Isoform-dependent Formation of Heteromeric Ca\(^{2+}\) Release Channels (Ryanodine Receptors)*

Received for publication, August 12, 2002, and in revised form, August 29, 2002
Published, JBC Papers in Press, September 3, 2002, DOI 10.1074/jbc.M208210200

Bailong Xiao‡, Haruko Masumiya§§, Dawei Jiang‡‡, Ruiwu Wang‡, Yoshitatsu Sei¶, Lin Zhang‡, Takashi Murayama**+, Yasuo Ogawa**+, F. Anthony Lai‡‡, Terence Wagenknecht§§, and S. R. Wayne Chen¶¶

From the §Cardiovascular Research Group, Departments of Physiology & Biophysics, and Biochemistry & Molecular Biology, University of Calgary, Calgary, Alberta T2N 4N1, Canada, the ¶Department of Anesthesiology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814, the **Department of Pharmacology, Juntendo University School of Medicine, Tokyo 113-8421, Japan, the ¶¶Department of Cardiology, Wales Heart Research Institute, University of Wales College of Medicine, Heath Park, Cardiff CF14 4XN, United Kingdom, and the §§Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, New York 12201

Three ryanodine receptor (RyR) isoforms, RyR1, RyR2, and RyR3, are expressed in mammalian tissues. It is unclear whether RyR isoforms are capable of forming heteromeric channels. To investigate their ability to form heteromeric channels, we co-expressed different RyR isoforms in HEK293 cells and examined their interactions biochemically and functionally. Immunoprecipitation studies revealed that RyR2 is able to interact physically with RyR3 and RyR1 in HEK293 cells and that RyR1 does not interact with RyR3. Co-expression of a ryanodine binding deficient mutant of RyR2, RyR2 (I4827T), with RyR3 (wt) restored \(^{3}H\)yanodine binding to the mutant. Interactions between RyR isoforms were further assessed by complementation analysis using mutants RyR2 (I4827T) and RyR2 (E3987A), RyR3 (I4732T), RyR3 (E3885A), and RyR1 (E4032A), all of which are deficient in caffeine response. Caffeine-induced Ca\(^{2+}\) release was restored in HEK293 cells co-transfected with mutants RyR2 (I4827T) and RyR3 (E3885A), RyR2 (E3987A) and RyR3 (I4732T), or RyR2 (I4827T) and RyR1 (E4032A), but not with RyR1 (E4032A) and RyR3 (I4732T), indicating that mutants of RyR2 and RyR3, or RyR2 and RyR1, but not RyR1 and RyR3, are able to complement each other. Co-expression of RyR3 (wt) and a pore mutant of RyR2, RyR2 (G4824A), produced regulatable single channels with intermediate unitary conductances. These observations demonstrate that RyR2 is capable of forming functional heteromeric channels with RyR3 and RyR1, whereas RyR1 is incapable of forming heteromeric channels with RyR3.

Ryanodine receptors (RyRs)\(^{3}\) belong to a superfamily of Ca\(^{2+}\) release channels, which also includes the inositol 1,4,5-trisphosphate receptors (IP\(_{3}\)Rs). These channels are located in the sarcoplasmic reticulum of muscle or non-muscle cells and play an essential role in muscle contraction and Ca\(^{2+}\) signaling (1). Three RyR isoforms, RyR1, RyR2, and RyR3, have been identified and characterized in mammalian tissues. They are the products of three distinct genes and share 66–70% amino acid sequence identity. Each RyR isoform exhibits a unique tissue distribution. RyR1 is primarily expressed in skeletal muscle, whereas RyR2 is predominantly expressed in heart and brain. RyR3 expression has been detected, although at a relatively low level, in a variety of tissues, including brain, diaphragm, and smooth muscles (2–4). However, recent studies using RNase protection assays revealed that all three RyR genes are widely expressed and that some tissues express two or all three RyR isoforms (5). For instance, both RyR1 and RyR3 are expressed in the diaphragm, whereas all three RyR isoforms are detected in brain and smooth muscle tissues.

The finding that multiple RyR isoforms are co-expressed in the same tissue raises a possibility that RyRs may exist as heteromeric channels in addition to homomeric channels. Early biochemical studies showed that the purified RyR1 from rabbit skeletal muscle is a homotetramer composed of four identical subunits each with a molecular mass of ~400 kDa (6). Recently, RyR3 was purified from diaphragm skeletal muscle in which RyR1 was also expressed by immunoprecipitation using an RyR3-specific antibody. The purified RyR3 was shown to be devoid of RyR1 even though RyR1 is co-expressed with RyR3 in the same muscle fibers and in large excess (7–9). These observations indicate that RyR1 and RyR3, although co-localized, exist only in the form of homotetramers. The same has been shown to be true for the \(\alpha\)- and \(\beta\)-RyRs, the non-mammalian counterparts of RyR1 and RyR3 (3, 10). Immunoprecipitation of RyR3 from brain tissue using an RyR3-specific antibody did not co-precipitate RyR2 (the major RyR isoform expressed in the brain), demonstrating that RyR2 and RyR3 also exist as homotetramers in the brain (11). These observations have led to the general belief that RyRs may exist only in the form of homotetramers in contrast to IP\(_{3}\)Rs. Immunoprecipitation studies have shown that the type 1, 2, and 3 IP\(_{3}\)R isoforms can form heteromeric channels among one another (12, 13).

It is clear that RyR1 and RyR3 expressed in mammalian skeletal muscle or \(\alpha\)-RyR and \(\beta\)-RyR expressed in non-mammalian skeletal muscle are incapable of forming heteromeric channels. The question of whether RyR2 and RyR3, or RyR2 and RyR1, or RyR1 and RyR3, all in different combinations, may form heteromeric channels requires further investigation.

\* This work was supported in part by research grants from the Canadian Institutes of Health Research and the Heart and Stroke Foundation of Alberta, Northwest Territories and Nunavut (to R.R.W.C.) and by Grant AR40615 from the National Institutes of Health (to T.W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked \"advertisement\" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by postdoctoral fellowships from the Heart and Stroke Foundation of Canada and the Alberta Heritage Foundation for Medical Research (AHFMR).

¶¶ AHFMR Senior Scholar. To whom correspondence should be addressed. Tel.: 403-220-4235; Fax: 403-283-4841; E-mail: swenchen@ucalgary.ca.

The abbreviations used are: RyR, ryanodine receptor; IP\(_{3}\), inositol 1,4,5-trisphosphate receptor; CHAPS, 3-[3-Cholamidopropyl)dimethyl-ammonio]-1-propane sulfonate; PBS, phosphate-buffered saline; wt, wild type; CICR, Ca\(^{2+}\)-induced Ca\(^{2+}\) release; pS, picosiemens.

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and RyR1, are capable of forming heteromeric channel is, however, debatable, because it is unknown whether RyR2 and RyR3, or RyR2 and RyR1, expressed in the brain are co-localized in the same cells. All three RyR isoforms have also been detected in smooth muscle cells. Functional studies have shown that specific inhibition of either RyR1 or RyR2 synthesis abolished Ca2+ -induced Ca2+ release triggered by depolarization and that abolition of RyR2 by gene knock-out enhances the activity of other RyR isoforms in vascular smooth muscle cells (14, 15). One possible explanation for these observations is that RyR isoforms form heteromeric channels in these cells. To test this possibility, we co-expressed different RyR isoforms in HEK293 cells and examined the existence of heteromeric RyR channels. To do so, we utilized c-Myc antibody epitope-tagged RyR2 or RyR3 and RyR mutants that are deficient in [3H]ryanodine binding or caffeine response, or have different single-channel conductances to distinguish the RyR isoforms structurally and functionally. These RyR isoforms, tagged or non-tagged, wt or mutants, were co-expressed in HEK293 cells, and the formation of heteromeric RyR channels was examined by immunoprecipitation, [3H]ryanodine binding, and single-channel recordings in planar lipid bilayers. Our results reveal for the first time that RyR2 are capable of forming functional heteromeric channels with RyR3 or RyR1 in HEK293 cells.

EXPERIMENTAL PROCEDURES

Materials—[3H]Ryanodine was obtained from PerkinElmer Life Sciences, and ryanodine was obtained from Calbiochem. Anti-RyR antibody (anti-RyR (34C)) was purchased from Affinity Bioreagents Inc. Brain phosphatidylserine and soybean phosphatidylcholine were from Avanti Polar Lipids. Synthetic 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine was obtained from Northern Lipids. 3-(3-Cholamidopropyl)dimethyloammonio-1-propane sulfonate (CHAPS) and other reagents were purchased from Sigma.

Cell Culture and DNA Transfection—HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium as described previously (16). HEK293 cells grown on 100-mm tissue culture dishes for 18–20 h after subculture were transfected with 12–16 μg of wild type or mutant RyR cDNAs using Ca2+ -phosphate precipitation (17).

Site-directed Mutagenesis—Point mutations, RyR1 (E4032A), RyR2 (E3987A), RyR2 (G4624A), and RyR3 (E3885A), were constructed as described previously (18–20). The RyR2 (14827T) and RyR3 (14732T) mutations were generated by the overlap extension method using the polymerase chain reaction (PCR) (22). The NruI (14237T)-NoI (vector) fragment that contains the RyR2 (14827T) mutation was used to replace the corresponding wild type fragment in the full-length mouse RyR2 cDNA. The EcoRI (14322)-NoI (vector) fragment that contains the RyR3 (14732A) mutation was used to replace the corresponding wild type fragment in the RyR3 cDNA without the cDNA fragment that contains the c-Myc tag was removed from the PCR product generated by the overlap extension method, and was used to replace the corresponding region in the BsiWI (8664)-NoI (vector) fragment that was subsequently subcloned into the full-length mouse RyR2 cDNA. All point mutations and insertions were confirmed by DNA sequencing.

Preparation of Cell Lysate from Transfected HEK293 Cells—Preparation of cell lysate from transfected HEK293 cells was performed as described previously (19), with some modifications. HEK293 cells grown in 60-mm culture dishes were harvested three times with PBS (137 mM NaCl, 8 mM NaH2PO4, 1.5 mM KH2PO4, 2.7 mM KCl) plus 2.5 mM EDTA and were harvested in the same solution by centrifugation. Transfected HEK293 cells were solubilized in lysis buffer containing 25 mM Tris/50 mM Hepes (pH 7.4), 137 mM NaCl, 1% CHAPS, 0.5% soybean phosphatidylcholine, 2.5 mM dithiothreitol, and a protease inhibitor mix (1 mM benzamidine, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride) on ice for 1 h. Cell lysate was obtained after removing the unsolubilized materials by centrifugation twice in a microcentrifuge at 4 °C each for 30 min.

Immunoprecipitation and Immunoblotting Analysis—Cell lysates were incubated with protein G- or protein A-Sepharose (30 μl) that was pre-washed with PBS and pre-bound with 5–10 μg of anti-c-Myc, anti-RyR1 (13C2) (24), anti-RyR2, or anti-RyR3 antibodies at 4 °C for 17–19 h. The anti-RyR2 antibody was produced in rabbits against a synthetic peptide, QLKRLHHTRYGE, which corresponds to residues 4460–4472 of rabbit RyR2. Generation of the anti-RyR3 antibody was described previously (11). The protein G- or protein A-agarose beads were washed with ice-cold lysis buffer three times, each time for 10 min. The immunocomplexes bound to the agarose beads were solubilized by addition of 30 μl of 2× Laemmli sample buffer (25) plus 5% β-mercaptoethanol and were boiled at 100 °C for 2 min. The solubilized immunocomplexes (10–15 μl) were separated by 6% SDS-PAGE. The SDS-PAGE-resolved proteins were then transferred to nitrocellulose membranes at 45 μA for 1 h in the buffer (26). The nitrocellulose membrane was blocked for 0.5 h with PBS containing 0.5% Tween 20 and 5% skim milk powder. The blocked membrane was incubated with primary antibodies, anti-RyR (34C), anti-RyR2, anti-RyR3, or anti-RyR3 (FP8) (16) for 2–4 h, washed three times each time for 15 min with PBS containing 0.5% Tween 20. The membrane was then incubated with the secondary anti-mouse or anti-rabbit IgG (H&L) antibodies conjugated with alkaline phosphatase for 30–40 min. After washing three times each time for 15 min, the bound antibodies were visualized by the alkaline phosphatase-mediated color reaction using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as the substrates.

[3H]Ryanodine Binding to Immunoprecipitates—The c-Myc-tagged RyR proteins expressed in HEK293 cells were immunoprecipitated by protein G-agarose that was pre-bound with anti-c-Myc antibody. The immunoprecipitates were washed three times with lysis buffer. Equilibrium [3H]ryanodine binding was carried out in a binding mixture (300 μl) containing 15 μl of immunoprecipitates (agarose beads), 25 mM Tris/50 mM Hepes (pH 7.4), 500 mM KCl, 0.8 mM CaCl2, 5 μM caffeine, 5 mM [3H]ryanodine, and the protease inhibitor mix was incubated at 37 °C for 2–3 h. The binding mix was filtered through Whatman GF/B filters presoaked with 1% polyethyleneimine. The filters were immediately washed four times with 5 ml of ice-cold washing buffer containing 25 mM Tris (pH 5.0) and 250 mM KCl. The radioactivity associated with the filters was then determined by liquid scintillation counting. Non-specific binding was determined by measuring [3H]ryanodine binding in the presence of 50 μM unlabeled ryanodine. All binding assays were done in duplicate.

Ca2+ Release Measurements in Transfected HEK293 Cells and Single Channel Recordings—Free cytosolic Ca2+ concentration in transfected HEK293 cells was measured using the fluorescent Ca2+ indicator dye fluo-3-AM as described previously (19). Recombinant RyR proteins were purified from whole cell lysate by sucrose density gradient centrifugation and were used for single-channel recordings as described previously (19). Free Ca2+ concentrations were calculated using the computer program of Fabiato and Fabiato (27).

RESULTS

RyR2 Interacts Physically with RyR3 in HEK293 Cells—To test the possibility that RyR2 is capable of forming heteromeric channels with RyR3, we first investigated whether RyR2 interacts with RyR3 physically. RyR2 and RyR3 have a comparable molecular weight and migrate similarly in SDS-PAGE. To facilitate specific detection and isolation of RyR2 and RyR3, we inserted a c-Myc tag into RyR2 and RyR3. The c-Myc-tagged RyR2, RyR2 (c-Myc), or c-Myc-tagged RyR3, RyR3 (c-Myc), was expressed in HEK293 cells either alone or in combination with the untagged RyR3 (wt) or untagged RyR2 (wt), respectively. Immunoprecipitation was carried out using the anti-c-Myc antibody, and the immunoprecipitates were separated and probed with either the anti-RyR (34C) antibody, which recognizes all three RyR isoforms, or an RyR3-specific antibody, anti-RyR3 (FPS8). Fig. 1 shows that the anti-c-Myc antibody was able to pull down the RyR2 (c-Myc) protein from a lysate of HEK293 cells transfected with RyR2 (c-Myc) (Fig. 1A, lane 1), but not RyR3 (wt) protein from cells transfected with RyR3 (wt) (Fig. 1A, lane 2), indicating that the anti-c-Myc antibody is specific. The anti-c-Myc antibody also pulled down a major band, which should contain the RyR2 (c-Myc) protein, from HEK293 cells co-transfected with RyR2 (c-Myc) and RyR3 (wt) (Fig. 1A, lane 3). The same anti-c-Myc immunoprecipitates were blotted with

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cells was monitored continuously before and after addition of 0.25 mM caffeine at room temperature for 1 h. Fluorescence intensity of the fluo-3 loaded B and antibody (34C) antibody (Fig. 1A, lane 1). The immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with anti-RyR (34C) antibody (A and C), anti-RyR (FP8) antibody (B), or anti-RyR2 antibody (D). The anti-c-Myc antibody did not precipitate the RyR3 (A and B, lane 2) or RyR2 (C and D, lane 2) proteins, although they are present in the cell lysate. E and F, HEK293 cells were transfected with RyR2 (c-Myc) (12 μg) or RyR3 (c-Myc) (12 μg) plus RyR2 (6 μg) (A and B) or transfected with RyR3 (c-Myc) (12 μg), RyR2 (12 μg), or RyR3 (c-Myc) (6 μg) plus RyR2 (6 μg) (C and D). The immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with anti-RyR (34C) antibody (A and C), anti-RyR (FP8) antibody (B), or anti-RyR2 antibody (D). The anti-c-Myc antibody did not precipitate the RyR3 (A and B, lane 2) or RyR2 (C and D, lane 2) proteins, although they are present in the cell lysate. E and F, HEK293 cells were transfected with RyR2 (c-Myc) (12 μg) or RyR3 (c-Myc) (12 μg) (F). 18–20 h after transfection, cells were harvested and loaded with 10 μM fluo-3-AM at room temperature for 1 h. Fluorescence intensity of the fluo-3-loaded cells was monitored continuously before and after addition of 0.25 mM caffeine (letter C), 50 μM ryanodine (letter R), and 2.5 mM caffeine (letter C). Similar results were obtained from three separate experiments.

The anti-RyR3 (FP8) antibody (Fig. 1B). This antibody recognized a major band in the anti-c-Myc immunoprecipitate from HEK293 cells co-transfected with RyR2 (c-Myc) and RyR3 (wt) (Fig. 1B, lane 3). No significant signal was detected by the anti-RyR3 (FP8) antibody in the immunoprecipitate from cells transfected with RyR2 (c-Myc) alone (Fig. 1B, lane 1). These data indicate that the anti-RyR3 (FP8) antibody is specific and that RyR3 (wt) was co-precipitated with RyR2 (c-Myc) by the anti-c-Myc antibody. It should be noted that caffeine and ryanodine induced Ca2+ release from intracellular Ca2+ stores in HEK293 cells transfected with RyR2 (c-Myc) or RyR3 (c-Myc), indicating that both RyR2 (c-Myc) and RyR3 (c-Myc) are functional (Fig. 1, E and F).

We also performed the reciprocal experiment in which the RyR3 (c-Myc) was immunoprecipitated by the anti-c-Myc antibody and the presence of RyR2 (wt) in the anti-c-Myc immunoprecipitates was detected by Western blotting using an RyR2-specific antibody. As shown in Fig. 1C, the anti-c-Myc antibody pulled down the RyR3 (c-Myc) (lanes 1 and 3) but not the RyR2 (wt) (lane 2). The anti-RyR2 antibody did not co-react with the RyR3 (c-Myc) (Fig. 1D, lane 1) but recognized a major band in the anti-c-Myc immunoprecipitate from HEK293 cells co-transfected with RyR2 (wt) and RyR3 (c-Myc). Several minor bands were also detected by the anti-RyR (34C) antibody in the anti-c-Myc immunoprecipitates (Fig. 1B, lane 3; Fig. 1C, lanes 1 and 3). They are most likely degradation or aggregation products of RyR2, because RyR2 (c-Myc) or RyR2 (wt) show a single band (Fig. 1A, lane 1; Fig. 1D, lane 3). Thus, although the anti-c-Myc antibody is unable to pull down RyR2 (wt), it is able to precipitate the RyR2 (wt) in the presence of RyR3 (c-Myc). Taken together, these results demonstrate that RyR2 and RyR3 interact physically with each other.

**RyR2-RyR3 Complex Is Capable of Binding [3H]Ryanodine—**

[3H]ryanodine binding has been widely used as a functional assay for RyR channel activity, because ryanodine binds to the open state of the channel (28). To investigate whether the RyR2-RyR3 complex observed in HEK293 cells is functional, we isolated the complex and determined its [3H]ryanodine binding capability. To specifically measure [3H]ryanodine binding to the RyR2-RyR3 complex, but not to individual RyR2 or RyR3, we utilized a c-Myc-tagged RyR2 mutant, RyR2 (I4827T, c-Myc), that is equivalent to the central core disease mutation I4898T in RyR1 (29). It has been shown that mutant RyR1 (I4898T) does not bind [3H]ryanodine when expressed alone but displays significant [3H]ryanodine binding after co-expressed with wt RyR1 (29), most likely as a result of formation of mutant RyR1 (I4898T)wt RyR1 complexes. Similarly, we found that the RyR2 (I4827T, c-Myc) mutant isolated by anti-c-Myc immunoprecipitation showed no significant [3H]ryanodine binding (Fig. 2B), while [3H]ryanodine binding was readily detected in anti-c-Myc immunoprecipitates from HEK293 cells co-transfected with RyR2 (wt) and RyR2 (I4827T, c-Myc) (Fig. 2B). It should be noted that mutant RyR2 (I4827T, c-Myc) is expressed in HEK293 cells (Fig. 2A) and that the lack of [3H]ryanodine binding to RyR2 (I4827T, c-Myc) is unlikely due to the lack of expression of the mutant. It should also be noted that the anti-c-Myc antibody does not precipitate [3H]ryanodine.
binding activity from HEK293 cells transfected with RyR2 (wt) alone (data not shown).

We reasoned that, if RyR2 is capable of interacting with RyR3, co-expression of RyR3 (wt) with RyR2 (I4827T, c-Myc) should also be able to restore some $[^3H]$ryanodine binding to the mutant. As shown in Fig. 2B, the anti-c-Myc antibody was able to precipitate $[^3H]$ryanodine binding activity from HEK293 cells co-transfected with RyR3 (wt) and RyR2 (I4827T, c-Myc). Because the anti-c-Myc antibody does not precipitate $[^3H]$ryanodine binding activity from cells transfected with RyR3 (wt) alone (data not shown), the $[^3H]$ryanodine binding detected in the immunoprecipitates must result from binding to the RyR3 (wt)/RyR2 (I4827T, c-Myc) complexes. These observations indicate that RyR2 and RyR3 can form a functional complex.

**RyR2 Is Able to Form Functional Heteromeric Channels with RyR3 in HEK293 Cells**—The observed physical and functional RyR2-RyR3 interaction could occur between homomeric RyR2 and homomeric RyR3 channels or via the formation of heteromeric RyR2-RyR3 hybrid channels. To distinguish these possibilities, we made use of the RyR2 (I4827T) mutant and a Ca$^{2+}$-sensing mutant of RyR3, RyR3 (E3885A) (20). Neither mutant when expressed alone in HEK293 cells exhibited caffeine-induced Ca$^{2+}$ release (Fig. 3, A and B). If RyR2-RyR3 interaction occurs only between homomeric RyR2 and homomeric RyR3 channels, the RyR2 (I4827T)-RyR3 (E3885A) mutant complex formed after co-expression in HEK293 cells should remain insensitive to caffeine. On the other hand, if RyR2-RyR3 interaction occurs between monomeric RyR2 and monomeric RyR3, co-expression of RyR2 (I4827T) and RyR3 (E3885A) in HEK293 cells may produce hybrid channels that are caffeine-sensitive, because these two mutations are located in different regions of RyR and one mutant may be able to complement the defect of the other. As shown in Fig. 3, HEK293 cells co-transfected with different ratios of RyR2 (I4827T) and RyR3 (E3885A) displayed caffeine-induced Ca$^{2+}$ release (Fig. 3, C–E), indicating the formation of heteromeric RyR2-RyR3 hybrid channels. The immediate drops in fluorescence signals after addition of caffeine are due to fluorescence quenching by caffeine.

**Co-expression of RyR2 and RyR3 in HEK293 Cells Produces Single Heteromeric Channels**—To demonstrate the formation of RyR2-RyR3 heteromeric channels at the single-channel level, we utilized a pore mutant of RyR2, RyR2 (G4824A). We have shown that mutation RyR2 (G4824A) reduces the single-channel conductance by ~97% and that co-expression of RyR2 (wt) and RyR2 (G4824A) mutant produces wt/mutant hybrid channels with intermediate single-channel conductances (21). If RyR2 is able to form single heteromeric channels with RyR3, one would expect that co-expression of RyR2 (G4824A) and RyR3 (wt) would also result in the synthesis of single hybrid channels with intermediate single-channel conductances. To test this hypothesis, we analyzed the single-channel conductances of the co-expressed channels after incorporation into planar lipid bilayers. A total of 42 single channels were detected. Based on their single-channel conductances, they can be separated into six groups (Fig. 4), displaying single-channel conductances of 799 ± 1.9 pS (n = 7), 524 ± 5.1 pS (n = 9), 267 ± 6.8 pS (n = 11), 178 ± 4.7 pS (n = 6), 77 ± 1.7 pS (n = 8), and 22 pS (n = 1), similar to those observed after co-expression of RyR2 (wt) and RyR2 (G4824A) (21). The 799-pS (Fig. 4A) and the 22-pS (Fig. 4F) single channels most likely represent the homotetrameric RyR3 (wt) and the homotetrameric RyR2 (G4824A) mutant channels, respectively, whereas single channels with intermediate single-channel conductances most probably represent the RyR3 (wt)/RyR2 (G4824A) hybrid channels with different subunit ratios of RyR3 (wt) to RyR2 (G4824A). For instance, the 524-pS conductance may result from hybrid channels formed by three RyR3 (wt) and one RyR2 (G4824A) subunits (Fig. 4B), whereas hybrid channels with one RyR3 (wt) and three RyR2 (G4824A) subunits may produce the 77-pS conductance (Fig. 4E). The 267-pS (Fig. 4C) and 178-pS (Fig. 4D) channels may be formed by two RyR3 (wt) and two RyR2 (G4824A) subunits arranged in two possible configurations (parallel and diagonal). These results clearly demonstrate that RyR2 and RyR3 are capable of forming single heteromeric channels.

**Single RyR2-RyR3 Heteromeric Channels Are Sensitive to Modulation**—To assess whether RyR2-RyR3 heteromeric channels are regulatable, we examined the effect of Ca$^{2+}$, ATP, caffeine, Mg$^{2+}$, and ryanodine on single RyR3 (wt)/RyR2 (G4824A) hybrid channels with intermediate single-channel conductances.
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Fig. 5. Ligand gating properties of a single RyR2/RyR3 hybrid channel. Single-channel activities of an RyR2 (G4824A)/RyR3 hybrid channel with a unitary conductance of 269 pS were recorded in a symmetrical recording solution containing 250 mM KCl and 25 mM Hepes (pH 7.4). The trans chamber was connected to the input of the headstage amplifier, and the cis chamber was held at virtual ground. Addition of EGTA to the cis chamber inhibited single-channel activities, indicating that the channel was incorporated into the bilayers with its cytoplasmic side facing the cis chamber. Channel activities in the presence of 125 mM free cis (cytoplasmic) Ca$^{2+}$ (a), and after sequential additions (cis) of CaCl$_2$ (b), EGTA (c), ATP (d), caffeine (e), MgCl$_2$ (f), CaCl$_2$ (g), and ryanodine (h) are shown. The open probability (Po), arithmetic mean open time (To), and the arithmetic mean closed time (Tc) are indicated on the top of each panel. A short line to the right of each current trace indicates the baseline. The holding potential was +40 mV. Openings are upward.

conductances. Fig. 5a shows the channel activity of a single RyR3 (wt)/RyR2 (G4824A) heteromeric channel with a unitary conductance of 269 pS (probably comprised of two RyR3 (wt) and two RyR2 (G4824A) subunits). The heteromeric channel was sensitive to Ca$^{2+}$ (Fig. 5, b, c, and g), activated by ATP (Fig. 5d) and caffeine (Fig. 5e), inhibited by Mg$^{2+}$ (Fig. 5f), and modulated by ryanodine (Fig. 5h). Similar ligand responses were also observed in other single-hybrid channels with different unitary conductances (not shown). Hence RyR2 and RyR3 are able to form functional and regulatable single heteromeric channels in HEK293 cells.

RyR2 Interacts Physically with RyR1 in HEK293 Cells—The finding that RyR2 interacts physically with RyR3 raises the question of whether RyR2 is also able to interact with RyR1. To address this question, we co-expressed RyR2 (c-Myc) and RyR1 in HEK293 cells and examined possible interactions between them by immunoprecipitation and Western blotting. Because RyR2 migrates faster than RyR1 in SDS-PAGE, RyR1-RyR2 interaction could be readily detected by immunoprecipitation followed by SDS-PAGE. Fig. 6a shows that the anti-c-Myc antibody did not pull down the RyR1 protein from HEK293 cells transfected with RyR1 alone (Fig. 6a, lane 2), but an anti-RyR1 (13C2) antibody did (Fig. 6a, lane 1). The anti-c-Myc antibody also did not cross-react with RyR1 on Western blot (Fig. 6a, lane 3). On the other hand, the anti-c-Myc antibody precipitated two RyR bands from HEK293 cells co-transfected with RyR2 (c-Myc) and RyR1 (Fig. 6b, lane 2). The bottom band (indicated by an arrowhead), which was recognized by both the anti-RyR (34C) and the anti-c-Myc antibodies, corresponds to RyR2 (c-Myc), whereas the top band (indicated by an arrow), which was recognized by the anti-RyR (34C) antibody but not by the anti-c-Myc antibody, corresponds to RyR1 (Fig. 6b, lanes 2 and 4). These data indicate that RyR1 was co-precipitated with RyR2 (c-Myc) by the anti-c-Myc antibody. It should be noted that the anti-RyR1 (13C2) antibody, although raised against RyR1-specific sequence, exhibits some cross-reactivity with the RyR2 and RyR3 isoforms in both immunoprecipitation and immunoblotting (data not shown). Thus, the presence of RyR2 (c-Myc) in the anti-RyR1 (13C2) immunoprecipitate (Fig. 6b, lane 2) and in immunoblotting (lane 3) indicates the position of RyR1, whereas arrowheads point to the position of RyR2 (c-Myc).

We also examined whether RyR1 interacts with RyR3 when co-expressed in HEK293 cells. Unlike the double bands seen in Fig. 6b (lane 2), only a single RyR band was detected by the anti-RyR (34C) antibody in the anti-c-Myc immunoprecipitate from cells co-transfected with RyR1 and RyR3 (c-Myc) (Fig. 6c, lane 2), although a comparable amount of RyR1 protein was present in the lysate (Fig. 6c, lane 1). This band corresponds to RyR3 (c-Myc), because it was also recognized by the anti-c-Myc antibody (Fig. 6c, lane 4). These results indicate that RyR1 was not co-precipitated with RyR3 (c-Myc) by the anti-c-Myc antibody. A faint band, corresponding to RyR3 (c-Myc), was detected in the anti-RyR1 (13C2) immunoprecipitate (Fig. 6c, lane 3). This is most likely due to direct immunoprecipitation of RyR3 (c-Myc) by the nonspecific anti-RyR1 (13C2) antibody.

Formation of Functional Heteromeric RyR2/RyR1 Channels in HEK293 Cells—Functional interaction between RyR2 and RyR1 was demonstrated by co-expression studies using the RyR2 (I4827T) mutant and a Ca$^{2+}$-sensing mutant of RyR1, RyR1 (E4032A) (18), similar to the co-expression studies of RyR2 (I4827T) and RyR3 (E8858A) described in Fig. 3. Both mutants, RyR2 (I4827T) and RyR1 (E4032A), when expressed individually in HEK293 cells exhibited no caffeine-induced Ca$^{2+}$ release (Fig. 7, A and B). However, HEK293 cells co-transfected with different ratios of RyR2 (I4827T) and RyR1 (E4032A) displayed caffeine-induced Ca$^{2+}$ release (Fig. 7,
It is commonly believed that RyRs exist in the form of homotetramers. This is unexpected, given the existence of multiple RyR isoforms and their similar tetrmeric structure, and the fact that another type of Ca$^{2+}$ release channels related to RyRs, IP3Rs, are able to form heteromeric complexes (3, 4, 12, 13). Structural incompatibility for forming heteromers or non-overlapping cellular distribution of RyR isoforms may account for a lack of detectable heteromeric RyR channels. The purpose of this study is to investigate the ability of RyR isoforms to form heteromeric channels when co-expressed in HEK293 cells. Our results demonstrate for the first time that RyR2 is able to form heteromeric channels with RyR3 and RyR1.

Based on the following observations, it is unlikely that the observed interactions between RyR2 and RyR3 and between RyR2 and RyR1 in HEK293 cells are the result of overexpression.
of heteromeric RyRs in the brain, although it is possible, may be much less efficient than that of homomeric channels. As a result, heteromeric channels may represent only a small portion of the total RyR population in cells that express multiple RyR isoforms and thus may not be readily detected.

Recently, it has been shown that several immune cells, including U937, SKW6.4, SupT1, and Jurkat treated with transforming growth factor-β, express multiple RyR isoforms (30). We have attempted to determine whether heteromeric RyR channels exist in these cells. We have performed immunoprecipitation studies on cell lysates prepared from these immune cells using RyR2- and RyR3-specific antibodies. We found that the levels of RyR expression in these cells were too low to allow us to determine whether the cross-activity was due to co-precipitation or nonspecific antibody binding (data not shown). Similar results were also observed using microsomal membranes isolated from bovine uterus. Better RyR isoform-specific antibodies would be required for determining the existence of heteromeric RyR channels in native cells or tissues.

Although no heteromeric RyR channels have been isolated biochemically, their presence has been functionally implicated in vascular smooth muscle cells where the expression of all three RyR isoforms has been detected (14, 31). For instance, in rat portal vein myocytes, which express all three RyR isoforms, inhibition of either RyR1 or RyR2 by isoform-specific antisense oligonucleotides blocked depolarization-induced Ca2+ sparks and global Ca2+ response (14). These observations suggest that both RyR1 and RyR2 are required to form the functional Ca2+ release units in these cells, although how these Ca2+ release units are formed by two different RyR isoforms is unclear. These functional Ca2+ release units could be composed of homotetrameric RyR1 and RyR2 channels somehow coupled both structurally and functionally, or of heteromeric RyR1/RyR2 channels. Our finding that RyR2 is able to form heteromeric channels with RyR1 when expressed together makes the latter possibility likely. Functional Ca2+ release units comprised of multiple RyR isoforms have also been implicated in cerebral artery smooth muscle cells. It was reported that the frequency of Ca2+ sparks and spontaneous transient outward currents in cerebral artery smooth muscle cells isolated from RyR3-deficient mice was significantly higher than that in wild type cells (15). These data suggest that RyR3 is part of the functional Ca2+ release units formed by RyR2 or RyR1 and regulates their activity negatively.

These functional studies of Ca2+ release units in smooth muscles not only implicate the existence of heteromeric RyR channels, but also provide some clues as to their physiological roles. RyR1 is known to interact physically with the plasma membrane and be activated by Ca2+ influx through the L-type Ca2+ channels upon depolarization. Thus, by forming heteromeric RyR1/RyR2 channels, functional Ca2+ release units with strong CICR (a property of RyR2) could be targeted specifically to the plasma membrane via physical interaction with the L-type Ca2+ channels (a property of RyR1), and thus coupled to depolarization.

The physiological role of RyR3 in smooth muscle is largely undefined. The observation that abolition of RyR3 increases the frequency of Ca2+ sparks in smooth muscles suggests that RyR3 may normally inhibit the activity of Ca2+ release units (15). RyR3 expressed in smooth muscle appears to function differently from that expressed in skeletal muscle. It does not produce Ca2+ sparks (14, 34) and becomes active only under the condition of sarcoplasmic reticulum Ca2+ overload (35). Considering these unique properties of smooth muscle RyR3 and our observation that RyR3 is able to form heteromeric channels with RyR2, it is possible that RyR3 in some smooth muscles may function as a suppressor of RyR2-mediated Ca2+ release by forming non-sparking RyR2-RyR3 heteromeric channels with a decreased sensitivity to activation by luminal Ca2+. Further investigation is required to determine the molecular basis that confers these unique properties of smooth muscle RyR3 and whether smooth muscle RyR3 suppresses the activity of RyR2.

Acknowledgments—We thank the Immunology Core Facilities at the Wadsworth Center of the New York State Department of Health for providing the anti-c-Myc antibody, Dr. Paul D. Allen for providing the RyR1 cDNA, Dr. Wayne R. Giles and the Ion Channels and Transporters Group for continued support, Dr. Paul Schnetkamp for the use of his laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, for providing the anti-c-Myc antibody, Dr. Paul D. Allen for providing the RyR1 cDNA, Dr. Wayne R. Giles and the Ion Channels and Transporters Group for continued support, and Dr. Paul Schnetkamp for the use of his laboratory manual.

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