Characterization of Recombinant Rabbit Cardiac and Skeletal Muscle Ca\(^{2+}\) Release Channels (Ryanodine Receptors) with a Novel \([^3H]\)Ryanodine Binding Assay*

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A rapid assay for high affinity \([^3H]\)ryanodine binding to 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS)-solubilized recombinant or native Ca\(^{2+}\) release channel proteins (ryanodine receptor, RyR) was devised. The key to preservation of high affinity \([^3H]\)ryanodine binding sites in the presence of increasing concentrations of CHAPS was the addition of phosphatidylcholine. This assay was used to characterize the equilibrium and kinetic properties of \([^3H]\)ryanodine binding to recombinant skeletal (RyR1) and cardiac (RyR2) Ca\(^{2+}\) release channels and the effects on binding of physiological modulators including ATP, Ca\(^{2+}\), and Mg\(^{2+}\). Both RyR1 and RyR2 had a single high affinity ryanodine binding site and low affinity sites, but \([^3H]\)ryanodine binding to recombinant RyR2 was not sensitive to ATP activation or Ca\(^{2+}\) inactivation and was less sensitive to Mg\(^{2+}\) inhibition. The \([^3H]\)ryanodine binding assay was used to estimate the expression level of recombinant RyR2 and RyR1, and to show that RyR2 can be expressed at very high levels in HEK-293 cells. Analysis of the properties of recombinant RyR2 and RyR1 by measurement of intracellular Fura-2 fluorescence revealed that the different properties of RyR2 and RyR1 are retained in the recombinant expressed proteins.

Ca\(^{2+}\) release channels (ryanodine receptors or RyR)\(^{1}\) from sarcoplasmic reticulum are formed as homotetramers of 565,000-Da subunits (1, 2). The activities of native Ca\(^{2+}\) release channels from skeletal (RyR1) and cardiac muscle (RyR2) are modulated by a variety of physiologically relevant agents and by a series of pharmacological compounds (for details, see Refs. 3–5). Ryanodine binds to Ca\(^{2+}\) release channels with high affinity and was used in the original identification of Ca\(^{2+}\) release channel proteins (6). At concentrations below 10 \(\mu M\), ryanodine locks the channel into a subconductance state and at concentrations above 10 \(\mu M\) it blocks conductance. Ryanodine is often used as a probe of conformation to indicate the open state of the channel.

A major goal in studies of ryanodine receptors is to understand structure/function relationships through expression and analysis of mutant forms of both skeletal muscle (RyR1) and cardiac (RyR2) isoforms. In order to carry out such studies, a series of useful assays for ryanodine receptors expressed in homologous cell culture have been developed, including planar bilayer assays (7, 8), Ca\(^{2+}\) photomery (9), and Ca\(^{2+}\) imaging (10). In this paper, we describe modifications to the \([^3H]\)ryanodine binding assay (11) which makes it useful for microscale analyses of expressed ryanodine receptors. We also describe the expression of a rabbit cardiac ryanodine receptor (RyR2) clone in HEK-293 cells and we characterize some of its properties through use of the \([^3H]\)ryanodine binding assay and through Ca\(^{2+}\) photomery.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases, and other DNA modifying enzymes were from Boehringer Mannheim, New England Biolabs, Promega, and Amersham Pharmacia Biotech; Fura-2 acetoxymethyl ester (AM) was from Molecular Probes; caffeine, AMPPCP, and protease inhibitors were from Sigma; \([^3H]\)Ryanodine was from NEN Life Science Products; unlabeled ryanodine and thapsigargin were from Calbiochem; CHAPS was from Bio-Rad; and phosphatidylcholine (PC) was from Avanti Polar Lipids. The expression vector pcDNA 3.1(−) was from Invitrogen. Monoclonal antibody 34C was a kind gift from Dr. Judith Airey (12). Monoclonal antibody C3-33 for cardiac ryanodine receptor was from Affinity Bioreagents. Horseradish peroxidase-conjugated goat anti-mouse secondary antibody (IgG) was from Promega, and SuperSignal Ultra chemiluminescent substrate was from Pierce. Nitrocellulose membranes were from Schleicher & Schuell. All other reagents were of reagent grade or highest grade available.

Sarcoplasmic Reticulum Microsome Preparation—Heavy sarcoplasmic reticulum microsomes were isolated from rabbit heart and from twitch skeletal muscle as described previously (13). Microsomes were suspended in 0.25 M sucrose, 10 mM Tris-HCl, pH 8.0, and stored at −70 °C.

Construction of Full-length RyR1 and RyR2 cDNA for Expression—The expression of cDNA encoding rabbit skeletal muscle ryanodine receptor was described previously (7–9). Eight overlapping clones of RyR2 (14) were subcloned into the EcoRI site in the pBluescript vector and labeled as pBS-CRR1 to pBS-CRR8. A polymerase chain reaction fragment containing nucleotide 12351–13140 (pBS-CRR9) was synthesized from a cardiac cDNA library. These nine clones were used for construction of full-length expression plasmid pcDNA-CRR as follows. The SacII (−53)–EcoRI (1590) fragment from pBS-CRR1 and the EcoRI (1590)–EcoRI (4387 to vector) fragment of pBS-CRR2 were ligated into SacII–EcoRI sites of pBluescript vector to form pBS-CRR12. The HindIII (2859)–EcoRI (5443) fragment from pBS-CRR3 and the EcoRI (5443)–EcoRI (8270 to vector) fragment from pBS-CRR4 were ligated into HindIII–EcoRI sites of the vector to yield pBS-CRR34. The EcoRI (7950 to vector)–EcoRI (10075) fragment of pBS-CRR5 and the EcoRI (10075)–NdeI (11072) fragment from pBS-CRR6 were ligated into EcoRI–NdeI sites of the modified pBluescript vector (pBS42) (9) to form pBS-CRR7. The NdeI (11072)–Smal (MCS, 12649) fragment from pBS-CRR7 were ligated into pBS-CRR6 cut with NdeI (11072)–EcoRV (MCS, of the vector to form pBS-CRRB7. The AvaII (11008)–ClaI (MCS) (16225) fragment from pBS-CRR9 and the fragment PstI (12346)–AvaII (13008) from pBS-CRR8 were ligated to the PstI and ClaI sites of the vector to

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1 The abbreviations used are: RyR, ryanodine receptor; AMPPCP, β,γ-methyleneadenosine 5′-triphosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; mAβ, monoclonal antibody; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PAGE, polyacrylamide gel electrophoresis; MCS, multiple cloning site.
form pBS-CRR89.

The XhoI (3111)-AhdI (8191) fragment from pBS-CRR34 and the fragment AhdI (8191)-BamHI (10563) from pBS56 were ligated into XhoI-BamHI sites in the vector to form pBS-CRR3–6. The fragment BamHI (10563)-PstI (12946) from pBS56 was ligated into pBS-CRR89. MscI-PstI (12946) to form pBS-CRR9.

The SacII (–53)–BglII (3954) fragment from pBS-CRR12 and the fragment BglII (3954)-Sall (10563) fragment from pBS-CRR3–6 were ligated into SacII–SalI sites in the vector to form pBS-CRR1–6. The SacII sites in pBS-CRR1–6 was changed to NotI by blunt-ending with Klenow fragment and ligated into the blunt-ended fragment with a NotI linker to from pBS-CRR1–6. The cells were transformed with plasmids containing pBS-CRR (linker–BamHI) for E. coli S100 (5555) fragment from pBS-CRR1–6 and the Ndel (11072)-BstBI (15355) fragment from pBS-CRR6–9 was ligated into NotI-BstBI sites of pBS2 to yield pBS-CRR, and the Ndel (3555)-Ndel (11072) fragment from pBS-CRR56 was inserted into pBS-CRRs to yield pBS-CRR. Finally, the entire cDNA sequence encoding RyR2 from pBS-CRR was excised with NotI and BstBI (blunt-ended with Klenow) and subcloned into the NotI-HindIII (blunt-ended with Klenow) sites of pcDNA 3.1(−).

Cell Culture and DNA Transfection—HEK cells were maintained and DNA transfection was carried out by the calcium phosphate precipitation method described previously (8, 15).

Immunocytochemical Staining—HEK-293 cells transfected with RyR1 cDNA or RyR2 cDNA were stained with mAb 543 and mAb C3-33 to detect RyR1 or RyR2 by immunofluorescence staining. The cells adhering to the coverslips were washed three times with 6 ml of washing buffer. Filters were washed three times with 6 ml of washing buffer. Filters were washed three times with 6 ml of washing buffer. Filters were washed three times with 6 ml of washing buffer. Filters were washed three times with 6 ml of washing buffer. Filters were washed three times with 6 ml of washing buffer. Filters were washed three times with 6 ml of washing buffer. Filters were washed three times with 6 ml of washing buffer. Filters were washed three times with 6 ml of washing buffer. Filters were washed three times with 6 ml of washing buffer. Filters were washed three times with 6 ml of washing buffer. Filters were washed three times with 6 ml of washing buffer. Filters were washed three times with 6 ml of washing buffer. 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RyR1 cDNA (horseradish peroxidase-conjugated anti-mouse IgG. D) and secondary filters were probed with mAb 34C (E) and C3-33 (A) in HEK-293 cells transfected with pcDNA vector (blocks those channels that were responsive to caffeine. tive to these two agents and that ryanodine binds only to shown). The results illustrate that recombinant RyR2 is sensi-

Thapsigargin—We measured Ca2+ release in HEK-293 cells in which caffeine-induced Ca2+ release was from intracellular stores, through endogenous leaks that be-

Caffeine-induced Ca2+ Release from HEK-293 Cells Transfected with RyR1 cDNA—We measured Ca2+ release in HEK-293 cells in which caffeine was washed out. Subsequent addition of 20 mM caffeine caused one Ca2+ release similar to that observed in untransfected cells, and abolished caffeine-induced Ca2+ release (Fig. 2B), demonstrating that caffeine-induced Ca2+ release was from thapsigargin-sensitive Ca2+ stores. Thapsigargin is a potent and specific inhibitor of sarcoldo-(endoplasmic reticulum Ca2+-ATPases. Ca2+ is probably released from intracellular stores, through endogenous leaks that become apparent following Ca2+ pump inhibition (26, 27). In untransfected HEK-293 cells, 1 μM thapsigargin caused a slow phase of Ca2+ release (n = 3, data not shown). In HEK-293 cells transfected with RyR2 cDNA, in which caffeine-induced Ca2+ release was evident, application of 1 μM thapsigargin induced Ca2+ release similar to that observed in untransfected cells, and abolished caffeine-induced Ca2+ release (Fig. 2B), demonstrating that caffeine-induced Ca2+ release was from thapsigargin-sensitive Ca2+ stores.

Characterization of Caffeine-induced Ca2+ Release from In-

Recombinant Ryanodine Receptor, [3H]Ryanodine Binding

FIG. 1. Expression of RyR1 and RyR2 in HEK-293 cells. Immunocytochemical staining was carried out to detect transient expression in HEK-293 cells transfected with pcDNA vector (A), RyR2 (B), and RyR1 cDNA (C). Cells on the coverslips were fixed with 4% formalde-

FIG. 2. Fluorescence measurements of Ca2+ release by caffeine from internal stores into the cytoplasm of HEK-293 cells trans-

FIG. 2A shows that 20 mM caffeine caused a rapid Ca2+ release in HEK-293 cells transfected with RyR2 cDNA. This response could be obtained repeatedly, provided that previously applied caffeine was washed out. Subsequent addition of 50 μM ryanodine did not cause significant intracellular Ca2+ release in three of four experiments over a period of 2–3 min. The first application of 20 mM caffeine after the addition of ryanodine induced a release phase that was similar to the previous one, indicating that ryanodine did not have any direct effect on caffeine-induced Ca2+ release. Further caffeine challenges greatly diminished and eventually could not stimulate any Ca2+ release from the internal store, even in the absence of ryanodine in the extracellular solution (Fig. 2A). Similar results were also obtained in HEK-293 cells transfected with RyR1 cDNA for caffeine and ryanodine (data not shown). In untransfected cells, 20 mM caffeine and 50 μM ryanodine did not cause any significant Ca2+ release (data not shown). The results illustrate that recombinant RyR2 is sensitive to these two agents and that ryanodine binds only to open-state channels, induced by caffeine. Ryanodine then blocks those channels that were responsive to caffeine.
and the mean half-rise time ($t_{1/2}$) in the 20 mM caffeine responses. These two values, calculated from independent transients, are presented in Table I. Significant differences in both peak and $t_{1/2}$ between RyR2 and RyR1 cDNA-transfected cells indicate that recombinant RyR2 and RyR1 have different kinetics of caffeine-induced Ca$^{2+}$ release.

Characterization of a $[^3H]$Ryanodine Binding Assay—The detergent CHAPS is widely used in solubilization and purification of muscle sarcoplasmic reticulum Ca$^{2+}$ release channels (6, 29). We solubilized rabbit skeletal muscle microsomes at a concentration of 0.4 mg/ml in 1% CHAPS (16 mM), which is 2-fold higher than its critical micelle concentration. Equilibrium binding experiments were carried out following 10-fold dilution with 0.1% CHAPS in the binding buffer, and results were compared with controls with no CHAPS in the binding buffer. Scatchard plot analysis showed that $B_{\text{max}}$ for the solubilized protein was significantly lower than that for controls (3.6 versus 7.7 pmol of ryanodine/mg of protein) ($p < 0.05$) with no alteration of $K_J$ (Fig. 4A and Table II). Although 0.1% CHAPS in binding buffer has been reported to stabilize $[^3H]$ryanodine binding to RyR (30), our observation of a decrease in the number of high affinity binding sites suggested that endogenous lipid molecules were stripped away from the solubilized RyR protein and replaced by CHAPS molecules (31). To avoid the formation of a preponderance of CHAPS:RyR protein micelles, we tested the effect of addition of exogenous phospholipids, since PC has been used to stabilize the cardiac ryanodine receptor during its purification from dog heart sarcoplasmic reticulum in the presence of CHAPS (22). The addition of 5 mg/ml PC in the solubilization buffer led to a higher recovery of high affinity $[^3H]$ryanodine binding sites. We observed a $B_{\text{max}}$ of 11.8 pmol/mg of protein with an unchanged $K_J$ (Fig. 4A and Table II), indicating that the ryanodine binding function could be preserved by increasing the phospholipid concentration to form mixed micelles (detergent:protein:phospholipid).

Table I

Comparison between the properties of caffeine-induced Ca$^{2+}$ release from RyR1 and RyR2 cDNA transfected HEK-293 cells

|            | RyR2 | RyR1 | n   | p  |
|------------|------|------|-----|----|
| Threshold value | 0.03–0.1 mM | 16   | 0.3–0.52 mM | 16  |
| EC$_{50}$ | 1.05 ± 0.10 mM | 16   | 2.75 ± 0.24 mM | 16  | <0.05 |
| Slope | 1.29 ± 0.10 | 16   | 1.91 ± 0.12 | 16  | <0.05 |
| $t_{1/2}$ | 0.44 ± 0.02 s | 6    | 0.86 ± 0.08 s | 25  | <0.05 |
| Peak amplitude | 0.89 ± 0.05 | 22   | 0.55 ± 0.03 | 32  | <0.05 |

* Averaged from individual experiments.
ment of transfected cells that were ~80–90% confluent with 1% CHAPS alone did not abolish [3H]ryanodine binding (see below), suggesting that increased endogenous lipid might help to preserve [3H]ryanodine binding. In five independent experiments (of which two were with ~40% confluent cells and three were with ~80% confluent cells), an average $B_{\text{max}}$ of 0.18 pmol/mg of protein with a $K_d$ of 2.2 nM (Table II) was observed when cells were solubilized with 1% CHAPS and 5 mg/ml PC.

The $K_d$ value was similar to that of native RyR1.

We did not observe any specific [3H]ryanodine binding in pcDNA vector-transfected cells after solubilization with 1% CHAPS and 5 mg/ml PC (Fig. 4B), nor any specific [3H]ryanodine binding following solubilization of RyR1 cDNA-transfected HEK-293 cells with 1% Zwittergent 3-14 in the presence of 5 mg/ml PC (Table II). Zwittergent 3-14 is a detergent known to disrupt the tetrameric structure of RyR (32).

The effect of CHAPS and PC on the extraction of protein and on [3H]ryanodine binding was determined by assaying different concentrations of CHAPS in solubilizing and binding buffers. We increased the concentration of CHAPS in the presence of PC, but maintained a PC:CHAPS ratio of 0.5:1 except for 2% CHAPS, in which the PC concentration was maintained at 5 mg/ml. The presence of PC in the solubilizing buffer increased both [3H]ryanodine binding and protein concentration in the lysate from ~80% confluent cells (Fig. 5, A and B). At 1% CHAPS in the presence of 5 mg/ml PC, solubilization of protein began to plateau. When the same batch of cells was solubilized with CHAPS alone, protein solubilization was increased, but [3H]ryanodine binding decreased (Fig. 5, A and B). Increasing concentrations of CHAPS in the binding buffer also inhibited [3H]ryanodine binding (Fig. 5C). CHAPS inhibition of [3H]ryanodine binding was marked at 0.4% (6 mM) or above, where the critical micelle concentration was exceeded. These results suggest that both endogenous and exogenous lipids are important in maintaining the function of solubilized RyR1 protein in the presence of CHAPS, and indicate that this assay for [3H]ryanodine binding can be used in the characterization of recombinant RyR proteins.

**High Affinity Equilibrium Binding of [3H]Ryanodine—Scatchard analysis of equilibrium [3H]ryanodine binding to recombinant RyR1 showed a single binding site with $K_d = 2.2$ nM (Tables II and III). Recombinant RyR2 showed a similar single high affinity binding site with $K_d = 2.1$ nM (Table III). The values are similar to those reported previously for native RyR1 and RyR2 (21, 33). We obtained similar $K_d$ values for skeletal (2.2 ± 0.1 nM, $n = 4$) (Table II) and cardiac muscle (2.1 ± 0.3 nM, $n = 4$) using sarcoplasmic reticulum microsomes, under identical conditions. $B_{\text{max}}$ was 0.18 ± 0.02 and 0.76 ± 0.07 pmol/mg of protein for recombinant RyR1 and RyR2, respectively. There was no specific binding in lysates isolated from pcDNA-transfected HEK cells.

**Association and Dissociation Kinetic Studies of High Affinity Binding Sites**—The association kinetics for high affinity binding were measured for recombinant RyR1 and RyR2, with 10 nM [3H]ryanodine. Ryanodine binding reached saturation within 60 min in both RyR1 and RyR2. The association rate constant ($k_1$), calculated from the slope of the pseudo-first-order plot was similar for recombinant RyR1 and RyR2 (Fig. 6A and Table III) using the equation listed in the legend to Table III.

The rate of dissociation of [3H]ryanodine from the high affinity binding sites in recombinant RyR1 and RyR2 was also examined by 50-fold dilution into binding buffer B without added ATP and Ca$^{2+}$ so that rebinding of [3H]ryanodine was prevented. The first-order plots were linear for both RyR1 and RyR2 (Fig. 6B), indicating single high affinity ryanodine binding sites, which do not have cooperativity. The dissociation rate constant, $k_{-1}$, was similar for recombinant RyR1 (0.01056 min$^{-1}$) and RyR2 (0.01106 min$^{-1}$) (Table III). The dissociation constant ($K_{f}$) for ryanodine binding to high affinity sites calculated from the kinetic rate constants for association and dissociation ($K_{f} = k_{-1}/k_{1}$) was virtually the same for RyR1 (1.8 nM) and RyR2 (1.8 nM) and was similar to that obtained from Scatchard analysis (Table III).

In studies with skeletal and cardiac muscle sarcoplasmic reticulum, low affinity binding sites for ryanodine were identified, and occupation of low affinity sites decreased the dissociation rate of bound ryanodine from high affinity sites, suggesting that positively cooperative interactions occur between high and low affinity sites (20, 21). To determine whether this characteristic is preserved in recombinant RyR1 and RyR2, we tested for low affinity sites by 50-fold dilution in binding buffer B containing 40 μM unlabeled ryanodine, without added ATP and Ca$^{2+}$. A dramatic decrease in [3H]ryanodine dissociation from high affinity sites was observed in both recombinant RyR1 and RyR2.

**Table II**

| RyR1          | $B_{\text{max}}$ | $K_d$  |
|---------------|------------------|--------|
| Native Control | 7.7 ± 0.3        | 3.1 ± 0.5 |
| +CHAPS        | 3.6 ± 0.3$^a$    | 2.6 ± 0.2 |
| +CHAPS + PC   | 11.8 ± 1.8$^a$   | 2.4 ± 0.2 |
| Expressed     |                  |         |
| +CHAPS        | Variable          |        |
| +CHAPS + PC   | 0.18 ± 0.02      | 2.2 ± 0.4 |
| HEK-293 + CHAPS | 0               | 0       |

$^a p < 0.05$ when compared with control. Data were obtained from three independent experiments.

$^a$ Values were variable, depending on the amount of cells, as described under “Results.”
and RyR2 when 40 μM ryanodine was present in the dissociation buffer (Fig. 6B). The \( k_{-1} \) values were 0.00167 min\(^{-1}\) and 0.00115 min\(^{-1}\) for recombinant RyR1 and RyR2, respectively (Table III). These results suggest that recombinant RyR1 and RyR2, like the native channels, possess both high and low affinity binding sites and that there is positive cooperativity between these binding sites.

**Effects of Ca\(^{2+}\), AMPPCP, and Mg\(^{2+}\) on \([\text{H}]\)Ryanodine Binding**—The effects of the physiological modulators of muscle Ca\(^{2+}\) release channels, Ca\(^{2+}\), ATP, and Mg\(^{2+}\), were tested on recombinant RyR1 and RyR2. The Ca\(^{2+}\) dependence of \([\text{H}]\)ryanodine binding and the effect of 1 mM AMPPCP on the Ca\(^{2+}\) dependence of \([\text{H}]\)ryanodine binding to recombinant RyR2 are shown in Fig. 7A and for RyR1 in Fig. 7B. At low Ca\(^{2+}\) concentrations (pCa \( \geq 7 \)) in the binding buffer without ATP, no significant binding was detected in either recombinant RyR1 or RyR2. \([\text{H}]\)Ryanodine binding was activated by increasing Ca\(^{2+}\) concentrations, reaching optimal binding at about pCa 5. At higher Ca\(^{2+}\) concentration (pCa \( < 4 \)), \([\text{H}]\)ryanodine binding was decreased in RyR1. However, binding to RyR2 was not sensitive to inactivation by high Ca\(^{2+}\) concentration up to pCa 2.0. Thus, the Ca\(^{2+}\) dependence of \([\text{H}]\)ryanodine binding was bell-shaped in RyR1 but not in RyR2. The EC\(_{50}\) and Hill coefficient for Ca\(^{2+}\)-activation in RyR2 and RyR1 and the IC\(_{50}\) and Hill coeff-
HEK-293 cells were added into 4.5 ml of binding buffer B with 10 nM ARyR2 containing different Ca²⁺ of 1 mM AMPPCP. [3H]ryanodine binding was shifted to the left in RyR1 (Fig. 7A), indicating a cooperative action of Ca²⁺ in Fig. 8. The IC₅₀ value was 3.1 ± 1.2 mM (n = 3) for RyR1 and 8.7 ± 0.7 mM (n = 3) for RyR2 (p < 0.05), indicating a significant difference in the mechanism by which Mg²⁺ affects the [3H]ryanodine binding. In both proteins, Mg²⁺ is proposed to displace Ca²⁺ competitively from its activation site(s) (5). The slopes were 0.8 ± 0.1 for RyR1 and 1.1 ± 0.06 for RyR2, suggesting that a single binding site for Mg²⁺ occurs in both RyR2 and RyR1.

**TABLE IV**

| pCa | EC₅₀ | Slope | IC₅₀ | Slope |
|-----|------|-------|------|-------|
| RyR2 | | | | |
| 0 mM AMPPCP | 6.01 ± 0.06 | 2.0 ± 0.3 | | |
| 1 mM AMPPCP | 6.09 ± 0.11 | 2.2 ± 0.4 | | |
| RyR1 | | | | |
| 0 mM AMPPCP | 6.12 ± 0.11 | 2.1 ± 0.8 | 2.33 ± 0.28 | 1.1 ± 0.2 |
| 1 mM AMPPCP | 6.78 ± 0.17 | 1.5 ± 0.6 | 1.81 ± 0.21 | 1.6 ± 0.8 |

**DISCUSSION**

[3H]Ryanodine Binding—In this study, we found that the property of [3H]ryanodine binding to recombinant RyR1 was unstable in the presence of the detergent CHAPS. The addition of PC, however, resulted in the recovery of [3H]ryanodine binding activity, demonstrating that phospholipids are essential for maintenance of recombinant RyR1 structure and function in the solubilized state (6). While native RyR1 from sarcoplasmic reticulum could be dissolved in CHAPS and purified to homogeneity without lipids and without loss of ryanodine binding, the highest [3H]ryanodine binding ability was obtained after the purified RyR1 was incorporated into liposomes (6).

Our modified assay has proven to be procedurally simple and efficient for Ca²⁺ inactivation in RyR1 are listed in Table IV. The Hill coefficient for activation was close to 2 in both cases, indicating a cooperative action of Ca²⁺ on [3H]ryanodine binding. These results are all in agreement with those obtained in skeletal and cardiac muscle sarcoplasmic reticulum (3, 5).

In the presence of 1 mM AMPPCP, the Ca²⁺ activation of [3H]ryanodine binding was shifted to the left in RyR1 (Fig. 7B), but was not changed in recombinant RyR2 (Fig. 7A). The EC₅₀ values calculated from three independent experiments are listed in Table IV. The inactivation curve was shifted to the right in RyR1 with IC₅₀ from 2.33 to pCa 1.81 (p < 0.05) (Fig. 7B and Table IV). These results suggest that the sensitivity to ATP was higher in recombinant RyR1 than in RyR2, consistent with those reported in muscle (34, 35).

Mg²⁺ at millimolar concentrations inhibits Ca²⁺ release and [3H]ryanodine binding in skeletal muscle sarcoplasmic reticulum and, to a lesser extent, in cardiac muscle sarcoplasmic reticulum (3, 5). The inhibition of [3H]ryanodine binding to recombinant RyR1 and RyR2 by increased Mg²⁺ concentrations is shown in Fig. 8. The IC₅₀ value was 3.1 ± 1.2 mM (n = 3) for RyR1 and 8.7 ± 0.7 mM (n = 3) for RyR2 (p < 0.05), indicating a significant difference in the mechanism by which Mg²⁺ affects the [3H]ryanodine binding. In both proteins, Mg²⁺ is proposed to displace Ca²⁺ competitively from its activation site(s) (5). The slopes were 0.8 ± 0.1 for RyR1 and 1.1 ± 0.06 for RyR2, suggesting that a single binding site for Mg²⁺ occurs in both RyR2 and RyR1.

**TABLE IV**

| pCa | EC₅₀ | Slope | IC₅₀ | Slope |
|-----|------|-------|------|-------|
| RyR2 | | | | |
| 0 mM AMPPCP | 6.01 ± 0.06 | 2.0 ± 0.3 | | |
| 1 mM AMPPCP | 6.09 ± 0.11 | 2.2 ± 0.4 | | |
| RyR1 | | | | |
| 0 mM AMPPCP | 6.12 ± 0.11 | 2.1 ± 0.8 | 2.33 ± 0.28 | 1.1 ± 0.2 |
| 1 mM AMPPCP | 6.78 ± 0.17 | 1.5 ± 0.6 | 1.81 ± 0.21 | 1.6 ± 0.8 |

**FIG. 6.** First-order plots of the association and dissociation of 10 nM [3H]ryanodine to solubilized recombinant RyR1 and RyR2. A, aliquots of 0.5 ml of solubilized proteins from transfected HEK-293 cells were added into 4.5 ml of binding buffer B with 10 nM [3H]ryanodine and incubated at 37 °C. After various periods of time, aliquots were removed and assayed for binding. The data were plotted according to Equation 1 under “Experimental Procedures.” The slope (kₐₙ) was 0.07038 for RyR1 and 0.07363 for RyR2. The correlation coefficients were 0.93 for RyR1 and 0.97 for RyR2. B, aliquots of 0.25 ml of solubilized proteins were incubated with 1 ml binding buffer B and 10 nM [3H]ryanodine at 37 °C for 2 h until equilibrium was reached. Aliquots were diluted 50-fold (1 ml to 49 ml) in binding buffer B containing 0 or 40 mM unlabeled ryanodine, in the absence of ATP or Ca²⁺. First-order plots of specific binding (ln%) versus time are displayed for RyR2 (dilution buffer containing 0 (●) or 40 mM (▼) ryanodine) and RyR1 (0 (●) or 40 mM (+) ryanodine).

**FIG. 7.** Ca²⁺ dependence of [3H]ryanodine binding and effect of 1 mM AMPPCP. A, recombinant RyR1; B, recombinant RyR2. Aliquots of 25 μl of solubilized proteins from transfected HEK-293 cells were incubated for 2 h at 37 °C with 2.5 nM [3H]ryanodine in buffer B (pCa 5.7) containing different Mg²⁺ concentrations in a total volume of 0.25 ml. The experiments were carried out as described under “Experimental Procedures.” 100% corresponds to binding in the absence of Mg²⁺. Curve fitting yielded an EC₅₀ of 3.4 mM for RyR1 (slope 1.0) and 8.4 mM for RyR2 (slope 1.1).
to be highly reliable for the investigation of the properties of [3H]ryanodine binding to recombinant channels and the effects of modulators on [3H]ryanodine binding. One of the important applications of the assay has been to show that expression levels for functional expressed channels can be readily calculated, since the bulk of the expressed recombinant proteins are extracted for assay. After solubilization with 1% or 2% CHAPS in the presence of PC, the debris of transfected cells with RyR cDNAs had less than 5% of the [3H]ryanodine binding activity in solubilized proteins. Assuming that the expressed RyR proteins are all assembled into tetrameric complexes, that one tetramer has one high affinity binding site, and that a tetrameric receptor binds about 400 pmol of ryanodine/mg of protein (6, 7), we calculated that our solubilized recombinant protein in whole cell lysates is about 0.045% (0.18/400) of total cellular proteins for recombinant RyR1 ($B_{max}$ = 0.18 pmol/mg) and about 0.2% (0.76/400) for recombinant RyR2 ($B_{max}$ = 0.76 pmol/mg). By contrast, the expression of RyR1 was only 0.05% in isolated microsomes from transfected COS-1 cells (7). Thus, the expression of RyR protein in HEK-293 cells is indeed higher than in COS-1 cells (8). The high expression of RyR2 in HEK-293 cells could be confirmed directly by staining of gels with antibodies to RyR, demonstrating that recombinant RyR2 is more sensitive to caffeine than RyR1. This is in agreement with results obtained with skeletal and cardiac sarco(endo)plasmic reticulum (35, 40).

The time course of Ca$^{2+}$ release for recombinant RyR2 was faster than that for recombinant RyR1, and the peak amplitude of the Ca$^{2+}$ transient was higher for recombinant RyR2. This does not necessarily mean that the same dose of caffeine releases more Ca$^{2+}$ in RyR2 cDNA-transfected cells, since high concentrations of caffeine can release Ca$^{2+}$ completely from both skeletal and cardiac muscle sarcoplasmic reticulum (28). Thus, the different caffeine response for recombinant RyR1 and RyR2 might reflect different properties of these two channels. We do not know, however, to what extent the differences in expression level between RyR1 and RyR2 cDNA-transfected cells will contribute to the differences in time course. The Hill coefficient for caffeine-induced Ca$^{2+}$ release in recombinant RyR2 was close to 1, implying that a single binding site exists for caffeine, while the value in recombinant RyR1 was greater than unity, indicating a cooperative action. The caffeine binding site has been proposed to be located in the first 4000 amino acids (37), so the precise site of molecular interaction of caffeine with RyR is still not known. These differences in caffeine activation might be exploited to pinpoint the site of caffeine activation in future studies of chimeric RyR molecules.

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