

# Tectoridins Modulate Skeletal and Cardiac Muscle Sarcoplasmic Reticulum Calcium-Release Channels<sup>1</sup>

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## ABSTRACT

The isoflavones tectoridin (TTR) and 3'-hydroxy TTR (3'-TTR) were isolated from an Ayurvedic herbal preparation *Vacã* and evaluated for their affinity and effect on ryanodine receptors (RyR) using junctional sarcoplasmic reticulum vesicles (JSRVs). In [<sup>3</sup>H]ryanodine displacement binding affinity assays, TTR and 3'-TTR exhibited IC<sub>50</sub> values of 17.3 ± 1.3 μM (K<sub>d</sub> = 6.7 ± 0.4 μM) and 6.6 ± 1.4 μM (K<sub>d</sub> = 2.4 ± 0.2 μM), respectively, for fast skeletal muscle RyR (RyR1) compared with an IC<sub>50</sub> value for ryanodine of 6.2 ± 0.4 nM (K<sub>d</sub> = 2.4 nM). TTR demonstrated a 3-fold higher affinity for cardiac RyR (RyR2) [IC<sub>50</sub> value of 5.2 ± 0.6 μM (K<sub>d</sub> = 0.95 ± 0.3 μM)] than for RyR1. The displacement isotherms for both TTRs paralleled that for ryanodine, consistent with the notion that all three are likely binding to similar site(s) on the receptors. Calcium efflux from and

calcium influx into JSRVs were used to measure function effects of TTRs on binding to RyR. In calcium efflux assays, TTR (up to 1 mM) enhanced the release of <sup>45</sup>Ca<sup>2+</sup> from JSRVs in a concentration-dependent manner (EC<sub>50act</sub> of 750 μM). Higher concentrations deactivated (partially closed) RyR1. 3'-TTR had similar effects, but was approximately 2-fold more potent, exhibiting an EC<sub>50act</sub> value of 480 μM. Using passive calcium influx assays, TTR activated and deactivated RyR1 in a time- and concentration-dependent manner. The aglycone tectorigenin also was effective in displacing [<sup>3</sup>H]ryanodine from RyR1 but not from RyR2. These results demonstrate that TTRs are capable of interacting at ryanodine binding sites to differentially modulate fast skeletal and cardiac calcium-release channels.

Release of calcium ions from internal sarcoplasmic reticulum (SR) is a key step in the cascade of events leading to striated muscle contraction (Berridge, 1997; Franzini-Armstrong and Protasi, 1997). Of the two types of calcium-release channels present on SR, namely inositol 1,4,5-trisphosphate receptors and ryanodine receptors (RyR), the latter is the major calcium-release channel involved in excitation-contraction coupling (Bers, 1991; Catterall, 1991). Thus, regulating RyR may be a strategy for beneficially altering the concentrations of intracellular calcium.

The plant alkaloid ryanodine is the best known exogenous ligand of RyR. At the single channel level, its effects are quantal, occurring in three discrete steps. Nanomolar con-

centrations increase open probability without changing conductance, low micromolar concentrations reduce conductance but increase open probability (modified state), and higher concentrations of the alkaloid lead to irreversible channel closure (Holmberg and Williams, 1990; Buck et al., 1992). Similar effects are also observed at the multichannel level (using SR membrane vesicles) where low concentrations of ryanodine activate or open RyR, whereas higher concentrations deactivate or close them (Lattanzio et al., 1987; Hummerickhouse et al., 1993). Ryanodine cannot be considered a therapeutic lead compound because it activates and deactivates RyR at relatively similar concentrations and it is extremely toxic (Waterhouse et al., 1987; Besch et al., 1994). Also, its complex structure (heptacyclic, polyhydroxy diterpene) limits facile chemical synthesis; to date, only its less active C<sub>3</sub> epimer has been produced (Ruest and Deslongchamp, 1993). The need to identify additional compounds with ryanodine-like actions, but simpler pharmacodynamics and chemical structures, is warranted.

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**ABBREVIATIONS:** SR, sarcoplasmic reticulum; RyR, ryanodine receptors; TTR, tectoridin; 3'-TTR, 3'-hydroxy TTR; RyR1, skeletal muscle ryanodine receptor; RyR2, cardiac muscle ryanodine receptor; JSRV, junctional sarcoplasmic reticulum vesicles; TLC, thin-layer chromatography; CV, crude sarcoplasmic reticular membrane vesicles; PMSF, phenylmethyl sulfonyl fluoride; JSRV, junctional SR membrane vesicles; E1c, CHCl<sub>3</sub> extract.

In the Ayurvedic literature, ground rhizomes of *Acorus calamus* Linn (referred to as *Vacā*) are indicated for treatment of the symptoms of several illnesses including inflammation and constipation. *Vacā* is also used as an expectorant, stomachic, and anticonvulsant (Nadkarni, 1954). Recently, Panchal et al. (1989) showed that ethanolic extracts of rhizomes of *Acorus calamus* Linn provided protection against strychnine-induced convulsions in frogs. These researchers also found that extracts of *Acorus calamus* Linn produce negative chronotropic and inotropic effects in frog hearts. Neither the mechanism(s) underlying the decrease in rate and force of cardiac contractions nor the component(s) responsible for these effects has yet been defined.

Some years ago, we reported that at low concentrations ( $\leq 1 \mu\text{M}$ ), ryanodine induces transient negative inotropic effects on cat ventricular papillary muscles (Sutko et al., 1979). Very recently, Ju and Allen (1999) showed biphasic chronotropic effects of ryanodine on bullfrog cardiac nodal tissue. Based on these reports, we hypothesized that the negative inotropic and chronotropic effects of extracts of *Acorus calamus* Linn on cardiac muscle might be attributable to component(s) with ryanodine-like actions.

This study was undertaken to isolate principal constituents from extracts of *Acorus calamus* Linn that may bind to and functionally modulate calcium flux via RyR calcium-release channels.

## Experimental Procedures

### Materials

Ryanodine used in this study was isolated from chipped *Ryania* wood supplied by Integrated Biotechnology Corporation (Carmel, IN) and purified by chromatography to  $\geq 98\%$  (Bidasee et al., 1993). Ground rhizomes of *Acorus calamus* Linn were obtained from Star West Botanical Inc. (Rancho Cordova, CA) in 1993 under the label *Vacā*. [ $^3\text{H}$ ]Ryanodine (specific activity 87 Ci/mmol) and  $^{45}\text{CaCl}_2$  (specific activity 2.7 Ci/mmol) were purchased from NEN Life Science Products (Boston, MA). Methohexital sodium (Brevital) was obtained from Eli Lilly & Co. (Indianapolis, IN). Precoated silica gel plates (with fluorescence indicator) were obtained from Sigma (St. Louis, MO). All other reagents and solvents used were of analytical grade.

### Isolation and Characterization of Ryanodine-Like Constituents from *Vacā*

Briefly, 100 g of *Vacā* was extracted by stirring overnight in 500 ml of ethanol. The next day, the ethanol was filtered and rotary evaporated to dryness to produce 1.1 g of residue. This residue (E1) was dissolved in 50 ml of water and extracted three times with 75 ml of chloroform ( $\text{CHCl}_3$ ), each time retaining both the aqueous and organic layers. The pooled aqueous fractions were then freeze-dried, producing 410 mg of a pale yellow powder designated E2w. On rotary evaporation, the  $\text{CHCl}_3$  extract (E1c) produced 550 mg of an oily liquid.

E2w was separated further into various fractions by redissolving in 2 ml of  $\text{CHCl}_3$ /methanol (MeOH) (90:10) and chromatographing on a silica gel column (50 g). Sequential elution with six solvent combinations of increasing polarity (100 ml each) was as follows:  $\text{CHCl}_3$  containing 2 drops of triethylamine, the same but with 2% MeOH, 4% MeOH, 6% MeOH, 10% MeOH, and 15% MeOH. Fractions eluted from the column with the latter two solvent combinations were pooled and rotary evaporated to dryness affording 210 mg (E2w1). E2w1 was separated further by semipreparative, high-performance liquid chromatography using MeOH/water (42:58) as the mobile phase into three compounds: E2w1a (~2.0 mg), E2w1b (25 mg), and E2w1c (40 mg).  $R_f$  values of these three compounds on thin-layer

chromatography (TLC) plates using  $\text{CHCl}_3$ /MeOH/40% aqueous methylamine (85 parts:15 parts:2 drops) as the mobile phase were E2w1a (0.15), E2w1b (0.36), and E2w1c (0.44).

Crystallization of E2w1c from MeOH/ $\text{CHCl}_3$  (90:10) produced pale white needles, m.p. 250 to 252°C (uncorrected). Melting point of E2w1b (blue-white flakes after freeze-drying from dioxane) was 120°C (uncorrected). Carbon, hydrogen, nitrogen elemental analysis, NMR, and mass spectrometry were used to elucidate the chemical structures of E2w1b and E2w1c.

### Preparation of JSRVs from Rabbit Skeletal Muscle (RyR1)

Crude sarcoplasmic reticular membrane vesicles (CVs) were prepared as described previously (Humerickhouse et al., 1993, 1994). Briefly, after induction of anesthesia with methohexital sodium, fast-twitch skeletal muscles (perivertebral and hind limb) from rabbits were removed, homogenized at speed setting 4.5 with a Kinematica PT-600 homogenizer (Polytron, Patterson, NJ) in isolation buffer [0.3 M sucrose, 10 mM imidazole-HCl, 230  $\mu\text{M}$  phenylmethylsulfonyl fluoride (PMSF), 1.1  $\mu\text{M}$  leupeptin, pH 7.4], and then centrifuged at 7500 $g_{av}$  for 20 min. The supernatant was discarded, and the pellet was resuspended in fresh isolation buffer, homogenized for a second time at speed setting 5.5, and centrifuged at 11,000 $g_{av}$  for 20 min. The supernatant was filtered through gauze (cheese cloth), and CVs were obtained by sedimentation at 85,000 $g_{av}$  for 30 min. The CVs were resuspended in isolation buffer, quick-frozen in dry ice-acetone, and stored at  $-80^\circ\text{C}$ .

JSRVs were prepared by layering CVs onto discontinuous sucrose gradients (1.5, 1.2, 1.0, and 0.8 M sucrose in isolation buffer, top to bottom layers, respectively) and centrifuged at 110,000 $g_{av}$  for 2 h. The vesicles that sedimented to the interface between 1.2 and 1.5 M sucrose were collected, resuspended in 30 ml of isolation buffer, and harvested by centrifugation at 110,000 $g_{av}$  for 2 h. The resultant pellet was resuspended in fresh isolation buffer, quick-frozen, and stored at  $-80^\circ\text{C}$ . Protein concentrations were determined later (Lowry et al., 1951).

### Preparation of CVs from Canine Heart (RyR2)

SR membrane vesicles were prepared from canine heart as described (Humerickhouse et al., 1993). Briefly, after deep anesthesia with methohexital sodium, hearts were removed from two dogs and placed into ice-cold saline solution. Ventricular tissues were collected, stripped of adhering adventitia, and homogenized in a buffer consisting of 10 mM  $\text{NaHCO}_3$ , 230  $\mu\text{M}$  PMSF, and 1.1  $\mu\text{M}$  leupeptin, pH 7.4, using a Kinematica PT-600 homogenizer (Polytron) at a speed setting of 6.0 ( $3 \times 30$  s). The homogenate was then centrifuged twice at low speeds (first at 8,500 $g_{av}$  and then at 12,000 $g_{av}$ ) for 20 min each to remove mitochondria, nuclei, and other contaminating debris. Crude SR vesicles were harvested by sedimenting the supernatant at 27,500 $g_{av}$  for 30 min. The vesicles were resuspended in buffer containing 0.6 M KCl, 30 mM histidine, 230  $\mu\text{M}$  PMSF, and 1.1  $\mu\text{M}$  leupeptin, pH 7.4, and then centrifuged at 46,000 $g_{av}$  for 30 min. The pellet of membrane vesicles was resuspended in storage buffer (0.25 M sucrose, 10 mM histidine, 230  $\mu\text{M}$  PMSF, and 1.1  $\mu\text{M}$  leupeptin, pH 7.4) at a concentration of 8 to 10 mg/ml, quick-frozen, and stored at  $-80^\circ\text{C}$  until use.

### Pharmacological Profiles of Purified Ryanodine-Like Constituents from *Vacā*

The principal ryanodine-like compounds isolated and characterized from *Vacā* were the two isoflavones TTR (E2w1c) and its congener 3'-hydroxy TTR (3'-TTR; E2w1b).

**Relative Binding Affinity Assays.** The affinities of TTR and 3'-TTR for RyR1 and RyR2 RyR were assessed from their ability to compete with [ $^3\text{H}$ ]ryanodine for binding to these receptors. Briefly, JSRVs prepared from rabbit fast skeletal muscle or membrane vesicles from canine ventricular muscle (0.1 mg/ml) were incubated in binding buffer, called buffer A for later comparison, consisting of 500

mM KCl, 20 mM Tris-HCl, 0.2 mM CaCl<sub>2</sub>, pH 7.4, at 37°C, containing 6.7 nM [<sup>3</sup>H]ryanodine with increasing concentrations of either TTR or 3'-TTR (up to 200 μM) for 2 h at 37°C. Nonspecific binding was determined simultaneously by incubating vesicles with 500 μM TTR or 3'-TTR as appropriate. This concentration of 3'-TTR and TTR was chosen for determining nonspecific binding because it routinely displaces ≥96% of [<sup>3</sup>H]ryanodine bound to RyR. At the end of the incubation time, the samples were filtered through GF/C filters (0.45 μ; Whatman International, Maidstone, England) using a cell harvester (model M-24R; Brandel Biomedical Research, Gaithersburg, MD), and the vesicles remaining on the filter paper were washed three times with 3 ml of ice-cold binding buffer (pH 7.4 at 0°C). The filters were then placed in scintillation cocktail, vortexed, and allowed to stand overnight before liquid scintillation counting to determine the amount of [<sup>3</sup>H]ryanodine bound. This protocol was chosen for equilibrium displacement binding, because in previous studies, we found under these conditions that >80% of Ca<sup>2+</sup>-dependent ryanodine binding to RyR occurred (Bidasee et al., 1994; Emmick et al., 1994). Also, under these conditions 6.7 nM [<sup>3</sup>H]ryanodine binds primarily to high-affinity ryanodine binding site(s) on RyR (Zhang et al., 1999). Displacement curves, IC<sub>50</sub>, and K<sub>d</sub> values were calculated using the binding analysis programs PrismPad 2.0 (PrismPad Software Inc., San Diego, CA) and EBDALigand (MacPherson, 1985). For comparison, displacement binding with the prototype drug ryanodine was run concurrently in each assay.

Because TTR possesses an isoflavone moiety, experiments were also conducted to determine whether the decrease observed in [<sup>3</sup>H]ryanodine binding might be attributable to the ability of TTR to interact with hyperreactive sulfhydryls on RyR. For these studies, binding was conducted as described above, except that 2 mM glutathione (reduced form) was added to the binding buffer before beginning the incubation (Zable et al., 1997).

**Passive Calcium Flux Assays.** The abilities of TTR and 3'-TTR to alter ensemble functional patency ("openness") of RyR1 were assessed using passive calcium efflux as well as passive calcium influx assays.

*Passive calcium efflux assays.* JSRVs prepared from rabbit fast skeletal muscles (3.5 mg/ml) were preincubated for 2 h at 22°C in calcium loading buffer [140 mM NaCl, 20 mM HEPES, 1.1 mM Ca<sup>2+</sup> (spiked with 0.25 μM <sup>45</sup>Ca<sup>2+</sup>), 0.1 mM EGTA, and 1 mM MgCl<sub>2</sub>, pH 7.0 at 22°C] in the presence of varying concentrations of TTR and 3'-TTR (up to 5 mM). At the end of the preincubation, passive calcium (<sup>45</sup>Ca<sup>2+</sup>) efflux through RyR1 was determined by diluting the vesicles (5 μl) 100-fold into an efflux buffer (140 mM NaCl, 20 mM HEPES, 0.2 mM Ca<sup>2+</sup>, 1 mM EGTA, and 1 mM MgCl<sub>2</sub>, pH 7.0 at 22°C). Efflux was allowed to continue for 3 s and then stopped by additionally diluting the vesicles 6-fold into an ice-cold stop solution (140 mM NaCl, 20 mM HEPES, 0.1 mM EGTA, 5 mM MgCl<sub>2</sub>, 25 μM ruthenium red, and 250 μM LaCl<sub>3</sub>) and rapidly filtering. The vesicles on the filters were then washed three times with 3 ml of rinse solution (identical with stop solution except without ruthenium red), and the <sup>45</sup>Ca<sup>2+</sup> remaining inside the vesicles was determined by liquid scintillation counting. In this assay, the amount of calcium efflux occurring through RyR1 in 3 s is inversely related to the amount of calcium (<sup>45</sup>Ca<sup>2+</sup>) remaining in the vesicles. Because calcium efflux may be different from different vesicular preparations (reflecting, for example, different densities of RyR), drug-induced releases were taken as those greater than the amount released in the absence of TTR and 3'-TTR. For comparison, ryanodine-induced calcium releases from JSRV preparations were conducted in parallel. Maximum releasable calcium (full agonist efficacy) was defined as before (Bidasee and Besch, 1998) from the amount of release that occurs in the presence of 60 μM C<sub>10</sub>-O<sub>eq</sub> guanidinopropionyl ryanodine and 1 mM β,γ-methylene adenosine 5'-triphosphate (AMP-PCP) determined for each vesicle preparation.

*Passive calcium influx assays.* To further characterize the deactivating (partial channel closing) effects of TTR on its binding to RyR1, we developed and used passive calcium influx assays (Besch et al.,

1995). Briefly, JSRVs (5.0 mg/ml) were resuspended in binding buffer called buffer B (250 mM KCl, 15 mM NaCl, 20 mM HEPES, 25 μM CaCl<sub>2</sub>, pH 7.0) along with either 6.0, 60, or 600 μM TTR. The samples were then incubated for up to 3 h at 37°C. After various incubation times (5, 10, 15, 30, 60, and 120 min), aliquots of the vesicles (5 μl) were diluted 100-fold into an influx buffer (250 mM KCl, 15 mM NaCl, 20 mM HEPES, 0.5 mM CaCl<sub>2</sub>, 0.125 μM <sup>45</sup>Ca<sup>2+</sup>, and 0.1 mM EGTA), and calcium influx was allowed to proceed for 5 s. This provides time-effect data by giving a snapshot of the ensemble patency of RyR1 channels that had been achieved during varying preincubation periods. Additional influx was terminated immediately by diluting the JSRVs 6-fold into an ice-cold stop solution (250 mM KCl, 15 mM NaCl, 20 mM HEPES, 0.1 mM EGTA, 5 mM MgCl<sub>2</sub>, 25 μM ruthenium red, and 250 μM LaCl<sub>3</sub>) and rapidly filtering. The vesicles were then washed three times with 3 ml of the stop buffer, and <sup>45</sup>Ca<sup>2+</sup> content inside the vesicles was determined by liquid scintillation counting. Nonspecific influx was determined by identical incubation of JSRVs in binding buffer to which 25 μM ruthenium red and 250 μM LaCl<sub>3</sub> had been added before initiation of the preincubation. In this assay, the amount of calcium (<sup>45</sup>Ca<sup>2+</sup>) fluxed into the vesicles is related directly to the ensemble functional patency (openness) of RyR1 when the snapshot is taken; the greater the amount of calcium (<sup>45</sup>Ca<sup>2+</sup>) found inside the vesicles, the more activated (opened) the RyR1 had been and vice versa.

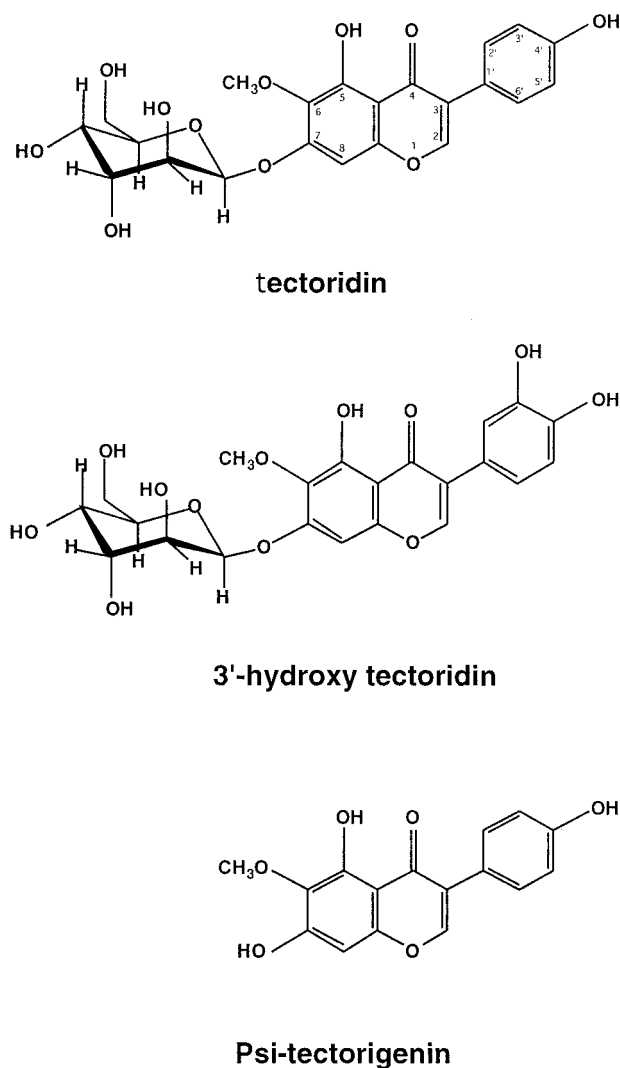
## Results

### Isolation and Identification of Ryanodine-Like Constituents from Vacă

During a preliminary screen, we found that an ethanol extract of the *Acorus calamus* Linn obtained under the Ayurvedic label, Vacă, displaced [<sup>3</sup>H]ryanodine from RyR1 in a concentration-dependent manner. We then set out to isolate and characterize the constituent(s) in this extract (E1) of Vacă responsible for this activity.

E1 contained several UV-active (365 nm) compounds with TLC R<sub>f</sub> values ranging from 0.3 to 0.9. The less polar of these compounds (TLC R<sub>f</sub> ≥ 0.7) were removed by dissolving E1 in water and then extracting it with chloroform. The major components of E1c were *cis/trans*-asarones (by comparison with authentic *cis/trans*-asarones) and in binding affinity; up to a concentration of 1000 μg/ml, these compounds did not demonstrate the ability to displace [<sup>3</sup>H]ryanodine from RyR1 (data not shown). We have not yet identified the other minor compounds present in E1c, although they also are likely to be essential oils (Mazza, 1985).

Analysis of the water-soluble components of Vacă (from ethanol extracts that had been previously extracted with chloroform (designated E2w) revealed the presence of three major UV-active compounds with R<sub>f</sub> values on TLC plates of 0.15, 0.36, and 0.44. Using reversed-phase (C<sub>18</sub>) high-performance liquid chromatography, we isolated and purified these three compounds and assigned them the labels E2w1a (2 mg), E2w1b (40 mg), and E2w1c (25 mg), respectively. Using carbon, hydrogen, nitrogen elemental analysis, <sup>1</sup>H and <sup>13</sup>C NMR spectrometry and mass spectrometry, E2w1c and E2w1b were identified as the isoflavones TTR (4*H*-1-benzopyran-4-one-3-(4'-hydroxy-phenyl)-5-hydroxy-6-methoxy-7-(β-D-glucopyranosyloxy) and its congener 3'-TTR (4*H*-1-benzopyran-4-one-3-(3', 4'-dihydroxy-phenyl)-5-hydroxy-6-methoxy-7-(β-D-glucopyranosyloxy), respectively (Fig. 1). We have yet to fully elucidate the structure of E2w1a.



**Fig. 1.** Chemical structures of the isoflavonoids isoflavones TTR and 3'-TTR isolated from *Vacá*. Tectorigenin is the 7-hydroxy aglycone of TTR. Configurations shown represent two-dimensional projections of three-dimensional, globally minimized structures using the algorithms of the software package CS ChemDraw Ultra, version 5 (Cambridge Software Corp., Cambridge, MA).

#### Relative Binding Affinities of TTR, 3'-TTR, and Ryanodine for RyR1

The affinities of the aqueous extract Ew2 and its principal constituents, TTR and 3'-TTR, were determined from their abilities to compete with 6.7 nM [<sup>3</sup>H]ryanodine for binding sites on RyR1, using displacement binding affinity assays (Fig. 2). In preliminary assays, E2w (water-soluble extract) displaced [<sup>3</sup>H]ryanodine from RyR1 binding site(s) in a concentration-dependent manner exhibiting an IC<sub>50</sub> value of 18 ± 2.0 μg/ml (Fig. 2, Δ). From this crude extract, 3'-TTR and TTR were purified to homogeneity. In displacement binding affinity assays, 3'-TTR (□) and TTR (●) also competed with [<sup>3</sup>H]ryanodine from binding sites on RyR1, exhibiting IC<sub>50</sub> values of 3.2 ± 1.1 and 8.0 ± 0.2 μg/ml. Because molecular masses are available for these compounds, their IC<sub>50</sub> values were calculated as 6.7 ± 1.4 μM (mol. wt. 478) and 17.3 ± 1.3 μM (mol. wt. 462) for 3'-TTR and TTR, respectively. Using the equation (Cheng and Prusoff, 1973):

$$K_d = IC_{50} / (1 + (L)/K_L) \quad (1)$$

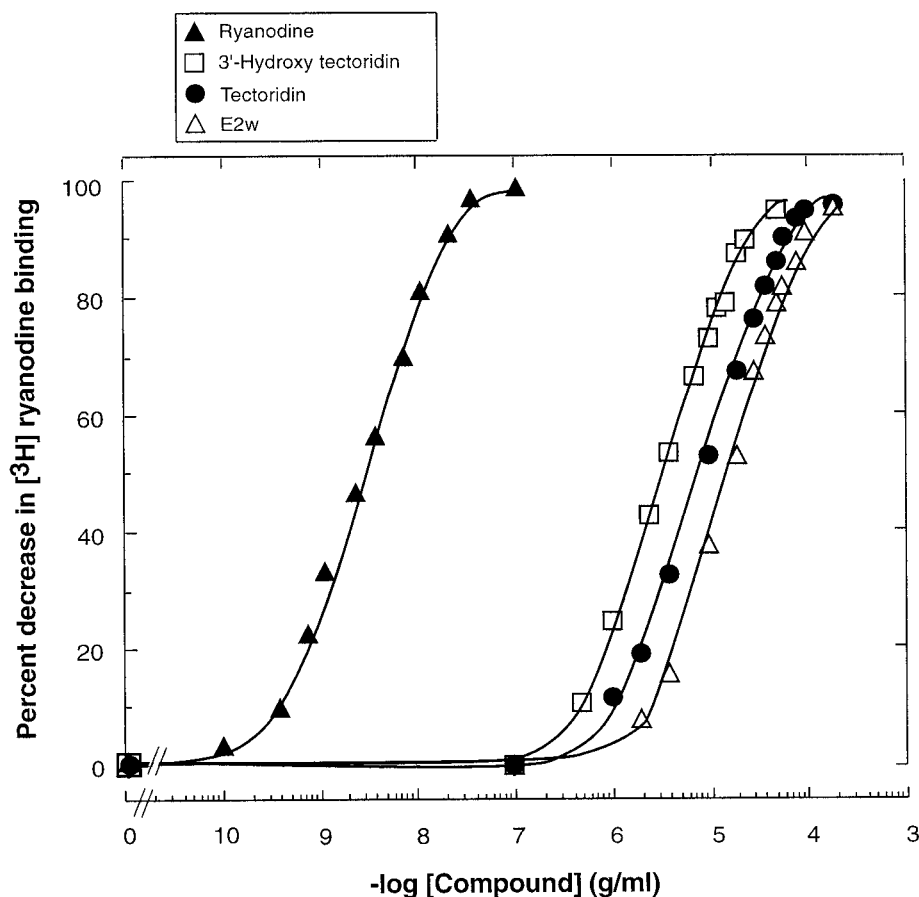
where IC<sub>50</sub> is the concentration of drug that displaces 50% of the [<sup>3</sup>H]ryanodine, *L* is the concentration of [<sup>3</sup>H]ryanodine (6.7 nM), and *K<sub>L</sub>* is the equilibrium dissociation constant of [<sup>3</sup>H]ryanodine (2.4 nM for RyR1; see Bidasee and Besch, 1998), the *K<sub>d</sub>* values for 3'-TTR and TTR are 2.4 ± 0.2 and 6.2 ± 0.4 μM, respectively. For comparison, the displacement isotherm for ryanodine is shown and, in this assay, exhibits an IC<sub>50</sub> value of 3.1 ± 0.2 ng/ml (IC<sub>50</sub> = 6.3 nM given mol. wt. of 493 and *K<sub>d</sub>* = 2.4 nM).

Although both TTR and 3'-TTR display affinities some three orders of magnitude less than that of ryanodine for RyR1, their binding isotherms paralleled that of ryanodine. Their displacement curves also appear saturable, indicating a finite population of high-affinity ryanodine binding site(s).

#### Relative Binding Affinities of TTR and Ryanodine for RyR2

The affinity of TTR for RyR2 was also estimated using equilibrium displacement binding affinity assays. As shown in Fig. 3 (●), TTR displaced [<sup>3</sup>H]ryanodine from RyR2 with an IC<sub>50</sub> value of 5.2 ± 0.6 μM. Thus, the affinity of TTR for RyR2 is almost three orders of magnitude less than that of ryanodine (IC<sub>50</sub> of 4.8 ± 0.2 nM and *K<sub>d</sub>* = 1.2 ± 0.1 nM). Using the Cheng-Prusoff equation above, TTR exhibits a *K<sub>d</sub>* value of 0.93 ± 0.3 μM for RyR2. Its binding isotherm also parallels that of ryanodine, suggesting that both compounds are binding to similar sites on the receptor. These data also indicate that TTR has an affinity for RyR2 3.5 times greater than its affinity for RyR1. Similar characteristics have also been noted with ryanodine in this as well as in previous studies (Humerickhouse et al., 1993). In addition, these data show that TTR displays apparent saturation kinetics, indicating a limited quantity of binding sites on RyR2 with affinity for isoflavones.

Various compounds with conjugated carbonyls (e.g., quinones) are known to alter the binding of [<sup>3</sup>H]ryanodine to RyR by interacting with hyperreactive sulfhydryl groups (oxidation/reduction reactions) (Abramson et al., 1988; Feng et al., 1999). Because TTR contains a conjugated carbonyl functionality, we sought to determine whether the decrease in [<sup>3</sup>H]ryanodine seen with TTR might be attributable to its ability to interact with sulfhydryl groups on RyR. The addition of 2 mM reduced glutathione (a nonspecific sulfhydryl reagent) to the binding buffer had no significant effect on the ability of TTR to displace [<sup>3</sup>H]ryanodine from RyR2 (Fig. 3, ○). Similar results were observed with RyR1 (data not shown). These results suggest that TTR decreases the binding of [<sup>3</sup>H]ryanodine from RyR by direct competition with [<sup>3</sup>H]ryanodine for binding site(s) on the receptors rather than by complexing with accessible sulfhydryl groups, altering the secondary structure of the receptors and limiting ryanodine access to its binding site(s). Higher concentrations of glutathione could not be used because we found that they directly inhibit binding of [<sup>3</sup>H]ryanodine to RyR. Such inhibitory effects of glutathione on [<sup>3</sup>H]ryanodine binding have been observed in previous studies (Zable et al., 1997). Although similar experiments with 3'-TTR have not yet been performed, there seems little reason to anticipate that reduced glutathione will alter the ability of this isoflavonoid to displace [<sup>3</sup>H]ryanodine from RyRs.



**Fig. 2.** Relative affinities of ryanodine ( $\blacktriangle$ ), 3'-TTR ( $\square$ ), TTR ( $\bullet$ ), and the ethanol extract of *Vacã*, E2w ( $\triangle$ ) for SR calcium-release channel from rabbit skeletal muscles (RyR1). Affinities were measured using displacement binding affinity assays. Skeletal muscle SR membrane vesicles (0.1 mg/ml) were incubated for 2 h at 37°C in the presence of 6.7 nM [ $^3$ H]ryanodine and increasing concentrations of test compound (up to 200  $\mu$ g/ml) in a buffer consisting of 500 mM KCl, 20 mM Tris-HCl, and 0.2 mM CaCl<sub>2</sub>, (pH 7.4 at 37°C). After incubation, the vesicles were filtered and washed with 3  $\times$  3 ml of the respective ice-cold buffer. Nonspecific binding was determined simultaneously by incubating vesicles with a concentration of test compound five times higher than that used in the displacement assay. [ $^3$ H]Ryanodine bound to RyR1 was determined by liquid scintillation counting. The data shown for each compound represent the mean of at least three experiments with at least two different membrane preparations. S.D. values were <7% for these experiments and are omitted for clarity.

### Functional Effects of TTR and 3' TTR on RyR1

Two different experimental protocols with JSRVs were used to evaluate the effects of 3'-TTR and TTR on binding to RyR1 at the multichannel level. These are passive calcium efflux and passive calcium influx assays. Passive calcium efflux assays are particularly useful for assessing the ability of compounds to activate (i.e., open) RyR1. To observe the activating or opening effects of ryanodine or other drugs, vesicles must be loaded with calcium. This requires conditions that coincidentally suppress ryanodine binding. To overcome this problem, high concentrations of drugs are required.

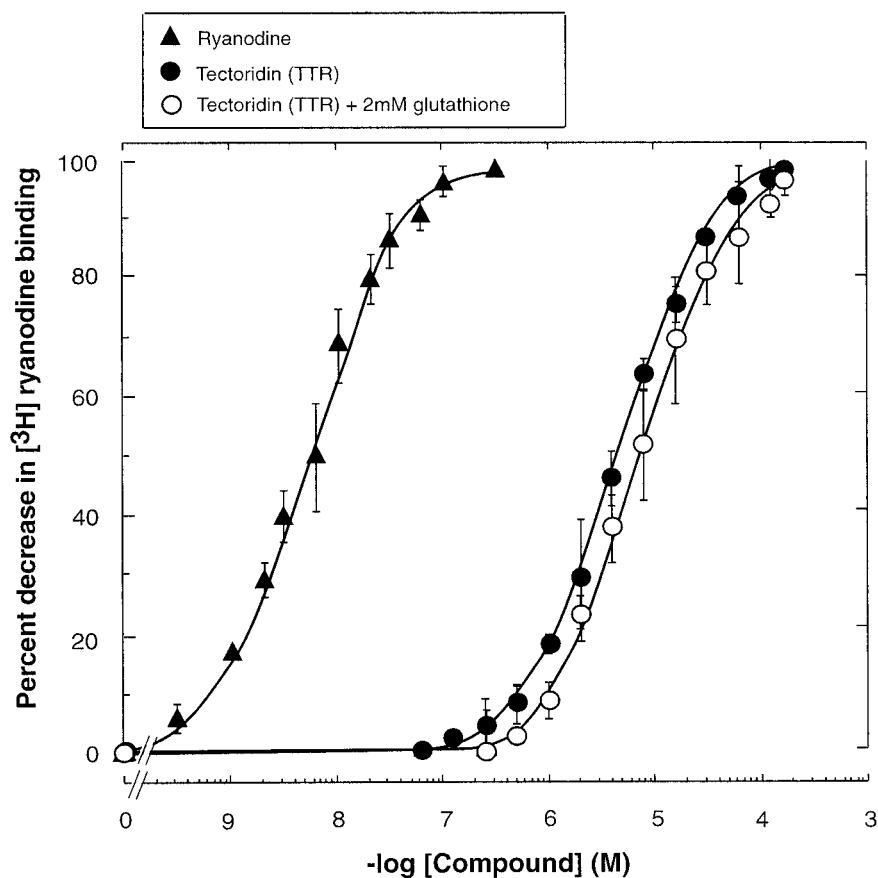
This calcium efflux assay, however, is less applicable for evaluating the ability of compounds to deactivate or close RyR1, especially for compounds with lower affinities and limited aqueous solubility, as seen with TTR and 3'-TTR. To more closely investigate the channel-deactivating effects of 3'-TTR and TTR, we developed and used passive calcium influx assays. In this assay, an intermediate concentration of Ca<sup>2+</sup> is used in binding buffer to partially activate (open) RyR1 in the control buffer, accentuating the ability to observe drug-induced alterations in the openness of the channels.

**Passive Calcium Efflux Studies: Channel-Activating Effects of TTR and 3'-TTR on RyR1 Are Emphasized in Passive Calcium Efflux Assays.** Because several different vesicular preparations were used for these studies, to account quantitatively for variations among preparations, calcium releases were calibrated as a function of maximum-releasable calcium (see *Experimental Procedures*). The calcium efflux buffer contains approximately 50 nM free cal-

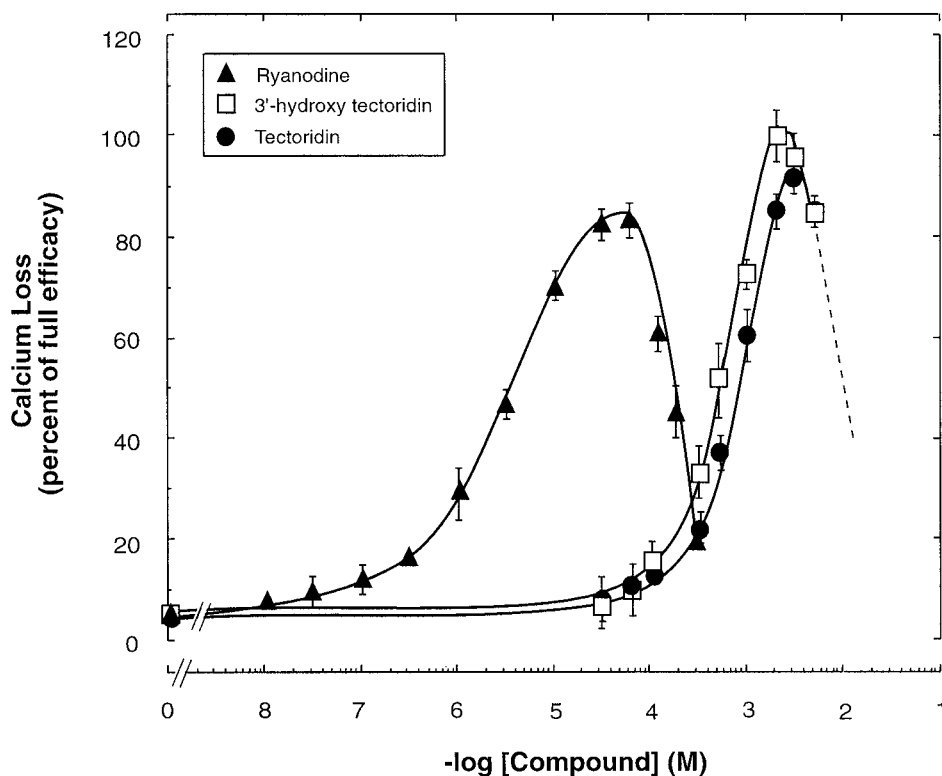
cium, which promotes closure of RyR1 and, as expected, vesicles diluted into this low calcium buffer in the absence of drug release less than 8% of their intraluminal calcium in 3 s (data not shown).

Like ryanodine, TTR and 3'-TTR enhanced calcium release from the vesicles (reflecting activation or opening of RyR1) in a concentration-dependent manner (Fig. 4). Concentration-effect curves of the isoflavonoids were shifted rightward to that of ryanodine. Although ryanodine exhibited an EC<sub>50act</sub> value of 2.3  $\mu$ M and triggered release of a maximum of 83% of the intravesicular calcium load at an optimal concentration of 60  $\mu$ M, 3'-TTR exhibited an EC<sub>50act</sub> value of 480  $\mu$ M and at 2 mM triggered complete loss of intravesicular calcium. TTR was slightly less potent than 3'-TTR, exhibiting an EC<sub>50act</sub> value of 750  $\mu$ M and at its optimal concentration of 3 mM, released a maximum of 93% intravesicular calcium. Although variations between maximal calcium release by 3'-TTR and TTR were not statistically significant ( $P < .05$ ), both maxima were significantly greater than that of ryanodine ( $P < .05$ ). Also, the slopes of the activation curves for 3'-TTR and TTR appeared to be steeper than that of ryanodine, suggesting that both compounds are more effective than ryanodine in activating RyR1.

TTR and 3'-TTR at concentrations higher than 3 mM appear to impede calcium efflux from the membrane vesicles, suggesting channel deactivation (closing) characteristics. Although we could readily determine an EC<sub>50deact</sub> value for ryanodine (210  $\mu$ M), it was not possible to ascertain experimental EC<sub>50deact</sub> values for TTR and 3'-TTR because of their limited aqueous solubilities. However, by extrapolation we



**Fig. 3.** Relative affinities of ryanodine ( $\blacktriangle$ ) and TTR ( $\bullet$ ) for SR calcium-release channel from canine cardiac muscles (RyR2). Experimental conditions were comparable with those described in the legend to Fig. 2. As indicated by the open circles, the addition of 2 mM reduced glutathione to the binding buffer did not significantly alter the ability of TTR to displace  $[^3\text{H}]$ ryanodine from RyR2.



**Fig. 4.** Effects of 3'-TTR ( $\square$ ), TTR ( $\bullet$ ), and ryanodine ( $\blacktriangle$ ) on  $\text{Ca}^{2+}$  efflux from rabbit skeletal muscle JSRVs (RyR1). Vesicles (3.5 mg/ml) were equilibrated with  $\text{Ca}^{2+}$  (containing trace amounts of  $^{45}\text{Ca}^{2+}$ ) by incubation for 2 h at room temperature in the presence of varying concentrations of the test compounds.  $\text{Ca}^{2+}$  efflux was initiated by diluting 5  $\mu\text{l}$  of incubation medium into 0.5 ml of efflux solution. Efflux was terminated after 3 s by the addition of 3 ml of ice-cold stop solution and immediate filtration. Values within each curve are mean  $\pm$  S.D. determined from at least three different efflux experiments using two different membrane preparations.

estimate their  $\text{EC}_{50\text{deact}}$  values at  $\sim 10$  mM (Fig. 4, dashed line).

Normalizing the calcium efflux profiles of TTR and 3'-

TTR based on their affinities relative to that of ryanodine suggest that TTR and 3'-TTR are more effective than ryanodine at calculated equivalent receptor half-occupancies

TABLE 1  
Activating potencies of tectoridin and 3'-TTR relative to that of ryanodine for RyR1

Compound	Affinity IC <sub>50</sub>	Activation Potency EC <sub>50act</sub>	Calculated Fold Loss in Affinity <sup>a</sup>	Expected EC <sub>50act</sub> Based on Fold Loss in Affinity <sup>b</sup>	Fold Increase in EC <sub>50act</sub> <sup>c</sup>
	nM	μM		μM	
Ryanodine	6.2	2.3	1.0	2.3	1.0
TTR	17300	750	2790	6417	8.6
3'-TTR	6600	480	1064	2447	5.1

<sup>a</sup> Affinity of compound/affinity of ryanodine.

<sup>b</sup> Affinity of compound × EC<sub>50act</sub> of ryanodine.

<sup>c</sup> Expected EC<sub>50act</sub> of compound/experimental EC<sub>50act</sub> of compound.

in their ability to activate RyR1, by 9- and 5-fold, respectively (Table 1).

**Passive Calcium Influx Assays.** To provide a wider margin for investigating the channel-deactivating effects of TTR, we used passive calcium influx assays. For this assay, an incubation buffer called buffer B (250 mM KCl, 15 mM NaCl, 20 mM HEPES, and 25 μM Ca<sup>2+</sup>, pH 7.0, at 37°C) was used (Zimanyi et al., 1991). As shown in Fig. 5A, after 2 h of incubation in buffer B, JSRVs bound 23% less [<sup>3</sup>H]ryanodine than JSRVs incubated in buffer A (500 mM KCl, 20 mM Tris-HCl, 200 μM CaCl<sub>2</sub>, pH 7.0, at 37°C). This difference, significant at  $P < .05$ , was secondary to altered calcium concentration. Increasing the calcium concentration in buffer B from 25 to 200 μM without changing any other constituents increased [<sup>3</sup>H]ryanodine binding by 27%, an amount equivalent to that produced with buffer A. These data confirm that the differences in [<sup>3</sup>H]ryanodine binding under these two ionic conditions is dependent primarily on the concentration of Ca<sup>2+</sup>. The apparent affinity of ryanodine for RyR1 under all three buffer conditions was similar (IC<sub>50</sub> ≈ 6.5 nM; Fig. 5B).

In our calcium influx assays, preincubation conditions are adjusted to achieve channels activated to 75% maximum. These conditions permit fairly ready access to ryanodine binding site(s), especially compared with supramaximal concentrations necessitated in calcium efflux assays. We anticipated and experimentally confirmed that lower concentrations of TTR would be more effective in calcium influx assays than in calcium efflux assays. Because the ryanodine EC<sub>50act</sub> was approximated by 600 μM TTR in calcium efflux assays, we used 600, 60, and 6 μM TTR in calcium influx assays. At these lower concentrations, time-effect data become crucial (Fig. 6). TTR at 6.0 μM did not significantly alter snapshot calcium influx until after more than 15 min of incubation. Appearing thereafter was a progressive increase in snapshot calcium influx after 30 and 60 min. After 1 h of preincubation with 6 μM TTR, snapshot calcium influx increased to a maximum of 18% over controls (JSRV not treated with TTR). These data clearly indicate that RyR1 channels that had been opened to 75% with 25 μM Ca<sup>2+</sup> were activated by 6.0 μM. The calibration scale for apparent channel opening as determined from [<sup>3</sup>H]ryanodine bound to RyR1 as a function of buffer calcium is indicated by on the right abscissa (Fig. 6) (see also Chu et al., 1990; Emmick et al., 1994). Although channel deactivation appears to intervene after 2 h of incubation with 6 μM TTR, this tendency did not achieve significance. These data suggest a rate that does not permit near-equilibrium binding in less than 2 h with 6 μM TTR.

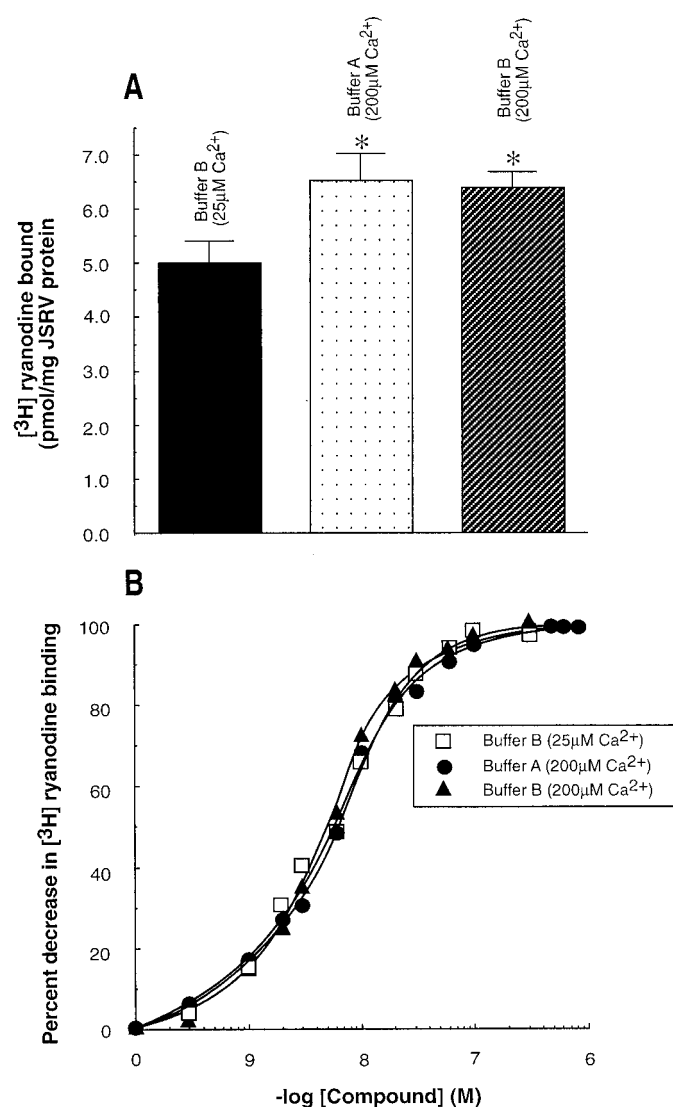
With a 10-fold higher concentration (60 μM TTR), the activating and deactivating effects of TTR on RyR1 become

readily apparent (Fig. 6, ●). After 5 min of preincubation with 60 μM TTR, snapshot calcium influx had increased by 18% over control. Additional increases in incubation time resulted in a marked time-dependent decrease in calcium influx to a nadir of 68% of full opening by 30 min. The deactivation appears stable for up to 2 h. With 600 μM TTR, only a monotonic, time-dependent decrease in snapshot calcium influx was evident; any preceding channel activation could not be discerned (Fig. 6, ●). After 5 min of incubation, calcium influx had already declined by 12%, revealing only 60% of full channel opening. Additional increases in incubation times with 600 μM TTR resulted in an additional time-dependent decrease in snapshot calcium influx. After 2 h, 600 μM TTR had effected almost complete closure of RyR1, allowing snapshot calcium influx of only 16%.

For comparison, the time-dependent closure of RyR1 channels with the known calcium channel modulator ryanodine is also shown (Fig. 6, ▲). After 5 min of incubation, ryanodine (1 μM) had reduced snapshot calcium influx by 15% (i.e., it had reduced channel activation to 54% of maximum). As the preincubation period with 1 μM ryanodine increased, snapshot calcium influx was diminished further, reaching 88% channel deactivation after 2 h of preincubation.

Thus, in calcium efflux and calcium influx assays, both ryanodine and TTR display channel-activating and -deactivating effects. Activation is experimentally emphasized in calcium efflux assays, whereas deactivation is more readily evident in calcium influx assays, apparently attributable to calcium concentration of the incubation medium. Although we have not fully characterized the functional effects of 3'-TTR, its structural similarity to TTR would support its similar actions on RyR1.

Because the deglycosylated derivative of TTR, tectorigenin, is commercially available, we sought to extend these studies and perhaps gain structure-activity insight using this aglycone. We assessed the affinity of tectorigenin from its ability to compete with 6.7 nM [<sup>3</sup>H]ryanodine for binding sites on RyR1 and RyR2 (Fig. 7). Tectorigenin displaced [<sup>3</sup>H]ryanodine from binding sites on RyR1 in a concentration-dependent manner. Near-maximal displacement was evident at 50 μM tectorigenin; displacement at 100 and 200 μM was not significantly higher. Thus, tectorigenin apparently competed for approximately half the available ryanodine binding sites on RyR1. Whether this is secondary to solubility constraints remains to be determined. Interestingly, displacement by tectorigenin appeared to be RyR isoform-specific. At concentrations up to 200 μM, tectorigenin exerted no ability to displace [<sup>3</sup>H]ryanodine from its binding sites on RyR2 (Fig. 7).



**Fig. 5.** Effects of incubation buffer calcium concentration on [<sup>3</sup>H]ryanodine to JSRVs. **A**, binding of [<sup>3</sup>H]ryanodine to JSRVs using two different ionic strength buffers with varying concentrations of calcium. Briefly, JSRVs (0.1 mg/ml) were incubated for 2 h at 37°C with 6.7 nM [<sup>3</sup>H]ryanodine either in buffer A (500 mM KCl, 20 mM Tris-HCl, and 200 μM CaCl<sub>2</sub>, pH 7.0) or in buffer B (250 mM KCl, 15 mM NaCl, 20 mM HEPES, pH 7.0) with 25 or 200 μM Ca<sup>2+</sup>. At the end of this time, the vesicles were filtered and washed with 3 × 3 ml of the respective ice-cold buffer. [<sup>3</sup>H]Ryanodine bound to ryanodine receptors on JSRVs was determined by liquid scintillation counting. Nonspecific binding was determined simultaneously by incubating vesicles with 1 μM ryanodine. Data shown are mean ± S.D. for four experiments done in duplicate using three different membrane preparations. Asterisks indicate significant differences at *P* < .05. **B**, displacement of [<sup>3</sup>H]ryanodine from high-affinity binding sites on RyR1 using buffer A (●), buffer B [5 μM Ca<sup>2+</sup>] (▲), or buffer B [200 μM Ca<sup>2+</sup>] (□). Briefly, JSRVs were incubated in respective buffer for 2 h at 37°C with 6.7 nM [<sup>3</sup>H] ryanodine and varying concentrations of ryanodine up to 300 nM. At the end of this time, the vesicles were filtered and washed, and [<sup>3</sup>H]ryanodine bound was determined by liquid scintillation counting. Nonspecific binding was determined simultaneously by incubating vesicles with 1 μM ryanodine. Data shown are mean ± S.E. for at least six experiments done in duplicate using three different membrane preparations. S.D. values were <6% for these experiments and are omitted for clarity.

## Discussion

The principal finding of this study is that TTR isoflavonoids bind to and activate and deactivate striated muscle

RyR. Furthermore, the data also support an isoform-specific effect of tectorigenin; it displaced [<sup>3</sup>H]ryanodine from binding sites on RyR1 but not those on RyR2.

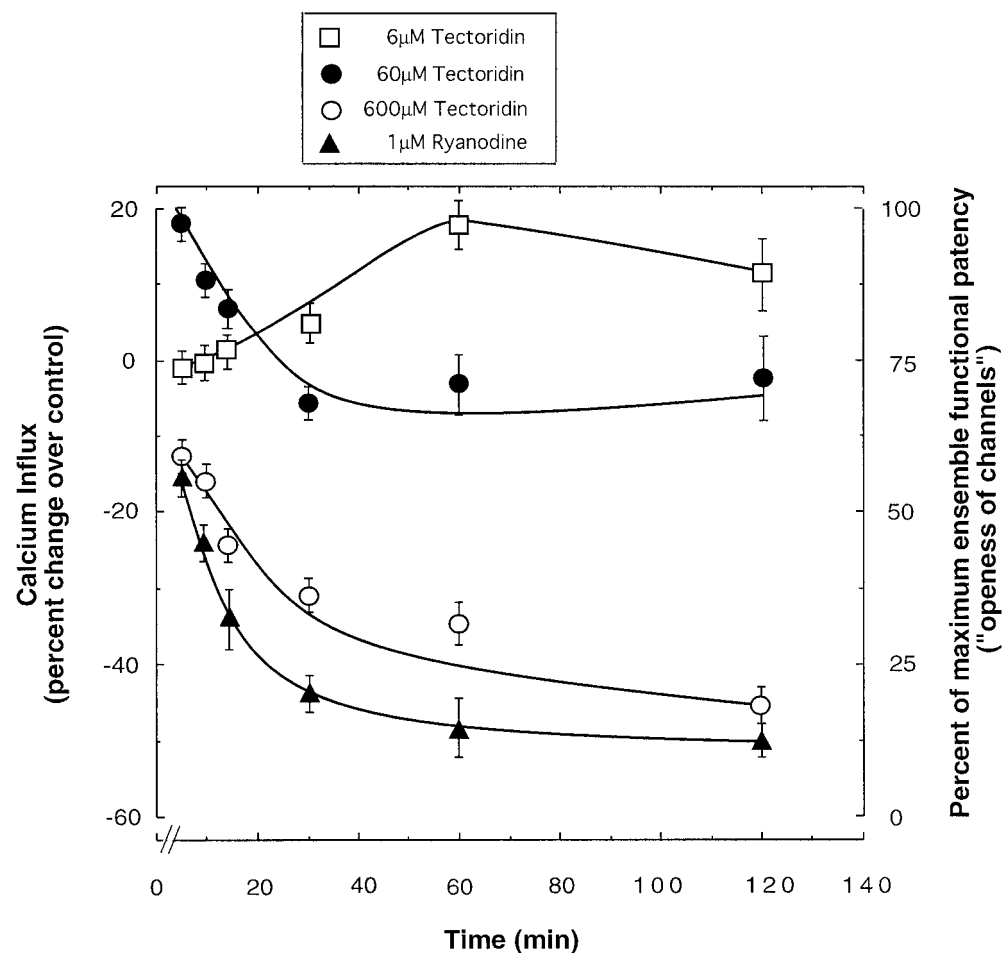
Compared with the molecular structure of ryanodine, TTRs are chemically simple molecules. Nevertheless, these compounds have been found in these studies to compete effectively with ryanodine for binding site(s) on striated muscle RyR. These results suggest that TTR and 3'-TTR possess unique chemical moieties in common with ryanodine or have functionalities with similar conformations. Removal of the sugar moiety on TTR significantly decreases affinity for RyR (*K<sub>d</sub>* decreased from 6.2 to greater than 100 μM). Thus, the glucose on TTR and 3'-TTR may play an important role in their ability to bind to RyR. It deserves mention that like glucose, the β-face of the ryanodine molecule also contains a cluster of hydroxyl functionalities. Recently, it was shown that oxidation of the hydroxyls on the C<sub>4</sub>, C<sub>10</sub>, and C<sub>12</sub> carbons of the ryanodine molecule also significantly reduce affinity for RyR (Jefferies and Casida, 1994).

Also of interest is the observation that the aglycone tectorigenin appears to have preferential affinity for RyR1 of fast skeletal muscles. The unexpected finding is especially intriguing because few isoform-specific exogenous ligands of RyR have been described. Imperatoxin activator was initially indicated to be specific for RyR1 (Valdivia et al., 1992); however, additional studies appear to contradict that notion (Tripathy et al., 1998). The early data support the notion that TTR or analogs thereof may be useful as lead compounds for ligands with specificity among RyR isoforms. In a recent study using rat hepatocytes, Tomonaga and coworkers (1992) found that tectorigenin was capable of mobilizing calcium from intracellular stores. However, these investigators found that the structurally related compounds genestein (also an isoflavone) and quercetin (a flavone) can also mobilize calcium from intracellular stores. Because quercetin appears to act on sarco(endo)plasmic reticulum Ca<sup>2+</sup>/Mg<sup>2+</sup>-ATPases, they inferred that the mode of action of tectorigenin might also be via inhibition of calcium pumps. This study demonstrates tectorigenin actions in the absence of calcium pump activity but does not rule out multiple modes of action on sarco(endo)plasmic reticulum Ca<sup>2+</sup>/Mg<sup>2+</sup>-ATPases.

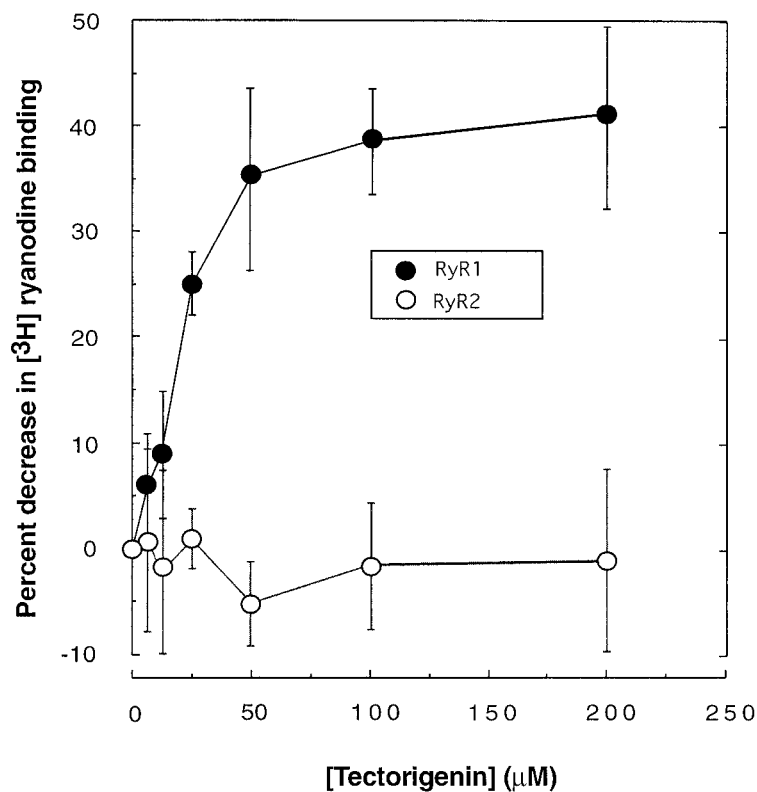
In previous studies we found that passive calcium efflux assays were sufficient to assess the functional effects of ryanoids on its binding to RyR1. However, efflux assays do not present optimal conditions for assessing channel deactivation effects, especially for those compounds that possess lower affinities (micromolar range) and have limited aqueous solubilities. To circumvent this restriction, we modified and optimized a calcium influx protocol described previously by Sutko and coworkers (Lattanzio et al., 1987) to better assess the ability of TTRs to promote closure of RyR1. Using the latter assay, we observed that TTR activates (opens) and deactivates (closes) RyR1 in a time and concentration manner.

In this assay, 1 μM ryanodine also exerts its deactivating effects on RyR1 after only 5 min of preincubation. These data suggest a fairly rapid rate of binding of ryanodine to RyR1 under conditions of the influx assay (low intravesicular calcium and high ambient ionic strength). It is relevant that two recent studies showed that micromolar luminal calcium concentrations can increase open probability of RyR1 activated by ATP and calcium (Herrmann-Frank and Lehmann-Horn, 1996; Tripathy and Meissner, 1996).





**Fig. 6.** Effect of TTR on snapshot calcium influx through RyR1 of JSRVs from rabbit skeletal muscles. In this assay, membrane vesicles (5.0 mg of protein/ml) were incubated with varying concentrations of TTR [6  $\mu$ M ( $\square$ ), 60  $\mu$ M ( $\bullet$ ), and 600  $\mu$ M ( $\circ$ )] in buffer B containing 250 mM KCl, 15 mM NaCl, 20 mM HEPES, 25  $\mu$ M  $\text{CaCl}_2$  (pH 7.0) for varying preincubation periods at 37°C. After the indicated intervals, the TTR-bound vesicles (5  $\mu$ l) were diluted into an influx buffer containing 250 mM KCl, 15 mM NaCl, 20 mM HEPES, 0.5 mM  $\text{CaCl}_2$ , 0.1 mM EGTA, and  $^{45}\text{Ca}^{2+}$  (0.125  $\mu$ M  $^{45}\text{Ca}^{2+}$  or 1  $\mu$ l  $^{45}\text{Ca}$ /ml influx buffer), and influx was allowed to proceed for 5 s. Influx was terminated immediately by diluting the vesicles 40-fold into an ice-cold stop solution (250 mM KCl, 15 mM NaCl, 20 mM HEPES, 0.1 mM EGTA, 5 mM  $\text{MgCl}_2$ , 250  $\mu$ M  $\text{LaCl}_3$ , and 25  $\mu$ M ruthenium red). The vesicles were then filtered rapidly and washed with  $3 \times 3$  ml of stop buffer, and  $\text{Ca}^{2+}$  content of the vesicles was determined by liquid scintillation counting. Data shown for each concentration represent the mean  $\pm$  S.D. for at least three experiments with at least two different membrane preparations. Ryanodine ( $\blacktriangle$ ) was used in this experiment as a positive control, because this compound is a known deactivator of RyR.



**Fig. 7.** Relative affinity of tectorigenin for SR calcium-release channel from rabbit skeletal muscles (RyR1) and canine cardiac muscles (RyR2). Experimental conditions were comparable with those described in the legend to Fig. 2. Nonspecific binding was determined simultaneously by incubating vesicles with 1  $\mu$ M ryanodine. [ $^3\text{H}$ ]Ryanodine bound to RyR1, and RyR2 was then determined by liquid scintillation counting. The data shown for each compound represent the mean of two experiments done in duplicate with two different membrane preparations.

In this study, we isolated TTRs from ground rhizomes of *Acorus calamus* Linn sold under the label "Vacā" (Uragandha in Sanskrit, Bacc or Gorbacc in Hindi), and their identities were established by routine physiochemical methods. However, because TTR and its congener 3'-TTR have not been described previously as secondary metabolites of *Acorus calamus* Linn (Araceae family), these data suggest that the TTR and 3'-TTR we isolated might have originated from other plant sources. If so, likely sources include *Belamchanda* and *Iris* species (Iridiaceae family), because both are indigenous to the Asian continent and each has been reported to contain TTR (Agarwal et al., 1984; Kim, 1999). During field collection, *Iris* rhizomes could be harvested inadvertently along with *Acorus* rhizomes because both are phenotypically similar. Although the plant origin of the TTR and 3'-TTR used in this study may be thus indeterminate, their presence is certain, given our physiochemical confirmations. Thus this study illuminates some vagaries of basic research into herbal preparations.

In summary, these data demonstrate that the isoflavones TTR and 3'-TTR bind to and modulate RyR. Furthermore, the aglycone of TTR, tectorigenin, likewise competes with [<sup>3</sup>H]ryanodine for binding to RyR, apparently in an isoform-specific manner. Functional studies are underway to evaluate the validity of these observations and to characterize whether isoform specificity of binding is reflected in functional specificity of tectorigenin on RyR1 and RyR2.

#### Acknowledgments

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