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Functional properties of ryanodine receptors from rat dorsal root ganglia

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Abstract The properties of ryanodine receptors (RyRs) from rat dorsal root ganglia (DRGs) have been studied. The density of RyRs (B_{max}) determined by [^{3}H]ryanodine binding was 63 fmol/mg protein with a dissociation constant (K_d) of 1.5 nM. [^{3}H]Ryanodine binding increased with caffeine, decreased with ruthenium red and tetracaine, and was insensitive to millimolar concentrations of Mg^{2+} or Ca^{2+}. DRG RyRs reconstituted in planar lipid bilayers were Ca^{2+}-dependent and displayed the classical long-lived subconductance state in response to ryanodine; however, unlike cardiac and skeletal RyRs, they lacked Ca^{2+}-dependent inactivation. Antibodies against RyR3, but not against RyR1 or RyR2, detected DRG RyRs. Thus, DRG RyRs are immunologically related to RyR3, but their lack of divalent cation inhibition is unique among RyR subtypes.

Key words: Calcium release channel; Ryanodine receptor; Dorsal root ganglion; Calcium-induced calcium release; [^{3}H]Ryanodine binding; Planar lipid bilayer

1. Introduction

Intracellular Ca^{2+} release channels/ryanodine receptors (RyR) have a pivotal role in the signal transduction process known as Ca^{2+}-induced Ca^{2+} release (CICR). This process is central to excitation–contraction coupling in mammalian muscle cells whereby calcium entry via voltage-gated dihydropyridine-sensitive calcium channels located on the sarcolemma triggers a massive calcium release from an intracellular calcium storage organelle that is the sarcoplasmic reticulum [1]. CICR has now been reported to occur in neuronal dorsal root ganglion (DRG) cells [2] and the presence of RyRs in DRGs has been demonstrated by immunological methods [3,4]. However, the pharmacological and single channel properties of RyRs from DRG have not been clearly defined.

Previously, both IP_3-gated channels and RyR-gated channels were postulated to share a role in releasing calcium from DRG intracellular stores as experiments revealed that increases in intracellular Ca^{2+} could be inhibited by intracellular perfusion with heparin [5] or ryanodine [6]. Additional studies showed that intracellular Ca^{2+} release in intact DRG cells could be induced by caffeine [2,4,6,7] or cADP ribose [8] and inhibited by ryanodine [2,4,6], procaine, and dantrolene [6]. Furthermore, the ryanodine-sensitive intracellular Ca^{2+} stores appear to be important for Ca^{2+}-induced Cl^- currents [9] and for generation of store-operated Ca^{2+} currents [10]. Recently, it was demonstrated that activation of a metabotropic glutamate receptor, $mGluR_5$, induces Ca^{2+} release from ryanodine-sensitive stores, resulting in Cl^- currents and non-specific cation currents [11]. These studies of intact DRG cells entice speculation about the functional properties of the RyRs underlying the observations.

In the present study, we have measured the single channel properties of RyRs from rat DRG. Single channel activity was measured by incorporation of DRG microsomes into planar lipid bilayers. We also used [^{3}H]ryanodine binding to determine the density of RyRs and to obtain their pharmacological profile with regards to classical modulators of RyRs such as Ca^{2+}, Mg^{2+}, caffeine, ruthenium red, and tetracaine. Finally, we also examined the immunoreactivity of these DRG RyRs with antibodies to RyR1 (skeletal muscle type), RyR2 (cardiac type), and RyR3 (brain type). The data show that DRG RyRs share some common functional characteristics with the other well-known mammalian skeletal (RyR1) and cardiac (RyR2) isoforms [12,13]; surprisingly however, they lack Mg^{2+}- and Ca^{2+}-dependent inactivation.

2. Materials and methods

2.1. Materials

[^{3}H]Ryanodine (60–80 Ci/mmol) was purchased from Dupont Nuclear (Wilmington, DE, USA). Bovine brain phosphatidylethanolamine (PE) and phosphatidylserine (PS) were from Avanti Polar Lipids (Birmingham, AL, USA). The RyR2 monoclonal antibody was from Affinity Bioreagents, Inc. (Golden, CO, USA) and the RyR1 polyclonal antibody was from Upstate Biotechnologies (Lake Placid, NY, USA). The RyR3 polyclonal antibody was the kind gift of Dr. Vincenzo Sorrentino (University of Siena, Italy). Appropriate peroxidase-conjugated secondary antibodies and the chemiluminescence detection kit were from Pierce (Rockford, IL, USA). Pre-cast linear gradient polyacrylamide gels were from Bio-Rad Laboratories (Hercules, CA, USA). Caffeine and all other reagent grade chemicals were from Sigma Chemical (St. Louis, MO, USA).

2.2. Preparation of DRG microsomes

Newly weaned Sprague–Dawley rats were anesthetized by intraperitoneal injection of pentobarbital (200 mg/kg) and intact DRGs were carefully teased from the periphery of the entire spinal column with the aid of a dissecting microscope (this method has been approved by the Animal Care and Use Committee at the University of Wisconsin). The whole length of vertebral column was removed by cutting the column at the base of the skull and the top of the hip. The isolated column was then cut in half at the middle and placed in iced DMEM (Dulbecco's modified Eagle's medium). Soft tissue from the ventral side of the column was removed and a cut was made down the entire

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Abbreviations: CICR, Ca^{2+}-induced Ca^{2+} release; DRG, dorsal root ganglion; P_o, open probability; RyR, ryanodine receptor

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column length between the vertebral body and the transverse processes. A cut lengthwise through the middle of the spinal cord resulted in four pieces of cervical–thoracic and thoracic–lumbar column which was then placed in iced DMEM. Starting at the caudal end of each piece, the spinal cord was carefully lifted, the nerves that connect the cord to DRGs were cut using a dissecting microscope, and rows of DRGs in each half were isolated, extracted, and placed in iced DMEM. From each rat ~30 DRGs were obtained along the whole column length. Isolated DRGs from two rats (wet weight 0.1 g) were combined by low speed centrifugation in a tabletop centrifuge and then quickly frozen. For microsome preparation, DRGs from a total of eight rats were rapidly thawed and disrupted using a Polytron probe to break the tough outer coating and disrupt cells (20 μm probe, three times for 15 s each at low speed) in a solution containing 50 mM Tris–HCl (pH 7.2), 0.3 M sucrose, 1 mM dithiothreitol, 30 mM MOPS (pH 7.0), 2 μM leupeptin, and 0.8 μM benzamidine at 4°C. The Polytron homogenate was then further homogenized in a Potter-Elvejem type glass homogenizer to be sure all visible clumps of tissue were removed. The total homogenate was spun at $1000 \times g$ for 20 min and the guaze-filtered supernatant was then spun at $100\,000 \times g$ for 30 min. The pellet was resuspended in the above homogenization medium to a protein concentration of 4 mg/ml. Typically four rats yielded 2 mg of total membrane protein. Protein concentration was determined by the Bradford method using bovine serum albumin as the standard.

2.3. [^{3}H]Ryanodine binding

High affinity [^{3}H]ryanodine binding (K_d 5–10 nM) to rat DRG microsomes was measured as previously described [14] with minor modifications. 60 μg of microsomal protein was added to an incubation medium containing 7 nM [^{3}H]ryanodine in 0.5 M KCl, 30 mM MOPS (pH 7.2), and 10 μM $CaCl_2$. The incubation took place in a volume of 0.1 ml at 36°C for 90 min. Modulators, including tetracaine, Mg^{2+}, and caffeine, were added to reaction mixtures from 10× stocks. When different levels of cytosolic free [Ca^{2+}] were desired, Ca:EGTA mixtures were added to the above medium using the stability constants for Ca^{2+}-EGTA taken from Fabiato [15]. At the end of the incubation period, bound and free [^{3}H]ryanodine were separated by rapid filtration onto Whatman GF/C glass fiber filters and the filters were washed twice with cold distilled water using a Brandel M-24R harvester (Gaithersburg, MD, USA). The filters were placed in liquid scintillation cocktail and counted in a Beckman LS6500 β-counter. Non-specific [^{3}H]ryanodine binding was determined in the presence of 100 μM unlabeled ryanodine and has been subtracted

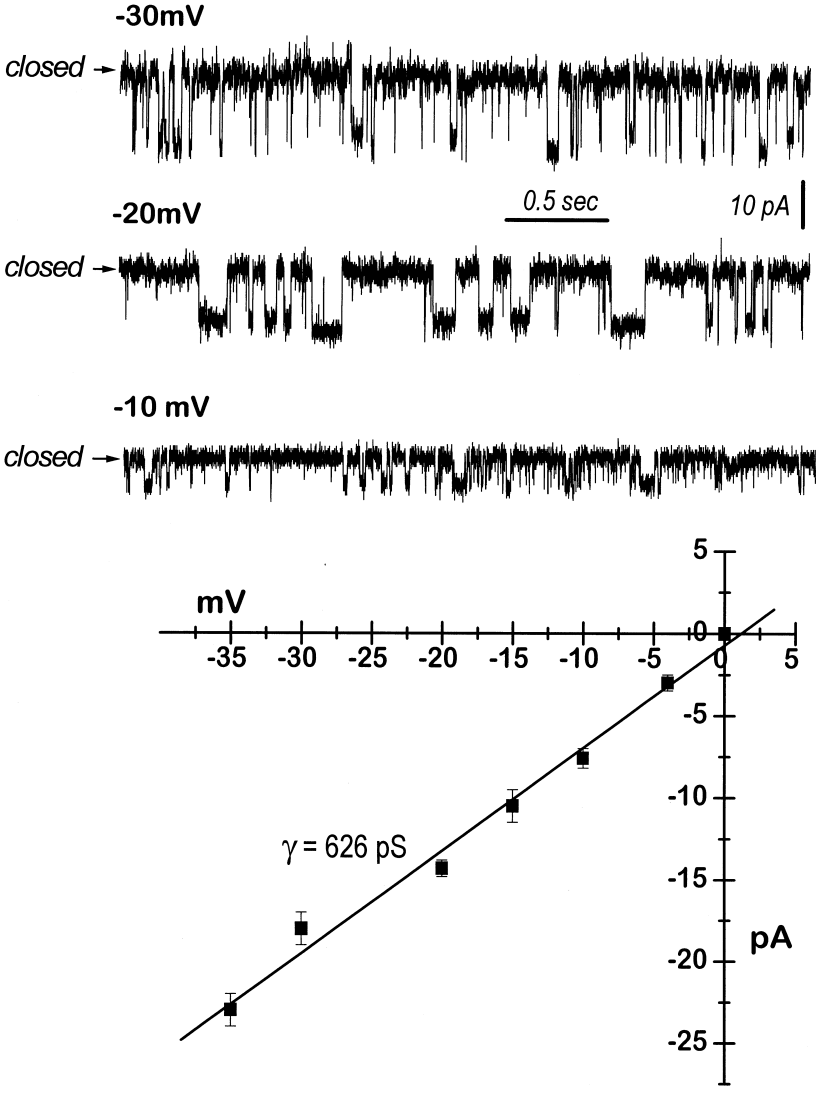

Fig. 1. Detection of single RyR channels isolated from rat DRG and reconstituted in planar lipid bilayers. 40–100 μg of DRG microsomes was added to the *cis* chamber in which the command electrode was positioned. Channel openings were stimulated by nominally free bath Ca^{2+} in our standard recording conditions (symmetrical 300 mM cesium methanesulfonate and 20 mM MOPS, pH 7.2). The holding potential was varied from −40 mV to 0 mV. At these voltages, Cs^+ flows in the physiological direction from the *trans* (luminal) to the *cis* (cytosolic) chamber. Channel openings correspond to downward deflections and each representative trace is 2 s in length.

from all reported values. Mathematical fitting of data was accomplished with Origin software (version 6.0, Microcal Inc., Northampton, MA, USA).

2.4. Planar bilayer technique

DRG RyRs were reconstituted into Muller-Rudin planar lipid bilayers as previously described [14,16]. Bilayers were composed of PE and PS (1:1) dissolved in *n*-decane at a concentration of 25 mg/ml. DRG microsomes (40–100 μg) were added to a 500 μl chamber (*cis* side) which corresponded to the cytosolic face of the channel. Thus the *trans* chamber corresponded to the lumenal face of the channel. The recording solution in the *cis* chamber was 300 mM cesium methanesulfonate and 20 mM MOPS, pH 7.2. The *trans* solution was the same except that cesium methanesulfonate was 50 mM before fusion and 300 mM after fusion. Agar–KCl bridges were used to connect the chambers to Ag–AgCl electrodes and the *cis* voltage command electrode was connected to the headstage of a 200A Axopatch amplifier while the *trans* chamber was held at virtual ground. In this configuration, Cs^+ flows from the lumenal (*trans*) to the cytoplasmic (*cis*) side at negative holding potentials when the Cs^+ concentration gradient has been dissipated. Channel activity in the presence or absence of modulators was recorded at a sampling rate of 5 kHz using a 16-bit VCR-based acquisition and storage system. To analyze the data, the records were played and filtered through an eight-pole low pass Bessel filter set at 1.5 kHz and finally digitized at 4 kHz using a Digidata 1200 AD/DA interface. Data acquisition and analysis were carried out with Axon Instruments (Burlingame, CA, USA) hardware and software (pClamp version 6.0).

2.5. SDS–PAGE and Western blot analysis of RyR

To examine whether the DRG microsomal RyR was immunologically similar to cardiac RyR2, brain RyR3, or skeletal RyR1, microsomal samples were subjected to SDS–PAGE. Separated proteins were either Coomassie blue-stained or transferred to nitrocellulose membranes for Western blot analysis. Blots were probed first with the desired RyR antibodies and then with an appropriate peroxidase-conjugated secondary antibody. Detection and quantification of protein–antibody complexes was carried out using a chemiluminescent detection kit (Pierce, Rockford, IL, USA).

3. Results and discussion

3.1. Reconstitution of single DRG RyR channels in planar lipid bilayers

Microsomes isolated from rat DRGs were reconstituted into planar lipid bilayers and the activity of RyRs was re-

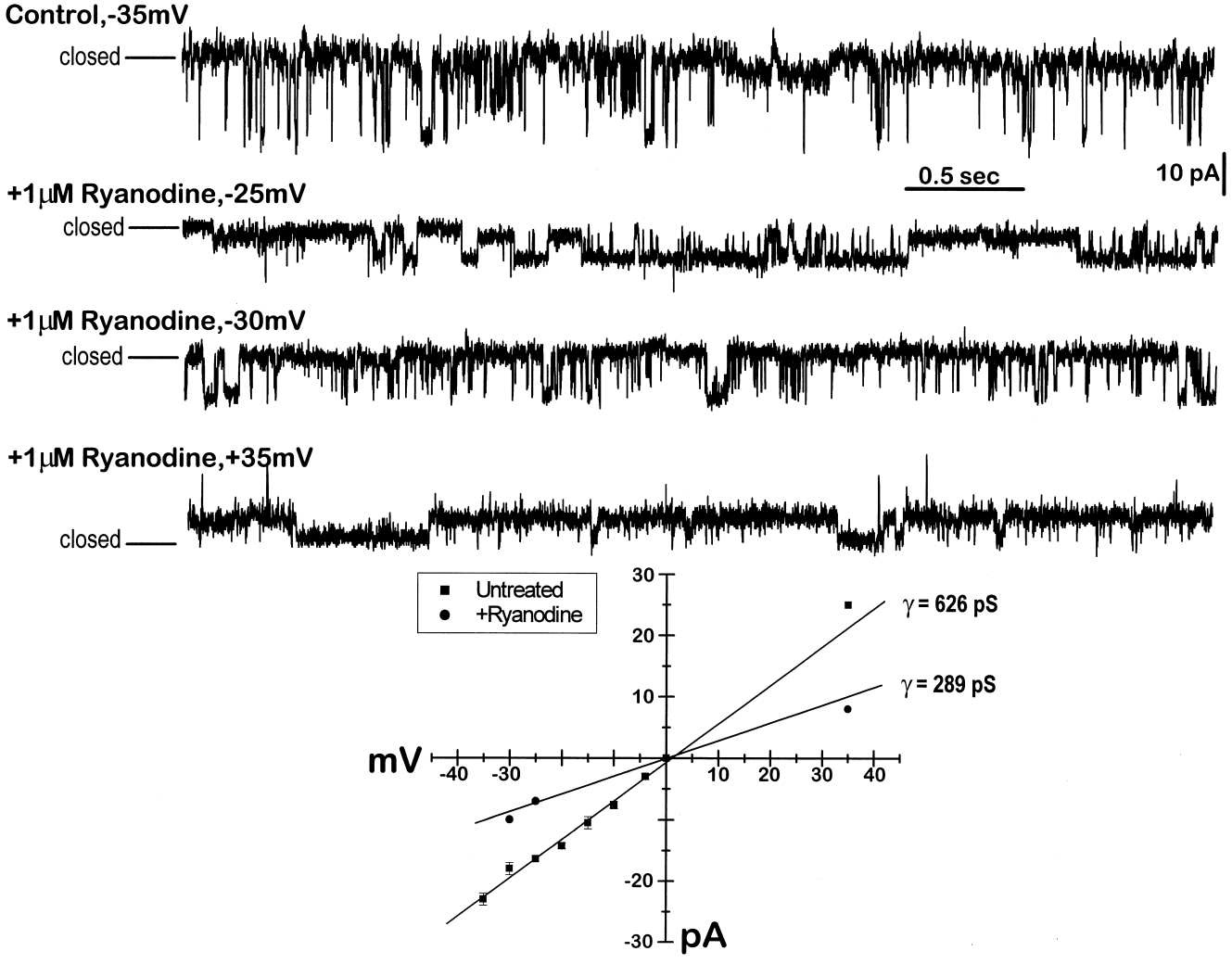

Fig. 2. Effect of ryanodine on RyRs isolated from rat DRG and reconstituted in planar lipid bilayers. 40–100 μg of DRG microsomes was added to the *cis* chamber in which the command electrode was positioned with the same bath solutions as in Fig. 1. Channel openings were stimulated by 10 μM calcium added to the *cis* chamber. Before the addition of ryanodine, the holding potential was again varied to obtain baseline current–voltage relations. 1 μM ryanodine was added to the *cis* chamber from a 100 μM stock. Up to 10 min passed before changes in the RyR gating properties were noted. Each representative trace is 2 s in length and the current–voltage relations before and after the addition of ryanodine are shown in the graph at the bottom.

corded for the first time. We used recording solutions considered standard in studies of cardiac or skeletal RyRs [14,16]. The use of Cs^+ prevents RyR inactivation caused by large Ca^{2+} gradients, increases the signal-to-noise ratio of the observed currents ($G_{Cs^+}/G_{Ca^{2+}} \sim 2$), and blocks interference from K^+ channels present in the SR membranes [17]. Methanesulfonate, on the other hand, blocks Cl^- channels that may also be present in the microsomes. Fig. 1 shows single channel recordings of a fast-flickering, Ca^{2+}-dependent, large conductance cation channel present in this preparation. The slope conductance for Cs^+ was 626 pS, a value typical for other RyRs [12,13]. Furthermore, addition of 1 μM ryanodine to the cytoplasmic face of the channel profoundly modifies both the open channel current amplitude and the mean open time

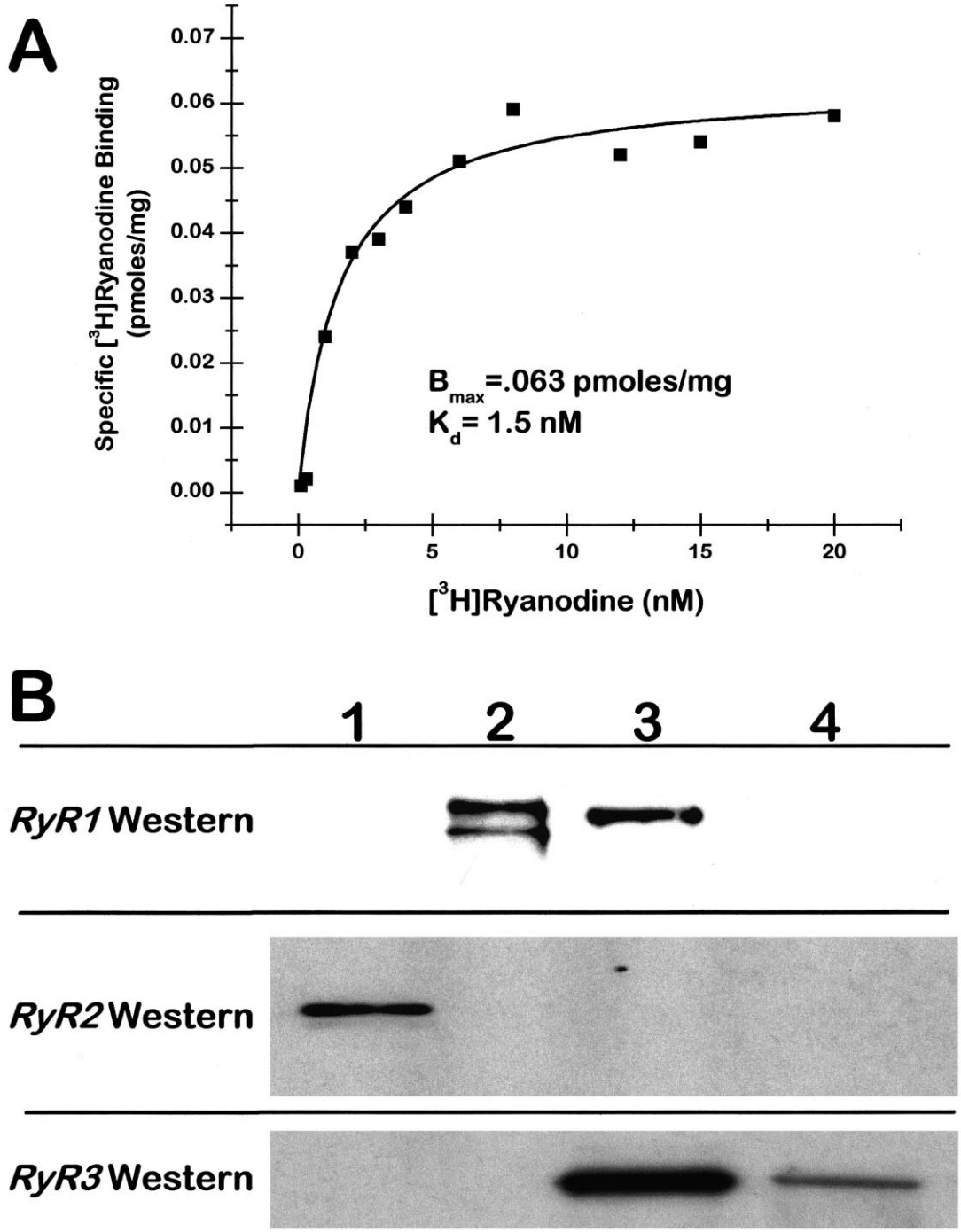

Fig. 3. A: [^{3}H]Ryanodine saturation binding curve. 60 μg of rat DRG microsomes was incubated with the indicated concentration of [^{3}H]ryanodine for 120 min at 36°C in medium containing: 1 M KCl, 30 mM MOPS (pH 7.2), and 30 μM $CaCl_2$. Non-specific binding was determined in the presence of 10 μM unlabeled ryanodine and has been subtracted from each point. The B_{max} and K_d were determined by curve fitting with Microcal Origin software (version 6.0). B: Western blot analysis of DRG microsomes with RyR isoform-specific antibodies. Microsomal proteins were separated by 7% SDS–PAGE, transferred to nitrocellulose, and then probed with RyR antibodies as indicated. Lane 1, 30 μg of pig cardiac microsomes; lane 2, 30 μg of pig skeletal microsomes; lane 3, 30 μg of chicken skeletal microsomes; lane 4, 100 μg of rat DRG microsomes.

(Fig. 2). Ryanodine 'locks' the channel into a long-lived subconductance state that represents ~40% of the full conductance openings (Fig. 2, bottom panel). These current and kinetic modifications are the signature effects of ryanodine on skeletal and cardiac RyRs [18]. Thus, DRG microsomes have a ryanodine-sensitive large conductance cation channel kinetically indistinguishable from cardiac and skeletal RyRs.

3.2. Detection of high affinity [^{3}H]ryanodine binding in DRG microsomes

To determine if high affinity RyRs could be biochemically detected in DRG microsomes, saturation binding curves were carried out and an example result is shown in Fig. 3A. Both the hyperbolic curve fitting in Fig. 3A and the Scatchard linear regression analysis (not shown) revealed low density (B_{max} of 63 and 64 fmol/mg, respectively) and high affinity (K_d of 1.5 and 1.67 nM, respectively) binding sites present in these DRG microsomes. These results, combined with those in Fig. 1, demonstrate that the DRG microsomes are in fact a good medium in which to study the properties of RyRs found within sensory neurons.

Since experimental conditions that decrease or increase channel activity also modify [^{3}H]ryanodine binding in the same manner [12,13], the [^{3}H]ryanodine binding assay may be used as an index of channel activity. In subsequent experiments we used the [^{3}H]ryanodine binding assay to assess the effect of known RyR pharmacological modulators in a large population of receptors. Fig. 4 shows that [^{3}H]ryanodine binding was inhibited 63.9% by 5 μM ruthenium red (20.3 ± 5.3 and 7.3 ± 3.5 fmol/mg, control and ruthenium red-treated, respectively), and inhibited 56.9% by 1 mM tetracaine (8.75 ± 2.4 fmol/mg). Furthermore, 10 mM caffeine was able to increase binding 50% above control even though activating concentrations of Ca^{2+} (10 μM) had already been added to the incubation medium. However, unlike other typical mammalian RyRs, 3 mM Mg^{2+} was distinctly ineffective as a channel inhibitor (15.5 ± 6.6 fmol/mg, not statistically different from control values).

The enhancement of [^{3}H]ryanodine binding by caffeine and the inhibition by ruthenium red and tetracaine are typical characteristics of mammalian RyRs. The lack of inhibition by millimolar concentrations of Mg^{2+} however is not typical for the RyRs so far characterized. Among the RyRs of neuronal tissues, the RyR of sympathetic ganglia is inhibited by Mg^{2+} [19], suggesting that RyRs from sympathetic ganglia and those of DRG are not identical. This insensitivity to 3 mM Mg^{2+} is further intriguing given the physiological relevance that Mg^{2+} inhibition plays in mammalian RyR1, RyR2, and RyR3 [12,13].

3.3. Western blot analysis of RyRs in DRG microsomes

We next screened DRG microsomal proteins with isoform-specific RyR antibodies in Western blot analyses (Fig. 3). We used as positive control samples of pig cardiac SR (RyR2, lane 1), rabbit skeletal SR (RyR1, lane 2), and chicken skeletal SR (RyR1 and RyR3, lane 3). DRG microsomes were loaded in lane 4. Fig. 3A shows that a monoclonal antibody against a cytosolic segment of the rabbit skeletal RyR1 recognizes a ~450 kDa protein in both rabbit skeletal SR (lane 2) and in chicken skeletal microsomes (lane 3). RyR2 from pig cardiac SR does not cross-react with this antibody (lane 1) but does react with a mouse monoclonal RyR2 antibody. Conversely, this RyR2 antibody failed to recognize high molecular weight proteins in all the other samples (Fig. 3B). Finally, a RyR3 polyclonal antibody was the only antibody that reacted with the RyR of DRG microsomes (lane 4). The cross-reactivity of chicken skeletal microsomes with both RyR1 and RyR3 antibodies is expected since the chicken skeletal microsomes contain roughly equal amounts of α and β RyRs, which immunologically correspond to mammalian skeletal RyR1 and mammalian brain RyR3, respectively [20].

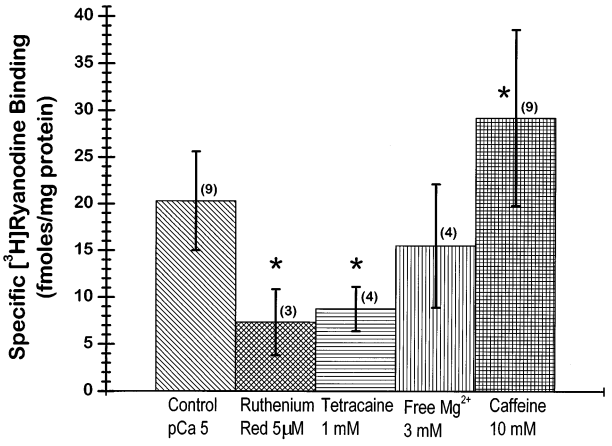

Fig. 4. Pharmacological modulation of [^{3}H]ryanodine binding to rat DRG microsomes. 60 μg of rat DRG microsomes was incubated with 7 nM [^{3}H]ryanodine for 90 min at 36°C in medium containing: 0.5 M KCl, 30 mM MOPS (pH 7.2), 10 μM Ca^{2+} and the indicated reagent. Modulators were added from 10× stocks and the resulting specific binding is shown. Data are expressed as the mean ± S.D. with the number of determinations indicated in parentheses. The significance of the difference between two experimental means was tested by an unpaired *t*-test. $P < 0.05$ was considered significant. * indicates statistical difference ($P < 0.05$) when compared with control. The effect of caffeine had a marginal ($P = 0.05$) significance.

These Western blot results suggest that RyRs of DRGs are immunologically similar to RyR3 and different from RyR1 or RyR2. As noted with RyRs from the microsomes of sympathetic ganglia [19], an extensive characterization of RyRs associated with microsomes of DRGs was hindered by the small amount of microsomes that can be obtained. Furthermore, the microsomes of DRGs had a lower density of RyRs as compared to those of sympathetic ganglia. Still, we were able to measure pharmacological modulation of [^{3}H]ryanodine binding although the results differ from intact DRG studies in which ruthenium red did not negatively impact Ca^{2+} release-induced Cl^- currents [21]. We also did not detect inhibition of DRG RyRs by dantrolene [6] or activation by cyclic ADP ribose [8], contrary to what are reported in studies using intact DRG cells (data not shown).

3.4. Calcium dependence of single channel activity and [^{3}H]ryanodine binding of DRG microsomes

In addition to the well-characterized effect of ryanodine as shown in Fig. 2, RyRs also have a typical Ca^{2+}-dependent activity [12,13]. Fig. 5 shows that RyRs from DRGs reconstituted into planar lipid bilayers also have Ca^{2+}-dependent activation similar to that observed in mammalian RyR1 and RyR2 revealing threshold levels of activity at *p*Ca 7 and maximal activity at *p*Ca 5–4. The results of six experiments in which free Ca^{2+} at the cytosolic face of the channel was progressively altered are depicted in Fig. 5. For this particular

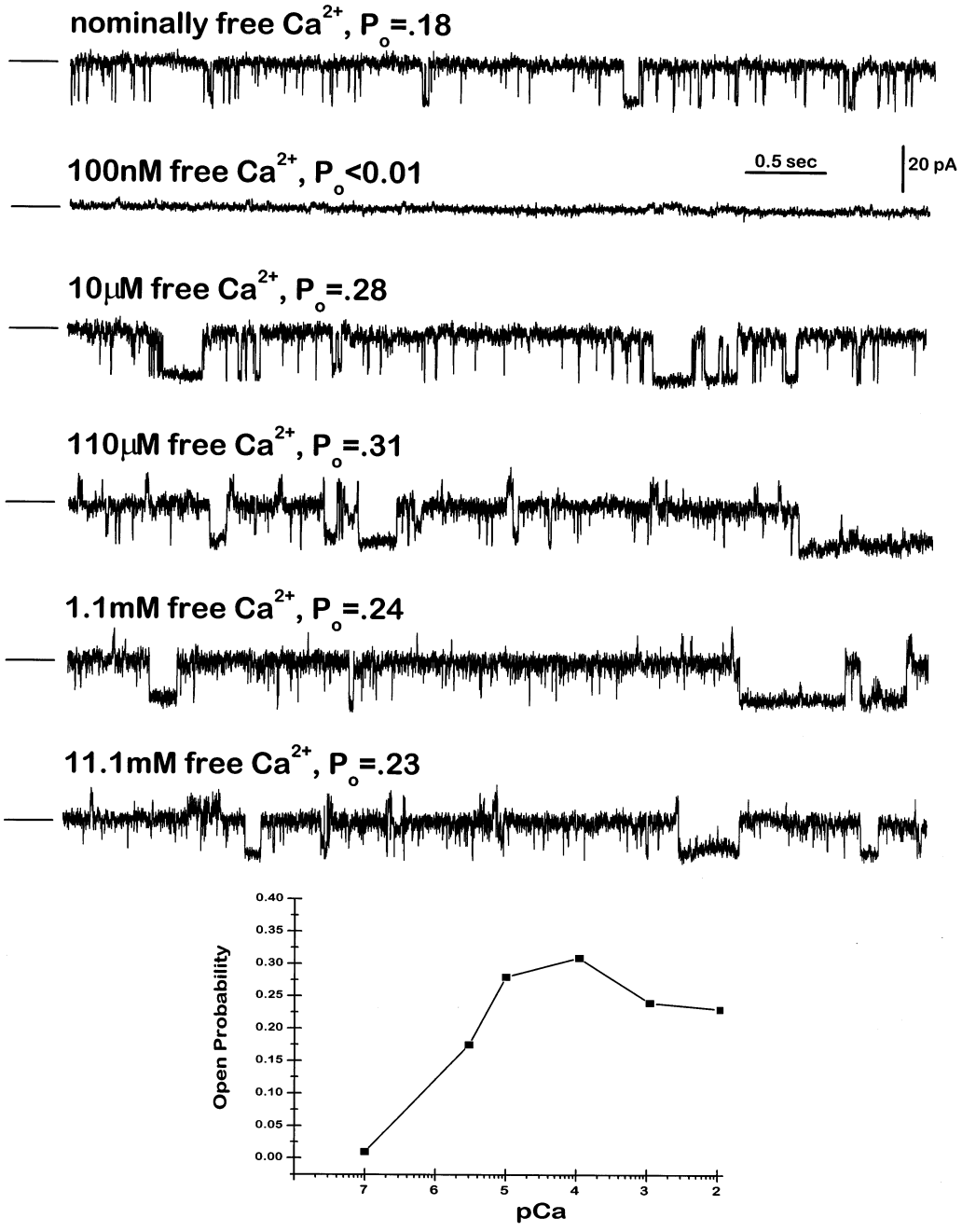

Fig. 5. Single channel activity in planar lipid bilayers in the presence of varying concentrations of cytosolic calcium. As in Fig. 1, 40–100 μg of DRG microsomes was added to the *cis* chamber containing cesium methanesulfonate and the command electrode. After channel incorporation and gradient dissipation, a constant −30 mV was applied, driving Cs^+ through the open channel from the lumen to the cytosol. Channel openings were then modulated by changing the free bath *cis* [Ca^{2+}] by adding predetermined amounts of EGTA and/or calcium chloride thus achieving the indicated final free [Ca^{2+}]. Each representative trace is 2 s in length and the P_o resulting from these maneuvers is graphically shown at the bottom.

channel, we were able to successfully develop the entire open probability (P_o)–*p*Ca relation in the range *p*Ca 7 to *p*Ca 2. Continuous gating was observed for 2–3 min at each Ca^{2+} concentration. When *p*Ca 2 was achieved, channel activity was observed in four out of six attempts. A summary plot of the channel's P_o is shown in the bottom panel. These results are consistent with those of Rousseau and Proteau [22] who reported that RyR3 from canine diaphragm did not inactivate at millimolar Ca^{2+} concentrations. However, Maruyama et al. [23] reported that RyR3 from rabbit diaphragm showed almost full openings at *p*Ca 6–3 and a marked decline in P_o at a [Ca^{2+}] of about 3 mM. Thus once the molecular structure and composition of rat DRG RyRs are found, it may reveal important Ca^{2+} modulatory regions by comparison to the other known RyRs.

Fig. 6 shows a comparison of the effect of Ca^{2+} on the binding of [^{3}H]ryanodine to cardiac, skeletal and DRG microsomes. Because of the low density of RyRs in DRG microsomes, experiments were carried out using 0.5 M KCl in order to increase the signal-to-noise ratio. Increasing [KCl] has been

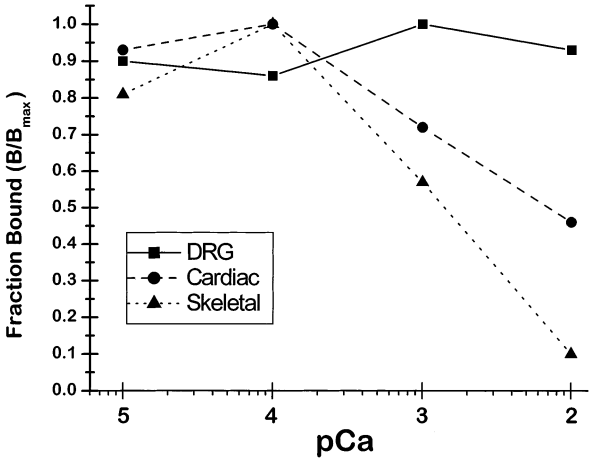

Fig. 6. Calcium-dependent effects on [^{3}H]ryanodine binding to rat DRG microsomes, pig cardiac microsomes, and pig skeletal microsomes. 60 μg of tissue-specific microsomes was incubated with 7 nM [^{3}H]ryanodine for 120 min at 36°C in medium containing: 0.5 M KCl, 30 mM MOPS (pH 7.2), and the concentration of free calcium as indicated (via $CaCl_2$:EGTA mixture). Shown are average specific binding levels from at least three determinations and the solid lines do not represent a curve fitting attempt.

shown to alter the Ca^{2+}-sensitive properties of RyRs, which in our case may explain why skeletal RyRs showed only moderate inactivation at *p*Ca 3 [16]. However, the extent of inactivation is simply right-shifted and stronger inactivation is obvious at *p*Ca 2 for skeletal RyR1, and less pronounced for RyR2, also in agreement with previous observations [16]. Remarkably, DRG RyRs again lack detectable calcium-dependent inactivation. This apparent lack of calcium-dependent inactivation is unique to these DRG RyRs and may reflect a broader insensitivity to divalent cations since a lack of strong inhibition in the presence of 3 mM Mg^{2+} was also observed (Fig. 4). Furthermore, the single channel activity in the presence of high [Ca^{2+}] suggests that in the intact DRG cells, where a large number of channels would be present, CICR may be substantial.

In summary, we have successfully isolated and reconstituted RyRs from rat DRGs and report for the first time their single channel properties. The large single channel conductance for Cs^+ (626 pS) and Ca^{2+}-dependent activation are characteristic of all RyRs. As verified in [^{3}H]ryanodine binding experiments, these channels respond to ryanodine and other pharmacological modulators in a fashion typical of mammalian skeletal RyR1 and cardiac RyR2. However, a notable exception to this trend would be their lack of sensitivity to millimolar Mg^{2+} and Ca^{2+} concentrations, more characteristic of the RyR3 in diaphragm. Along these lines, the rat DRG RyR is immunologically related to RyR3 as shown by Western blot analysis. These data are compelling evidence that rat DRGs contain RyRs with perhaps a unique molecular structure and composition which needs to be cloned and sequenced. Once that is done, the DRG RyR may help to reveal important regions of RyR regulation when compared to other known RyRs.

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