The functional heterogeneity of the ryanodine receptor (RyR) channels in avian cerebellum was defined. Heavy endoplasmic reticulum microsomes had significant levels of ryanodine and inositol 1,4,5-trisphosphate binding. Scatchard analysis and kinetic studies indicated the existence of at least two distinct ryanodine binding sites. Ryanodine binding was calcium-dependent but was not significantly enhanced by caffeine. Incorporation of microsomes into planar lipid bilayers revealed ion channels with pharmacological features (calcium, magnesium, ATP, and caffeine sensitivity) similar to the RyR channels found in mammalian striated muscle. Despite a wide range of unitary conductances (220–500 pico siemens, symmetrical cesium methanesulfonate), ryanodine locked both channels into a characteristic slow gating subconductance state, positively identifying them as RyR channels. Two populations of avian RyR channels were functionally distinguished by single channel calcium sensitivity. One population was defined by a bell-shaped calcium sensitivity analogous to the skeletal muscle RyR isoform (type I). The calcium sensitivity of the second RyR population was sigmoidal and analogous to the cardiac muscle RyR isoform (type II). These data show that there are at least two functionally distinct RyR channel populations in avian cerebellum. This leads to the possibility that these functionally distinct RyR channels are involved in different intracellular calcium signaling pathways.

Calcium release from internal Ca\(^{2+}\) stores is important to intracellular Ca\(^{2+}\) signaling in neurons. Intracellular Ca\(^{2+}\) release is mediated by 1,4,5-trisphosphate (IP\(_3\))- receptor and/or ryanodine receptor (RyR) Ca\(^{2+}\) channels. These specialized Ca\(^{2+}\) channels are ligand-activated and are distinctly distributed throughout the mammalian nervous system (1). Three different mammalian RyR isoforms have been clearly identified. Type I RyR is predominately found in mammalian skeletal muscle (2). Type II RyR is predominately found in mammalian heart (3). The third type of RyR was originally identified in mammalian brain (4). Interestingly, all three RyR isoforms are expressed in various regions of the mammalian brain (5). In nonmammals, the expression of multiple RyR isoforms in the same tissue is quite common. For example, two RyR isoforms have been identified in amphibian (frog) and avian (chicken) skeletal muscles (6, 7). Like the RyRs in mammalian brain, the RyRs in avian brain are expressed differentially throughout the brain but are particularly concentrated in the Purkinje neurons of the cerebellum (8, 9). Avian cerebellum expresses at least two RyR isoforms (\(\alpha\) skeletal and \(\beta\) skeletal and/or cardiac isoforms) (9, 10). The morphological diversity of RyR channels in the avian cerebellum suggests that the different RyR channels may be functionally heterogeneous, as has been suggested to be the case during normal development of avian skeletal muscle (11). In this paper, we tested the hypothesis that functionally heterogeneous RyR exist in avian cerebellum.

We show here that avian cerebellum contains RyR channels that vary widely in unitary conductance and are regulated by Ca\(^{2+}\), Mg\(^{2+}\), ATP, and caffeine. Individual RyR channels were functionally classified into two categories based on their Ca\(^{2+}\) sensitivity. The physiological significance of these two functionally distinct RyR isoforms is unknown. However, such functionally heterogeneous RyR channels may underlie the complex spatiotemporal dynamics of intracellular Ca\(^{2+}\) signaling in brain.

### MATERIALS AND METHODS

Membrane Preparation—Endoplasmic microsomal membranes were prepared from adult chicken cerebellum as described by Ashley (12) with modifications. Tissue from 20–40 animals was cut into small pieces and suspended in 10 volumes of buffer A plus a protease inhibitor mixture. Buffer A contained 5 mM HEPES-KOH, pH 7.4, 0.32 M sucrose, and 1 mM dithiothreitol. The protease mixture contained phenylmethysulfonyl fluoride (1 mM), leupeptin (1 μg/ml), and trypsin inhibitor (10 μg/ml). The tissue was disrupted with a Teflon-glass homogenizer. The suspension was centrifuged at 1,000 × g for 10 min. The pellet was rehomogenized and centrifuged again (1,000 × g, 10 min). Supernatants were combined and centrifuged at 17,000 × g for 50 min. The supernatant of the 17,000 × g spin was then centrifuged at 100,000 × g for 1 h. The resulting microsomal pellet (P3) was resuspended in 2–4 ml of buffer A and layered on top of a discontinuous sucrose gradient (20, 32, and 40% sucrose) and centrifuged overnight. The microsomal subfractions at the 20% interface (P3-1), 20–32% (P3-2), 32–40% interface (P3-3), and 40% (P3-4) were collected and resuspended in buffer A plus the protease mixture (minus leupeptin). Microsomal samples in small aliquots were quickly frozen in liquid nitrogen and then stored at –80 °C until use. The protein concentration was determined by the bicinchoninic acid method (13).

Ryanodine Binding—Membranes (100–200 μg of protein) were incubated for 60–90 min at 37 °C in a buffer containing 1 mM KCl, 100 mM Ca\(^{2+}\), 10 mM Heps-KOH, 1 mM phenylmethylsulfonyl fluoride in a final volume of 0.25 ml (pH 7.4). The [\(^3\)H]ryanodine (76.2 Ci/mmol; DuPont) was added at concentrations ranging from 1 to 200 nM. Nonspecific binding was determined in the presence of 8 μM ryanodine. The reaction was stopped by filtration on Whatman GF/B filters with two washes with 4 ml of ice-cold 1 mM KCl with 10 mM Heps-KOH. Radio-
activity was quantified by liquid scintillation. Binding assays were performed in duplicate.

Planar Lipid Bilayer Experiments—Single Ca\(^{2+}\) release channels studied by incorporating microsomes from the P3–3 fraction into planar lipid bilayers (14, 15). Planar phospholipid bilayers of the Mueller-Rudin type were formed from a mixture of 1-palmitoyl 2-oleoyl phosphatidyethanolamine, phosphatidylserine, and phosphatidylcholine (Avanti Polar Lipids Inc., Birmingham, AL) in the ratio 5:3:2 dissolved in decane at 50 mg/ml. Microsomes were added to one side of the bilayer called the cis chamber that corresponds to the cytosolic channel side. Microsome fusion was promoted by establishing a cesium methanesulfonate gradient across the bilayer (400 mM cis versus 25 mM trans) (16). Experimental solutions also contained 20 mM HEPES-Tris, 1–10 μM free Ca\(^{2+}\), pH 7.4. After incorporation of a channel, the trans side was grounded and concentrated cesium methanesulfonate (4 mM) was added to so that the cesium methanesulfonate concentrations on each side of the bilayer were equal. Pharmacological agents were applied to the cis side. Where appropriate, the Ca\(^{2+}\) concentration was adjusted by EGTA and N-hydroxyethyl-ethylenediamine-triacetic acid buffers using the Catlig solution mixing program. The Ca\(^{2+}\) concentrations were confirmed by a calcium electrode.

Single channel currents were digitally recorded on video cassettes using a modified pulse code modulation audio processor (Sansui PC-X11, 14 bits, DC to 14 kHz) after a low pass filtering stage (1 or 2 kHz; 8 pole Bessel, Frequency Devices Co. Haverhill, MA). The mean open probability (P\(_o\)) and channel amplitudes were calculated from records during 3–5 min using the pClamp program (Axon Instruments, Foster City, CA). The Ca\(^{2+}\) dependence data were fit using the following equation (17),

\[
P_o = P_{\text{max}} / (1 + (K_1)^n \cdot [\text{Ca}^{2+}]) + (\text{Ca}^{2+} \cdot K_2)]
\] (Eq. 1)

where P\(_{\text{max}}\) corresponds to P\(_o\) value at maximal activation by Ca\(^{2+}\); K\(_1\) is the Ca\(^{2+}\) concentration at which half-maximal activation occurs, K\(_2\) is the Ca\(^{2+}\) concentration at which half-maximal block occurs, and n is the Hill coefficient.

RESULTS

Ryanodine and IP\(_3\) Binding to Endoplasmic Microsomes from Avian Cerebellum—Ryanodine and IP\(_3\) binding in the endoplasmic reticulum microsome fraction (P3) was defined. Microsomes in the P3 fraction were subfractionated (P3–1, P3–2, P3–3, and P3–4) on a sucrose gradient. The ryanodine and IP\(_3\) binding in specific fractions is shown in Fig. 1A. Each microsomal fraction contained both \[^{3}H\]IP\(_3\) and \[^{3}H\]ryanodine binding sites. The P3–3 subfraction (P3–3) was richer in both compared with the other microsomal fractions. Compared with binding in the P3 fraction, the P3–3 subfraction was 220% richer in ryandine binding and 233% richer in IP\(_3\) binding. The co-migration of ryanodine and IP\(_3\) binding suggests that the RyR and IP\(_3\)R proteins cannot be easily separated using standard biochemical techniques.

Binding isotherms and Scatchard analysis of the ryanodine binding in subfraction P3–3 are shown in Fig. 1B. The P3–3 subfraction (Fig. 1B) showed two classes of \[^{3}H\]ryanodine binding sites (K\(_D\) = 1.39 ± 0.97 nM (n = 4) and 133 ± 65.8 nM (n = 3); B\(_{\text{max}}\) = 0.36 ± 0.14 (n = 4) and 1.45 ± 0.11 (n = 3) pmol/mg, respectively). Repeated observations were done in duplicate, and each duplicate determination was done on different fresh membrane preparations.

Ryanodine Binding Sites—The high affinity binding site was comparable with that reported in endoplasmic microsomes from mammalian and avian brain (18–21, 40). This is the first report on the properties of a low affinity ryanodine binding site in brain tissues. Low affinity sites have been previously described for RyRs of mammalian skeletal and cardiac muscle (22, 23) and amphibian skeletal muscle (15). To confirm the presence of low affinity binding sites, displacement and disso-
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![Diagram](http://example.com/diagram.png)

**FIG. 2.** A, displacement experiment demonstrating biphasic nature of ryanodine binding. Inset, Scatchard transformation of the data. [3H]Ryanodine binding was performed in the presence of 4 nM [3H]ryanodine and increasing concentrations of cold ryanodine. B, dissociation kinetics of 4 nM [3H]ryanodine binding. The dissociation was started by the addition of a 10-fold excess of incubation medium in the presence (open circles) or the absence (closed circles) of 10 μM of cold ryanodine. Each point is the average of two determinations done in duplicate.

Modulation studies in the presence of an excess of cold ryanodine were performed. As shown in Fig. 2A, addition of increasing concentrations of cold ryanodine displaced [3H]Ryanodine in a biphasic fashion (three separate experiments with three different membrane preparations). Scatchard plots (Fig. 2A, inset) could only be fitted assuming the presence of a second low affinity site. The high affinity constant was 2.0 ± 0.74 nM (n = 3). The low affinity constant was more than 115 nM for the example shown in Fig. 2A and higher than 1 μM for the two other experiments.

Dissociation kinetics were done in the presence of an excess of incubation medium with or without 10 μM cold ryanodine (two experiments with two different membrane preparations). As shown in Fig. 2B, the dissociation kinetics in the presence of an excess of cold Ry was biphasic without complete dissociation (open circles). The mean dissociation times were 1.1 min and much longer than 120 min, respectively. In the absence of cold Ry (closed circles) dissociation kinetics was monophasic with a mean dissociation time of 35.2 min. These dissociation data are consistent with previous findings in muscle RyR studies (22) and strongly suggest allosteric interaction among sites of different affinities. These binding data also suggest that the presence of low affinity binding sites is a very conserved intrinsic property of ryanodine receptors.

Modulation of Ry Binding by Calcium and Caffeine—The Ca2+ dependence of ryanodine binding in the P3-3 and P3-2 subfractions was determined at low [3H]ryanodine concentration (2 nM) and in the absence of other channel agonists (Fig. 3). The Ca2+ dependence was sigmoidal with apparent affinity constants of 1.08 μM (P3-3; n = 4). When normalized to the maximum binding, the Ca2+ dependence in both fractions was nearly identical (Fig. 3B). Similar Ca2+ activation constants (1.6 μM) were obtained by Murayama and Ogawa (24) for the purified RyR (α isoform) from bullfrog and by Zimanyi and Pessah (40) for the rat brain RyR. Our ryanodine binding results indicate that avian cerebellum contains Ca2+-sensitive ryanodine binding sites. The use of 2 nM concentration of Ry in these experiments implies that the observed calcium dependence is associated to a large extent with the high affinity Ry binding site.

Modulation of Ry binding by caffeine (0–10 mM) was studied at pCa 8 and 5 (four experiments with four separate membrane preparations). As shown in Fig. 4, a small but not significant enhancement of Ry binding was observed only at pCa 8 in the presence of 1 mM caffeine. To confirm these findings, parallel experiments performed with rabbit skeletal muscle RyR in the presence of the same solutions, showed significant increments of Ry binding that depended strongly on the caffeine and calcium concentrations (not shown). These results indicate that under the conditions of these experiments (high ionic strength) little if any further enhancement of Ry binding is obtained in the presence of caffeine above that obtained with calcium.

Functional Characterization of Single RyR Channels—The function of individual RyR channels from avian cerebellum was investigated by fusing RyR-enriched microsomes (P3-3) into planar lipid bilayers. Single channel currents were monitored using Cs+ as a charge carrier. The channels examined were selective for divalent ions (Ca2+/Mg2+ ratio = 3.3) as were RyR channels in avian skeletal muscle (ratio 3.8; Ref. 25). However, using Cs+ as charge carrier significantly increased the signal-to-noise ratio because Cs+ conductance through the RyR is quite large and allowed precise control of calcium concentrations. The Cs+ conductance through the avian RyR channels ranged from 180 to 530 pS (Fig. 5A). A wide range of conductances was also reported for fish RyR channels (26). The avian RyR channels could not be clearly divided into groups with distinct conductances. The small conductance channels (180–250 pS) were noisy and observed infrequently. The larger conductance RyR channels (250–550 pS) were frequently observed. The large variability in conductance may suggest that the microsomes contained a mixture of RyR and non-RyR channels that conduct Cs+. However, all the Cs+ conducting channels tested were ryanodine-sensitive. The action of ryanodine on channels of different conductances is illustrated in Fig. 5 (B and C). Ryanodine locked both channels into the characteristic slow gating subconductance state (27, 28). Thus, it appears that avian cerebellum contains RyR channels, which vary significantly in their ion conduction properties.

All the RyR channels tested were sensitive to Ca2+, Mg2+, ATP, and caffeine. The pharmacological sensitivity of a representative RyR channel is illustrated in Fig. 6. In the absence of other ligands, single channel activity at pCa 6.5 for this particular channel was quite low. The addition of ATP (1 mM, pCa 6.5) increased channel activity. Application of caffeine and additional Ca2+ (pCa 4.5) activated the channel further. The addition of 1 mM Mg2+ to the active channel (2 mM caffeine, 1 mM ATP, pCa 4.5) reduced single channel activity dramatically. This pharmacological profile of the channels is similar to that reported for RyR channels in avian, mammalian, and amphibian striated muscles (15, 16, 25).

The general pharmacological profile illustrated in Fig. 6 was representative of all channels tested, and all exhibited Ca2+, Mg2+, ATP, and caffeine sensitivity.
Fig. 3. A, calcium dependence of [3H]ryanodine binding to the RyRs present in subfraction P3–3 (open circles) and P3–2 (filled circles). Absolute total [3H]ryanodine binding expressed as fmol/mg. The values are given as the means ± S.E. from five different experiments in five different preparations. Membranes were incubated in the presence of 2 nM [3H]ryanodine and at the different Ca2+ concentrations shown. B, the same data sets normalized to maximal binding.

Mg2+, ATP and caffeine sensitivity. Differences in single channel conductance and Ca2+ sensitivity between channels were clear and are described in detail in this paper. Differences between Mg2+, ATP, and caffeine sensitivity were not discernable and thus were not used to classify channels.

Ca2+ Dependence of Avian RyR Channels from Cerebellum—The RyR channels are key effectors for intracellular Ca2+ signaling in many cell types (29, 30) where it is thought that their gating is precisely regulated by small changes in the intracellular Ca2+ concentration. Because Ca2+ is key to RyR regulation, the Ca2+ dependence of individual RyR channels was defined in the planar bilayers one channel at a time. The Ca2+ sensitivity of individual RyR channels followed one of two patterns that are illustrated in Fig. 7 and 8.

Some RyR channels were very active at relatively low Ca2+ concentrations (pCa 6.3; Fig. 7), whereas others were not (Fig. 8). Channels that were active at pCa 6.3 were classified as high affinity channels. Channels that were not active at pCa 6.3 were classified as low affinity channels. The open probability (Pₒ) of the high affinity channels significantly decreased as Ca2+ was elevated to pCa 4 (Fig. 7). The Pₒ of low affinity channels did not significantly decrease as Ca2+ was elevated to pCa 4 (Fig. 8). Thus, the two Ca2+ sensitivity patterns were distinguishable at both low (pCa 6.3) and high Ca2+ concentrations (pCa 4).

The two Ca2+ sensitivity patterns are compared in Fig. 9. The data points (mean ± S.E.) were fit by curves generated by Equation 1 assuming the existence of two Ca2+ binding sites on the RyR channel. The Pₒ was normalized to mean Pₒ at pCa 5. The Ca2+ dependence of the high affinity channel was bell-shaped, similar to that described for the mammalian type I RyR (31). The high affinity channels activated with an apparent Kₒ of 0.9 μM and were blocked by Ca2+ with an apparent Kᵦ of 78.5 μM.

The low affinity channels had a sigmoidal Ca2+ dependence over the same Ca2+ concentration range. The sigmoidal Ca2+ dependence was similar to that described for the mammalian type II RyR (32). The low affinity channels were activated with an apparent Kₒ of 3.7 μM. Peak activation of both high and low affinity channels occurred near pCa 5. The apparent affinity constant for the Ca2+ dependence of ryanodine binding was approximately 1 μM (in between the Ca2+ sensitivities of the two RyR classes; 0.9 and 3.7 μM). Thus, it would not be unreasonable to conclude that the binding data reflect the average Ca2+ sensitivity in a large RyR population that contains at least two populations of RyR channels with different Ca2+ sensitivities for activation.

DISCUSSION

In this study the possibility that avian RyR channels from cerebellum were functionally heterogeneous was tested. The Ca2+ sensitivity of the avian RyR was examined in an enriched microsomal fraction using two experimental approaches. First, the average Ca2+ sensitivity of large RyR channel populations was defined in ryanodine binding assays. Second, the Ca2+ sensitivity of individual RyR channels was defined by incorporating single RyR channels into planar lipid bilayers.

The Ca2+ sensitivity of ryanodine binding in that fraction did not reveal any heterogeneity. However, individual RyR channels in bilayers showed significant heterogeneity in Ca2+ sensitivity. Two patterns of Ca2+ sensitivity were defined at the single channel level. One class of RyR channels was characterized by a bell-shaped Ca2+ dependence with channel activation occurring at relatively low Ca2+ concentrations (Kₒ = 0.9 μM). The other class of RyR channels was characterized by a sigmoidal Ca2+ dependence with channel activation occurring at higher Ca2+ concentrations (Kₒ = 3.7 μM). Thus, the data show that RyR channels in the avian cerebellum are heterogenous with respect to Ca2+ sensitivity.

Ryanodine Binding Studies—The ryanodine binding data show for the first time that avian cerebellum endoplasmic reticulum contains ryanodine receptors with high and low affinity ryanodine binding sites. Displacement and dissociation...
kinetic studies confirmed a strong allosteric interaction among sites of different affinities. Similar results have been reported in ryanodine binding studies on amphibian and mammalian skeletal muscle sarcoplasmic reticulum (15, 33). The functional manifestation and/or significance of multiple classes of ryanodine binding sites has not been clearly defined. It has been suggested that the multiple binding sites may correlate to the complex action of ryanodine on the channel. Some authors have correlated the high affinity binding site with the characteristic subconductance state induced by low ryanodine concentration (22, 33–35) and the low affinity binding sites with the closed state obtained at high ryanodine concentrations (>50 μM).

The Ca²⁺ dependence of ryanodine binding was first defined here using Ca²⁺ as the sole agonist (i.e., no caffeine or ATP). The apparent association constant for ryanodine binding in the avian cerebellum endoplasmic reticulum was 11 μM. Other ryanodine binding studies, performed under different experimental conditions, had revealed RyRs with different Ca²⁺ sensitivities (24, 36). The Ca²⁺ sensitivity of ryanodine binding in mammalian cardiac and skeletal muscle sarcoplasmic reticulum is $K_{0.5}$ = 29 and 52 μM, respectively (37). Amphibian skeletal muscle contains two RyR isoforms (α and β) (24) with relatively high Ca²⁺ sensitivity. The purified bullfrog β RyR isoform is about 20 times more Ca²⁺-sensitive ($K_{0.5}$ = 0.08 μM) than the α RyR isoform (1.6 μM) in the presence of caffeine (10 mM) and AMPOPCP (1 mM) (24). In mammalian and nonmam-
Fig. 8. Representative single channel records and the Ca$^{2+}$ sensitivity of a RyR channel. This channel represents the class of RyR channels characterized by low Ca$^{2+}$ affinity and a sigmoidal Ca$^{2+}$ sensitivity. The $P_o$ was calculated from 4 min of recordings at each Ca$^{2+}$ concentration (indicated). Open events are shown as upward current deflections. Curve fit was performed using Equation 1. The best least square fitting as obtained with the parameters $K_o$, $K_w$, and $n_0$ indicated on plot.

Fig. 9. Two patterns of RyR channel Ca$^{2+}$ sensitivity. Open circles represent the Ca$^{2+}$ sensitivity pattern illustrated in Fig. 7. Filled circles represent the Ca$^{2+}$ sensitivity pattern illustrated in Fig. 8. The $P_o$ values were normalized to the maximum value of $P_o$ in each individual experiment and then averaged. The data points are the means ± S.D. from three (filled circles) and four (open circles) channels, respectively. Curve fit was performed using Equation 1. The fitting parameters were $K_o = 0.86 \mu M$, $K_w = 78.5 \mu M$, and $n_0 = 1.3$ for the open circles. The fitting parameters were $K_o = 3.7 \mu M$ and $n_0 = 2.2$ for the filled circles.

The study of caffeine modulation on Ry binding showed a small but not significant enhancement of Ry binding at suboptimal calcium concentrations (pCa 8) and in the presence of 1 mM caffeine. In contrast, parallel experiments performed with rabbit skeletal muscle RyR showed significant increments of Ry binding that depended strongly on the caffeine and calcium concentrations (not shown). The lack of caffeine effect on binding at optimal calcium concentration is not new for brain receptors. Padua et al. (39) have shown that ligand modulation of binding at optimal calcium concentration in brain can only be resolved at relatively low ionic strengths. The low level of ryanodine binding at such low ionic strengths in our preparation made such experiments impractical. In order to decrease the saturation of the binding we did experiments at suboptimal calcium concentration. However, our results suggest that at suboptimal calcium, caffeine did not enhance the binding significantly.

Several studies have attempted to predict steady state single channel activity using measurements of $[^3H]$Ry equilibrium binding. The clearly different Hill numbers for the Ca$^{2+}$ dependence of binding and single channel open probability and the apparently different results on caffeine effects on binding and on single channel activation indicate that the assumed correlation between binding and $P_o$ is not as simple as expected.

The different Hill values for binding and $P_o$ would not be intuitively predicted. However, the reported values appear genuine and are not likely due to experimental error. The differences could be due to any number of factors. Among them, the proportion of different RyR channel types in the preparation and the bilayer fusion probability of particular RyR channel types is unknown, and environmental (vesicle versus bilayer) or ionic conditions (high versus low ionic strength) may impact function and/or binding. In addition, unavoidable single channel filtering constraints mean that very brief channel openings (less than about 0.7 ms) cannot be resolved resulting in an overestimate of single channel open and closed durations. Also, all ryanodine binding proteins in the vesicle preparation may not be functional channels. In addition, binding studies were performed in the presence of 2 nM $[^3H]$ryanodine, conditions after which mostly the high affinity Ry binding sites are revealed. Thus, it is not surprising that the Hill numbers resulting from fundamentally different methodologies are not equal.

Heterogeneity in Single Channel Conductance—In mammalian and avian striated muscles and mammalian brain, single RyR channel conductance is typically clustered around a single value (25). In saturating concentrations of charge carrier, the mammalian type I, type II, and brain RyR channels have Ca$^{2+}$ conductances of 145, 148, and 140 pS, respectively. If $K^+$ is the charge carrier, then the conductances are 770, 745, and 800 pS, respectively (18).

The conductance of single RyR channels from avian cerebellum endoplasmic reticulum was not clearly clustered around a single value. Although the conductance of any particular channel was constant, conductance varied widely from channel to channel (180–530 pS). In fish skeletal muscle, there are two populations of RyR channels distinguishable by conductance and Ca$^{2+}$ sensitivity (26). Here, avian RyR channels were not divided by conductance because it was impossible to assign clearly defined groups. There was also no apparent correlation between conductance and Ca$^{2+}$ or Ry sensitivity. The heterogeneity of RyR conductances found here could reflect subtle but important structural differences in the RyR permeation pathways. Alternatively, the differences in conductance could reflect some form of regulation of permeation by some extrinsic unidentified factor (e.g., a closely associated regulatory protein).

Heterogeneity of RyR Channel Ca$^{2+}$ Sensitivity—Single RyR channels from mammalian (31), amphibian (17), and fish (26) display two patterns of Ca$^{2+}$ sensitivity, bell-shaped and sigmoidal. The type I RyR channels in mammalian skeletal muscle have a bell-shaped Ca$^{2+}$ sensitivity (31). The type II RyR channels found in mammalian cardiac muscle have a sigmoidal Ca$^{2+}$ sensitivity over the same concentration range (31). These two patterns emerged here in the same preparation. The single channel data show that one population of RyR channels had a bell-shaped Ca$^{2+}$ dependence, whereas another RyR population had a sigmoidal Ca$^{2+}$ dependence. Thus, the Ca$^{2+}$ sensitivity of the first avian brain RyR population may be analogous to that of the type I mammalian RyR channel. The Ca$^{2+}$ sen-
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Significance—The complex spatiotemporal nature of intracellular Ca\(^{2+}\) signaling in cerebellum may be correlated to a rich diversity of intracellular Ca\(^{2+}\) release channels. However, defining the function of channel types in brain has been quite difficult. This study provides the first experimental evidence that functionaly different RyR channels exist in avian cerebellum. The two RyR populations have different patterns of Ca\(^{2+}\) sensitivity. This means that these channels will respond differently to a particular Ca\(^{2+}\) signal. For example, the inactivating channel has the activation and inactivation properties adequate for them to participate in the generation of calcium waves activated by calcium induced calcium release mechanisms. These types of channels will endow the neurons with the ability to signal calcium changes far away from their point of origin. Such diversity of calcium release pathways are fundamental to sustain the complex spatiotemporal nature of Ca\(^{2+}\) signaling in brain.

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