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Characterization of the Ryanodine Receptor-Ca^{2+} Release Channel from the Thoracic Tissues of the Lepidopteran Insect *Heliothis virescens*

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Abstract. The existence of invertebrate forms of the RyR has recently been confirmed (Takeshima et al., 1994, Puente et al., 2000). However, information on the functional properties of this insect RyR is still limited. We report the functional characterization of a RyR from the thoracic muscle of *H. virescens* (Scott-Ward et al., 1997). A simple purification protocol produced membranes from homogenized prefrozen *H. virescens* thoracic muscle with a [^{3}H]-ryanodine binding activity of 1.19 ± 0.21 pmol/mg protein (mean ± SE; $n = 4$). [^{3}H]-Ryanodine binding to the *H. virescens* receptor was dependent on the ryanodine concentration in a hyperbolic fashion with a K_D of 3.82 nM ($n = 4$). [^{3}H]-ryanodine binding was dependent on [Ca^{2+}] in a biphasic manner and was stimulated by 1 mM ATP. Millimolar caffeine did not stimulate [^{3}H]-ryanodine binding to *H. virescens* membranes in the presence of either nanomolar or micromolar Ca^{2+}. A protein of at least 400 KDa was recognized in *H. virescens* membrane proteins by a specific anti-*H. virescens* RyR antibody. Discontinuous density sucrose gradient fractionation of microsomal membranes produced vesicles suitable for single-channel studies. Ca^{2+}-sensitive, Ca^{2+}-permeable channels were successfully inserted into artificial lipid bilayers from *H. virescens* membrane vesicles. The *H. virescens* RyR-channel displayed a Ca^{2+} conductance of ~110 pS and underwent a persistent and characteristic modification of ion handling and gating following addition of 100 nM ryanodine. The gating of *H. virescens* channels was sensitive to ATP and ruthenium red in a manner similar to mammalian RyR. This is the first report to describe the single channel and [^{3}H]-ryanodine binding properties of a native insect RyR.

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Key words: Insect — Ryanodine — Ryanodine receptor — Calcium channel — Sarcoplasmic reticulum

Introduction

The release of Ca^{2+} from mammalian muscle sarcoplasmic reticulum (SR) plays a crucial role in excitation-contraction (E–C) coupling and occurs through the ryanodine receptor-Ca^{2+} release channel or RyR (Pessah et al., 1986). In skeletal muscle, clusters of RyR embedded in the SR form physical contacts with dihydropyridine receptors (DHPR) in the T-tubule sarcolemmal membrane, resulting in the triad membrane structures seen by electron microscopy or EM (Block et al., 1988). Depolarization of the sarcolemma induces a conformational change in the DHPR that activates the RyR (Rios, Ma & Gonzalez, 1991), releasing Ca^{2+} from a pool within the SR. The protein contacts between the two membrane systems form the characteristic "foot" structures visible in EM (Franzini-Armstrong, 1970). The cytoplasmic region of the RyR has been identified as the major component of these "feet" (Inui, Saito & Fleischer, 1987).

The rabbit skeletal muscle RyR-Ca^{2+} release channel (RyR1) has been biochemically isolated and shown to be a ligand-gated, Ca^{2+}-selective channel composed of four identical 565 KDa subunits (Hymel et al., 1988). The plant alkaloid ryanodine binds at nanomolar concentrations to RyR in a manner dependent on receptor state (Rousseau, Smith & Meissner, 1987). This interaction has allowed [^{3}H]-ryanodine to be utilized to determine RyR location, concentration and function (Lai et al., 1988). At the single channel level ryanodine radically modifies the ion handling properties of the channel, resulting in a reduced conductance substate with high open probability or P_O (Imagawa et al., 1987). Ca^{2+} ions are endogenous ligand of RyR1 and effect a concentration-dependent biphasic variation in channel P_O and ryano-

dine binding activity. The detailed pharmacology of a variety of other modulators, including ATP, caffeine, Mg^{2+} and small endogenous proteins, has also been reported (Zucchi & Roncatestoni, 1997). The channel is permeable to both monovalent and divalent cations but is Ca^{2+}-selective (Smith et al., 1988).

Functionally and structurally distinct isoforms of the ryanodine receptor are resident in the sarcoplasmic and endoplasmic reticulum membranes of a variety of species (Ogawa et al., 1999). Similar to mammalian cardiac muscle, E–C coupling in invertebrate muscle requires extracellular Ca^{2+} together with the induced release of Ca^{2+} from the SR (Gyorke & Palade, 1993). This process is likely to be mediated by isoforms of the RyR (Loesser, Castellani & Franzini-Armstrong, 1992). A high molecular weight Ca^{2+}-release channel that binds ryanodine at low nanomolar concentrations has also been identified in lobster muscles (Olivares & Airpse, 1993). The single channel, $^{45}Ca^{2+}$ flux and ryanodine binding properties of the membrane bound and purified lobster RyR were however measurably different to those of the vertebrate isoforms (Seok et al., 1992; Quinn et al., 1998; Zhang, Williams & Sitsapesan, 1999). The functional properties of high molecular weight ryanodine binding proteins from crayfish (Formelova et al., 1990) and *C. elegans* (Kim et al., 1992) have also been reported.

In addition to triads, insect flight muscle and other invertebrate muscle types contain single membrane contacts or "dyads" between the T-tubules and the SR (Loesser et al., 1992). Ultrastructural studies of grasshopper muscle reveal that these dyads contain clusters of "foot structures" which are similar in appearance to those seen in mammalian skeletal muscle (Loesser et al., 1992). Use of the ryanodine containing insecticide *Ryania* (Pepper & Carruth, 1945), prepared from the stemwood of *Ryania speciosa*, implies the existence of an insect ryanodine receptor. The dietary application or intrathoracic injection of purified ryanodine induces flaccid muscle paralysis in a range of insect species (Edwards et al., 1948, Jefferies et al., 1992).

The level of information on the presence and properties of potential RyR in insect tissues is somewhat limited. Housefly and cockroach muscle membranes bind [^{3}H]-ryanodine in a similar manner to vertebrate RyR (Lehmberg et al., 1994). The gene encoding a 5126 amino acid protein from *D. melanogaster* has been cloned and found to have 45% identity with the rabbit *ryr*1 (Takeshima et al., 1994). cDNAs encoding the carboxy-terminal 1172 amino acids from an RyR have been isolated from the lepidopiteran pest *Heliothis virescens* (*H. virescens*) and shown to have 51 and 78% identity with the equivalent regions in the rabbit skeletal and *D. melanogaster* RyR (Puente et al., 2000). We present here the identification and characterization of a ryanodine receptor-Ca^{2+} release channel from the thoracic tissues of *H. virescens.* [^{3}H]-ryanodine binding, single channel techniques and western blotting were used to establish the presence and functional properties of this insect RyR. Part of this work appears in abstract form (Scott-Ward et al., 1997).

Materials and Methods

Materials

[^{3}H]-ryanodine was purchased from NEN Life Sciences. Unlabeled ryanodine was purchased from Calbiochem (Nottingham, UK). All other chemicals were of AnalaR or best available grade from BDH (Poole, UK) or Sigma (Poole, UK). Counting scintillant was purchased from Amersham International (Amersham, UK). Phosphatidylethanolamine was purchased from Avanti Polar Lipids (Alabaster, Alabama). Adult *H. virescens* were grown and harvested at Zeneca Agrochemicals (Jealotts Hill, UK). The polyclonal anti-rabbit RyR1 was purchased from Upstate Biotechnology.

Isolation of Muscle Microsomal Membranes

Rabbit white skeletal muscle was removed from the hind legs and back of adult white rabbits and dissected into small pieces on ice. Adult whole *H. virescens* were snap frozen in liquid nitrogen and shaken while still frozen to separate the body segments. Frozen thoraces, which are rich in flight muscle, were isolated *en mass* by fractionation of the differently sized body parts through a series of precooled metal sieves. Thoraces were stored in liquid nitrogen and used within 30 days of isolation. Microsomal membranes were prepared from both tissues in the same manner (all manipulations at +3°C). Fresh rabbit skeletal muscle (25 g) or 40 g frozen *H. virescens* thoraces were homogenized for 60 sec in 350 ml 0.3 M sucrose, 0.2 M KCl, 0.1 mM EGTA, 2 mM PMSF, 25 mM KPIPES; pH 7.4 (solution A). After low speed centrifugation (*Spin 1*) of the homogenate for 20 min at 8,000 × g_{av}, the supernatant was recentrifuged (*Spin 2*) at 100,000 × *g* for 50 min. The resultant pellet was resuspended in a minimum volume (3–5 ml) of solution A using a Polytron homogenizer and then frozen in liquid N_2 as aliquots. The method of Bradford (1976) was used to assay the concentration of protein in all membrane samples.

Preparation of Sarcoplasmic Reticulum Vesicles

Microsomal membranes were prepared as described above. These were then resuspended in 0.3 M (~10%) sucrose and loaded onto a discontinuous sucrose gradient (10, 20, 30, 40% w/v) containing 0.4 M KCl, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 EGTA, 5 mM KPipes (pH 7.2) and centrifuged for 140 min at 100,000 × *g*. Membranes at the 10/20, 20/30 and 30/40% sucrose solution interfaces were removed, diluted in 8 to 10 volumes of ice-cold 0.4 M KCl and immediately centrifuged at 100,000 × *g* for 50 min. The pellets were resuspended in a minimum volume of 0.3 M sucrose, 0.1 M KCl, 50 μM EGTA, 10 mM TRIS-HEPES (pH 7.4) with hand-held glass homogenizers and then frozen and stored in liquid N_2.

SDS-Polyacrylamide Electrophoresis and Silver Staining of Proteins

Protein samples (5–20 μg per lane) were analyzed according to the SDS-PAGE electrophoresis method of Laemmli (1970). Microsomal

membrane fractions were loaded on 5% polyacrylamide minigels cast using a 5-fold concentrate of loading buffer (5% SDS, 25% sucrose, 5 mM EDTA, 5 mM TRIS-HCl (pH 6.8), 0.5% bromophenol blue and 200 mM dithiothreitol (DTT). Electrophoresis was carried out at 40 V for 45 min and then 125 V for ~1.5 hr on ice. High range (45 to 200 KDa) rainbow markers (Sigma-Aldrich, UK) were used to track the migration of proteins of different molecular weight through the gel. Gels were stained with an Instaview silver staining kit (BDH, UK) according to the manufacturer's instructions.

Antibody Production

Rabbit anti-*H. virescens* RyR antibodies were prepared from a 120 amino acid tract of the *H. virescens* RyR expressed as a maltose binding protein fusion protein in *Escherichia coli* (E. Puente, A. Dinsmore and J. Windass (*unpublished*)).

Western Transfer and Immunoblot Staining

Proteins were electrophoresed as described under SDS-Polyacrylamide Electrophoresis. The SDS-polyacrylamide gel containing the proteins was transferred onto PVDF paper in TRIS-Glycine, 10% methanol, 0.01% SDS, using a water-cooled Bio-Rad apparatus and a power supply set 400 mA/gel for 3 hr. The membranes were incubated in 5% nonfat milk, 0.5% Tween-20 with a 1 in 2,000 dilution of primary antibody and then a 1 in 2,000 dilution of the horse radish peroxidase-conjugated secondary antibodies as shown. Detection of antibody recognition was performed using ECL chemiluminescent detection (Amersham Life Science, UK).

[^{3}H]-Ryanodine Binding Assay

Binding of [^{3}H]-ryanodine to membrane fractions was carried out by a method developed from that used by Holmberg & Williams (1990). For equilibrium binding, membranes (25 to 50 μg protein for rabbit membranes and 50 to 200 μg protein for *H. virescens* membranes) were incubated with 5 nM [^{3}H]-ryanodine for 60 min at 37°C with shaking (5 Hz) in a solution containing 1 M KCl and 25 mM KPIPES (pH 7.4) (binding buffer) plus reagents as described, in a final assay volume of 500 μl. In assays where the effects of additional substances were being investigated, these were added from stock solutions or dissolved in the binding buffer to the correct concentration prior to the addition of the [^{3}H]-ryanodine or membranes. [^{3}H]-ryanodine was added from a 1.42 μM stock in ethanol. Addition of the relevant membranes in a 10 μl volume was used to initiate the reaction. Nonspecific binding was determined from duplicate assays in which 5 μM unlabeled ryanodine was added prior to the start of the reaction. Specific binding was greater than 80% in all assays. Binding was terminated by the addition of 5 ml cold binding buffer followed by immediate filtration through a Whatman GF/B filter presoaked in binding buffer containing 2% Polymin P. To remove residual unbound [^{3}H]-ryanodine the filter paper was washed with two further 5 ml aliquots of ice-cold binding buffer. Radioactivity remaining on the filter was determined by placing the filter in 10 ml UltraGold MV scintillant (Canberra-Packard, UK) and counting for 2 min in a liquid scintillation counter. All assays were done in duplicate.

Data were analyzed and displayed using the PRISM computer program (Graph Pad Prism). Saturation isotherms (Fig. 4) were fitted to the data according to Eq. 1, from which values for K_D ([^{3}H]-ryanodine concentration at which 50% of predicted maximum binding is achieved) and B_{MAX} (predicted maximum [^{3}H]-ryanodine binding) have been estimated.

$$b = \frac{[R] \times B_{MAX}}{[R] + K_D} \tag{1}$$

b represents the [^{3}H]-ryanodine binding activity (in pmol/mg) at the total [^{3}H]-ryanodine concentration [R]. All other dose-dependent binding data (Figs. 5, 6 and 7) were fitted with Eq. 2 describing a variable sigmoidal dose-reponse curve.

$$b = \frac{b_{MAX} - b_{MIN}}{1 + 10^{n(log\ EC_{50} - \log [M])}} \tag{2}$$

Where b is the [^{3}H]-ryanodine binding activity at the concentration of modulator [M] and b_{MIN} and b_{MAX} are the minimum and maximum binding activities, respectively. EC_{50} is the concentration of ligand at which b is at 50% and n is the Hill Coefficient (derived from the linear portion of the slope).

Planar Lipid Bilayer Methods

Planar lipid bilayers were formed from phosphatidylethanolamine suspended in *n*-decane across a 200 μm hole in a partition separating two chambers (*cis* chamber, 0.5 ml, *trans* chamber, 1 ml). Unless otherwise stated, the *cis* chamber was clamped at 0 mV holding potential relative to the *trans* at ground and current flow through channels in the bilayer from *trans* to *cis* chambers was monitored and recorded via an operational amplifier. On bilayer formation in symmetrical 250 mM CsPIPES (pH 7.4), an osmotic gradient was created by addition of 100 μl 3M CsCl or KCl to the *cis* chamber. Membrane vesicles (0.5 to 5 μl) were then added and, after stirring, a second aliquot of CsCl/KCl added to induce fusion of vesicles with the bilayer. The potential was held at +30 mV relative to ground to help visualize incorporation of fast gating, high conductance potassium-permeable channels. On incorporation of channels, a *trans* to *cis* Ca^{2+} gradient was introduced by perfusion of the *cis* chamber with 250 mM HEPES/125 mM TRIS, ~7 μM free Ca^{2+} (pH 7.4) and the *trans* chamber with 250 mM Ca^{2+} glutamate (~50 mM free Ca^{2+}; pH 7.4). Incorporated channel activity was monitored using an oscilloscope and stored unfiltered on DAT tape when appropriate. Additional ligands were administered to the *cis* chamber from stock solutions (100 mM EGTA, 100 mM ATP, 0.1 mM ryanodine, 100 μM ruthenium red).

Data Acquisition and Analysis

For analysis, data were filtered through a low-pass 8-pole Bessel filter at 600 Hz and digitized at 4 kHz using an AT based computer system (SATORI (version 3.2), Intracel, UK). Open probabilities (P_O) were determined from 1 to 3 min steady-state recordings at 0 mV holding potential by 50% threshold analysis, with cursors set manually for open and closed states. In multiple channel experiments, the global open probability of the channels (nP_O) was determined as previously described (Boraso & Williams, 1994). For comparison with single channel experiments *nPo/n* values were used, where *n* represents the number of open channel levels observed in the presence of activating ligands ($CaCl_2$ or ATP) in the *cis* chamber and nP_O the global open probability. Single channel current amplitudes at different holding potentials were determined by assigning cursors manually to open and closed levels observed during ⩾20 second steady-state recordings filtered and digitized into SATORI as described above. Single channel conductances were obtained from the slope of linear regressions fitted

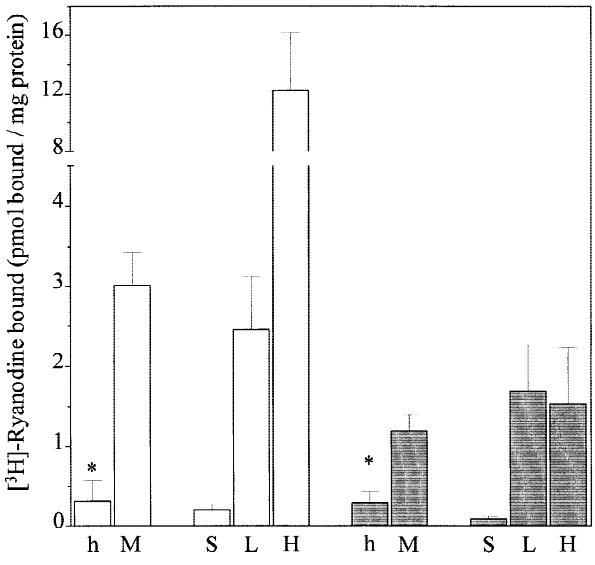

Fig. 1. Isolation of membrane fractions that bind [^{3}H]-ryanodine from *H. virescens* flight muscle and rabbit skeletal muscle. Microsomal membranes (M) were isolated by centrifugation of muscle homogenates (h) as described. Further separation of *H. virescens* (*shaded*) and rabbit (*clear*) membranes through a discontinuous density sucrose gradient produced membrane bands at the 10/20% (S), 20/30% (L) and 30/40% (H) interfaces. Binding was determined as described in the presence of 5 nM [^{3}H]-ryanodine. All values are the mean ± SE of binding to 4 membrane preparations.

to current (I) data for a holding potential (V) from −40 to +10 mV. For display purposes, single channel recordings were filtered at 300 Hz. Graphical displays of conductance data were best fit with an equation describing a third order polynomial:

$$I = A + (B \times V^1) + (C \times V^2) + (D \times V^3) \quad (3)$$

Results

Isolation of Membranes Containing a Lepidopteran Ryanodine Receptor

The [^{3}H]-ryanodine binding activities of membrane fractions isolated from frozen *H. virescens* thoracic flight muscle and fresh rabbit skeletal muscle were determined using an assay protocol optimized for low concentrations of RyR (Fig. 1). Homogenization of the pellets isolated after high-speed centrifugation produced *H. virescens* microsomal membranes (M) that bound 1.19 ± 0.21 pmol of [^{3}H]-ryanodine per mg protein at nanomolar ryanodine concentration. This indicates one or more isoforms of RyR are present in *H. virescens* thoracic muscles. Enrichment of binding activity in the microsomal fraction was estimated to be approximately 4-fold. The ryanodine binding to *H. virescens* microsomal membranes is approximately 3 times higher than to housefly thorax membranes (Lehmberg & Casida, 1994). The quantities of total membrane protein isolated (≥30 mg protein per preparation) were sufficient to allow further characterizations. Membranes isolated from fresh rabbit skeletal muscle by an identical protocol bound [^{3}H]-ryanodine with an activity of 3.00 ± 0.49 pmol/mg protein.

Isolation Of Membrane Fractions Containing SR Membrane Vesicles

Fractionation of rabbit microsomal membranes on a discontinuous density sucrose gradient produced heavy sarcoplasmic reticulum (HSR) membranes at the 30/40% sucrose interface with a further enriched mean binding activity of 12.23 ± 3.98 pmol/mg (Fig. 1). Similar fractionation of *H. virescens* microsomal membranes did not significantly enrich the binding activity. *H. virescens* SR membranes isolated from the 20/30% and 30/40% interfaces bound 1.69 ± 0.56 and 1.53 ± 0.70 and pmol ryanodine per mg protein, respectively. Increasing the centrifugation time to 4 hr did not appear to increase the binding activities of membrane fractions at the 20/30 and 30/40% w/v interfaces (*data not shown*). Unfractionated microsomal membranes were used to characterize the ryanodine binding properties of the *H. virescens* and rabbit skeletal receptors.

Immunological Identification of a *H. virescens* RyR Protein

Examination of silver stained proteins separated on SDS-polyacrylamide gels reveals that proteins of ≥400 KDa are present in microsomal membranes of rabbit skeletal muscle and *H. virescens* thoracic muscle (Fig. 2*A*). Western blot analysis with anti-RyR antibodies reveals these high molecular weight proteins are antibody-specific immunogens. The anti-*H. virescens* RyR antibody is specific for a 120 amino acid region in the *H. virescens* RyR C-terminus and recognized the single polypeptide of ≥400 KDa from *H. virescens* membranes (Fig. 2*B*). A second, much weaker, band indicates recognition of a protein of ~210 KDa. The commercial anti-rabbit RyR1 antibody recognizes a characteristic doublet band of high molecular weight proteins in rabbit but not *H. virescens* membranes (Fig. 2*C*). The multiple banding patterns are most likely due to recognition of proteolyzed RyR. Similar patterns were observed in previous studies on rabbit RyR1 from HSR membranes using other anti-RyR1 antibodies (Wu et al., 1997). The anti-rabbit RyR1 antibody also weakly recognized a single high molecular weight protein from sheep cardiac HSR, together with material of lower molecular weight most likely derived from the denatured RyR (Fig. 2*C*). No sheep cardiac membrane proteins were recognized by the anti-*H. virescens* RyR antibody (Fig. 2*B*). The varying pattern of recognition between different antibodies

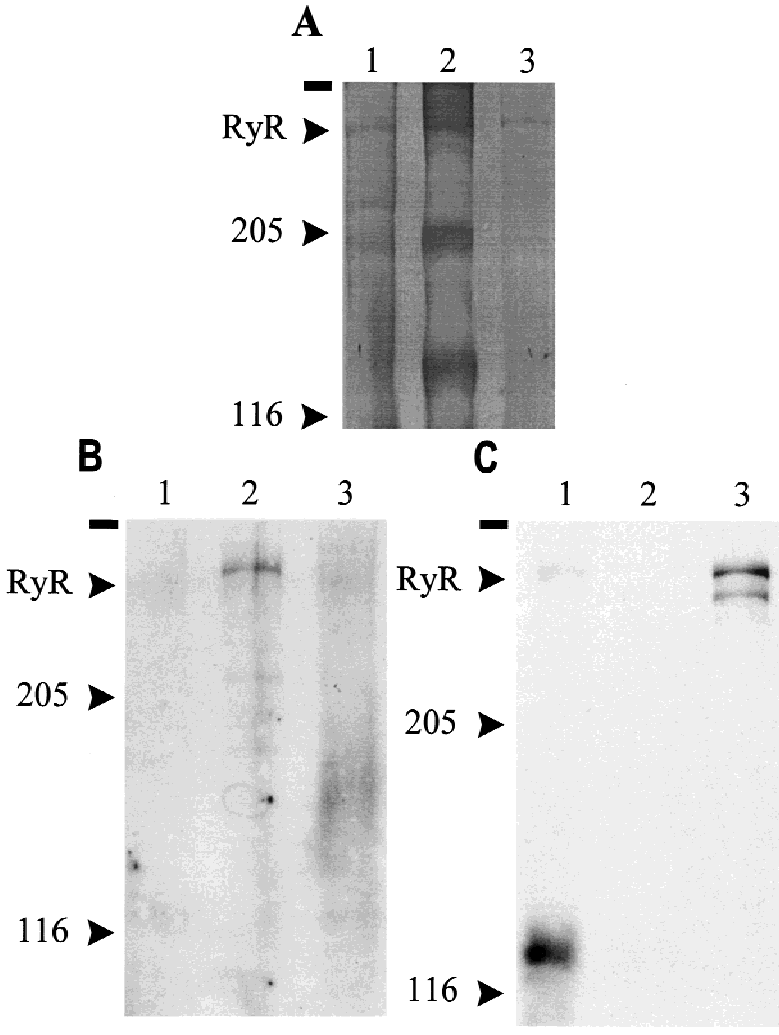

Fig. 2. Silver stain and immunoblot analysis of RyR proteins. Microsomal membrane proteins (M, 10 μg/lane) were electrophoresed through 5% SDS polyacrylamide gels and (*A*) were silver stained as described. Proteins from separate SDS-PAGE gels were electrophoretically transferred onto PVDF membranes and probed with a polyclonal anti-*H. virescens* RyR1 antibody (*B*) and a polyclonal anti-rabbit RyR antibody (*C*), both at 1 in 2,000 dilution. In all figures lane 1 contains sheep cardiac HSR membrane proteins, lane 2 *H. virescens* microsomal membrane proteins and lane 3 rabbit skeletal muscle microsomal membrane proteins. The position of molecular weight markers is given to the left of the immunoblots (205 KDa, myosin; 116 KDa, β-galactosidase). The position of potential RyR proteins is indicated on all figures (~400 KDa).

indicates *H. virescens* and rabbit skeletal RyR are immunogenically distinct.

The Effect of RyR Modulators on [^{3}H]-Ryanodine Binding Activity

The effect of specific concentrations of established RyR ligands and modulators on [^{3}H]-ryanodine binding activity is shown in Fig. 3. Consistent with studies on RyR1 (Pessah et al., 1986), specific ryanodine binding to both rabbit and *H. virescens* membrane preparations is dependent on the presence of both micromolar $CaCl_2$ and molar KCl. In the presence of 50 μM $CaCl_2$ and 1 M KCl both membrane preparations bind picomolar levels of [^{3}H]-ryanodine with high affinity (*A*, Fig. 3). This binding is effectively abolished on addition of 5 mM EGTA, both in the presence (*C*) and absence (*B*) of 1 M KCl. The free Ca^{2+} concentration was ≤10 nM in the presence of 5 mM EGTA and 50 μM $CaCl_2$. [^{3}H]-ryanodine binding in the presence of 50 μM $CaCl_2$ only (*D*) could have been stimulated by K^+ ions contributed by the KPIPES buffer. Addition of 1 mM ATP stimulates $CaCl_2$/KCl induced binding to *H. virescens* and rabbit membranes by 64 and 52%, respectively (*E*). In contrast, substituting 20 mM caffeine for ATP results in enhanced binding to only rabbit membranes (*F*). Addition of 5 mM $MgCl_2$ at constant $CaCl_2$ and KCl concentrations inhibits binding to *H. virescens* membranes by 83% and rabbit membranes by 86% (*G*). The addition of the channel antagonist ruthenium red to 5 μM reduces ryanodine binding to both membrane preparations by over 95% (*H*). Ryanodine binding to house fly membranes varied in a similar fashion in response to the above RyR effectors (Lehmberg & Casida, 1994). It should be noted that preliminary studies revealed specific [^{3}H]-ryanodine binding was highly dependant on KCl concentration, with a sharp increase between 0.5 and 1 M ($n = 2$, *not shown*). Hence 1 M KCl was included in all other experiments in order to maintain suitable assay sensitivity while ensuring results were obtained under conditions comparable to those used

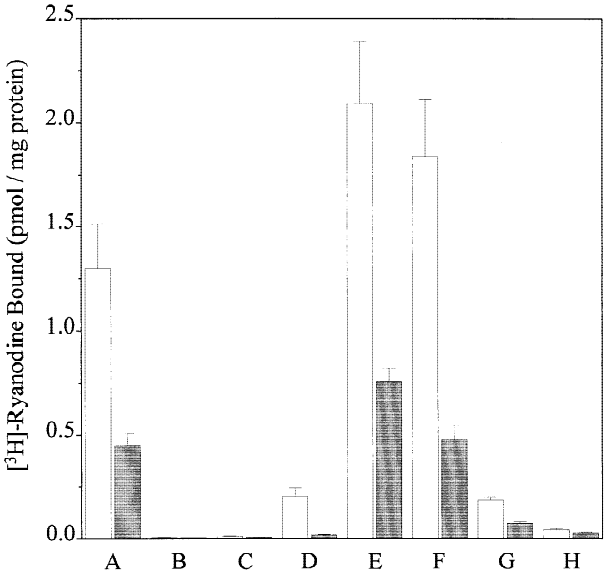

Fig. 3. [^{3}H]-Ryanodine binding to microsomal membrane fractions in the presence of different cations and RyR modulators. *H. virescens* (*shaded*) and rabbit (*clear*) membranes were assayed for binding activity as described in the presence of (A) 50 μM $CaCl_2$, 1 M KCl; (B) 5 mM EGTA; (C) 1 M KCl, 5 mM EGTA; (D) 50 μM $CaCl_2$; (E) 50 μM $CaCl_2$, 1 M KCl, 1 mM ATP; (F) 50 μM $CaCl_2$, 1 M KCl, 20 mM caffeine; (G) 50 μM $CaCl_2$, 1 M KCl, 5 mM $MgCl_2$; (H) 50 μM $CaCl_2$, 1 M KCl, 5 μM ruthenium red. All values are the mean ± SE of binding to 4 membrane preparations. [^{3}H]-ryanodine binding to *H. virescens* and rabbit membranes under the conditions in (A) was 0.45 ± 0.07 and 1.30 ± 0.22 pmol/mg, respectively.

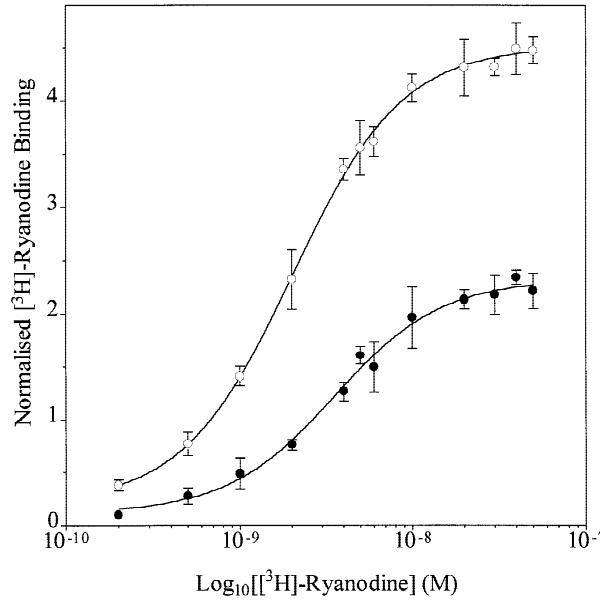

Fig. 4. The kinetics of equilibrium [^{3}H]-ryanodine binding to *H. virescens* (●) and rabbit (○) membranes. Specific binding was determined in the presence of 0 to 100 nM [^{3}H]-ryanodine as described. Nonspecific binding was assessed in the presence of 5 μM unlabeled ryanodine. It was established in separate experiments that apparent equilibrium binding to both membrane preparations was achieved after 1 hr incubation at 37°C. All values are the mean ± SE of binding to 4 membrane preparations.

in previous ryanodine binding studies on invertebrate RyR (Lehmberg & Casida, 1994).

Kinetics Of [^{3}H]-Ryanodine Binding

The data in Fig. 4 show the effect that increasing [^{3}H]-ryanodine concentration from 0.2 to 100 nM has on the level of high-affinity binding to membranes. The mean specific activities displayed represent equilibrium binding to four separate *H. virescens* and rabbit membrane preparations. The binding activities saturate according to hyperbolic kinetics. This indicates that a single class of high affinity ryanodine sites exists on each receptor isoform. Nonspecific binding in 5 μM unlabeled ryanodine increases linearly with [^{3}H]-ryanodine concentration but was never more than 20% of the total binding (*data not shown*). *H. virescens* and rabbit isoforms bind ryanodine with apparent affinity constants (K_D) of 3.82 ± 0.39 and 2.04 ± 0.12 nM, respectively (mean ± SD, n = 4) while B_{MAX} values for *H. virescens* and rabbit membranes are 2.41 ± 0.17 and 4.57 ± 0.32 pmol/mg (mean ± SD, n = 4), indicating that there was almost 50% fewer functional *H. virescens* binding sites at saturating [^{3}H]-ryanodine concentrations. Assay conditions of 500 μM $CaCl_2$, 1 mM ATP and 1 M KCl were used to ensure that as many functional receptors were activated as possible, thus exposing the maximum number of ryanodine binding sites. The K_D and B_{MAX} values obtained from binding to rabbit membranes are comparable to those reported in previous studies on membrane-bound rabbit RyR1 (Ogawa, Kurebayashi & Murayama, 1999). Preliminary experiments addressing the time course of [^{3}H]-ryanodine association revealed binding to both receptor isoforms had peaked by 1 hr incubation at 37°C (*data not shown*). The [^{3}H]-ryanodine concentration used in all other experiments (5 nM) was 2.5 and 1.3-fold higher than the K_D values for rabbit skeletal and *H. virescens* RyR, respectively. This concentration was deemed suitable for characterization of the effects of modulators on high affinity binding because assay sensitivity is maintained. Previous studies suggest that binding to potential low affinity sites on both isoforms should be insignificant (Shoshan-Barmatz & Ashley, 1998). In addition, comparable concentrations of [^{3}H]-ryanodine have been used in previous binding studies on other invertebrate RyR (Lehmberg et al., 1994; Zhang et al., 1999).

The Effect of Variation in Ca^{2+} Concentration

Increasing the free Ca^{2+} concentration from 3 μM to 10 mM in 1 M KCl by addition of EGTA and $CaCl_2$ has a

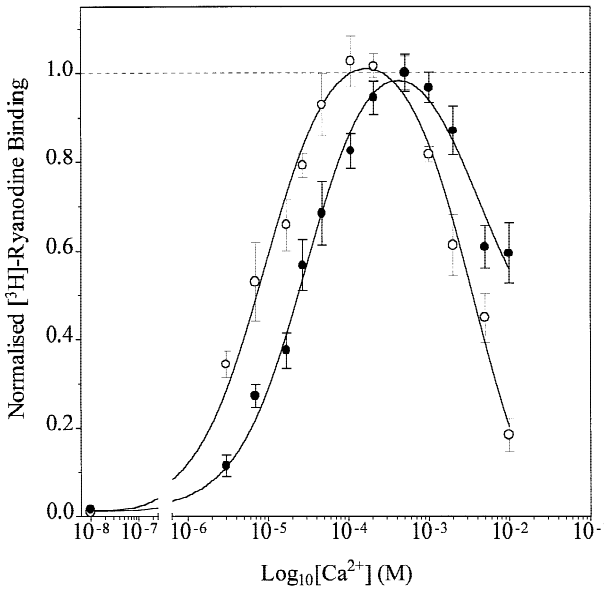

Fig. 5. The $[Ca^{2+}]$ dependence of $[^3H]$-ryanodine binding activity to *H. virescens* (●) and rabbit (○) microsomal membranes. All values are the mean ± SE of binding to 5 membrane preparations. All individual values are independently normalized relative to the mean binding obtained in 500 μM Ca^{2+}. Free Ca^{2+} concentration was calculated using the EQCAL computer program (Biosoft, UK) and adjusted using $CaCl_2$ and EGTA solutions. The concentration of contaminant Ca^{2+} was determined to be 7.1 μM using a Ca^{2+}-sensitive electrode. The pH was maintained at 7.4. Binding to *H. virescens* and rabbit membranes in 500 μM Ca^{2+} and 5 nM $[^3H]$-ryanodine was 0.74 ± 0.02 and 1.41 ± 0.11 pmol/mg.

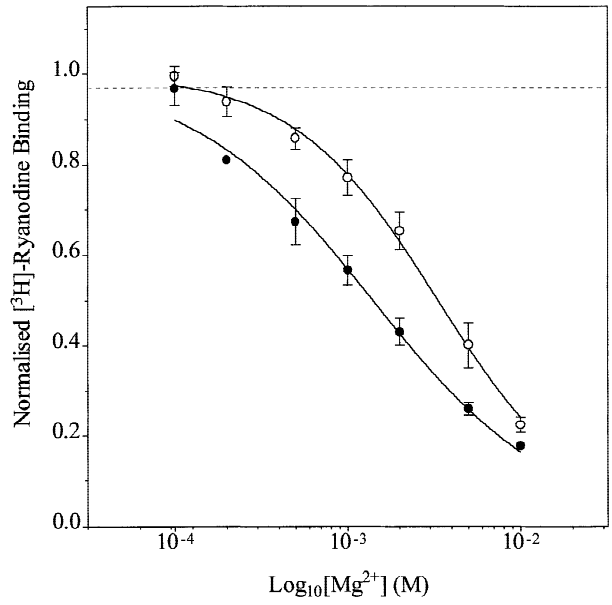

Fig. 6. Mg^{2+} inhibition of Ca^{2+}-sensitive $[^3H]$-ryanodine binding to *H. virescens* (●) and rabbit (○) membranes. The effect of Mg^{2+} on $[^3H]$-ryanodine binding in the presence of 100 μM $CaCl_2$, 1 M KCl (pH 7.4) and 5 nM $[^3H]$-ryanodine was assessed by addition of $MgCl_2$. All values are the mean ± SD of the binding to 3 membrane preparations. All individual values were independently normalized relative to the mean binding obtained in 100 μM $CaCl_2$, 0 mM $MgCl_2$. $[^3H]$-ryanodine binding to *H. virescens* and rabbit membranes in 100 μM Ca^{2+} was 0.59 ± 0.09 and 1.52 ± 0.26 pmol/mg (mean ± SD; $n = 3$), respectively.

biphasic effect on the binding activity at a fixed $[^3H]$-ryanodine concentration (Fig. 5). Variation in Ca^{2+} concentration also affects $[^3H]$-ryanodine binding to housefly, lobster and rabbit skeletal muscle membranes in a biphasic fashion (Pessah, Waterhouse & Casida, 1985; Zhang et al., 1999; Lehmberg & Casida, 1994). This indicates that, as in previously characterized RyRs, Ca^{2+} modulates *H. virescens* receptor activity in at least two ways, one stimulatory and one inhibitory. For the purposes of comparison, data points obtained from each membrane fraction were separately normalized with respect to the binding activity obtained at 500 μM $CaCl_2$ for either *H. virescens* or rabbit membranes. The means of the normalized binding to four separate membrane preparations are displayed on Fig. 5. Peak $[^3H]$-ryanodine binding to *H. virescens* membranes (0.78 ± 0.19 pmol/mg) is achieved at 500 μM free Ca^{2+}. In contrast, peak binding to rabbit membranes (1.49 ± 0.06 pmol/mg) is seen at 100 μM Ca^{2+}. The marginally lower Ca^{2+}-sensitivity of the *H. virescens* receptor is also reflected by comparison of the EC_{50} values of 30.0 ± 4.33 μM Ca^{2+} for *H. virescens* membranes and 8.69 ± 1.24 μM Ca^{2+} and rabbit membranes (mean ± SEM, $n = 4$). The Hill coefficient for activation is below 1.2 for both isoforms, indicating that Ca^{2+} stimulates binding through interaction at a single activation site on the receptor. Above 500 μM free Ca^{2+} dose-dependent inhibition of $[^3H]$-ryanodine binding activity occurs. Inhibition of optimal binding to rabbit membranes at 10 mM $CaCl_2$ is 81%. The *H. virescens* receptor is less sensitive to Ca^{2+} dependent inhibition as optimal binding is inhibited by only 55% at 10 mM $CaCl_2$. Accurate determination of the IC_{50} values for the inhibitory effects of millimolar Ca^{2+} will require the acquisition of further binding data.

Inhibition of $[^3H]$-Ryanodine Binding by Magnesium

The dose-dependent inhibition of $[^3H]$-ryanodine binding on addition of magnesium chloride from 0.1 to 10 mM in 100 μM $CaCl_2$ and 1 M KCl to membrane fractions is shown in Fig. 6. This inhibition follows sigmoidal dose-response kinetics with Hill coefficients of 0.79 ± 0.11 and 0.92 ± 0.19 for *H. virescens* and rabbit isoforms, respectively. Notably binding to *H. virescens* membranes is significantly more sensitive to Mg^{2+} concentration than rabbit membranes at the selected Ca^{2+} concentration, since IC_{50} values for the $MgCl_2$ inhibition are 1.40 ± 0.39 mM and 3.33 ± 0.66 mM, respectively. Mg^{2+} ions appear more effective at inhibiting Ca^{2+}-induced $[^3H]$-ryanodine binding to the *H. virescens* RyR.

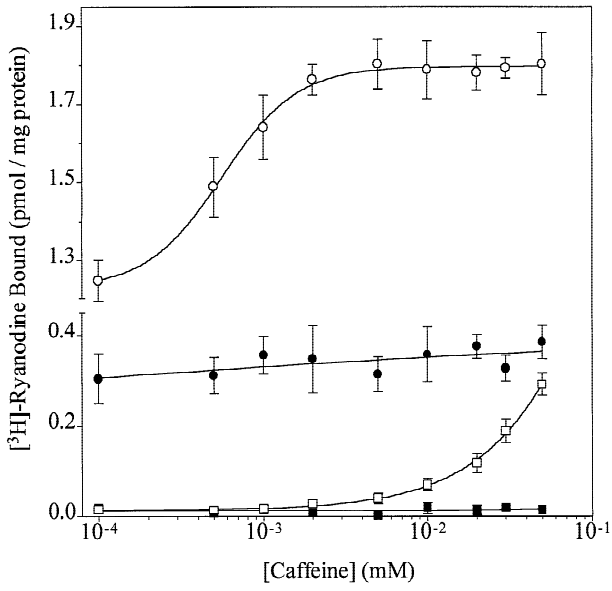

Fig. 7. Insensitivity of *H. virescens* membrane [^{3}H]-ryanodine binding to caffeine stimulation. The effect of 0 to 50 mM caffeine on [^{3}H]-ryanodine binding to *H. virescens* (●,■) and rabbit (○,□) microsomal membranes in presence (■,□) and absence of 1 mM EGTA (●,○) was assessed. Binding to 3 separate membrane preparations was determined in the presence of 1 M KCl, pH 7.4 and 5 nM [^{3}H]-ryanodine (mean ± SD; $n = 3$). Free Ca^{2+} concentration was determined to be ~7 μM in the absence of EGTA, and ≤15 nM in the presence of EGTA.

These data are consistent with a single, low affinity receptor site for Mg^{2+}. At 10 mM Mg^{2+}, inhibition of binding to both isoforms is greater than 75%. Mg^{2+} ions did not independently stimulate [^{3}H]-ryanodine binding to the *H. virescens* RyR as no measurable binding activity could be detected upon addition of 5 mM $MgCl_2$ in the presence of 4 mM EGTA (≤10 nM free Ca^{2+}, ~3.5 mM free Mg^{2+}). The effect of Mg^{2+} on binding to rabbit membranes is in accordance with previous studies (Pessah et al., 1986). In 1986 Smith and coworkers demonstrated Mg^{2+} exerts its effects through reducing the open probability of the Ca^{2+}-activated RyR-channel (Smith et al., 1986), as do Ba^{2+} ions (Laver, Baynes & Dulhunty, 1997). In light of our Ca^{2+} dose-response data, the differing potencies of Mg^{2+} on rabbit and *H. virescens* RyR isoforms at fixed Ca^{2+} concentrations supports the theory that the inhibition is due to Mg^{2+} and Ca^{2+} competing for the divalent cation activation site (Laver et al., 1997).

The Effect of Caffeine on [^{3}H]-Ryanodine Binding

Caffeine and caffeine analogues are known activators of mammalian muscle RyR, binding to these receptor at distinct sites from those recognized by ryanoid, divalent cations and adenine-nucleotides (Pessah, Stambuk, & Casida, 1987; Rousseau et al., 1987; McGarry & Williams, 1994). Figure 7 shows that Ca^{2+}-dependent [^{3}H]-ryanodine binding to *H. virescens* membranes is insensitive to caffeine ($n = 3$). Concentrations of up to 50 mM caffeine stimulates dose-dependent [^{3}H]-binding only to rabbit membranes in either nanomolar (≤20 nM) or contaminant (~7 μM) Ca^{2+} ($n = 3$). Increasing the caffeine concentration to 50 mM enhanced contaminant Ca^{2+}-activated ryanodine binding by 31% to a maximum of 1.80 pmol/mg. The EC_{50} for caffeine is 0.56 ± 0.160 mM (mean ± SD, $n = 3$). Lowering free Ca^{2+} to nanomolar levels resulted in higher concentrations of caffeine being required to stimulate binding with 0.29 ± 0.04 pmol [^{3}H]-ryanodine per mg protein at 50 mM caffeine. The relative shift to the right of the binding activation curve following a reduction in free Ca^{2+} concentration indicates the action of caffeine is dependent on Ca^{2+}. The caffeine insensitivity of the *H. virescens* RyR is due either to the absence of a millimolar affinity caffeine binding site or a lack of measurable effect following the binding of caffeine. These possibilities could be due to the natural state of the receptor or due to alteration of the receptor on preparation. Previous studies have however also indicated that [^{3}H]-ryanodine binding to housefly membranes is insensitive to caffeine (Lehmberg et al., 1994).

Fusion of SR Membrane Microsomes with Planar Lipid Bilayers

To study the single channel properties of the *H. virescens* and rabbit skeletal muscle ryanodine receptors, channels were incorporated into phosphatidylethanolamine planar lipid bilayers from SR membrane fractions added to the *cis* chamber under fusion conditions. Channels conducting chloride and potassium ions incorporated from *H. virescens* and rabbit membranes in a time-dependent fashion. After 2 to 6 min of channel incorporation a *trans* to *cis* Ca^{2+} gradient was introduced by perfusion of both chambers as described. This revealed the occasional incorporation of *H. virescens* Ca^{2+} channels with current amplitudes between 3.75 and 4.45 pA and openings of millisecond duration at 0 mV (Fig. 8*a*). Ca^{2+} channels with similar current amplitudes and millisecond duration openings were regularly incorporated from rabbit skeletal SR membrane fractions (Fig. 8*b*) with current recordings obtained being visually similar to those previously displayed for the rabbit RyR1-channel with Ca^{2+} as the permeant ion (Smith et al., 1986). Under post-perfusion conditions Ca^{2+} is the only cation remaining with potential for net *trans-cis* channel permeation at 0 mV as $TRIS^+$ ions can only show a net ion flux in the opposite direction. Due to low levels of incorporation and bilayer instability during perfusion, only 23 single or multiple *H. virescens* Ca^{2+} channels were successfully

studied at the single channel level. This was the result of over 1,000 attempted incorporations using 28 membrane preparations, all of which bound [^{3}H]-ryanodine at levels within the mean values for *H. virescens* SR membranes displayed in Fig. 1. Reproducible Ca^{2+} channel incorporations were achieved from only two of these SR membrane preparations. This has naturally limited the scope of single channel studies that might offer further insights into the properties of the *H. virescens* RyR-channel and compliment the receptors [^{3}H]-ryanodine binding characteristics.

Single Channel Recordings from *H. virescens* Ca^{2+} Channels

Figure 8 shows eight-second continuous recording of current fluctuations from typical *H. virescens* and rabbit skeletal muscle Ca^{2+} channels in contaminant cytosolic Ca^{2+} (~7 μM) at 0 mV holding potential. Channel openings are represented by brief upward current deflections to the fully open state (dotted line "O") and are characteristic of those reported for other RyR-channels recorded under similar conditions (Smith et al., 1986; Zhang et al., 1999). The mean open probabilities (P_O) of the *H. virescens* and rabbit Ca^{2+} channels under these conditions are 0.05 ± 0.03 and 0.02 ± 0.01 (mean ± SEM, $n = \geqslant 7$). In three out of 23 successful incorporations, single *H. virescens* Ca^{2+}-channels with a high P_O (⩾0.5) were observed, indicating the existence of at least two classes of channel activity (Fig. 8*c*). The current amplitudes of *H. virescens* and rabbit Ca^{2+}-channels are 4.32 ± 0.11 and 3.72 ± 0.05 pA (mean ± SEM, $n = \geqslant 6$) respectively, at 0 mV. Clearly defined openings to substate levels of varying amplitude and duration occurred regularly within the gating activity of three of the *H. virescens* Ca^{2+}-channels successfully studied (Fig. 8*d*). No other obviously different types of Ca^{2+}-channel activity from that described above were observed.

Modification of the *H. virescens* Ca^{2+}-Channel Activity by Ryanodine

The characteristic effect of ryanodine on the single channel activity of various mammalian isoforms of the RyR is well-documented (Sutko et al., 1997). An almost identical characteristic and reproducible modification of both *H. virescens* and rabbit Ca^{2+}-channel activity occurs 2 to 5 min after addition of 100 nM ryanodine to the *cis* chamber (Fig. 9*a* and 9*b*). A fractional conductance state of high open probability was induced in both channel isoforms and remained for the duration of the experiment (⩽5 min). Perfusion of the ryanodine from the *cis* chamber after modification did not restore normal gating activity (Fig. 9). The fractional conductance of the modified state at 0 mV is 35.9 ± 0.6 and 37.8 ± 1.5% of the fully open state for *H. virescens* and rabbit Ca^{2+}-channels, respectively. Occasional closing events from the modified states of both channels were also observed. No modification in channel gating activity was observed for the duration of the experiment (12–15 min) after addition of 1 μM ryanodine in the presence of 4 mM EGTA (i.e., ⩽10 nM free Ca^{2+}). Under such conditions

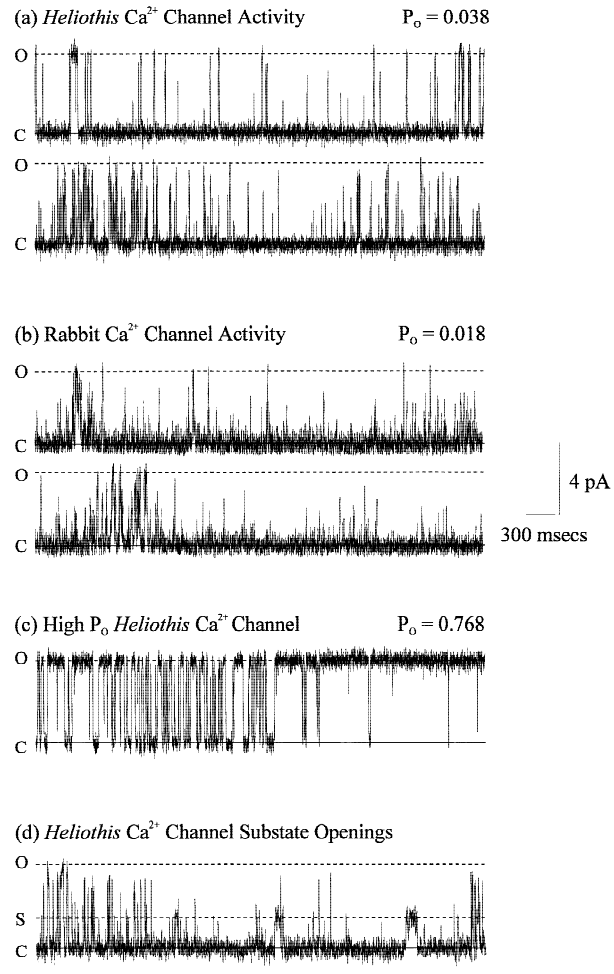

Fig. 8. *H. virescens* and rabbit skeletal SR Ca^{2+}-release channel activity in planar lipid bilayers. Under the postperfusion conditions (*cis*; 250 mM TRIS-HEPES, pH 7.4, *trans*; 250 mM Ca^{2+} glutamate, 10 mM TRIS-HEPES, pH 7.4) Ca^{2+} flows from the *trans* to *cis* chamber at 0 mV with upwards deflections denoting channel openings. The dotted ("O") and solid ("C") lines represent the fully open and closed levels, respectively. The concentration of Ca^{2+} in the *cis* chamber was ~7 μM (contaminant). Representative *H. virescens* and rabbit SR Ca^{2+} channel fluctuations are shown in (*a*) and (*b*), respectively (8 sec of continuous recording). The activity of a predominantly open *H. virescens* SR Ca^{2+} channel under identical conditions is shown in (*c*). A *H. virescens* SR Ca^{2+} channel containing openings to a clearly defined substate level is shown in (*d*). The individual open probabilities (P_O) for each channel were determined from ⩾2 min of continuous channel recording as described.

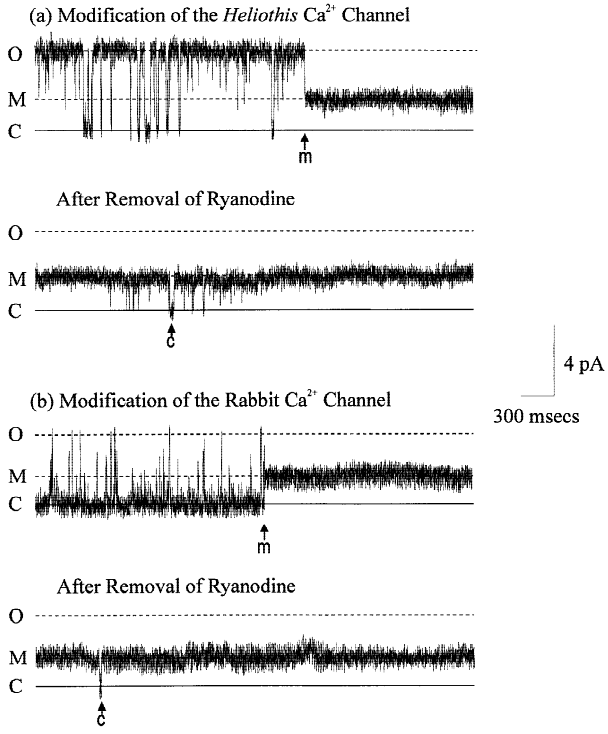

Fig. 9. Modification of SR-Ca^{2+} channel activity by ryanodine. The single channel current fluctuations of *H. virescens* (*a*) and rabbit skeletal (*b*) RyR in planar lipid bilayers are shown 2 to 5 min after the addition of 100 nM ryanodine to the solution in contact with the cytosolic side of the channel (0 mV). Both channel isoforms were reproducibly modified ("m") to a high open probability, reduced current amplitude state ("M"). Reversal of the modification was not observed for the remainder of the experiment. Perfusion of the ryanodine from the *cis* chamber did not reverse the modification.

either channel isoform was effectively closed with a P_O of close to 0 (*see below*).

The Ca^{2+} Conductance Properties of the *H. virescens* Ca^{2+} Channel

Figure 10*a* shows the effect of variation in holding potential from −20 to +20 mV on the current amplitude of the *H. virescens* RyR Ca^{2+} channel. The relationship between voltage (± 40 mV) and current for individual *H. virescens* and rabbit Ca^{2+} channels is displayed in Fig. 10*b* ($n = 3$). The slope conductance determined from a linear fit to the negative limb of the *I-V* plot are 115 ± 1 and 104 ± 2 pS for *H. virescens* and rabbit channels, respectively. These values are comparable with the previously determined conductances of lobster and rabbit skeletal RyR with Ca^{2+} as the permeant ion (Zhang et al., 1999). Due to the rectification of current at positive voltages caused by the presence of $TRIS^+$ in the *cis* chamber, the reversal potential for both channels isoforms is predicted to be above 50 mV, but cannot be reliably determined.

The Sensitivity of the *H. virescens* Ca^{2+} Channel to Ca^{2+}, ATP and Ruthenium Red

The open probability of the rabbit RyR1 is dependent on free Ca^{2+} concentration and affected by a range of endogenous and exogenous modulators (Zucchi & Roncatestoni, 1997). The channel current recordings shown in Fig. 11 are from a bilayer containing at least two *H. virescens* RyR-channels and demonstrate the dependence of channel open probability on the presence of ligands in the *cis* chamber. In five separate experiments a reduction in free Ca^{2+} from contaminant concentration (~7 μM) to less than 10 nM following addition of 4 mM EGTA closed the *H. virescens* Ca^{2+} channels, reducing mean P_O from 0.08 ± 0.05 (mean ± SEM, $n = 5$) to less than 0.001. The addition of 4 mM EGTA also lowered the P_O of rabbit Ca^{2+} channels to less than 0.001 (*data not shown*). On addition of $TRIS^+$-buffered ATP (pH 7.4) to the *cis* chamber instead of EGTA, the activity of *H. virescens* channels was increased by varying amounts. In three separate experiments, the addition of 1 mM ATP increased mean P_O from 0.07 ± 0.04 to 0.22 ± 0.11 (mean ± SD, $n = 3$). Following addition of ATP the free Ca^{2+} concentration in the *cis* chamber was calculated as ~0.7 μM. Further addition of 4 mM EGTA in the presence of ATP closed the *H. virescens* Ca^{2+} channels, reducing P_O to almost 0 as shown in Fig. 11. The closed state of the channel in the presence of ATP but with low nanomolar Ca^{2+} suggests that the mechanism of ATP action is Ca^{2+}-dependent. The addition of 1 μM ruthenium red alone to the *cis* chamber in the presence of contaminant Ca^{2+} completely closed the channel, reducing P_O to 0. The effects of modulators were observed in at least three separate experiments involving one or more *H. virescens* RyR-channels. The responses confirm that the cytosolic side of the channel is exposed to the *cis* chamber solution. Caffeine is a potent activator of rabbit RyR1, increasing single channel open probability in a Ca^{2+}-dependent manner (Rousseau et al., 1988). However, the addition of caffeine ($n = 2$) or its more potent isomazole analogue EMD 41000 (McGarry & Williams, 1994) ($n = 2$) caused bilayers containing *H. virescens* channels to break before recording was possible. The constraints imposed by the low level of *H. virescens* RyR-channel incorporation meant further single channel studies could not be pursued. It should be noted that both caffeine and EMD 41000 activated rabbit RyR-channels to varying degrees without breaking the bilayers in which they had been incorporated ($n = 5$, *data not shown*).

Discussion

This report is the first to describe the single channel, ryanodine binding and immunological characteristics of a membrane-bound insect RyR. It reveals that the tho-

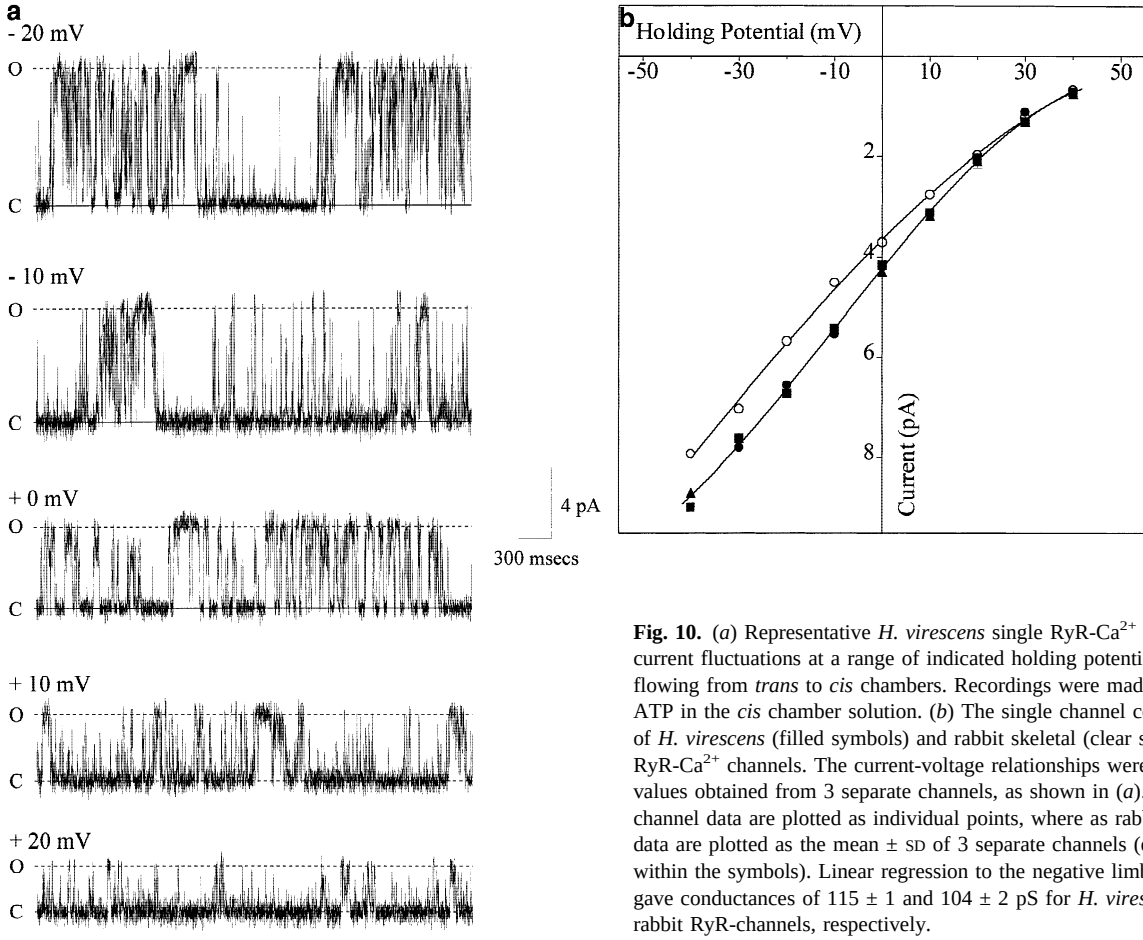

Fig. 10. (*a*) Representative *H. virescens* single RyR-Ca^{2+} channel current fluctuations at a range of indicated holding potentials, with Ca^{2+} flowing from *trans* to *cis* chambers. Recordings were made with 1 mM ATP in the *cis* chamber solution. (*b*) The single channel conductances of *H. virescens* (filled symbols) and rabbit skeletal (clear symbols) RyR-Ca^{2+} channels. The current-voltage relationships were plotted using values obtained from 3 separate channels, as shown in (*a*). *H. virescens* channel data are plotted as individual points, where as rabbit channel data are plotted as the mean ± SD of 3 separate channels (error bars are within the symbols). Linear regression to the negative limbs of the data gave conductances of 115 ± 1 and 104 ± 2 pS for *H. virescens* and rabbit RyR-channels, respectively.

racic tissues of *H. virescens* contain a functional RyR Ca^{2+}-release channel with similar properties to those reported for other receptor isoforms. Microsomal membranes isolated from adult *H. virescens* thoraces bound measurable levels of [^{3}H]-ryanodine and contained high molecular weight polypeptide of ⩾400 KDa recognized by an anti-*H. virescens* RyR antibody. This intimates that a functional RyR containing subunits of at least 3,500 amino acids in length is being expressed. Isolation of cDNA encoding the C-terminal 1172 residues of a *H. virescens* RyR by Puente and coworkers (2000) confirms a genetic origin for this isoform. The inability of the anti-*H. virescens* RyR antibody to recognize the rabbit isoform confirms that the sequence identity of 51% between isoforms is low enough to result in receptor specific antibodies.

Ca^{2+} channels displaying millisecond duration openings of high unitary conductance were reconstituted into planar lipid bilayers from gradient fractionated membranes. The gating and conductance properties of the *H. virescens* channel appear similar to those of the rabbit skeletal RyR as determined in this study. The current amplitude of the *H. virescens* RyR-channel decreased linearly with increasing holding potential from −40 to +20 mV, resulting in slope conductance 11% higher than that of the rabbit skeletal RyR. The conductance of the *H. virescens* channel is above the range previously reported for mammalian and invertebrate RyR (Smith et al., 1986; Zhang, et al., 1998). $TRIS^+$ ions in the *cis* chamber caused current to rectify at positive voltages resulting in a reversal potential (V_{rev}) of above 50 mV (Tinker & Williams, 1992). Accurate V_{rev} values cannot be reliably determined without further experimentation. The results confirm the Ca^{2+} conducting properties of the RyR are conserved in vertebrate and invertebrate isoforms.

Ryanodine bound specifically to *H. virescens* membranes and effected changes in the single channel properties of the *H. virescens* RyR in lipid bilayers. *H. virescens* and rabbit microsomal membranes have comparable [^{3}H]-ryanodine binding characteristics. K_D values measured for both isoforms are between 2 and 4 nM and comparable to values reported in other studies (Sutko et al., 1996). The hyperbolic dependence of binding on [^{3}H]-ryanodine concentration suggests the *H. virescens* RyR carries a single high affinity site. The 2-fold lower maximum density of *H. virescens* RyR binding sites (B_{MAX}) is probably the result of *H. virescens* membranes

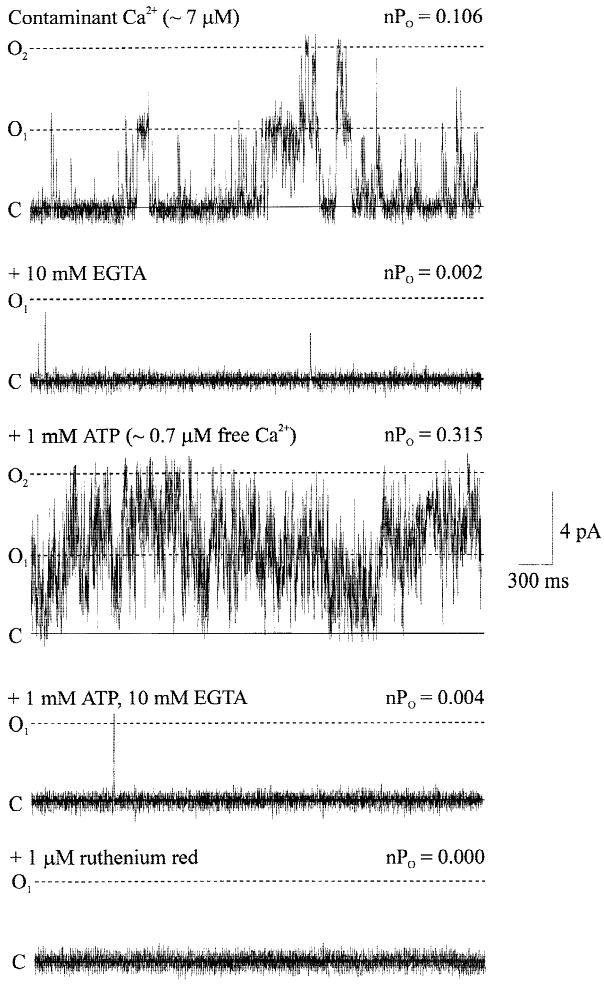

Fig. 11. The effect of classical RyR modulators on the channel activity of 2 *H. virescens* RyR at 0 mV holding potential. EGTA, ATP and ruthenium red were added to the *cis* chamber and the effects displayed typical of those observed in 2 other experiments. The dotted ("O_1", "O_2") and solid lines ("C") indicate the fully open and closed channel levels, respectively. The global open probability (nP_O) of both channels in each case was determined from ⩾2 min of channel recording. No effects on channel current amplitude were observed.

being prepared from thoracic tissues, rather than relatively pure mammalian skeletal muscle. Ryanodine binding proteins have also been identified in membranes prepared from housefly, cockroach and lobster tissue (Olivaries & Arispe, 1993; Lehmberg & Casida, 1994). At the single channel level ryanodine induced a sustained modification in the gating and conductance properties of both the *H. virescens* and rabbit Ca^{2+} channels. Both were modified to a 35 to 39% fractional conductance state with an open probability of ~1 less than 6 min after the addition of nanomolar concentrations of ryanodine to the *cis* chamber. Ryanodine modification is an established electrophysiological marker for the RyR Ca^{2+}-release channel (Rousseau et al., 1987). Modification did not occur when the *H. virescens* RyR-channel was closed (P_O = ~0), indicating the interaction of ryanodine is dependent on open probability (Tanna et al., 1998). The 20-fold difference in the effective concentrations of ryanodine at the binding and single channel levels may be attributed to differences in experimental conditions.

The results obtained in this study are consistent with the insecticidal activity of ryanodine arising primarily from its interaction with the insect RyR-Ca^{2+} release channel (Jefferies et al., 1992). Ryanodine modification of the *H. virescens* RyR-Ca^{2+}-release channel would eliminate controlled Ca^{2+}-release from the SR and uncouple membrane excitation from myofibril contraction, leading to paralysis. Plant extracts containing a mixture of ryanoids have been shown to cause over 95% mortality in *H. virescens* larvae (Yoshida & Toscano, 1994). However it remains a possibility that the action of ryanodine on other muscle proteins contributes to its toxic properties (Usherwood & Vais, 1995). Although ryanodine is ineffective on the mammalian skeletal muscle SR K^+ channel (Decarvalho & Cukierman, 1987), it does significantly lower the reversal potential (V_{rev}) and single channel conductance of a plasma membrane Ca^{2+}-activated K^+ channel from locust leg skeletal muscle in a dose-dependent manner (Vais, Rucareanu & Usherwood, 1996). These single channel actions of ryanodine were, however, different from those observed in this work.

[^{3}H]-ryanodine binding to the *H. virescens* RyR shows an absolute dependence on the concentration of Ca^{2+} present at the cytosolic face of the membrane. Increasing Ca^{2+} concentration from micromolar through to millimolar levels caused biphasic variation in ryanodine binding to both *H. virescens* membranes and rabbit membranes. Binding to *H. virescens* membranes was, however, less sensitive to Ca^{2+} concentration with a 3-fold higher EC_{50}. The Hill coefficients suggest the binding of one Ca^{2+} per receptor molecule is sufficient to activate either isoform. The open probability of the *H. virescens* RyR-Ca^{2+} channel was also dependent on the Ca^{2+} concentration. As with other isoforms of the receptor, lowering the Ca^{2+} concentration of the solution in contact with the sarcoplasmic face of the channel to nanomolar levels caused it to close. Previous studies reveal membrane-bound vertebrate cardiac and skeletal RyR to have EC_{50} values in the 0.2 to 10 μM Ca^{2+} range (Ogawa et al., 1999). Recent work on the lobster RyR suggests Ca^{2+} sensitivity is dependent on both the method of preparation and the study conditions (Seok et al., 1992; Quinn et al., 1998; Xiong et al., 1998; Zhang et al., 1999). Ca^{2+} may have a naturally lower affinity, or efficacy, for insect and possibly all invertebrate RyR isoforms.

Ryanodine binding to *H. virescens* membranes was inhibited by millimolar concentrations of Ca^{2+}. Many isoforms of the RyR are sensitive to inhibition by milli-

molar Ca^{2+}, with the degree of inhibition and effective Ca^{2+} concentration varying between isoforms (Xiong et al., 1998). In these studies, binding to the *H. virescens* receptor was less sensitive to Ca^{2+} inhibition than the rabbit receptor. It has been reported that higher concentrations of Ca^{2+} may also be required to inhibit the open probability and binding activity of the mammalian cardiac RyR (Chu et al., 1993). Inhibition may occur following low affinity binding of an additional Ca^{2+} ion to a low affinity divalent cation site (Lee, Xu & Meissner, 1994). Millimolar concentrations of the divalent cation Mg^{2+} also caused dose-dependent inhibition of ryanodine binding to both *H. virescens* and rabbit receptor isoforms. The *H. virescens* RyR is, however, more sensitive to Mg^{2+} inhibition at fixed Ca^{2+} concentrations with an IC_{50} 2.4-fold lower than that for the rabbit skeletal RyR. Previous work on rabbit RyR1 indicates that Mg^{2+} ions exert their effect by occluding the Ca^{2+} activation site (Laver et al., 1997). The marginal difference in Mg^{2+} sensitivity between isoforms may be the result of the previously discussed differences in Ca^{2+} sensitivity. A lower affinity for Ca^{2+} ions at the *H. virescens* receptor divalent cation activation site would make it easier for Mg^{2+} to displace Ca^{2+} and hence lower channel P_O and inhibit binding activity. Determination of the IC_{50} values at a range of micromolar Ca^{2+} concentrations coupled with single channel studies would define the mechanism of Mg^{2+} inhibition. As with other RyR isoforms, low micromolar concentrations of the channel antagonist ruthenium red lowered the ryanodine binding activity and open probability of the insect RyR-channel. Ruthenium red is believed to affect the mammalian RyR through interactions at a site in the ion conduction pore unique from the ryanodine binding site (Xu et al., 1999). The universal sensitivity of *H. virescens* RyR-channels to ligands in the *cis* chamber indicates incorporation was occurring in an orientation-specific manner with the sarcoplasmic "face" of the receptor in contact with the *cis* chamber solution. The directional incorporation of RyR into lipid bilayers is believed to be due to the receptors fixed orientation in isolated SR membrane vesicles (Miller & Rosenberg, 1979, Tomlins, Williams & Montgomery, 1984).

Ryanodine binding to the *H. virescens* RyR is unaffected by micromolar to millimolar concentrations of caffeine at the three free Ca^{2+} present in this study. These results indicate the effects of this ligand on RyR function are substantially different from those on the rabbit RyR1. Previous studies demonstrate that ryanodine binding to housefly thoracic membranes in the presence of 1 mM EGTA is unaffected by up to 20 mM caffeine (Lehmberg & Casida, 1994). In contrast, caffeine has been reported to cause muscle contraction in intact locust visceral muscle and also Ca^{2+}-dependent Ca^{2+} release from ryanodine-sensitive internal stores in honeybee photoreceptor cells (Walz et al., 1995). Despite 69% identity between the C-terminal 1170 amino acids of the *H. virescens* and lobster RyR isoforms, the Ca^{2+}-sensitivity of [^{3}H]-ryanodine binding to lobster RyR is measurably increased by millimolar concentrations of caffeine (Zhang et al., 1999). Binding studies, however, provide limited information on RyR properties and so it was therefore important to attempt to determine the effects, if any, of caffeine on *H. virescens* RyR function directly. The low efficiencies with which it has proved possible thus far to generate *H. virescens* RyR preparations suitable for single channel studies meant the potential effects of caffeine could not be investigated at this level. Differences in tissue protease activity or the stability of invertebrate and vertebrate RyR isoforms may also contribute to species-specific alteration in caffeine sensitivity during receptor preparation. The intermediate caffeine sensitivity of the cockroach [^{3}H]-ryanodine receptors suggests there could also be differences inherent to the invertebrate isoform in question (Lehmberg & Casida, 1994).

Low levels of channel incorporation restricted single channel studies on the *H. virescens* RyR. The density of [^{3}H]-ryanodine receptors in postgradient membrane preparations for this species was approximately 6 times lower than rabbit HSR membranes. However, the lower number of receptors in the insect SR membranes only partially explains the limited incorporation of RyR-channels. Studies by Hanke (1986) demonstrated that the ion channel and lipid composition of membranes also influences the ability of SR vesicles to fuse with bilayers under controlled conditions. Differences in the ion channel and membrane composition of rabbit and *H. virescens* SR vesicles may have contributed to a substantially lower level of *H. virescens* RyR incorporation. A difference in [^{3}H]-ryanodine binding to postgradient separation *H. virescens* and rabbit membranes indicates dissimilarities in their preparative and hence structural properties. It is reasonable to propose that such differences could also have affected RyR incorporation. Data presented by Palade & Gyorke (1993) reveals substantial differences between invertebrate and mammalian skeletal muscle structure, which support this suggestion. Measurement of $^{45}Ca^{2+}$ flux rates from *H. virescens* microsomes would provide another source of functional information and could potentially confirm the single channel properties reported here. Knowledge of the vesicle flux properties would assist in defining optimal channel study conditions and indicate what single channel studies would be most productive given the difficulty of performing such experiments. Although the $^{45}Ca^{2+}$ flux properties of the lobster RyR are known (Seok et al., 1992), measurements of $^{45}Ca^{2+}$ release through insect RyR have yet to be reported.

The results presented in this communication clearly

demonstrate that the thoracic tissues of *H. virescens* contain functional ryanodine receptor-Ca^{2+} release channels and that insect RyR are amenable to preparation and functional characterization. The [^{3}H]-ryanodine binding characteristics of *H. virescens* microsomal membranes complement the electrophysiological data from RyR-channels as exemplified by the effects of Ca^{2+}, ATP and ruthenium red on both binding activity and channel P_O. Sensitivity to Ca^{2+}, Mg^{2+} and ATP suggest the RyR may play an active role in insect muscle E–C coupling. Further studies under more physiologically relevant conditions are required to draw conclusions on the role and importance of the insect RyR in flight muscle E–C coupling. The recent cloning and expression of a *H. virescens* SR Ca^{2+}-ATPase also indicates the likelihood of a functioning system for the sequestration of Ca^{2+} from the cytoplasm back in to the SR (Lockyer et al., 1998).

Further characterization of the *H. virescens* RyR-channel properties in the presence of caffeine and ryanoid analogues is likely to shed more light on isoform-specific differences and contribute to understanding their structural basis. A recent report revealed heterologous expression of the C-terminus of *D. melanogaster* RyR in CHO cells produced ryanodine-sensitive ion channels (Xu et al., 2000). Development of a protocol for the stable heterologous expression of insect RyR and the isolation of functional insect RyR for detailed single channel studies will be defining steps in future research in this area.

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