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Novel ryanodine-binding properties in mammalian retina

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Abstract

The ryanodine receptor (RyR)/Ca^{2+} release channel mobilizes Ca^{2+} from internal calcium stores to support a variety of neuronal functions. To investigate the presence of such a protein in mammalian retina, we applied ryanodine binding, PCR and antibodies against known RyRs. Surprisingly, ryanodine-binding properties of retinal endoplasmic reticulum-enriched membrane fraction were vastly different from those of skeletal and cardiac muscles ryanodine-binding proteins. In common with the skeletal and cardiac muscle, ryanodine bound with high-affinity to two or more types of binding site (K_{d_1} = 20.6 and K_{d_2} = 114 nM); binding was strongly stimulated by high concentrations of NaCl; it was inhibited by tetracaine and the protein appeared to possess an ATP-binding site. Unlike cardiac and skeletal muscle, RyRs in retina binding was Ca^{2+}-independent; inhibited by caffeine and dantrolene; less sensitive to ruthenium red; and unaffected by La^{3+}. Also, in retina, ryanodine rapidly associated to and dissociated from its binding sites. Furthermore, although the protein bound the ATP analog BzATP, retinal ryanodine binding was not stimulated by nucleotides. Immunostaining of bovine retinal sections with anti-RyR2 showed a strong staining of amacrine, horizontal and ganglion cells. Finally, using RT-PCR, the three known RyR isoforms were identified in retina. However, consistent with the novel binding properties, the peptide maps yielded by trypsin treatment and Western blotting demonstrate different patterns. Together, the results suggest that retina expresses a novel ryanodine-binding protein, likely to be a ryanodine receptor. Its presence in retina suggests that this protein might play a role in controlling intracellular Ca^{2+} concentration.

Keywords: Caffeine; Dantrolene; Retina; Ryanodine receptor; Ryanodine

Abbreviations: DAB, 3,3′-diaminobenzidine; EDTA, ethylene-diaminetetraacetate; EGTA, ethylene glycol bis (aminoethylether) tetraacetate; Mops, 3-(*N*-morpholino) propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SR, sarcoplasmic reticulum; RyR, ryanodine receptor; Tricine, *N*-(2-hydroxy-1,1-bis (hydroxy-methyl)-ethyl)-glycine

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1. Introduction

Intracellular free Ca^{2+} ($[Ca^{2+}]_i$) plays an important role in regulating cell function in both contractile and non-contractile cells (Berridge, Lipp, & Bootman, 2000; Clapham, 1995). Intracellular $[Ca^{2+}]$ is regulated partially by mobilizing Ca^{2+} into and out of the endoplasmic reticulum (ER). In the ER, Ca^{2+} is taken up by Ca^{2+} pumps, and is released by two well-characterized Ca^{2+}-release channels: the inositol 1,4,5-triphosphate receptor ($InsP_3R$) (Berridge, 1993; Berridge et al., 2000) and the ryanodine receptor (RyR) (Coronado, Morrissette, Sukhareva, & Vaughan, 1994; Meissner, 1994; Shoshan-Barmatz & Ashley, 1998; Sutko & Airey, 1996). RyR exhibits ryanodine-sensitive Ca^{2+} release channel activity that is activated by Ca^{2+} binding to a high-affinity site and inactivated by Ca^{2+} binding to a low-affinity site. In addition to Ca^{2+}, the RyR is also activated by adenine nucleotides and caffeine, and is inhibited by Mg^{2+} and ruthenium red (for reviews see Coronado et al., 1994; Meissner, 1994; Shoshan-Barmatz & Ashley, 1998; Sutko & Airey, 1996). There are at least three RyR isoforms (RyR1–3), encoded by three distinct genes, which are widely distributed in different mammalian tissues, e.g., cardiac (Lai et al., 1992; Meissner, 2004; Otsu et al., 1990), skeletal muscle (Coronado et al., 1994; Meissner, 1994; Shoshan-Barmatz & Ashley, 1998; Sutko & Airey, 1996), brain (Ashley, 1989; Lai et al., 1992; McPherson & Campbell, 1990), and smooth muscle (Herrmann-Frank, Darling, & Meissner, 1991). In addition, the liver expresses a ryanodine-binding protein that differs from the three known RyR types in its binding properties, but its molecular nature has not yet been resolved (Shoshan-Barmatz, Pressley, Higham, & Kraus-Friedmann, 1991).

As in all neurons, intracellular Ca^{2+} in retinal neurons plays a critical role in transmitter release and in regulating a variety of signal transduction processes. Intracellular $[Ca^{2+}]$ is regulated by several mechanisms including Na/Ca exchanger (Schnetkamp, 2004), adenosine receptor (Hartwick, Lalonde, Barnes, & Baldridge, 2004; Stella, Bryson, Cadetti, & Thoreson, 2003) $InsP_3$ receptor (Wang, Sterling, & Vardi, 1999), and RyR. In invertebrate eyes, RyR contributes to light adaptation (Akopian & Witkovsky, 2002; Akopian, Gabriel, & Witkovsky, 1998; Arnon et al., 1997; Baumann, 2000; Walz, Baumann, Zimmermann, & von Ciriacy-Wantrup, 1995). In lower vertebrates, RyR and $InspP_3R$ were immunolocalized to several cell types (Akopian et al., 1998; Krizaj, Lai, & Copenhagen, 2003; Krizaj, Liu, & Copenhagen, 2004), and physiological recordings from ganglion cells showed that the activity of these receptors modulates GABAergic responses (Akopian et al., 1998). However, none of these studies pertain to mammalian retina in which anatomical and physiological connections are intensively studied. To start to understand RyR function in mammalian retina, we applied several approaches including, immunostaining, RT-PCR and characterization of ryanodine binding to retinal fractions enriched with endoplasmic reticulum. Surprisingly, we found that retina expresses novel ryanodine-binding properties distinguished from those of known RyRs, including Ca^{2+}-independence, inhibition by caffeine, and different kinetic parameters.

2. Materials and methods

2.1. Materials

ATP, BSA, EDTA, EGTA, $LaCl_3$, Mops, trypsin and Tris were obtained from Sigma (St. Louis, MO). Benzoyl–benzoyl–ATP (BzATP), and [α-^{32}P]BzATP were synthesized and purified as described previously (Zarka & Shoshan-Barmatz, 1993) with some modifications to scale down the amount of BzATP synthesized. [α-^{32}P]ATP was obtained from Amersham and [^{3}H]ryanodine (60 Ci/mmol) was purchased from NEN® Life Science Products Inc. (Boston, USA). Unlabeled ryanodine was obtained from Calbiochem. Ruthenium red (98% pure) was from Fluka. Monoclonal anti-RyR 34C antibodies were obtained from the Developmental Studies Hybridoma Bank (University of Iowa). A polyclonal, affinity purified antibody against a cardiac RyR was kindly provided by S. Fleischer (Vanderbilt University), and a monoclonal anti-cardiac antibody (MA3-916) was from Affinity Bioreagents (ABR) (Golden, CO, USA). Human retina and skeletal muscle Marathon-ready cDNA were obtained from BD Biosciences Clontech (CA, USA).

2.2. Membrane preparations

Retinal membrane fractions were prepared from about 10 freshly isolated or frozen bovine or rabbit

retinas by 10 strokes in a motor-driven glass–Teflon homogenizer (1000 rpm) in homogenization buffer containing 0.32 M sucrose, 10 mM Hepes (pH 7.4), and the protease inhibitors 0.1 mM PMSF, 0.5 μg/ml leupeptin, and 0.5 μg/ml aprotinin. In some experiments, BSA (0.1%) was added to all but the final suspension buffer. The homogenate was centrifuged at $1000 \times g$ for 15 min, and the obtained supernatant was centrifuged at $10{,}000 \times g$ to yield pellet P_{10} (which showed very low ryanodine-binding activity). The supernatant was centrifuged for 30 min at $44{,}000 \times g$ to yield pellet P_{44}. The subsequent supernatant was centrifuged once again for 60 min at $160{,}000 \times g$ to yield pellet P_{160}. The obtained pellets were resuspended in sucrose buffer. The P_{44} fraction, isolated from fresh or frozen retina expresses similar binding properties, and was used in most of the experiments. Sarcoplasmic reticulum (SR) membranes were prepared from rabbit fast twitch skeletal muscle (Shoshan-Barmatz & Zarka, 1988) or from canine heart (Chamberlain, Livitsky, & Fleischer, 1983) as described previously. All the membranes were frozen in liquid nitrogen, and stored at −70 °C. Protein concentration was determined by the standard Lowry procedure (Lowry, Rosenbrough, Farr, & Randall, 1951).

Trypsin treatment of skeletal or cardiac muscle SR and retina P_{44} fraction (5 mg/ml) was carried out by incubation with trypsin (1:700, w/w) for 1–5 min in a solution containing 0.5 M sucrose and 10 mM Tricine, pH 8.0 as described previously (Shoshan-Barmatz & Zarka, 1988). Proteolysis was stopped by addition of a sample buffer for SDS-PAGE and the samples were run on the same gel.

2.3. [^{3}H]Ryanodine binding

Unless otherwise specified, retinal or SR membranes were incubated for 20–60 min at 30 or 37 °C in a standard binding solution containing 0.5 or 1 M NaCl, 20 mM Mops, pH 7.4, 50 μM free [Ca^{2+}], and 5–120 nM [^{3}H]ryanodine. Unbound ryanodine was separated from protein-bound ryanodine by vacuum filtration of the sample through nitrocellulose filters (0.3 μm), followed by two washes with 4 ml ice-cold buffer containing 0.2 M NaCl, 10 mM Mops, pH 7.4, and 50 μM $CaCl_2$. The radioactivity retained on the dried filters was measured by liquid scintillation counting. Specific binding of [^{3}H]ryanodine was defined as the difference between the binding in the absence and the presence of 100 μM unlabeled ryanodine.

2.4. Photoaffinity labeling of ATP-binding proteins by [α-^{32}P]BzATP

Retinal microsomal membranes (1 mg/ml) were irradiated with UV light for 3 min in the presence of 1 μM [α-^{32}P]BzATP (4×10^6 to 4×10^7 cpm/nmol) in 50 μl of 20 mM Mops, pH 7.4, 0.4 M NaCl, and other reagents as indicated in the figure legends. The irradiated membranes were immediately diluted 1:1 with a buffer containing 125 mM Tris–HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, and 2% (v/v) 2-mercaptoethanol, and incubated for 3 min at 90 °C. The samples were analyzed by SDS-PAGE as described below. Autoradiography of the dried gels was carried out using Kodak X-Omat film.

2.5. Oligonucleotide primers, reverse transcription, and PCR amplification

Total RNA was extracted from rabbit, mouse or rat retina using the Micro RNA isolation kit following the method described by the vendor (Boehringer–Mannheim). First-strand cDNA was synthesized from 1 to 2 μg of total RNA using random primers (AB-Gene) for templates 1–7 and non-degenerate sense and antisense primers (8 and 9; Table 3) (Oswel DNA Service, Southampton, U.K.), and reverse transcription (RT) (50U, EpiCenter). RT was performed according to standard procedures. Subsequent PCR amplification of specific cDNAs was performed with several primer pairs designed for unique sequences in each *RyR* subtype (Table 3) using Taq polymerase (1.25 U, Fail Safe, Roche). Reaction conditions were 30 cycles of 30 s at 95 °C, 30 s at 54–66 °C (according to the primers used), and 1.5–4 min at 72 °C. The amplified products were analyzed directly by electrophoresis on 1% (w/v) agarose gels, and the products obtained using primers 8 and 9 (see Table 3) were also analyzed by Southern hybridization. No PCR products were obtained when cDNA was omitted.

2.6. Gel electrophoresis and immunoblot analysis

Analysis of protein profiles by SDS-PAGE was performed using the discontinuous buffer system of

Laemmli (Laemmli, 1970) in 1.5 mm thick slab gels containing a gradient of 3–6 or 3–10% (w/v) acrylamide. The separated proteins were stained with Coomassie blue or electrophoretically transferred onto nitrocellulose membranes using Tris–glycine, pH 8.3. The membranes were blocked with 5% (w/v) non-fat dry milk and 0.1% (v/v) Tween-20 in 10 mM Tris–HCl, pH 7.8, and 0.15 M NaCl, and incubated with the primary antibodies followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG as a secondary antibody.

2.6.1. Tissue preparation and immunostaining

Bovine retinas were obtained from a local slaughterhouse. Eyecups were immersion-fixed for 1 h in 4% (w/v) paraformaldehyde in phosphate buffer (PB; 0.1 M Pi, pH 7.4). Following cryoprotection by incubation overnight with 30% sucrose in PB at 4 °C, retinas were separated from the back of the eye, mounted in tissue freezing medium (Electron Microscopy Sciences, Ft. Washington, PA), and 20% sucrose (1:2), frozen in liquid nitrogen and sectioned vertically (12 μm thickness) with a cryostat. Sections were incubated for 1 h in a blocking solution (phosphate buffer containing 5% sucrose (SPB), 10% normal goat serum, and 0.4% Triton-X 100), and then in the primary antibody (1:1000 in blocking solution) for 16–20 h at 4 °C. Following three washes with SPB the sections were incubated for 2 h with a secondary antibody conjugated to horseradish peroxidase (HRP), and developed with 3,3′-diaminobenzidine (DAB) (0.5 mg/ml) and 0.015% hydrogen peroxide. Sections were washed again, mounted with glycerin, and viewed with a light microscope (Polyvar II, Leica).

3. Results

The binding of [^{3}H]ryanodine to different retinal membranous fractions is summarized in Table 1. High binding capacity was obtained with membranous fractions P_{44} and P_{160} representing heavy and light microsomal fractions, respectively. When BSA was included in the preparation of retinal membrane fractions, ryanodine binding to the P_{44} fraction was increased by about 2-fold (Table 1). This may have been due to stabilization of RyR and/or protection from degradation, given that the RyRs are highly sensitive to proteases (Shoshan-Barmatz & Zarka, 1988). The P_{44} and P_{160} were enriched with ER membranes as evidenced by the following. These fractions: (1) contained protein bands at the expected molecular mass (∼540 kDa) that immunoreacts with antibodies against RyR commonly used as an ER marker (see Figs. 6, 10 and 11); (2) exhibited protonophore FCCP insensitive Ca^{2+} uptake, suggesting that the uptake is not by mitochondria (Table 1); (3) exhibited Ca^{2+}-dependent formation of a 100 kDa phosphorylated protein, representing the phosphorylated intermediate (E-P) in the transport cycle (data not shown); (4) did not contain mitochondria since Tom−20, a specific OMM protein, was not detected with specific antibodies (data not shown).

3.1. Ryanodine binding to retinal membranes is Ca^{2+}-independent

All ryanodine receptor/Ca^{2+}-release channels that have been studied after incorporation into a bilayer

Table 1
Ryanodine binds to different retinal membrane fractions

Membrane fraction	Ryanodine bound pmol/mg protein		Ca^{2+} accumulation nmol/mg protein
	−BSA	+BSA	−BSA
P_{44}	0.41 ± 0.07 (n = 5)	0.99 ± 0.05 (n = 8)	19.6 ± 3 (n = 3)
P_{160}	0.40 ± 0.05 (n = 4)	0.46 ± 0.03 (n = 4)	17.8 ± 2 (n = 3)

Bovine retinal membrane fractions (P_{44} and P_{160}) (1.5 mg/ml) prepared with or without BSA were incubated in a reaction mixture containing 40 nM ryanodine, 1 M NaCl and 50 μM $CaCl_2$, for ryanodine binding. After 20 min at 30 °C, samples were assayed for bound [^{3}H]ryanodine as described in Section 2. Membranes (0.2 mg/ml) were assayed for Ca^{2+} accumulation in a reaction mixture for Ca^{2+} uptake containing 10 mM Mopes, 0.1 M NaCl, 3 mM $MgCl_2$, 3 mM ATP, 0.5 mM EGTA, 0.5 mM $CaCl_2$ and 50 mM Pi. After 5 min at 30 °C, uptake was terminated by rapid Millipore filtration followed by a wash with 5 ml of 0.15 M NaCl. Values represent the mean ± S.E.M. of n different independent experiments.

Table 2
Ca^{2+} and Mg^{2+} only slightly affect ryanodine binding to retinal membranes

Assay conditions	Ryanodine bound			
	SSR	CSR	Retinal P_{44}	
	[NaCl]: 0.5 M	[NaCl]: 0.5 M	[NaCl]: 0.5 M	[NaCl]: 1 M
Control (pmol/mg protein)	3.9 ± 0.8	1.03 ± 0.03	0.157 ± 0.01	0.264 ± 0.02
EGTA (1 mM) (% control)	1.3 ± 4	3 ± 1	71 ± 8	100 ± 4
$MgCl_2$ (5 mM) (% control)	43 ± 8	85 ± 8	85 ± 10	ND

Retinal membranes P_{44} fraction (1.5 mg/ml), skeletal muscle SR (SSR), or cardiac SR (CSR) (1 mg/ml) membranes were incubated in a reaction mixture for ryanodine binding containing 20 nM [^{3}H]ryanodine, 0.5 M NaCl or 1 M NaCl, 50 μM $CaCl_2$, and the indicated compound. After 1 h at 37 °C, samples were assayed for bound [^{3}H]ryanodine, as described in Section 2. The results are the mean ± S.E.M. of three to four similar independent experiments. ND, not determined.

or by addressing their ryanodine-binding activity are activated by Ca^{2+} (Coronado et al., 1994; Meissner, 1994; Shoshan-Barmatz & Ashley, 1998; Sutko & Airey, 1996). In retinal membranes, ryanodine binding did not depend on Ca^{2+} (Table 2). EGTA completely diminished ryanodine binding to skeletal and cardiac muscle SR, but only slightly affected the binding to retinal RyR. As reported previously (Coronado et al., 1994; Shoshan-Barmatz & Ashley, 1998), Mg^{2+} (5 mM)-inhibited ryanodine binding to skeletal muscle SR by ∼60%, while binding to cardiac SR and retinal membranes was inhibited only by ∼15% (Table 2).

In SR, low concentrations of Ca^{2+} stimulate ryanodine binding while high concentrations inhibit the binding (Coronado et al., 1994; Meissner, 1994; Shoshan-Barmatz & Ashley, 1998; Sutko & Airey, 1996) (Fig. 1A). In contrast, Ca^{2+}, at concentrations ranging from 10 μM to 10 mM, hardly affected ryanodine binding in retina (Fig. 1A). In skeletal SR, La^{3+}, known to bind to divalent cation-binding sites (Hadad, Zable, Abramson, & Shoshan-Barmatz, 1994), inhibits ryanodine binding to SR RyR. In retina, La^{3+} only slightly affected binding to RyR (Fig. 1B).

Similar to cardiac and skeletal muscle membranes (Coronado et al., 1994; Meissner, 1994; Shoshan-Barmatz & Ashley, 1998; Sutko & Airey, 1996), the binding of ryanodine to retinal RyR was dramatically increased by high concentrations of NaCl (Fig. 2). High salt concentration is thought to stabilize the protein in a conformation with high affinity for ryanodine, although the underlying mechanism of this effect remains unclear.

3.2. Effect of temperature and pH

The binding of ryanodine to SR is highly sensitive to temperature, increasing by over 5-fold with increasing temperatures from 4 to 37 °C (Fig. 3A and Buck, Zimanyi, Abramson, & Pessah, 1992). In retina, optimal ryanodine binding was detected at 20 °C and decreased by 50% at 37 °C (Fig. 3A).

Increasing the assay pH, 7.5 to 8.5 decreased binding in the SR by 60%, whereas binding was only slightly changed in the retina (Fig. 3B).

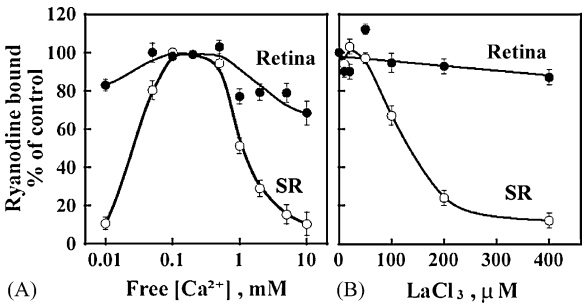

Fig. 1. Ryanodine binding to retinal membranes is Ca^{2+}-independent. (A) [^{3}H]Ryanodine binding to retinal membranes (P_{44}) (1.5 mg/ml) was assayed at 37 °C for 1 h as described under Section 2, except that 0.5 M NaCl and 20 nM (SR) or 40 nM (retina) of ryanodine, and the indicated concentrations of $CaCl_2$, were used. EGTA (0.2 mM) and Ca^{2+} were added to the binding solution, to obtain the indicated free Ca^{2+} concentration, as calculated using the computer program WinMAXC 2.05 (Bers, Patton, & Nuccitlli, 1994). In (B), ryanodine binding was assayed in the presence of 50 μM $CaCl_2$ as in (A), except that $LaCl_3$ was added at the indicated concentrations. Control activity (100%) for the experiment in (A) was 0.52 and 1.52 pmol/mg protein for retina and SR, respectively, and for the experiment in (B), it was 0.18 and 1.6 pmol/mg for retina and SR, respectively. Values represent the mean ± S.E.M. of three to four experiments carried out with different membrane preparations.

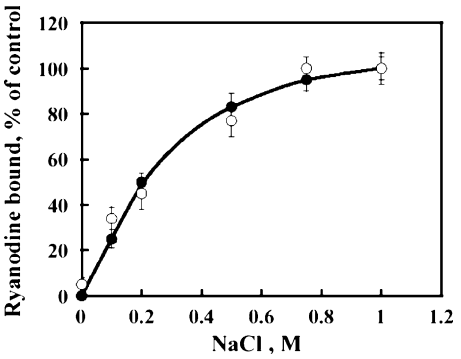

Fig. 2. Ryanodine binding to SR and retinal membranes is stimulated by NaCl. [^{3}H]Ryanodine binding to P_{44} (●) or SR (○) was assayed in the presence of 50 μM $CaCl_2$ and 20 nM ryanodine and the indicated concentration of NaCl, as described in Fig. 1. Control activities (100%) were between 0.29 and 0.45 pmol/mg protein (retina) and 2.9–3.6 pmol/mg protein (SR). Values represent the mean ± S.E.M. of three different independent experiments.

3.3. Retinal ryanodine binding displays fast kinetics

The time course of [^{3}H]ryanodine binding was first measured at 37 °C, in the presence of 1 M NaCl and 50 μM $CaCl_2$. Under these conditions, ryanodine binding to SR membranes showed relatively slow kinetics, reaching half-maximal binding after about 10 min, and a maximal value after about 60 min (Fig. 4A). In contrast, in retinal membranes, ryanodine rapidly associated with its binding sites, and this association appeared to be complete within 1 min, i.e., 60-times faster than in skeletal muscle (Fig. 4A). Decreasing the assay temperature from 37 to 20 °C increased the time for half-maximal retinal binding by about 4-fold (Fig. 4B). Kinetic analysis of ryanodine binding to retinal membranes at 20 °C (Fig. 4B) yielded an association rate constant (K_{obs}) of 1.12 min^{-1} (Fig. 4C) as compared to K_{obs} of 0.027 min^{-1} for SR at 37 °C (Shoshan-Barmatz & Zchut, 1993). Using this K_{obs}, a pseudo-first-order association rate constant (K_1) of 5.1×10^6 M^{-1} min^{-1} was calculated. Dissociation of bound ryanodine at equilibrium was initiated by a 10-fold dilution. A monophasic dissociation curve gave a K_{-1} of 0.67 min^{-1} (at 20 °C) (Fig. 4D) as compared to 0.012 min^{-1} for SR obtained at 37 °C (Shoshan-Barmatz & Zchut, 1993). Thus, in retina, both ryanodine association with and dissociation from its binding site are very rapid relative to skeletal or cardiac muscle RyRs (Coronado et al., 1994; Meissner, 1994; Shoshan-Barmatz & Ashley, 1998; Shoshan-Barmatz& Zchut, 1993; Sutko & Airey, 1996).

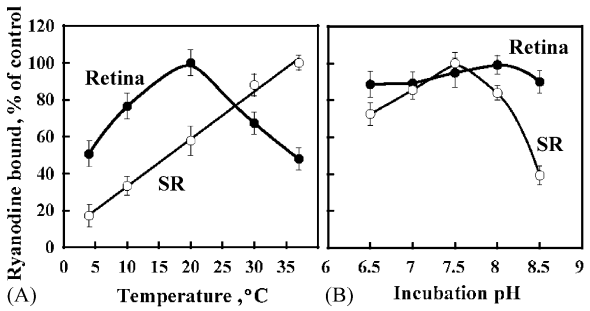

Fig. 3. Temperature and pH effects on ryanodine binding to retinal and SR membranes are different. P_{44} and SR membranes were assayed for [^{3}H]ryanodine binding at the indicated incubation temperature (A) or pH (B), as described in Section 2. For SR, incubation was for 2 h in the presence of 1 M NaCl and 20 nM [^{3}H]ryanodine, and for retina, incubation was for 30 min in the presence of 1 M NaCl and 40 nM of [^{3}H]ryanodine. Control activities (100%) were: 0.82 and 0.965 pmol/mg protein (retina) and 2.7 and 5.0 pmol/mg protein (SR) for (A) and (B), respectively. Values represent the mean ± S.E.M. of three to four different independent experiments.

The specific binding of [^{3}H]ryanodine to retinal membrane fraction P_{44} was assayed as a function of ligand concentration (Fig. 5). Scatchard plot analysis of [^{3}H]ryanodine binding to P_{44} yielded a non-linear curve, suggesting binding of ryanodine to two or more classes of high-affinity sites. The apparent dissociation constants (as calculated from the slopes of the linear parts of the curve) were: $K_{d_1} = 20.6 \pm 3$ nM and $K_{d_2} = 114 \pm 4$ ($n = 4$), and B_{max}, the total binding sites, was 2–2.6 pmol ryanodine bound/mg protein ($n = 4$). Using the association and dissociation constants we calculated a K_d of 131 nM (see Fig. 4), which is similar to the K_{d_2} calculated from saturation experiments (Fig. 5). This ryanodine-binding affinity is 2–20-fold lower than the affinity reported for brain (Lai et al., 1992; Otsu et al., 1990), cardiac (Meissner, 2004; Otsu et al., 1990) or skeletal muscle RyR (Coronado et al., 1994; Meissner, 1994; Shoshan-Barmatz & Ashley, 1998; Sutko & Airey, 1996). This is in agreement with the much higher association and dissociation rate constants determined for ryanodine binding to retinal membranes, as compared to those for skeletal and cardiac muscle receptors (Fig. 4).

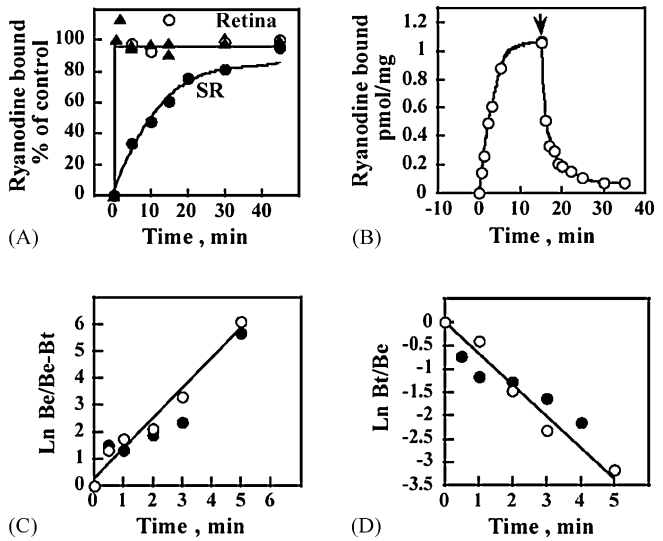

Fig. 4. Fast association and dissociation of ryanodine with and from its binding sites. (A) P_{44} and SR membranes were assayed for ryanodine binding (1 M NaCl, 20 nM (SR) or 40 nM (retina) [^{3}H]ryanodine at 37 °C) at the indicated incubation time as described in Section 2. (B) Ryanodine binding to P_{44} was assayed as in (A), except that the ryanodine concentration was 80 nM and the temperature was 20 °C (to slow down the association rate). Dissociation of bound ryanodine was initiated by 10-fold dilution with binding medium lacking ryanodine (at the time indicated by an arrow). (C and D) Kinetic analysis of ryanodine binding to P_{44} and its dissociation (assayed at 24 °C). (C) Association of ryanodine binding—ln (Be/Be–Bt) is plotted as a function of time, where Be indicates the amount of ryanodine bound at the plateau (0.95 pmol/mg) and Bt the bound ryanodine at the indicated time. The slope of this plot gives an observed association rate constant (K_{obs}) of 1.12 min $^{-1}$. (D) Dissociation of bound ryanodine was determined as in (B), where the residual bound ryanodine (Bt) after a 10-fold dilution was determined. The calculated dissociation rate constant K_1 from (D) was 0.67 min $^{-1}$. K_1 was calculated from K_{obs} as described previously (Weiland & Molinoff, 1981), using the following equation: $K_{obs} = K_1[L][R]/Be$, where [L] = ryanodine concentration; [R] = ryanodine-binding protein = B_{max} (2.6 pmol/mg). $K_1 = 5.1 \times 10^6$ M^{-1} min $^{-1}$. For (A), (C), and (D), different symbols represent different experiments.

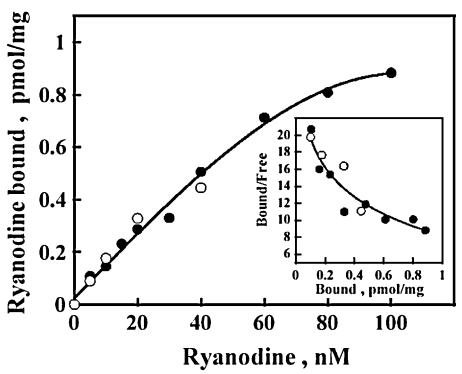

Fig. 5. Ryanodine-binding affinity of retinal RyR. [^{3}H]Ryanodine binding to retinal P_{44} fraction (1.5 mg/ml) was assayed as a function of ryanodine concentration for 30 min as described in Section 2. The data was analyzed by Scatchard plot (inset) which yielded a non-linear line fitted with K_{d_1} = 18 nM, K_{d_2} = 112 nM and B_{max} =1.4 pmol ryanodine bound/mg protein. Open and closed symbols indicate results from two different experiments using the same membrane preparation.

3.4. Effects of activators and inhibitors of cardiac and skeletal muscle RyR proteins on ryanodine binding to retinal RyR

In cardiac, skeletal muscle and brain RyR, adenine nucleotides increase ryanodine binding and the open-state probability of RyR channels (Shoshan-Barmatz & Ashley, 1998). Indeed, as shown in Fig. 6A, ATP and ADP stimulated ryanodine binding to skeletal muscle RyR by about 1.5-fold, while AMP affected ryanodine binding to a lesser extent. In contrast, adenine nucleotide in retinal P_{44}, did not stimulate, but rather inhibited, ryanodine binding (Fig. 6A). cGMP had no significant effect on ryanodine binding to either skeletal muscle or retinal P_{44} fraction (data not shown). To test for the presence of ATP-binding sites in retinal RyRs, the photoreactive ATP analogue, BzATP, was employed. Irradiation of retinal P_{44} with [α-^{32}P]BzATP resulted in covalent binding of the label to several membrane proteins, including two high molecular weight

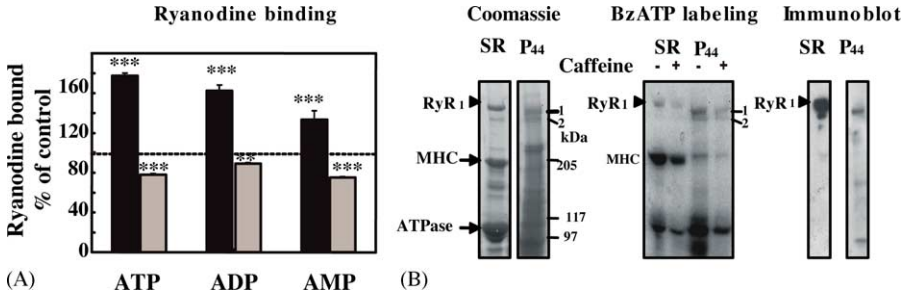

Fig. 6. Ryanodine binding to retinal RyR is not stimulated by nucleotides, even though RyR contains an ATP binding site. (A) [^{3}H]Ryanodine binding to retinal membranes (1.5 mg/ml) or SR (0.5 mg/ml) was assayed in the presence of 0.25 M NaCl, 20 nM (SR) and 40 nM (retina) ryanodine, 50 μM free Ca^{2+} (calculated as in Ref. (Bers et al., 1994) and 2 mM of the indicated nucleotide. After 1 h at 37 °C, samples were assayed for bound [^{3}H]ryanodine as described in Section 2. Control activity (100%) = 2.2 ± 0.3 pmol/mg of protein (n = 3) and 0.44 ± 0.4 pmol/mg of protein for SR (black bars) and retinal membranes (gray bars), respectively. Statistical analysis of nucleotide effect on ryanodine binding to SR and retina was performed by ANOVA and t-test; for all three pairs the differences were statistically significant ((**) $p < 0.01$ or (***) $p < 0.001$). (B) Retinal and SR membranes were labeled with 1 μM [α-^{32}P]BzATP in the absence and the presence of caffeine (10 mM) and then subjected to SDS-PAGE and immunoblot analysis as described under Section 2. In (B), RyR indicates ryanodine receptor monomer and MHC, myosin heavy chain. The protein bands labeled 1 and 2 represent RyR and dynein, respectively.

proteins in the range of the RyR subunit (∼500 kDa), and corresponding to the Coomassie-stained bands 1 and 2 (Fig. 6B). The [α-^{32}P]BzATP-labeled band 1 cross-reacted with anti-RyR antibodies recognizing all RyR isoforms (34C), and the second BzATP-labeled band 2 cross-reacted with anti-dynein specific antibodies. The latter was further identified as dynein by protein sequence analysis (data not shown; see Hadad, Martin, Ashley, Shoshan-Barmatz, 1999). BzATP labeling of both SR and retinal RyR was inhibited with caffeine. The results suggest that retinal RyR possesses at least one nucleotide-binding site.

In skeletal and cardiac muscle RyRs, caffeine acts as an agonist, increasing the open-state probability of the RyR channel and ryanodine binding (Coronado et al., 1994; Meissner, 1994; Shoshan-Barmatz & Ashley, 1998; Sutko & Airey, 1996). As expected, caffeine increased ryanodine binding to the skeletal muscle receptor; yet in retinal membranes, it strongly inhibited ryanodine binding (Fig. 7), regardless of the NaCl or Ca^{2+} concentrations used in the binding assay (data not shown). The hexavalent cation ruthenium red (RuR), known to inhibit ryanodine binding and channel opening (Coronado et al., 1994; Meissner, 1994; Shoshan-Barmatz & Ashley, 1998; Sutko & Airey, 1996), decreased ryanodine binding to retinal protein, but with much lower sensitivity as compared to skeletal muscle RyR. Half-maximal inhibition was obtained at about 10 and 60 μM for skeletal SR and retinal membranes, respectively (Fig. 8A). Various local anesthetics have also been shown to interact with RyRs and modify their activity (Shoshan-Barmatz & Zchut, 1993). Tetracaine decreased ryanodine binding to its receptors in both SR and retinal P_{44} membranes, but the retinal protein

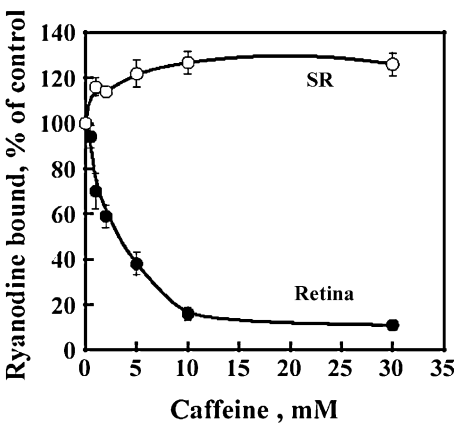

Fig. 7. Caffeine inhibits ryanodine binding to retinal RyR. Ryanodine binding to P_{44} retinal membranes (1.5 mg/ml) or skeletal muscle SR (1 mg/ml) was assayed for 1 h at 37 °C in the presence of 1 M NaCl (retina) or 0.25 M NaCl (SR) (to allow stimulation) and the indicated concentration of caffeine as described under Section 2. Control activity (100%) for SR was 2.25–3.3 pmol/mg of protein and for retina it ranged from 0.264 to 0.462 pmol/mg of protein. Open symbols, SR; closed symbols, retinal P_{44}. Values are mean ± S.E.M. of four different experiments.

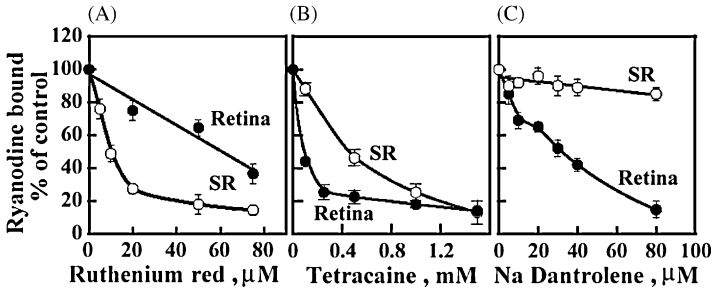

Fig. 8. Inhibition of ryanodine binding by tetracaine, ruthenium red and dantrolene. Ryanodine binding to P_{44} retinal membranes (1.5 mg/ml) or skeletal muscle SR (1 mg/ml) was assayed for 1 h at 30 °C in the presence of 1 M NaCl and the indicated concentration of ruthenium red (A), tetracaine (B) or Na dantrolene (C). Control activities (100%) were 4.2, 4.4, and 5.5 for SR (A, B and C, respectively), and for retina, between 0.495 and 0.67 pmol/mg of protein (for 3–4 different experiments). Values represent mean ± S.E.M.

was more sensitive (Fig. 8B). Dantrolene is a drug that suppresses intracellular Ca^{2+} release from the SR in skeletal muscle and is used as a therapeutic agent in individuals susceptible to malignant hyperthermia, presumably by modulating the activity of RyR1 (Krause, Gerbershagen, Fiege, Weisshorn, & Wappler, 2004). Dantrolene strongly inhibited the binding of ryanodine to retinal membranes (IC_{50} = 40 μM), but not to SR RyR (Fig. 8C).

3.5. *mRNAs encoding the three RyR isoforms are present in retina*

To determine if known isoforms of RyR are present in retina, we used specific primers to PCR amplify their mRNAs from mouse, rabbit, or rat retina (RT-PCR) or from retina Marathon cDNA (PCR) (Fig. 9 and Table 3). RNA isolated from skeletal muscle, heart, and whole brain, as well as skeletal muscle Marathon cDNA were used as positive controls. Primers were designed to match unique sequences in each isoform and that are located along the *RyR* genes (see Table 3). In retina, amplification was observed for all three isoforms *(RyR1, RyR2* and *RyR3*). Additionally, we performed RT-PCR with specific primers designed to differentially amplify the "short" and the "long" forms of RyR3 (274 and 674 bp product) (Miyatake et al., 1996), only the "short" form was identified in retina (data not shown). However, it should be mentioned that several other RyR1 and RyR2 primer pairs gave no PCR product in retina. This may have resulted from the template quality or reaction conditions, or it may reflect sequence differences between retina protein(s) and RyR1 or RyR2.

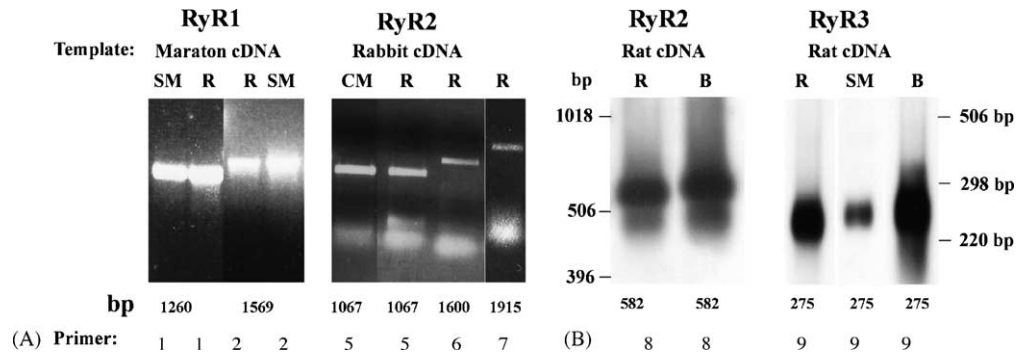

Fig. 9. RT-PCR and PCR analysis of RyRs specific transcripts in retina. (A) RT-PCR and PCR analysis for RyR1 and RyR2. Templates were either total RNA isolated from rabbit retinas or human Marathon cDNA. Primer sets are as those presented in Table 3. All obtained products were at their expected size and were not obtained when RNA was omitted. (B) Autoradiographs of blot hybridization analyses of *ryr2* or *ryr3* RT-PCR products. The amplified products (using primers and templates of Table 3) were analyzed directly by electrophoresis on 1% (w/v) agarose gels and Southern hybridization analysis. R, retina; SM, skeletal muscle; CM, cardiac muscle; B, brain.

Table 3
Sequence and position of the primers that successfully amplified RyR subtypes and the size of the expected PCR products

Sr. no.	Template source	Primer sequence	*RYR* type	Primer location	Expected product (bp)
1	Marathon cDNA (human)	F 5′-cggtggaggctggcgtggagtcat-3′; R 5′-tcagcaaagtgggcagcagtggtgtaga-3′	*RYR1*	381–1641	1260
2	Marathon cDNA	F 5′-agaggaggcagcagaaggggagaaagaa-3′; R 5′-gagcaggtcgatcaaggcggcatagaag-3′	*RYR1*	5860–7429	1569
3	Rat RNA	F 5′-ggaaagagattgtgaacctg-3′; R 5′-ctgtcaggaatggaaccact-3′	*RYR1*	1671–2158	487
4	Rat RNA	F 5′-ggtggccttcaacttcttcc-3′; R 5′-acttgctcttgttggtctcg-3′	*RYR1 RYR2*[a]	14692–14979	287
5	Rabbit RNA	F 5′-gctctggagcgtggc-3′; R 5′-ccgaggaagcttccag-3′	*RYR2*	917–1984	1067
6	Rabbit RNA	F 5′-gagttgttggcggctc-3′; R 5′-ctggcagcatgatcttgatc-3′	*RYR2*	1902–3502	1600
7	Rabbit RNA	F5′-catggttcgatgggc-3′; R 5′-ggtatcaacaggttgtgg-3′	*RYR2*	6495–8410	1915
8	Mouse RNA	F5′-tctgaattcatgaccctcctgcacttc-3′; R′-cactctagatgctgactctggaacttc-3′	*RYR2*	13174–13741	582
9	Mouse RNA	F 5′-caacagatgagcagcaaaga-3′; R′-aggtgatcaacaagtatgga-3′	*RYR3*	8219–8471	275 or 614

Total RNA was isolated from retina of rabbit, mouse or rat, and RT-PCR or PCR (on human Marathon cDNA) was performed as described under Section 2. Some of the results are presented in Fig. 9. All obtained products were at the expected size.
[a] These primers sequences are present in both RyR1 and RyR2.

3.6. Localization of RyR in retina

To localize RyR in the retina, we preformed immuno-staining of bovine retina with the affinity purified polyclonal anti-RyR2 antibody. Staining was strong in somas and primary dendrites of amacrine, ganglion, and horizontal cells (Fig. 10A). In the ganglion cell somas, staining appeared punctate, suggestive of ER staining (Fig. 10B). Western blot analysis of cardiac SR and of P_{44} and P_{160} membrane fractions with the antibodies used for retina immunostaining demonstrated the immunoreactivity of a ∼500 kDa protein band in the three fractions (Fig. 10C). The weaker band in the retinal fractions in comparison to cardiac SR may indicate a lower expression level in retina.

3.7. Peptide maps of retinal RyR are different from those of RyR1 and RyR2

All RyR isoforms are very sensitive to proteolysis (Shoshan-Barmatz & Ashley, 1998). Fig. 11 shows the tryptic peptide maps of RyRs in cardiac, skeletal muscle and retinal P_{44} membranes, as revealed using a monoclonal pan-RyR antibody (34C), which recognizes all known RyR isoforms (Airey et al., 1990), and also using monoclonal antibody that detects RyR2 but has only a weak affinity for RyR1 (Olivares et al., 1991). Immunoblot analyses showed that the tryptic profiles of the three proteins were different; the retinal RyR shares two tryptic fragments with skeletal RyR1 (320 and 200 kDa; Fig. 11A), and three fragments with cardiac muscle RyR (240, 155, and 130 kDa; Fig. 11B). However, several tryptic fragments of RyRs were unique to each membrane, indicating that the major retinal RyR is different from both RyR1 and RyR2.

4. Discussion

We have demonstrated here that retina expresses a ryanodine-binding protein whose binding properties are vastly different from those of known ryanodine receptor types 1–3. Although we cannot completely rule out the possibility that this ryanodine-binding protein is not related to the family of ryanodine receptors, the following results suggest that the ryanodine binding protein is likely a ryanodine receptor. (a) The RNAs of the three known RyR isoforms are present in retina

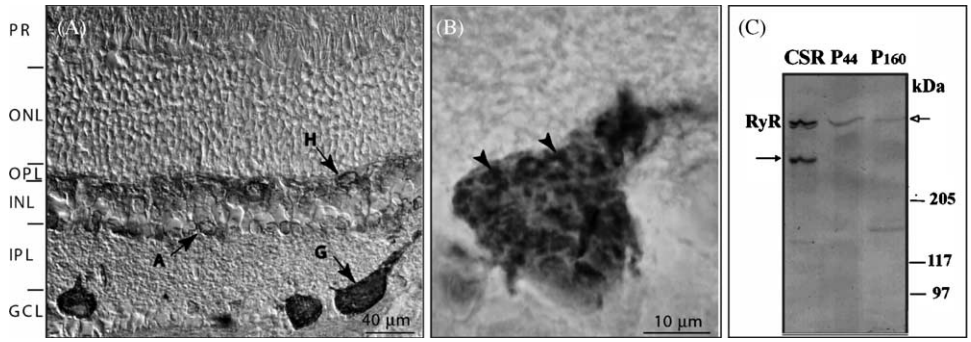

Fig. 10. Immunostaining of retina with anti-RyR2 antibodies. (A) Immunostaining of bovine retina with rabbit affinity purified polyclonal anti-RyR2 antibodies (visualized with DAB). Staining is strong in somas of horizontal (H), amacrine (A) and ganglion (G) cells. It is also often seen in primary dendrites of horizontal and ganglion cells. (B) A higher magnification of a ganglion cell showing that the stain is punctuate, consistent with it being localized to ER (arrowheads). (C) Rat cardiac SR (CSR; 30 μg), and retinal P_{44} and P_{160} fractions (50 ng) were subjected to SDS-PAGE (5% acrylamide) and subjected to Western blot analysis in panel (C) using anti-RyR2 antibodies used in panels (A) and (B). Closed and open arrows indicate RyR subunit and retinal polypeptide immuno-reacted with the antibodies, respectively.

(Table 3); (b) retinal sub-cellular membranous fractions express RyR proteins with the expected molecular mass (as revealed by Western blotting; Figs. 6, 10 and 11); (c) the peptide map of trypsin-treated P_{44} shares certain fragments with RyR1 and RyR2; (d) immunocytochemistry of bovine retina, using anti-RyR2, localized RyR to somas of amacrine, horizontal and ganglion cells, which are rich in endoplasmic reticulum (Fig. 10); (e) to our knowledge, only ryanodine receptors bind ryanodine with high affinity as that observed in this study. However, the differences in ryanodine-binding properties of the retinal membranes and the finding that the peptide maps showed that certain protein fragments in retina were different than those in skeletal muscle RyR1 or cardiac RyR2, as revealed by pan-RyR and anti-RyR2 antibodies (Fig. 11), suggest

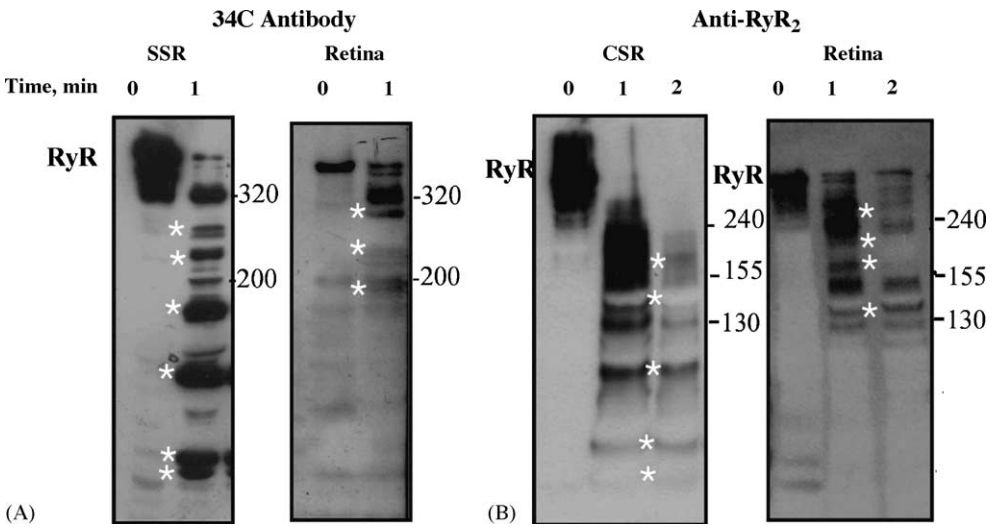

Fig. 11. The tryptic maps of retinal RyR, skeletal muscle RyR1 and cardiac RyR2 are different. Retinal P_{44} (retina), skeletal muscle (SSR) or cardiac (CSR) SR were treated with trypsin (1:700, w/w) for the indicated time as described under Section 2. Samples were subjected to SDS-PAGE (samples were run on the same gel), followed by immuno-staining using 34C antibody (A) or anti-RyR2 antibody (B). Immunoreactivity was visualized using peroxidase-conjugated secondary antibody and enhanced chemiluminiscent substrate was used for detection of HRP activity. White stars indicate peptides unique to RyR in retina, cardiac and skeletal muscle. The numbers indicate identical masses (in kDa) of the fragments.

that retina also expresses a novel type or splice variant of ryanodine-binding protein with unique regulatory mechanisms.

4.1. Retinal ryanodine-binding protein differs from RyR1–3

All three known RyR types show the same general characteristics of activation by low [Ca^{2+}] (<0.1 mM) and inhibition by high [Ca^{2+}] (>1 mM), stimulation by adenine nucleotides and caffeine, and inhibition by Mg^{2+}, RuR and tetracaine (Coronado et al., 1994; Meissner, 1994; Shoshan-Barmatz & Ashley, 1998; Sutko & Airey, 1996). Retinal RyR not only shares some of these characteristics with skeletal and cardiac muscle RyRs, but also differs from them in several important aspects. Similar to skeletal and cardiac RyRs, in retina, ryanodine binds to two or more classes of high-affinity sites in a manner stimulated by high NaCl concentrations. The retinal ryanodine binding, however, differs from that of the skeletal and cardiac muscle RyR as reflected by: (a) the fast association and dissociation of ryanodine with the retinal protein, (b) the Ca^{2+}-independent nature of the binding, (c) differential temperature and pH dependence, (d) the slight inhibition by La^{3+} or Mg^{2+}, (e) inhibition rather than stimulation by caffeine or nucleotides, and finally, (f) inhibition by dantrolene. These differences in the effects of ligands on ryanodine binding to retinal protein suggest an alteration in the ryanodine-binding sites.

4.2. Effects of drugs on ryanodine binding to the retinal protein

Caffeine is known to promote release of Ca^{2+} from intracellular stores and to stimulate ryanodine binding to skeletal and cardiac muscle SR, possibly by increasing the affinity of the high-affinity Ca^{2+}-binding sites (Coronado et al., 1994; Meissner, 1994; Shoshan-Barmatz & Ashley, 1998; Sutko & Airey, 1996). Since ryanodine binding in retina is Ca^{2+}-independent and caffeine inhibits ryanodine binding, the mode of action of caffeine must differ from that in cardiac and skeletal muscle. Similar inhibition was obtained for ryanodine binding to a RyR-like protein in the liver ER (Shoshan-Barmatz et al., 1991), where caffeine was shown to inhibit agonist-induced cytoplasmic Ca^{2+} oscillations in single rat hepatocytes (Sanchez-Bieno, Marrero, & Cobbold, 1994). Caffeine and other methylxanthines are commonly used as antagonists at adenosine receptors (Fredholm et al., 1994), but appear not to interact with adenine nucleotide/nucleoside sites on RyRs, suggesting that RyRs have distinct caffeine-binding sites (McGarryand & Williams, 1994). Indeed, it should be noted that caffeine was found to inhibit $InsP_3$-induced Ca^{2+} release (Missiaen, Parys, De Smsdt, Himpens, & Casteels, 1994). Furthermore, caffeine was shown to bind to the nucleotide-binding site of $InsP_3$ receptor (Maes et al., 1999; Missiaen et al., 1994). A similar effect of caffeine is demonstrated here, with the inhibition of BzATP binding to RyR in retina (Fig. 6B). Moreover, there is evidence that caffeine sites in the various RyRs may differ (Zhang, Williams, & Sitsapesan, 1999). Accordingly, the unique effect of caffeine on ryanodine binding to retinal membrane suggests that the retinal ryanodine-binding protein has a different caffeine-binding site than other RyRs.

The retinal RyR is more sensitive than skeletal RyR to tetracaine and is highly sensitive to sodium dantrolene. Dantrolene has been used in treatment of malignant hyperthermia, a genetic disease characterized by hypermetabolism in skeletal muscle following a triggering stimulus (Loke & MacLennan, 1998). The pathophysiology of this syndrome has been related to an alteration in RyR (Loke & MacLennan, 1998). Efforts to determine the site of action of this agent have yielded no definite conclusion; however, it is generally accepted that dantrolene modulates intracellular [Ca^{+}]. Still, whether dantrolene suppression of Ca^{2+} release occurs via direct interaction with RyR1 is not entirely clear. Some evidence suggests that RyR1 is not the target, but others have shown that dantrolene does bind to RyR1 and that this binding depends on the presence of adenine nucleotide triphosphate (Paul-Pletzer et al., 2002). The inhibition of the retinal and liver ryanodine-binding proteins by dantrolene (Shoshan-Barmatz et al., 1991) supports the notion that dantrolene specifically interacts with these Ca^{2+}-independent and caffeine-inhibited ryanodine-binding proteins.

4.3. Ca^{2+}-independence suggests the presence of an exposed ryanodine-binding site

RyR has both high-affinity activating and low-affinity inactivating Ca^{2+}-binding sites, as reflected in Ca^{2+} effects on Ca^{2+} release from SR vesicles,

RyR single channel activity, and ryanodine binding (Coronado et al., 1994; Hadad et al., 1994; Meissner, 1994; Shoshan-Barmatz & Ashley, 1998; Sutko & Airey, 1996). Ryanodine binding to retinal membranes was obtained in the absence of Ca^{2+} and was only slightly inhibited by high Ca^{2+} concentrations (20 mM). In addition, La^{3+} only slightly affected ryanodine binding to retinal membranes, and ruthenium red inhibited ryanodine binding in retina only at higher concentrations. Since La^{3+} and RuR interact with Ca^{2+}-binding sites, these results are in agreement with retinal RyR being Ca^{2+}-independent. The absolute dependence of ryanodine binding to RyR1-3 on Ca^{2+} was interpreted as follows: Ca^{2+} induces conformational changes in the protein that stabilize the channel in its open-state, exposing the ryanodine-binding sites (Coronado et al., 1994). Thus, the Ca^{2+}-independent binding of ryanodine to the retinal protein, together with the fast association and dissociation of such binding, suggest that ryanodine-binding sites are readily exposed and available without any requirement for Ca^{2+}-induced conformational changes in the protein. This is further supported by our observation that ryanodine binds at relatively low temperature, in contrast to skeletal and cardiac muscles RyRs, which require higher temperature to accelerate conformational changes in the protein and therefore expose ryanodine-binding sites (Buck et al., 1992). The Ca^{2+}-independent nature of retinal ryanodine binding may suggest that in vivo activation of retinal protein does not involve Ca^{2+}-induced Ca^{2+}release.

4.4. Possible identity of the retinal ryanodine-binding protein

BLAST analysis of the human genome provides little support for the existence of a new gene encoding a novel RyR. However, using RT-PCR, the expression of a RyR1-like mRNA in a number of non-muscle cells was demonstrated. For example, the RyR identified in parotid acinar cells differs in its functional properties from those of RyR1 (Zhang, Wen, Bidasee, Besch, & Rubin, 1997). Also, truncated forms and splice variants of RyRs have been described (Marziali, Rossi, Giannini, Charlesworth, & Sorrentino, 1996). These different splice variants might associate either as homomers or heterotetramers, thereby producing a large diversity of RyR channels. The alternative splicing sites identified in RyR1, RyR2 and RyR3 are located in the region that contains many potential regulatory sites (Zhang et al., 1993). It has been shown that a negatively charged region of the N-terminal portion of RyR1 is involved in Ca^{2+}-dependent regulation of the Ca^{2+}-release channel (Hayek et al., 2000). This region, known as D3, diverges between RyR1 and RyR2. Deletion of D3 had no effect on ryanodine binding, but modified the Ca^{2+} sensitivity of both high- and low-affinity binding sites, conferred resistance to inhibition by Mg^{2+}, and diminished stimulation of ryanodine binding by caffeine (Hayek et al., 2000). Therefore, retinal ryanodine-binding protein might represent a product of alternative splicing. Alternatively, the retinal ryanodine-binding protein may represent a heterotetrameric complex composed of different subunits representing either RyR1, RyR2 or RyR3; the mRNA of each is expressed in retina (Table 3). Commercially available anti-RyR antibodies are not absolutely specific for isoform or splice variants. Thus, co-immunoprecipitation could not be used to demonstrate RyR heterotetramers in retina.

To conclude, the retina expresses several ryanodine-binding proteins, some may be identical to the known RyR types. However, since retinal ryanodine-binding properties differ from those of known RyRs, and because the tryptic map is different, the major protein in retina that binds ryanodine must also be different in some structural aspects. These findings suggest the possibility that the mechanisms underlining $[Ca^{2+}]i$ regulation in retina are unique for their specific function in the circuit.

Acknowledgments

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