, 10 mM histidine, pH 7.4, and stored in small aliquots at −70° until used. JSRV fractions were identified as those fractions enriched in ryanodine-binding activity and exhibiting ryanodine-augmented ATP-dependent Ca^{2+} accumulation activity. The 1.0 M/1.5 M interface (fraction D) was typically most enriched in JSRV, although the 0.8 M/1.0 M interface (fraction C) was also enriched in JSRV. The small differences in functional effects of the ryanoids on these two fractions were completely accounted for by the differences in JSRV content, as judged from maximal ryanodine binding. For the various preparations of cardiac JSRV in the present study, this was 6–10 pmol/mg of protein.

Protein determination. Protein concentrations were determined according to the method of Lowry *et al.* (37), using bovine serum albumin as a standard.

Ryanodine relative binding affinity assay. Equilibrium ryanoid binding was determined by the following method, modified from that of Lattanzio *et al.* (12). Skeletal or cardiac SR vesicles (100 μg/ml) were incubated for 2 hr at 37° in the presence of 6.7 nM [^{3}H]ryanodine and the specified concentrations of the designated unlabeled ryanoids, in a binding buffer consisting of 0.5 M KCl, 20 mM Tris·HCl, pH 7.4, and 200 μM $CaCl_2$. Nonspecific binding was determined by adding 1 μM unlabeled ryanodine to the incubation medium. After incubation, samples were filtered under vacuum through Whatman GF/C filters (0.45 μm), using a Brandel model M-24R cell harvester. The protein was then rinsed twice with 3 ml of ice-cold binding buffer and placed in scintillation vials with 7 ml of scintillation cocktail. The samples were vortexed and allowed to stand overnight before bound [^{3}H]ryanodine was determined by liquid scintillation counting. Competitive displacement curves, IC_{50} values, and dissociation constants (K_d values) were calculated using the coupled binding/analysis programs EBDA/LIGAND (38). Relative binding affinities were determined by comparing the IC_{50} values and the K_d values of the ryanodine derivatives with the IC_{50} and K_d values of the unlabeled parent molecule, ryanodine.

Passive Ca^{2+} efflux. Ca^{2+} efflux from passively loaded skeletal JSRV was determined using the following method adapted from those of Meissner and co-workers (11, 39). Skeletal JSRV (3–5 mg/ml) were passively equilibrated with Ca^{2+} by incubation for 2 hr at room temperature in a loading solution containing 0.14 M NaCl, 20 mM HEPES, pH 7.0, 1.1 mM $CaCl_2$ (containing trace $^{45}Ca^{2+}$), 0.1 mM EGTA, and 1 mM $MgCl_2$. The specified concentrations of designated ryanoids were added at the beginning of the 2-hr incubation as 1/20 dilutions of 20-fold concentrated stocks in 10% methanol aqueous solutions.

After incubation the amount of Ca^{2+} loaded was determined by dilution of a 5-μl aliquot of the incubation medium into 3.0 ml of ice-cold stop solution (loading solution containing 10 μM ruthenium red and 5 mM $MgCl_2$) and immediate filtration under vacuum through Whatman GF/C glass fiber filters (0.45-μm pore diameter). The dilution tube was rinsed twice with 3 ml of ice-cold rinse solution (stop solution without ruthenium red), and these rinses were poured over the filter.

Ca^{2+} efflux was determined by diluting 5 μl of the JSRV-containing loading buffer 100-fold into efflux solution (loading solution containing 0.2 mM $CaCl_2$ and 1 mM EGTA) to initiate efflux. Efflux was terminated after 3 sec by addition of 3 ml of ice-cold stop solution. Filtration and washing were carried out as indicated above. Ca^{2+} remaining in the JSRV on the filters was determined by liquid scintillation counting. Experiments in which AMP-PCP was used were identical except that the loading buffer contained 1 mM AMP-PCP.

ATP-dependent Ca^{2+} accumulation assay. Ca^{2+} accumulation by cardiac JSRV was assessed as described (40). JSRV (15 μg of membrane protein/ml) were preincubated for 10 min at 37° in uptake buffer (0.1 M KCl, 50 mM histidine, pH 6.8, 6.5 mM $MgCl_2$, 3 mM Tris-oxalate, 0.5 mM EGTA, 0.5 mM $CaCl_2$, including trace $^{45}Ca^{2+}$) containing the specified concentrations of the designated ryanoids. Accumulation was initiated by addition of 5 mM Tris-ATP. At 0-, 10-, and 20-min time points after the addition of ATP, intravesicular Ca^{2+} was determined by filtering a 100-μl aliquot, under vacuum, through Whatman GF/C filters (0.45-μm pore diameter). Filters were rinsed twice

with 3 ml of ice-cold uptake buffer, and $^{45}Ca^{2+}$ remaining in the vesicles on the filter was determined by liquid scintillation counting.

TLC. TLC was performed on glass plates coated with silica gel containing a 254-nm fluorescent indicator (Sigma). The mobile phase consisted of chloroform/methanol/40% aqueous methylamine in a 85:15:2 ratio (31).

Silica gel column chromatography. Silica gel column chromatography was performed on Silicar (100–200 mesh, type 60 Å special) silica gel (Mallinckrodt, Paris KY), using the TLC mobile phase described above. The various ryanoids collected in the column effluents were identified by TLC, as described above.

HPLC. Analytical HPLC and preparative HPLC were carried out as described (41). Analytical HPLC used a Nova-Pak C18 (4-μm) 8-mm × 100-mm Radial-Pak radial compression liquid chromatography cartridge in a RCM 8-mm × 10-mm cartridge holder (Waters, Inc., Milford, MA). The mobile phase used was 50% aqueous methanol, at a flow rate of 1.0 ml/min.

Preparative HPLC was performed using a C18 μBondapak (10 μm) 25-mm × 100-mm semipreparative radial compression liquid chromatography cartridge with a C18 μBondapak Guard-Pak insert in a RCM PrePak 25-mm × 10-mm cartridge holder (Waters Inc.). A mobile phase of 50% aqueous methanol was used at a flow rate of 10 ml/min.

Structural analyses. Structures of the ryanoids used in the present studies were confirmed by proton NMR spectra recorded on a Bruker model WM-250. Chemical shifts were evaluated in δ ppm values, relative to deuterated chloroform (δ 7.26) or methanol (δ 3.35) as internal standards. The masses of the ryanoids were evaluated from spectra obtained with a ZAB-1F mass spectrometer. Physico-chemical data are given in the legend to Fig. 2.

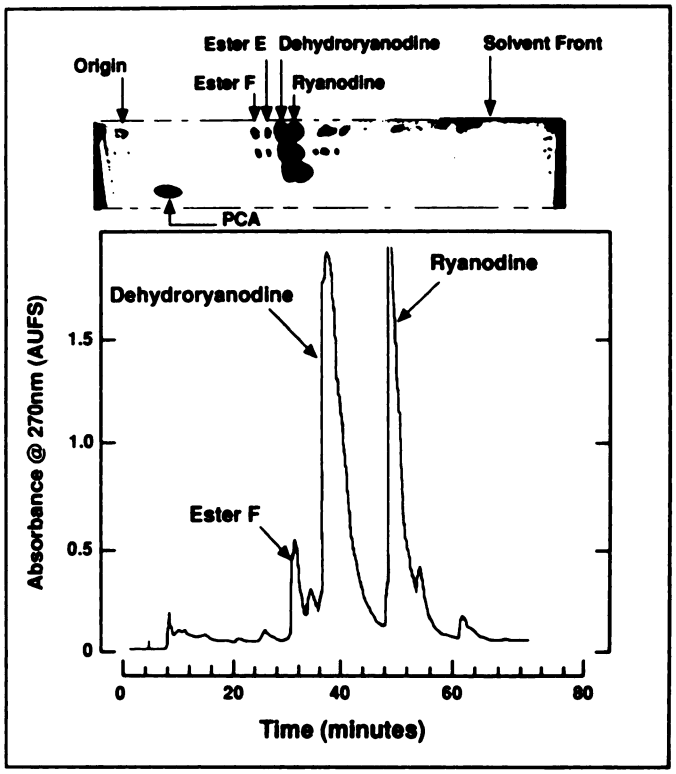

Fig. 1. Chromatograms of crude extracts of *R. speciosa* wood. *Upper*, typical TLC plate viewed under UV light. *Lanes* (from *top* to *bottom*) *1* and *2*, crude *Ryania* extracts; *lane 3*, reference commercial ryanodine (S.B. Penick & Co.); *lane 4*, pyrrolecarboxylic acid (*PCA*), the UV-absorbing C_3 substituent of ryanoids. The TLC chromatogram demonstrates the presence of UV-absorbing constituents both more polar and less polar than the major alkaloids ryanodine and dehydroryanodine. *Lower*, HPLC chromatogram of the same sample, confirming the presence of the ryanoids seen on the TLC chromatogram.

Results

Isolation and identification of the novel ryanoids. Esters E and F were originally noted on TLC chromatograms of organic solvent extracts of the crushed wood of *R. speciosa* Vahl, and their presence was confirmed by reverse phase HPLC of the extracts. In contrast to previously identified natural ryanodine metabolites, which are all less hydrophilic than ryanodine, both newly recognized compounds are more hydrophilic than ryanodine and its equally active congener dehydroryanodine (Fig. 1). Designation of the analogs as ester E and ester F was made in keeping with nomenclature previously established for secondary metabolites of *Ryania* (31).

Ester E and ester F were separated in bulk (by silica gel column chromatography) from the major constituents ryanodine and dehydroryanodine and from the other components present in the crude extracts. Fractions containing the isolated compounds ryanodine, dehydroryanodine, ester E, and ester F were collected individually and purified further by semipreparative HPLC. The purity of samples after freeze-drying was confirmed by analytical HPLC to be ≥98%. For esters E and F, HPLC was repeated until they were ≥99% pure. The structures of the ryanoids were determined by mass spectroscopy and proton NMR spectroscopy. Ester E was identified as C_{9ax}-hydroxyryanodine and ester F as C_{8ax}-hydroxy-C_{10}-epi-dehydroryanodine (Fig. 2).

Relative binding affinity of the ryanoids. The binding affinities of esters E and F were determined from their relative abilities to compete with 6.7 nM [^{3}H]ryanodine for binding to junctional SR membranes. At this low concentration of [^{3}H] ryanodine, interaction of these compounds with the receptor is selectively measured at only the high affinity ryanodine binding site (K_d = 1 to ~10 nM). Averaged competition displacement curves in skeletal JSRV are shown in Fig. 3 for ester E and ester F, as well as for unlabeled ryanodine. The shapes of the displacement curves were similar for all compounds. Mean K_d and IC_{50} values for each of the compounds and for pure dehydroryanodine in both skeletal and cardiac vesicles are given in Table 1. Ryanodine and dehydroryanodine yielded typical values, with K_d values of 4.4 nM and 5.4 nM, respectively. Ester E bound with 15-fold lower affinity than did ryanodine, and ester F bound with 58-fold lower affinity. Binding assays using cardiac JSRV produced an equivalent pattern, except that all compounds tested exhibited somewhat higher affinities for cardiac receptors (Table 1).

Activator and deactivator actions of the ryanoids on skeletal JSRV. A previous report by Sutko *et al.* (33) indicated that, of the several natural ryanodine analogs in *Ryania* extracts (31), the least polar (ester A) appeared to act selectively as an activator of SR Ca^{2+} release channels. We reasoned that the polar *Ryania* secondary metabolites ester E and ester F might also exhibit unidirectional actions and that they might be relatively selective for deactivation. To assess both activator and deactivator actions of the newly identified esters, their abilities to alter Ca^{2+} efflux rates from Ca^{2+}-loaded skeletal JSRV were evaluated. Skeletal JSRV passively equilibrated with 1.1 mM $CaCl_2$ and then exposed to an efflux solution designed to favor competence of the SR Ca^{2+} release channels showed slowed calcium efflux rates (Fig. 4). In preliminary

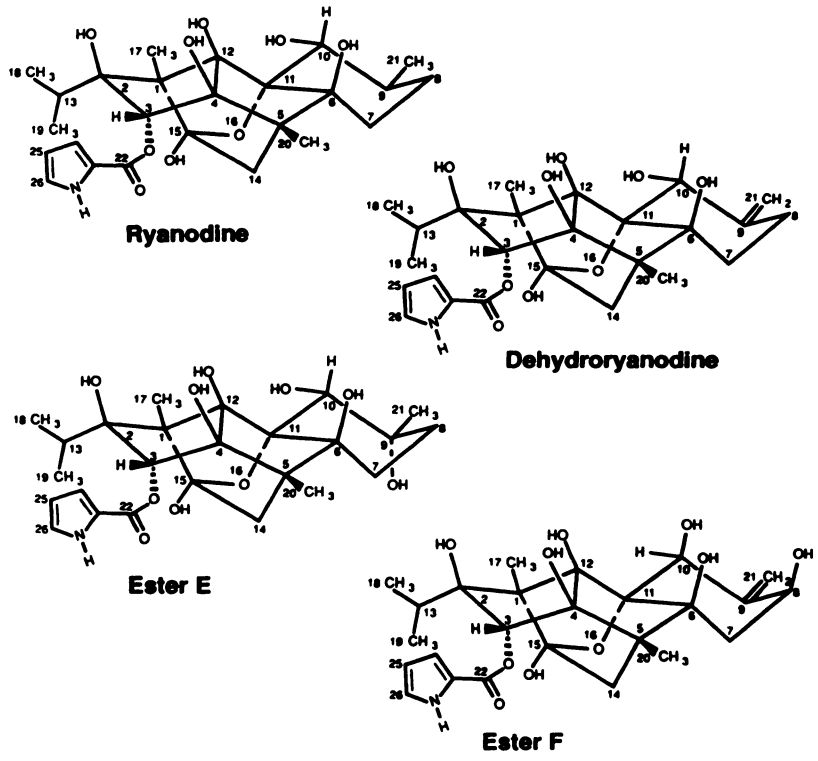

Fig. 2. Chemical structures of the ryanoids in the present study. Physico-chemical data were as follows. Ester E: R_F, 0.40; m.p. 195–197°; ^{1}H NMR (CD_3OD): δ 7.03, 6.87, and 6.24 (3 dd for pyrrole ring hydrogens), 5.63 (s, 1 H, HC3), 3.99 (s, 1 H, HC10), 2.59 (d, 13.9 Hz, 1 H, H_AC14), 2.24 (qn, 1 H, HC13), 2.15 (m, 2 H), 1.97 (d, 13.9 Hz, 1 H, H_BC14), 1.92 (m, 1 H), 1.62 (m, 1 H), 1.41 (s, 3 H, CH_3C1), 1.22 (s, 3 H, CH_3C9), 1.10 (d, 6.7 Hz, 3 H, CH_3C13), 0.91 (s, 3 H, CH_3C5), and 0.74 (d, 6.4 Hz, 3 H, CH_3C13); mass spectroscopy (m/z): 509 (M^+); exact mass calculated for $C_{25}H_{33}O_9N$ ($M^+ - H_2O$), 491.2155; found, 491.2147. Ester F: R_F, 0.36; m.p. 185–188°; ^{1}H NMR (CD_3OD): δ 7.02, 6.86, and 6.23 (3 dd for pyrrole ring hydrogens), 5.66 (s, 1 H, HC3), 5.24 (d, 1.6 Hz, 1 H, H_AC21), 5.21 (d, 1.6 Hz, 1 H, H_BC21), 4.53 (narrow m, 1 H, HC8), 4.42 (br s, 1 H, HC10), 2.62 (d, 13.9 Hz, 1 H, H_BC14), 2.33 (dd, 13.7 Hz and 4.3 Hz, 1 H, $H_{ax}C7$), 2.22 (qn, 6.5 Hz, 1 H, HC13), 1.84 (d, 13.9 Hz, 1 H, H_BC14), 1.77 (dd, 13.7 and 2.4 Hz, 1 H, $H_{eq}C7$), 1.34 (s, 3 H, CH_3C5), and 0.73 (d, 6.5 Hz, 3 H, CH_3C13); mass spectroscopy (m/z): 507 M^+ (weak) and 471 ($M^+ - 2H_2O$); exact mass calculated for $C_{25}H_{29}O_6N$ ($M^+ - 2H_2O$), 471.1893; found, 471.1905.

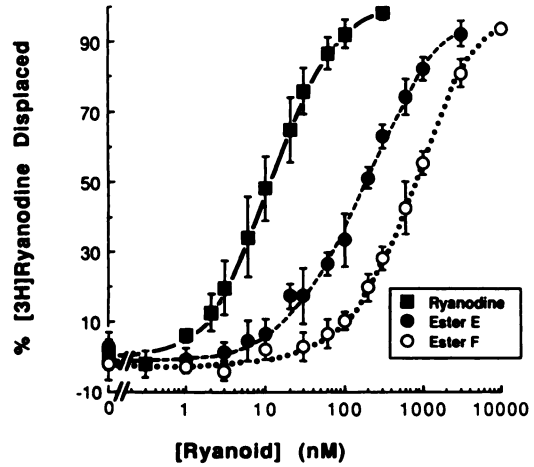

Fig. 3. Displacement of [^{3}H]ryanodine from the high affinity binding sites on the Ca^{2+} release channel/ryanodine receptor of skeletal JSRV by the unlabeled alkaloids ryanodine, ester E, and ester F. Equilibrium ryanoid binding was determined by incubating skeletal JSRV (100 μg/ml) for 2 hr at 37° in the presence of 6.7 nM [^{3}H]ryanodine and the specified concentrations of the designated unlabeled ryanoids, in a buffer consisting of 0.5 M KCl, 20 mM Tris·HCl, pH 7.4, and 200 μM $CaCl_2$, as described in Experimental Procedures. Relative IC_{50} and K_d values (see Table 1) were determined using the coupled binding/analysis programs EBDA/LIGAND (38). Values represent means ± standard deviations for at least four separate membrane preparations.

TABLE 1

Relative binding affinities of the ryanoids

Equilibrium ryanoid binding was determined by incubating JSRV (100 μg/ml) for 2 hr at 37° in the presence of 6.7 nM [^{3}H]ryanodine and the specified concentrations of the designated unlabeled ryanoids. Nonspecific binding was determined with 1 μM unlabeled ryanodine. IC_{50} and K_d values were calculated using the coupled binding/analysis programs EBDA/LIGAND (38). K_d estimates were based on initial assigned ryanodine K_d values of 4.0 nM for skeletal muscle JSRV and 1.0 nM for cardiac muscle JSRV. Values shown are means ± standard deviations.

Ryanoid	IC_{50}	K_d
	nM	nM
Skeletal JSRV ($n \geq 3$)[a]		
Ryanodine	11.9 ± 1.6	4.4 ± 0.8
Dehydroryanodine	16.1 ± 4.1	5.4 ± 0.1
Ester E	185 ± 31	65 ± 10
Ester F	749 ± 158	257 ± 53
Cardiac JSRV ($n \geq 2$)		
Ryanodine	4.9 ± 0.12	0.51 ± 0.01
Dehydroryanodine	6.9 ± 0.01	0.8 ± 0.03
Ester E	93 ± 6	12 ± 0.4
Ester F	461	57

[a] n, number of separate determinations on different membrane preparations; individual assays contained at least 10 duplicate points.

experiments, the calcium efflux rate in efflux medium without ryanoids was found to follow an approximately exponential decline, requiring at least 2 min to achieve steady state; control vesicles lost 21 ± 5% of their calcium load in a 3-sec efflux period (four experiments).

Increasing concentrations of ryanodine induced incrementally faster calcium efflux rates as SR Ca^{2+} release channels became increasingly activated. Half-maximal activation of efflux required a ryanodine concentration of 2.5 μM. The rightward shift of the concentration-effect curve for Ca^{2+} release (Fig. 4), compared with the binding displacement curve (Fig. 3), is explained by the suboptimal ryanoid binding milieu of the loading buffer mandated by the passive efflux assay. The lower ionic strength and pH and the higher free Ca^{2+} and Mg^{2+} concentrations all attenuate equilibrium ryanodine binding.

At its peak activator effect, ryanodine (30 μM) increased the calcium efflux rate >20-fold under the present conditions. A fraction of the intravesicular Ca^{2+} (ordinarily about 15–25% of

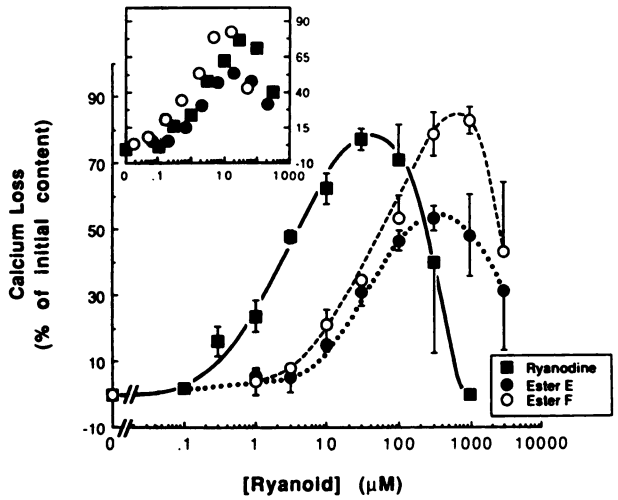

Fig. 4. Biphasic effects of the ryanoids on passive Ca^{2+} efflux from skeletal JSRV. The EC_{50} values for activation by ryanodine and by ester F lay about 100-fold to the left of those for deactivation; for ester E, the shift was only 20-fold. Skeletal JSRV (3–5 mg/ml) were passively equilibrated with Ca^{2+} by incubation for 2 hr at room temperature in a loading solution containing 0.14 M NaCl, 20 mM HEPES, pH 7.0, 1.1 mM $CaCl_2$ (trace $^{45}Ca^{2+}$), 0.1 mM EGTA, and 1 mM $MgCl_2$, in the absence or presence of the specified concentrations of the designated ryanoids. Ca^{2+} efflux was determined by diluting 5 μl of the JSRV-containing loading buffer into 500 μl of efflux solution (loading solution containing 0.2 mM $CaCl_2$ and 1 mM EGTA) to initiate efflux. Efflux was terminated after 3 sec by addition of 3 ml of ice-cold stop solution (loading solution containing 5 mM $MgCl_2$ and 10 μM ruthenium red) and immediate filtration, as described in Experimental Procedures. Values are means ± standard deviations (except where the variance was too small to be represented) from at least four different vesicle preparations. Overlapping error bars are not shown. Control (zero) values were set by subtracting the efflux occurring in 3 sec in the absence of any ryanoid. The mean value of zero efflux was 21 ± 5% for four separate JSRV preparations. *Inset*, data normalized for the observed differences in relative binding affinities among the ryanodine congeners (1:15:58; see Fig. 3). Normalization in this manner leaves ryanodine unmoved from its position on the *abscissa* and permits display of the effects of esters E and F as a function of equivalent occupancy of the high affinity receptor site. Error bars were omitted from the *inset* to improve clarity. *Inset* ordinate identical to main figure; abscissa, normalized ryanoid concentration. For further explanation, see text.

the total initial intravesicular Ca^{2+} load, depending on the relative purity of the JSRV, as indicated in the figures) was insensitive to the effect of ryanodine. This residual Ca^{2+} could be completely released by the Ca^{2+} ionophore A23187 (20 μM) within a few seconds (data not shown). Above 30 μM, the activator actions of ryanodine abated, and deactivation of the SR Ca^{2+} release channels became evident. At the highest ryanodine concentrations used (1 mM), the Ca^{2+} release channel was functionally closed and no significant loss of calcium was evident. This same general pattern of activation and deactivation was seen with esters E and F (Fig. 4). Details of the pattern of activation and deactivation of the Ca^{2+} release channel, however, differed among the ryanoids.

Activation concentration-effect curves for the three compounds appeared to be parallel, having a slope of 40% Ca^{2+} loss/decade increment in ryanoid concentration. The EC_{50} values for channel activation by ryanodine, ester E, and ester F were 2.5 μM, 63 μM, and 43 μM, respectively. Interestingly, the relative activator potency of ester F was higher than that of ester E, and it was substantially greater than would be predicted if there were a direct correlation between binding affinity and release activity. The activation potency ratio for ryanodine, ester E, and ester F was 1:25:17, compared with the relative binding affinity ratio of 1:15:58. Additionally, the magnitude of activation by these compounds was not equivalent. Taking the maximum efflux of Ca^{2+} in the presence of ester F as 1.0, the fractional efflux due to ryanodine at its peak effect was 0.95 and that due to ester E was 0.71.

To normalize the data for differences in ryanoid affinities, their concentration-effect curves were adjusted for their K_d values (Fig. 4, *inset*). This normalization shifted the relative functional effect curve for ester F to the left of that for ryanodine. Thus, at equivalent fractions of high affinity receptor occupancy, ester F displayed greater capacity to activate Ca^{2+} efflux than did ryanodine. Ester F induced half-maximal opening of the channel at an affinity-normalized ryanoid concentration of 0.77 μM, compared with 2.5 μM for ryanodine (Fig. 4, *inset*). The affinity-normalized activity of ester F for activating the SR Ca^{2+} release channel was therefore 3.2-fold greater than that of ryanodine. Ester E had an affinity-normalized activity approximately half that of ryanodine.

The relative potencies for channel deactivation by esters E and F in the Ca^{2+} efflux assay were also both less than that of ryanodine. The estimated deactivation EC_{50} values were 285 μM, 1300 μM, and 3100 μM for ryanodine, ester E, and ester F, respectively, yielding a relative potency ratio of 1:5:11. Again, the relative potencies of esters E and F were greater than predicted from simple extrapolation of their relative abilities to compete for binding at the ryanoid high affinity binding site. The affinity-normalized deactivator data yielded apparent half-effective concentrations for channel closure of 62 μM for ester F and 115 μM for ester E, compared with 286 μM for ryanodine, all at presumably equivalent fractions of receptor occupancy. The mean values for midpoints of the deactivation curves permit calculation of the quotient (CCR) of the mean values for midpoints of the deactivation and activation curves (Table 2).

For ryanodine the CCR of the apparent EC_{50} values for deactivation and activation of the Ca^{2+} release channel was >100. The CCR was slightly less for ester F (72), but for ester E it was only 21. The CCRs thus varied by >500% among these ryanoids. This indicates that the two actions of the compounds are not invariantly coupled; rather, they display functional independence. The closer coupling of deactivation and activation for ester E could well account for the decreased effectiveness of ester E in activating the SR Ca^{2+} release channel.

Deactivator actions of the ryanoids on cardiac JSRV. Deactivator effects of ryanoids on cardiac Ca^{2+} release channels can be readily evaluated, using ATP-dependent Ca^{2+} accumu-

TABLE 2
CCR of the Ryanoids
Incubation conditions were as indicated in the legends to Figs. 4 and 5.

Skeletal JSRV Ca^{2+} efflux assay	Apparent EC_{50} for activation (*A*)	Apparent EC_{50} for deactivation (*B*)	CCR (*B*/*A*)
	μM	μM	
Fig. 4			
Ryanodine	2.5	285	114
Ester E	63	1300	21
Ester F	43	3100	72
Fig. 6			
Ryanodine control	3.1	225	73
Ryanodine + AMP-PCP	0.038	85	2237

lation assays. (On the other hand, quantitation of activator actions of the ryanoids on cardiac SR is vitiated by the high basal activation of cardiac muscle JSRV Ca^{2+} release channels.) In the absence of ryanodine, cardiac JSRV exhibit only modest ability to accumulate Ca^{2+} when ATP is added to activate turnover of the SR Ca^{2+} pump. In confirmation of initial reports (42, 40) and many more since then, increasing concentrations of ryanodine enhanced net Ca^{2+} accumulation by cardiac JSRV (Fig. 5). (Ca^{2+} accumulation is displayed as descending in this figure, to facilitate comparisons with the data of Fig. 4.) Although each ryanoid apparently has full efficacy to close cardiac Ca^{2+} release channels, we did not have a large enough supply of esters E and F to test concentrations above 1 mM.

As was the case for deactivation of skeletal JSRV Ca^{2+} release channels (Fig. 4), both ester E and ester F exhibited potencies less than that of ryanodine. The EC_{50} values for deactivation of cardiac channels were 72 μM, 190 μM, and 700 μM for ryanodine, ester E, and ester F, respectively. The relative molar deactivator potency ratio in cardiac JSRV of about 1:3:10 differs considerably from the relative binding affinity ratio of 1:23:112 in the same cardiac JSRV. Thus, binding affinity of these ryanoids at the high affinity ryanodine binding site is a poor predictor of their ability to close the SR Ca^{2+} release channel. Separation of the capacity of the ryanoids to bind to the high affinity site of cardiac Ca^{2+} release channels from their ability to affect channel performance can be displayed graphically by normalizing for the difference in binding affinities among the ryanoids (Fig. 5, *inset*). At equivalent fractions of receptor occupancy, both of the ryanodine metabolites displayed substantially greater capacity to diminish Ca^{2+} efflux than did ryanodine. Ester F and ester E induced half-maximal closure of the channels at affinity-normalized ryanoid concentrations of 5.5 μM and 7.6 μM, respectively, compared with 72 μM for ryanodine.

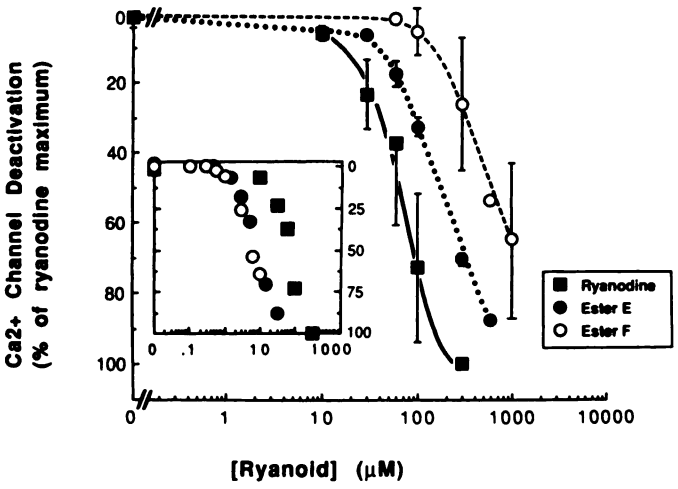

Fig. 5. Deactivator effects of the ryanoids on cardiac JSRV Ca^{2+} release channels, determined in ATP-dependent Ca^{2+} accumulation assays. The deactivation pattern among the ryanoids was similar to that for skeletal JSRV Ca^{2+} release channels. Cardiac JSRV (15 μg of membrane protein/ml) were preincubated for 10 min at 37° in uptake buffer containing 0.1 M KCl, 50 mM histidine, pH 6.8, 6.5 mM $MgCl_2$, 3 mM Tris-oxalate, 0.5 mM EGTA, 0.5 mM $CaCl_2$ (trace $^{45}Ca^{2+}$), and the specified concentrations of the designated ryanoids. Accumulation was then initiated by addition of 5 mM Tris-ATP. After 10 min, intravesicular Ca^{2+} was determined after filtration as described in Experimental Procedures. Values are means ± standard deviations (except where the variance was too small to be represented) from four different vesicle preparations. *Inset*, data normalized for the observed differences in relative binding affinities among the ryanodine congeners (1:23:112). Error bars were omitted from the *inset* to improve clarity.

Effects of AMP-PCP on activator and deactivator actions of ryanodine. Ryanodine-altered passive Ca^{2+} fluxes via JSRV Ca^{2+} release channels were sensitive to the presence of AMP-PCP (Fig. 6). Synergism between the two allosteric effectors of the channel was substantial. At a concentration of AMP-PCP (1 mM) that independently had only a limited net effect on channel permeability in the absence of ryanodine, the adenine nucleotide markedly enhanced the activity of ryanodine. The EC_{50} for ryanodine activation of the Ca^{2+} release channel in the presence of AMP-PCP, 0.038 μM, was almost 2 orders of magnitude less than that in the absence of AMP-PCP (Table 2). In preliminary experiments we have found that AMP-PCP may double equilibrium binding under the conditions used here for loading vesicles in preparation for efflux assays, i.e., 1.1 mM total Ca^{2+}, 1 mM Mg^{2+}, and intermediate ionic strength (0.14 M NaCl) (data not shown). These results confirm and extend the observations of others (11, 18, 20) showing that this adenine nucleotide augments or facilitates the effects of ryanodine.

The AMP-PCP data also demonstrate that nucleotide-induced augmentation of the effects of ryanodine occurs not only for activation of the channel but also for deactivation. However, the decrease in the apparent EC_{50} value for deactivation in the presence of 1 mM AMP-PCP was only 2.6-fold (Table 2). Therefore, AMP-PCP markedly diminishes concentration-coupling, effectively uncoupling deactivation from activation. In the presence of the nucleotide, maximal activation of the Ca^{2+} release channel occurred with as little as 1 μM ryanodine. Deactivation did not begin with less than a 60-fold higher concentration of ryanodine (Fig. 6). The variant CCRs (73 in the absence of AMP-PCP and >2000 in its presence) provide additional evidence that distinct binding sites or states

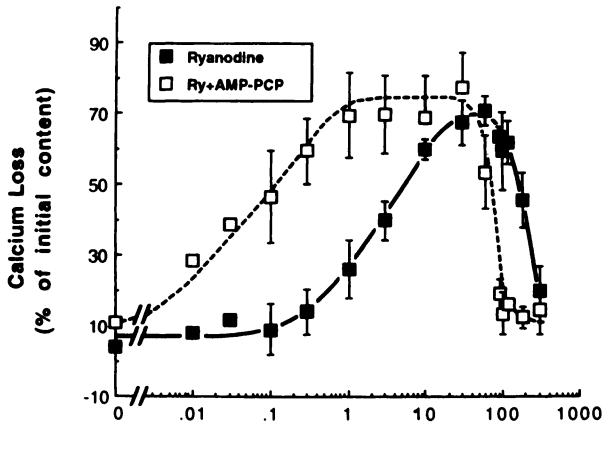

Fig. 6. Activator and deactivator effects of ryanodine on passive Ca^{2+} efflux from JSRV, in the absence (——) and presence (– – –) of 1 mM AMP-PCP. Activation EC_{50} values were 0.038 μM in the presence of AMP-PCP and 3.1 μM in its absence. Deactivation EC_{50} values were 85 μM and 225 μM, respectively. The CCR rose 31-fold, from 73 in the absence of the nucleotide to 2237 in its presence. Effects were measured as described in the legend to Fig. 4. Values are means ± standard deviations from at least four determinations per point.

are likely to be responsible for the two effects. Relative functional independence of the two binding sites is suggested, therefore, not only by the present data documenting differential intrinsic activities among natural ryanoids at the two sites but also by the differential CCRs between activator and deactivator effects of ryanodine in the presence of AMP-PCP. Relative functional independence of activation from deactivation is additionally substantiated by our preliminary reports on ester A (33) and C_{10eq}-*O*-aminoacyl-ryanodine derivatives possessing exclusively channel activator actions (28, 29).

Discussion

The results of the present investigations demonstrate that the novel, natural, *Ryania* secondary metabolites ester E and ester F bind specifically to high affinity sites of the SR Ca^{2+} release channel of JSRV and that they differentially induce activation and deactivation of these channels. The ryanoid CCR was almost 600% greater for ryanodine than for ester E; the CCR for ester F lay intermediate between them. Differential activating and deactivating effects were also documented for a single ryanoid by use of the adenine nucleotide AMP-PCP. This allosteric effector induced a 42-fold shift in the CCR for ryanodine (as well as for dehydroryanodine; data not shown). These findings establish that both relatively high affinity and pharmacologic diversity can exist among compounds active at the ryanodine binding site(s) of the SR Ca^{2+} release channel.

From the data of Fig. 3 and Table 1, the order of affinity of the ryanoids for the high affinity binding site on the SR Ca^{2+} release channel is ryanodine $\simeq$ dehydroryanodine $\gg$ ester E > ester F. The reduced affinities of esters E and F may be attributed to the structural differences between these analogs and their parent molecules. Ester E differs from its parent ryanodine by the addition of one functional group, an axial hydroxyl at C_9, projected to the α-plane of the molecule. This simple single alteration inhibits by 15-fold the binding of ester E to its receptor. Ester F differs from its parent molecule, dehydroryanodine, in two ways. First, the hydroxyl group at the C_{10} position is projected axially rather than equatorially. Second, ester F contains an additional hydroxyl group, projecting axially from the C_8 position. Although both changes in ester F place additional hydrophilic forces in the already hydrophilic β-plane of the dehydroryanodine molecule, their sum underlies the 60–100-fold reduced affinity of ester F.

In contrast to the relative order of binding affinities, the order of activator potencies (which presumably also reflects ryanoid actions at the high affinity site) is ryanodine $\gg$ ester F > ester E (Fig. 4). This inversion of potency order for high affinity binding (Fig. 3; Table 1) and high affinity functional effect is unexplained at present. Because the affinity values were estimated under conditions designed to maximize binding, whereas the functional studies were maximized for flux measurements, direct comparisons cannot easily be made between binding and functional data. Relative binding affinity assays using the assay conditions mandated by functional assays are poorly sensitive because [^{3}H]ryanodine binding is substantially reduced under these conditions. On the other hand, medium conditions optimal for the functional flux assays afford esters E and F little ability to competitively displace [^{3}H]ryanodine. We assume, as a first approximation, that the relative binding of the ryanoids is internally consistent under the differing conditions. Such an assumption permits reasonable interpretations of the initial data. Although it seems unlikely, the apparent differences in relative potencies might be due to shifts in the relative binding affinities, attributable only to the suboptimal binding conditions of the functional assays. Additional studies are underway to address these possibilities.

Given that the ryanoids share a proportional relationship between the binding affinities at the high and low affinity sites, the inversion of relative affinity order, compared with the activation potency order, for esters E and F confirms that ester F has a greater intrinsic activity for channel activation than does ester E. A compound with a lower relative affinity and a higher relative potency will, by definition, exhibit a higher intrinsic activity. Closer examination of the data, comparing the activation EC_{50} values for all three compounds, shows that at equivalent fractional occupancies of the high affinity receptor ester F is more effective than ryanodine and ester E is only slightly less effective (Fig. 4, *inset*).

Comparisons among the deactivator actions of the ryanoids introduce additional complexities. The deactivator potency order for both skeletal and cardiac JSRV Ca^{2+} release channels is ryanodine > ester E > ester F (see Figs. 4 and 5). This order is consistent with the interaction of the ryanoids at the high affinity binding site. However, functional data from many published studies suggest that a binding site with low affinity for ryanodine is responsible for its deactivator actions. Incubation conditions for the relative binding assay preclude the assessment of ryanoid action at low affinity sites. Differences in relative potencies identified at the low affinity deactivator site could be due to differential relative affinities of these compounds at that site. In the absence of data on this point, we assume that the relationship of relative binding affinities is internally consistent between the two sites. Thus, the differences in relative potencies may be taken to represent differences in intrinsic activities of the congeners. With both skeletal and cardiac Ca^{2+} release channels ester F possesses greater ability to deactivate the SR Ca^{2+} release channel than does ryanodine, at an equivalent fraction of occupancy of the high affinity binding sites.

When normalized for the differences in apparent K_d values, ester F displays greater capacity to activate as well as deactivate the SR Ca^{2+} release channel than does ryanodine. Ester E is a less effective channel activator than is ester F or ryanodine, whereas its ability to deactivate the SR Ca^{2+} release channel is only slightly less than that of ryanodine.

The effect of the ryanoids on Ca^{2+} efflux rates via the SR Ca^{2+} release channel (Fig. 4) can be explained as the algebraic sum of individual activation and deactivation concentration-effect curves for the ryanoids. Ryanodine itself produces full (or nearly full) activation because its activation curve does not appreciably overlap with its deactivation curve. With ester E the curves for the two components overlap substantially; deactivation begins well before maximal activation has been attained. This causes a significant decrease in the ability of ester E to activate the channel fully. The overlap could also be responsible for the attenuated slope of the deactivation curve for ester E. Ester F apparently exhibits little significant overlap between activation and deactivation curves and, hence, it not only activates maximally but also has a steep deactivation curve. The finding that these two components of the ryanoid concentration-effect curves apparently are shifted relative to

each other supports the suggestion that they are mediated by functionally independent sites.

Not only is the CCR of activation and deactivation inconsistent among the different ryanoids, it is alterable for a single ryanoid by simple manipulation of the incubation conditions. In the presence of the adenine nucleotide AMP-PCP, the CCR for ryanodine is >2000 (Table 2). This concentration-effect separation occurs because the activation curve is shifted 70-fold to the left, whereas the deactivation curve shifts <3-fold. Thus, neither for the novel ryanodine esters nor for ryanodine itself is coupling of activation and deactivation invariant. These data provide further evidence that functionally distinct binding sites or states are likely to be responsible for the two effects of ryanodine.

In summary, the close concentration-coupling that has been tacitly assumed for activator and deactivator effects of ryanoids no longer appears tenable. Alterations in the ryanodine molecule or even in the medium in which ryanodine is allowed to bind can preferentially affect the relative binding constants of the ryanoids for the SR Ca^{2+} release channel, as well as the potency of either the activator or the deactivator actions of these molecules. This strengthens the possibility that judicious chemical modification of ryanodine may produce derivatives that will selectively either only open or only close the SR Ca^{2+} release channel. This implication is supported by the results with ester A (33) and with our $C_{10}0_{eq}$-aminoacyl-ryanodine derivatives, which possess exclusively channel activator actions (28, 29, 43).

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Note added in proof. While this manuscript was under editorial consideration, we learned of work (Jeffries, P. R., W-W. Lam, R. F. Toia, and J. E. Casida. Ryania insecticide: structural assignments of four natural 8_{ax}-hydroxy-10-epiryanoids *J. Agric. Food Chem.* **40**:509–512 (1992)) suggesting a slight revision of the structure of ester A, confirmation of the structure of ester F, and reassignment of the abbreviation, ester E, to designate a ryanoid even more polar than ester F. In the present paper, we continue to use the abbreviations we have previously published (refs. 31, 32).

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Send reprint requests to: Henry R. Besch, Jr., Department of Pharmacology and Toxicology, Indiana University School of Medicine, 635 Barnhill Drive, Indianapolis, IN 46202–5120.