Ryanodine Receptor Type III (Ry3R) Identification In Mouse Parotid Acini

PROPERTIES AND MODULATION OF [%H]RYANODINE-BINDING SITES

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Immunoblot analysis and [%H]ryanodine binding were used to characterize and identify ryanodine receptors (RyRs) in nonexcitable mouse parotid acini. Western analysis revealed ryanodine receptor type III (Ry3R) to be the only detectable isoform in parotid microsomal membranes. Binding of [%H]ryanodine to microsomal fractions was dependent on Ca$^{2+}$, salt, pH, and temperature. At 23 °C, and in the presence of 0.5 M KCl and 100 mM Mg$^{2+}$, [%H]ryanodine bound specifically to membranes with high affinity (K$_a$ = 6 mM); maximum binding capacity (B$_{max}$) was 275 fmol/mg protein. Mg$^{2+}$ and ruthenium red inhibited [%H]ryanodine binding (IC$_{50}$ = 1.4 mM and 0.5 μM, respectively). 4-Chloro-3-ethylphenol enhanced the binding of [%H]ryanodine 2.5-fold; whereas ATP and caffeine were much less efficacious toward activating RyR (56% and 18% maximal enhancement, respectively). Bastadin, a novel modulator of the 12-kDa FK506 binding protein-RyR complex, increased [%H]ryanodine binding 3-4-fold by enhancing K$_a$. The immunosuppressant FK506 enhanced [%H]ryanodine receptor occupancy at >100 μM and antagonized the action of bastadin, suggesting that an immunophilin modulates RyR in parotid acini. These results suggest that Ry3R may play an important role in Ca$^{2+}$ homeostasis in mouse parotid acini.

Increases in cellular Ca$^{2+}$ occur in response to agonist stimulation in many cell types and are important in regulating a number of cellular functions. Two distinct classes of channels that mediate the release of Ca$^{2+}$ from intracellular stores have been identified: one is sensitive to the second messenger inositol 1,4,5-trisphosphate and the second is sensitive to ryanodine (1). Ryanodine receptors (RyRs) were first identified in skeletal and cardiac muscle junctional sarcoplasmic reticulum (SR) using radiolabeled ryanodine binding analysis (2, 3) where they function as Ca$^{2+}$ release channels during excitation-contraction coupling (4, 5). More recently, the availability of antibodies/antisera selective for the skeletal (Ry1R), cardiac (Ry2R), and brain (Ry3R) isoforms, in conjunction with molecular approaches, have clearly demonstrated expression of RyRs in a variety of nonmuscle tissues (6–8). In addition to expression in skeletal muscle, RyR has been shown to be expressed at low levels in cerebral cortex and hippocampus and exhibits especially high abundance in cerebellar Purkinje cells (9). Ry3R is expressed in cardiac tissue and throughout most of the brain, with relatively high levels of expression in the olfactory nerve layer, layer VI of the cerebral cortex, the dentate gyrus, cerebellar granule cells, the motor trigeminal nucleus, and the facial nucleus. The recently identified Ry3R isoform appears to be the major isoform in smooth muscle (10–12) and has also been found to be expressed in several nonexcitable cells, including HeLa and LLC-PKI cells, mammalian cells, and submandibular cells (7, 8). Interestingly, Ry3R was recently found to be generally expressed in mammalian brain at very low levels, raising questions about its precise function in Ca$^{2+}$ signaling (6).

 [%H]Ryanodine binding to receptors isolated from muscle SR and brain microsomes indicates that the RyRs are modulated by a number of physiologic and pharmacologic agents. Activators include micromolar Ca$^{2+}$, millimolar calcium and adenosine nucleotides, and nanomolar ryanodine. Inhibitors include micromolar ryanodine, nanomolar ruthenium red, and millimolar Mg$^{2+}$ or Ca$^{2+}$ (5). In addition, the immunosuppressive agents FK506 and rapamycin, which bind to immunophilins such as FKBP-12 (12-kDa FK506 binding protein) and inhibit the immunophilin response, have been implicated in the regulation of gating properties of the RyR in skeletal muscle (13–15), cardiac muscle (16), and neurons (17). Associated with the Ry1R, FKBP-12 has been shown to stabilize the closed conformation of the Ca$^{2+}$ release channel. FK506 was found to promote dissociation of FKBP-12 from the RyR complex (14) and to alter SR Ca$^{2+}$ transport and single channel gating behavior (15). The ability of FKBP to dissociate FKBP-12 from SR membranes was shown to be enhanced by macromolecular bactadins isolated from the marine sponge *Ianthella basta* (18). Like FK506, bastadins also appear to target FKBP-12. Unlike FK506, bastadins themselves do not dissociate FKBP-12 from the SR calcium release channel complex but instead facilitate FK506-induced dissociation (18).

Although RyRs have been well characterized in skeletal and heart muscle, less is known about RyRs in nonexcitable cells. The inability to observe caffeine-induced Ca$^{2+}$ release from intracellular stores has been misinterpreted to mean that RyRs
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are absent from some cell types. Results with caffeine are especially confusing since cells known to express the RyR isoform fail to respond to caffeine (10, 19), and various laboratories have reported differences in caffeine sensitivity in the same cell type (19, 20). Much of the evidence supporting the presence of RyRs in nonexcitable cells, including exocrine cells, has been based on functional studies (7, 21–23) with little information concerning expression of RyR protein (7, 11, 24). Furthermore, few studies have either demonstrated or characterized specificryanodine binding in microsomal fractions from nonexcitable cells (7, 25–27).

The focus of the present study is to identify the expression of RyR protein(s) in nonexcitable mouse parotid acini and to characterize its ability to bind [3H]ryanodine. Western blot analysis of microsomes isolated from primary acini reveals the expression of RyR protein without detectable levels of RyR or RyR protein. The data presented show that high-affinity binding of [3H]ryanodine to RyR from parotid acini is modulated in a similar fashion to RyR and RyR from muscle SR with important differences with respect to modulation by caffeine and adenine nucleotides.

### EXPERIMENTAL PROCEDURES

**Materials**—Reagents were obtained as follows. Benzamidine and 4-chloro-3-ethylphenol (4-CEP) were from Aldrich; purified sucrase, Silver Stain Plus, and dithiothreitol (DTT) were from Bio-Rad; caffeine, EDTA, EGTA, HEPES, MOPS, hyaluronidase, pepstatin A, phenylmethylsulfonyl fluoride (PMSF), leupeptin, adenosine 5'-[γ-3H]triphosphate (AMP-PCP), TRIS, horseradish peroxidase-conjugated goat anti-mouse secondary antibody, and ruthenium red were from Sigma; collagenase was from Worthington; horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody and from Amersham Corp.; CHAPS and chelatimunescence detection system were from Pierce; EDTA and ATP were from Boehringer Mannheim; radioiodinated ryanodine [9,21-3H] (68.4–84.0 Ci/mmol) was from DuPont NEN; purified ryanodine was from Calbiochem (La Jolla, CA) and Wako (Richmond, VA); universol scintillant was from ICN (Costa Mesa, CA); PFK06 was from Signal Transduction International (San Diego, CA); polyvinylidene difluoride (Immobilon-P) membranes were from Millipore (Bedford, MA); and bastardin (mixture) was from Calbiochem (San Diego, CA). Additional reagents of calcium cations were calibrated at mM concentration using atomic adsorption spectrometry. All other reagents were of analytical grade or higher. RyR antibody (34 °C) was a generous gift of Dr. J. L. Sutko; RyR antibody (C3–33) was a generous gift of Dr. G. Meissner; RyR antibodies were generous gifts of Dr. G. Meissner and Dr. V. Sorrentino.

**Acinar Cell and Tissue Preparation**—Acinar cells were isolated as described by Barmatz and Zarka (34). Protein was determined by the Hartree (35).

### Microsomal Membrane Preparation—

Microsomal membranes were prepared in homogenate medium without CHAPS according to Damiani and Margreth (29). EGTA and EDTA, fraction of skeletal muscle was prepared in homogenate medium without CHAPS according to Damiani and Margreth (29). EGTA and EDTA, each at 2 mM concentration, were substituted for 1 mM EDTA in homogenization buffer for the preparation of microsomes in some experiments. SR membranes used in Western blots as positive controls were isolated from rabbit fast skeletal muscle according to Saito, et al. (30), rat cardiac muscle according to Feher and Davis (31), and avian pectoralis muscle according to Airey, et al. (32).

[3H]Ryanodine Binding Assay—Equilibrium saturation experiments for [3H]ryanodine binding to mouse parotid acinar cell, brain, and skeletal muscle membranes were performed by titrating the radioligand concentration between 0.1 and 30 nM at constant specific activity in a final assay volume of 250 μl. Unless otherwise indicated, the binding media consisted of 0.5 mM KCl, 100 μM CaCl2, 20 mM HEPES, pH 7.4, and inhibitors:protinin (0.5 mg/ml), benzamidine (1 mM), leupeptin (1 μg/ml), pepstatin A (0.7 μg/ml), and PMSF (0.1 mM) with the protease substrate BSA (100 μg/ml) or 1 mM DTT. EC50 and IC50 values for modulators were obtained from concentration-response experiments of equilibrium binding of [3H]ryanodine added at a nonsaturating concentration of 6 nM. Modulators of [3H]ryanodine binding were added in equilibrium saturation experiments at a concentration that produced half-maximal response as determined above using a nonsaturating concentration of [3H]ryanodine. The tissue samples were incubated in binding media with or without modulators at 23 ± 0.5 °C for 16–22 h. The assay was terminated by rapid dilution of sample in 4 ml of wash buffer containing 0.5 mM KCl, 20 mM HEPES, pH 7.4, 100 μM Ca2+ and by passage of sample through a Whatman GF/F glass fiber filter followed immediately by 3 × 4 ml washes of filter with the same buffer; all procedures were completed within 1 min. The filters were dried overnight and placed in vials containing scintillant, and the bound [3H]ryanodine was measured by liquid scintillation counting using a Packard Tri-Carb 2200CA analyzer. Specific bound [3H]ryanodine was calculated as [3H]ryanodine binding minus nonspecific binding, measured for parallel assays in the presence of 10 μM unlabeled ryanodine, from the total bound. For acinar cell microsomal membranes, nonspecific binding averaged 81 and 57% at 0.3 and 20 nM, respectively, and was linear with the concentration of [3H]ryanodine. Free Ca2+ effects were obtained by titrating CaCl2 in binding media containing 1 mM EGTA using theoretical estimates derived from the computer program BADS (33). [3H]Ryanodine binding to proteins in the soluble fraction of acinar cells was determined by the PEG precipitation method as described by Shoshan-Barmatz and Zarka (34). Protein was determined by the Hartree modified method of Lowry (36).

Association and Dissociation Kinetics for [3H]Ryanodine Binding—For association kinetics, acinar cell microsomal membrane (0.4 mg of protein/ml) was incubated with 5 nM [3H]ryanodine in binding media with and without 10 μM cold ryanodine at 23 and 37 °C. At time intervals ranging between 10 min and 22 h, the reaction was terminated by rapid filtration as described above.

For dissociation kinetics, acinar cell microsomal membrane (0.4 mg of protein/ml) was incubated at 23 °C with 5 nM [3H]ryanodine in binding media with and without 10 μM cold ryanodine for 16 h until association was completed. Dissociation was initiated by diluting duplicate aliquots of each treatment group by 50-fold using binding media without ryanodine. Dissociation was terminated by filtering aliquots (250 μl) at the indicated intervals of time up to 20 h, and the bound radioactivity was assayed as above.

Analysis of Binding Data—EC50 and IC50 values for activators and inhibitors of [3H]ryanodine binding, respectively, were determined by linear analysis of log-log plots obtained using computer programs RADLIG. The equilibrium binding constants, Kd and Bmax, for [3H]ryanodine with and without effectors were derived by curvilinear analysis using the computer program RADLIG sub-routine EBDA (Elsevier-BIOSOFT) and depicted graphically using a Rosenthal (Scatchard) plot and linear analysis. Kinetic association and dissociation rate constants were calculated using sub-routine KINETIC of RADLIG. Mean treat-
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Fig. 1. Parotid acini express Ry₃R proteins by Western blot analysis. Constituent proteins from rabbit junctional sarcoplasmic reticulum, rat cardiac sarcoplasmic reticulum, avian pectoralis sarcoplasmic reticulum, or crude acini membranes were resolved by SDS-PAGE using 3–10% gradient gels and transferred overnight onto polyvinylidene difluoride membranes. Secondary antibody was visualized using chemiluminescent methods. Shown are the Ry₁R Western blot skm lane: 1 μg of rabbit skeletal junctional SR, and acr lane: 30 μg of crude acini membrane proteins; the Ry₂R Western blot crd lane: 5 μg rat cardiac SR, and acr lane: 30 μg of crude acini membrane proteins; and the Ry₃R Western blot avi lane: 5 μg of avian pectoralis SR, and acr lane, 20 μg of crude acini membrane proteins (arrow marks the relative mobility of Ry₃R protein). Ry₁R and Ry₂R antibodies were labeled using a goat anti-mouse secondary antibody (Sigma). Ry₃R antibody was labeled using a donkey anti-rabbit antibody (Amersham Corp.). Western analysis with each antibody was repeated at least three times on two independent preparations with the same results.

Electrophoreses and Western Blot Analysis—Constituent proteins from membrane preparations were resolved on 3–10% gradient gels by the method of Laemmli (37). Proteins were electroblotted overnight at 20 V followed by a 60-min fast transfer at 100 V. Nonspecific antibody binding was blocked by incubating blots for 1 h at 37 °C in TTBS buffer in the presence of 1% BSA and antibody. Resulting immunoblots were labeled with a donkey anti-rabbit antibody (Amersham Corp.). Western analysis with each antibody was repeated at least three times on two independent preparations with the same results.

Assay conditions of Ca²⁺, KCl concentrations, pH, and temperature were evaluated 1) for their effect on [³H]ryanodine binding to acinar cell membranes and 2) to select assay media conditions to test other known effectors of [³H]ryanodine binding to the RyR. An optimal range of free Ca²⁺ was required for the specific equilibrium binding of 6 nM [³H]ryanodine to membranes in assay media, pH 7.4, containing 0.5 mM KCl at 23 °C. Specific [³H]ryanodine binding was near detection limits in the presence of 1 mM EGTA (8 ± 5 fmol/mg of protein, n = 3) compared with controls in the presence of 100 μM CaCl₂ without added EGTA (123 ± 10 fmol/mg of protein, n = 13). Titration of CaCl₂ in the presence of 1 mM EGTA revealed a biphasic dependence of specific [³H]ryanodine equilibrium occupancy and Ca²⁺ with a threshold of approximately 10 nM and an optimum between 10 and 100 μM Ca²⁺ (Fig. 2A). The apparent IC₅₀ for Ca²⁺ was approximately 2 μM, and 10 mM Ca²⁺ inhibited binding by 86%.

When KCl or NaCl were removed from the binding media containing optimum Ca²⁺ (100 μM), specific equilibrium binding of 6 nM [³H]ryanodine was not detectable (Fig. 2B). [³H]Ryanodine binding increased proportionately with increase in concentration of KCl to 0.5 M, reached its maximum at 0.75 M KCl, and showed little or no change with increase in KCl concentration to 1 M. NaCl, at molar equivalence up to 1 M concentration, partially replaced KCl in effectiveness. Sucrose in molar equivalent to KCl or NaCl did not significantly stimulate [³H]ryanodine binding at concentrations <1 M.

Equilibrium binding of 6 nM [³H]ryanodine was also biphasic with respect to pH. When assay pH was buffered with HEPES between 7 and 8, ryanodine binding was between 70 and 90%, respectively, of its maximum at pH 7.6 (Fig. 2C). Test of pH effects below 7 (MOPS) and above 8 (TRIS) showed binding to decrease steeply to 20% of maximum at pH 6.2 and to be relatively unaffected in alkaline solution, with pH 8.8 giving 87% of maximum binding (data not shown).

Assay temperature dramatically influenced the time course and magnitude of [³H]ryanodine binding (Fig. 2D). At 37 °C in binding media (pH 7.4) containing 100 μM Ca²⁺ and 0.5 M KCl, maximum binding of 5 nM [³H]ryanodine occurred within 2 h with an association half-time of 45 min. Thereafter, total binding declined to approximately 50% by 4 h and to negligible levels by 22 h. At 23 °C, the rate of [³H]ryanodine (5 nM) association was significantly slower (half-time = 100 min; kₐss = 0.0069 min⁻¹) but reached a 2-fold higher level of occupancy that remained stable for at least 22 h (Fig. 2D). After equilibrating the membranes for 16 h and subsequent to a 50-fold dilution with binding media lacking ryanodine, the radioligand dissociated with a half-time = 595 min (kₛ = 0.0012 min⁻¹).

The time courses of association (Fig. 2D) and dissociation (Fig. 3) of 5 nM [³H]ryanodine at 23 °C were both best described by a mono-exponential equation. The association rate constant calculated according to the relationship kₐss = kₐss - kₛ[L] was 1.16 × 10⁶ min⁻¹ M⁻¹. The equilibrium dissociation constant calculated based on Kᵦ = kₛ/kₐss was 1 nM.

A standard assay condition that includes binding media (pH 7.4) with 100 μM Ca²⁺, 0.5 mM KCl and temperature at 23 °C was used to characterize equilibrium binding of [³H]ryanodine to mouse parotid acinar cell membranes. The distribution of specific binding of 6 nM [³H]ryanodine among acinar subcellular fractions was compared for two experiments. [³H]Ryanodine binding to the 10,000–100,000 × g particulate (microsomal) fraction comprised 26% of the total acinar cell specific binding. Percent distribution of [³H]ryanodine binding to the 250 × g and 250–10,000 × g particulate (putative nuclear and mitochondrial) fractions averaged 17 and 57% of the total, respectively. The ratio of specific binding activity for these fractions relative to the microsomal fraction (relative specific binding...
activity averaged 0.92 and 0.86. No binding was detectable in the 100,000 g soluble (cytosolic) fraction. Overall, [3H]ryanodine equilibrium binding in the standard assay media at 22 h averaged 132 ± 6 fmol/mg of protein for 27 preparations of microsomal membrane; the concentration of added [3H]ryanodine averaged 6.2 ± 0.06 nM. [3H]Ryanodine (6 nM) binding was a linear function of protein concentration from 0.1 to 1.0 mg of protein/ml (Fig. 4); assay concentrations of membrane used in this study were within this range. Since this is the first report of RyR in parotid acini, we chose to illustrate that the amount of specific binding is directly proportional to the amount of acini receptor in the assay.

As shown in Fig. 5A for three independent preparations of microsomes assayed in standard media at 23 °C, specific binding of [3H]ryanodine approached saturation near 20 nM. Non-specific binding was linear with increasing concentration of radionuclide but approached 45% of total binding at the higher concentrations of [3H]ryanodine. Nonlinear regression analysis of equilibrium binding curves reveals the data is best fit statistically by a mathematical model describing [3H]ryanodine binding to a single site and results in a linear Scatchard plot (Fig. 5B). The apparent $K_D$ was 6.2 ± 0.55 nM (n = 3), and maximum occupancy ($B_{max}$) was 275 ± 22 fmol/mg of protein (n = 3).

In Table I, [3H]ryanodine saturation binding to mouse parotid acinar microsomal membranes is compared with binding parameters measured with membrane preparations from mouse fast twitch skeletal muscle and whole brain tissues. Brain and parotid preparations differed from those derived from skeletal muscle in having a lower percentage of specific bound radioligand and greater than 10-fold less binding sites/mg of membrane protein. Brain had the highest affinity for [3H]ryanodine, having approximately 10-fold higher binding affinity than either parotid or muscle membranes.

In addition to Ca$^{2+}$, other compounds reported to modulate the ryanodine-sensitive release of Ca$^{2+}$ from muscle SR (caffeine, adenine nucleotide, ruthenium red, and MgCl$_2$), and from the endoplasmic reticulum of nonexcitable cells (4-CEP) were tested for their influence on [3H]ryanodine binding to acinar cell microsomes. Typical concentration-response curves of activators and inhibitors of specific [3H]ryanodine binding determined at equilibrium with a non-saturating concentration of 6 nM [3H]ryanodine are shown in Fig. 6, A and B, respectively. Concentration estimates of effectors producing half-maximal activation (EC$_{50}$) or inhibition (IC$_{50}$) are summarized in Table II. In the presence of optimal Ca$^{2+}$, AMP-PCP (2 mM) maximally enhanced [3H]ryanodine occupancy by 40% and exhibited an EC$_{50}$ of 1 mM. Caffeine (3–10 mM) enhanced occupancy by 25% although the activation was too small to calculate an EC$_{50}$. In marked contrast, 200–600 μM 4-CEP enhanced [3H]ryanodine receptor occupancy 2.5-fold, exhibiting an activation threshold of approximately 10 μM and an EC$_{50}$ of 50 μM.
The activating action of 4-CEP on receptor binding was sharply biphasic with 1 mM producing a 24% reduction of control occupancy. Ruthenium red was 50-fold less potent than unlabeled ryanodine toward competing with 6 nM [3H]ryanodine (IC50 of 10 and 500 nM, respectively); whereas Mg2+ was inhibitory at a concentration above physiological relevance (>1 mM). Table II summarizes EC50 and IC50 values for modulators of RyR in microsomes isolated from acinar cells.

The effects of caffeine and AMP-PCP in the absence and presence of an inhibitory concentration of MgCl2 are given in Table III. In the presence of 100 μM Ca2+, increases in [3H]ryanodine binding of 18 and 56% above controls were measured at 1 mM caffeine and 1 mM AMP-PCP, respectively. In the presence of 100 μM Ca2+, 1 mM Mg2+ reduced [3H]ryanodine binding by 40% and 10 mM caffeine could not fully restore occupancy to control levels. In contrast, 1 mM AMP-PCP in the presence of equimolar Mg2+ nearly restored occupancy to that seen with 1 mM AMP-PCP alone, revealing that the MgAMP-PCP complex maintains activating properties toward RyR.

Equilibrium saturation binding of [3H]ryanodine to acinar microsomes was examined in the presence and absence of modulators in binding media containing 100 μM Ca2+ and 0.5 mM KCl, which was found to be optimal for the binding of [3H]ryanodine to acinar microsomes (Table IV, Fig. 7). At a concentration shown to enhance occupancy by 50%, 4-CEP decreased the value of Kd (i.e. enhanced the apparent affinity of [3H]ryanodine for its binding site) nearly 4-fold and produced a small but significant reduction in Bmax. Interestingly, the macrocyclic bromotyrosine bastadins (20 μM) significantly enhanced [3H]ryanodine binding site affinity (4-fold) without a significant change in Bmax. Ruthenium red and Mg2+ significantly reduced [3H]ryanodine binding affinity (Kd from 6 to 8.5 and 10 nM, respectively) and lowered Bmax by 42% and 18%, respectively. The effects of Mg2+ on Kd were fully restored to the control value by caffeine, and the Mg-AMP-PCP complex enhanced Kd nearly 3-fold (Table IV). Although caffeine did not significantly reverse the actions of Mg2+ on Bmax, the Mg-AMP-PCP complex enhanced Bmax by 41%.

The immunosuppressive macrolactam, FK506, and bastadins have been shown to interact with the FKBP-12/Ry1 receptor complex found in skeletal muscle (18). These compounds were used to probe the possible involvement of immunophilins toward modulating [3H]ryanodine binding sites in acinar cell microsomes. The effect of dimethyl sulfoxide, used as a vehicle in studies with FK506 and bastadins, was examined and found to inhibit the equilibrium binding of 6 nM [3H]ryanodine by 9% and 37% at final concentrations of 1% and 5% (v/v), respectively. Bastadin enhanced [3H]ryanodine receptor occupancy in a concentration-dependent manner with maximum stimulation of >4-fold over dimethyl sulfoxide control observed between

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**Fig. 3.** Time course for dissociation of [3H]ryanodine. Microsomal membrane (0.4 mg/ml) was incubated with 5 nM [3H]ryanodine for 16 h at 23 °C with and without 10 μM cold ryanodine; aliquots were then quenched over time by 50-fold dilution in binding media without cold ryanodine and analyzed for specific bound [3H]ryanodine as described under “Experimental Procedures.” Data fit the exponential equation $y = 115.6e^{-0.0012x}$ with a correlation coefficient ($r$) = 0.997 and represent means of a single experiment performed in duplicate.

**Fig. 4.** Linear relationship between microsomal membrane protein concentration and specific bound [3H]ryanodine. Equilibrium binding of 6 nM [3H]ryanodine to increasing concentrations of membrane protein was assayed in standard binding media at 22 h and 23 °C. Data represent the mean of a single experiment performed in duplicate.

**Fig. 5.** Analysis of [3H]ryanodine equilibrium binding to mouse parotid acinar cell microsomal membrane in standard assay media at 22 h and 23 °C. A, total (○), specific (○), and nonspecific (●) binding in the presence of 0.3–20 nM [3H]ryanodine; and B, Rosenthal (Scatchard) plot of saturation data of the specific bound [3H]ryanodine. Data represent the mean ± S.E. of three experiments performed in duplicate using different microsomal membrane preparations.
100–200 μM when equilibrium binding was determined at the nonsaturating concentration of 6 nM [3H]ryanodine; whereas, FK506 was much less efficacious, producing a 2.0-fold increase in occupancy at 300 μM (Fig. 8). The EC50 for bastadin was 20 μM. Interestingly, FK506 negated the remarkable activity of bastadin toward activating [3H]ryanodine occupancy, resulting in a concentration-response relationship very similar to that seen with FK506 alone (Fig. 9).

**TABLE I**

Comparison of equilibrium saturation [3H]ryanodine binding to mouse membrane preparations by tissue type

| Tissue | [3H]Ryanodine range | Specific bound range | Apparent Kd (nM) | Bmax (pmol/mg protein) |
|--------|---------------------|----------------------|------------------|------------------------|
| Muscle | 0.1–20              | 96–91                | 9.3              | 3.75                   |
| Brain  | 0.3–50              | 87–43                | 0.86             | 0.15                   |
| Parotid| 0.3–20              | 81–56                | 6.2 ± 0.55 (3)   | 0.98 ± 0.02 (3)        |

**DISCUSSION**

In the present study, RyRs were characterized and identified in mouse parotid acinar cells by analysis of [3H]ryanodine binding and immunoblot analysis using antibodies selective toward the three known isoforms. Western analysis revealed that the major, perhaps only, RyR isoform expressed in mouse parotid acini was the so-called brain isoform, Ry3R. Data show that cells expressing Ry3R possess very low levels of Ry3R protein. Data also showed saturable, high affinity [3H]ryanodine binding in subcellular parotid membrane fractions that was Ca2+-, salt-, pH-, and temperature-dependent. Both kinetic and equilibrium binding studies with the microsomal membrane fraction revealed that [3H]ryanodine interacts with a single population of high affinity binding sites with Kd calculated between 1 and 7 nM in equilibrium and kinetic measurements in the standard assay media employed. Attempts to demonstrate different allosteric binding conformations of low and high binding affinities by using higher concentrations of [3H]ryanodine (∼30 nM) produced unacceptably high nonspecific binding (42). Given that parotid cells are composed of approximately 95% acinar cells, it is not likely that the specific [3H]ryanodine-binding sites or the immunoreactive protein toward Ry3R antibody originates from a contaminating cell other than acini. The contaminating cells would have to express very high levels of protein to account for the measurements made.

In general, the effects of temperature (42–45), alkaline pH (46–49), ions (species specific, Ca2+) (2, 44, 50, 51), and monovalent ions (46–48, 51, 52) on [3H]ryanodine binding reported here using parotid acinar cells were similar to those observed in excitable cells. [3H]Ryanodine binding showed a near absolute requirement for certain monovalent ions (K+ and Na+), even in the presence of optimum Ca2+. Since NaCl, at least partially, substituted for KCl, but sucrose, at a concentration close to physiologic molar equivalents, did not, the increase in [3H]ryanodine binding at 25 °C appears to be dependent on ionic strength as reported for smooth muscle (47), and not on osmolarity of the binding media, as reported for bullfrog skeletal muscle (53). There appears to be a temperature- and concentration-dependent monovalent ion effect on [3H]ryanodine binding with tissue-specific response. Concentrations of KCl and/or NaCl above 0.25 M are activating at 25 °C in rabbit skeletal muscle (51), bullfrog skeletal muscle (53), smooth muscle (47), and, as reported here, in acinar cells. At 37 °C, concentrations of KCl and/or NaCl are activating in rat heart muscle, but inhibitory in rabbit skeletal muscle sarcoplasmic reticulum (52). The higher sensitivity of the receptor for K+ over Na+ in parotid acinar cells, observed with less and varying magnitude in other cell types (47, 52), suggests that the larger size of the K+ cation may be a factor distinguishing the activation efficiency of monovalent ions.

The pharmacology of 1) inhibition by competing cold ryanodine (47, 49, 54, 55), ruthenium red (RuRD, ○), and MgCl2 (●) and (B) activation by AMP-PCP (45, 46, 49, 54), and 2) activation by AMP-PCP (45, 46, 49, 52, 54) and caffeine in excitable cells (47, 49, 52, 54, 56) was qualitatively similar to that found in mouse parotid acinar cells. Marked differences in binding assay conditions used by investigators preclude direct quantitative comparisons of the magnitude and potency of each effector in the parotid acinar cell with other cell types. The very low efficacy of caffeine and AMP-PCP toward enhancing [3H]ryanodine occupancy may reflect an inherently low sensitivity of the Ry3R complex to these agents compared with Ry1R and Ry2R isoforms expressed in striated muscle and is consistent with a lack of caffeine responsiveness of Ry3R in other cell types (10, 19). Deletion of Ry3R expression using gene targeting has revealed a small response to caffeine (25 μM) and is maintained in dyspedic myotubes, which has been attributed to expression of Ry1R (57, 58). However, this interpretation is based solely on the presence of Ry1R mRNA in selected dyspedic myotubes (57). Since the presence of Ry3R protein has not been positively identified in dyspedic muscle...
muscle, this does not preclude the possibility that in the absence of RyR, other effects of caffeine on the Ca\textsuperscript{2+} permeability of plasma membrane and mitochondria become apparent. However, the small effects of caffeine and AMP-PCP seen in acinar microsomes to partially and completely overcome MgCl\textsubscript{2} inhibition of \[^{3}H\]ryanodine binding, respectively, are consistent with the proposed roles of Ca\textsuperscript{2+}, ATP, and Mg\textsuperscript{2+} to modulate Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release via the ryanodine-sensitive calcium channel (59, 60).

4-Chloro-phenolic compounds have been shown to release Ca\textsuperscript{2+} from ruthenium red-sensitive intracellular stores of both excitable (61, 62) and nonexcitable cells (63). We sought to verify that 4-CEP specifically interacts with the ryanodine-sensitive calcium release channel of nonexcitable cells (63) by testing for 4-CEP-enhanced \[^{3}H\]ryanodine binding to mouse parotid acinar cell membranes. Our results show 4-CEP to increase \[^{3}H\]ryanodine binding to parotid acinar cell membranes. This supports the reported role of 4-CEP in elevating levels of cytosolic Ca\textsuperscript{2+} in excitable (61, 62) and nonexcitable cells (63). We sought to test for 4-CEP-enhanced \[^{3}H\]ryanodine binding to mouse parotid acinar cell membranes producing 50\% maximum activation (EC\textsubscript{50}) or inhibition (IC\textsubscript{50}).

- \[^{3}H\]Ryanodine (6 nM) was incubated in the presence of membranes (0.4 mg/ml) in standard assay medium for 22 h at 23 °C. Values reported are the mean ± S.E. of their mean of (N) independent experiments or the mean of single or replicate experiments (individual replicate means in parentheses) performed in duplicate.

### TABLE II

| Treatment | Specific bound \[^{3}H\]ryanodine | Control |
|-----------|-----------------------------------|---------|
| Calcium (free) | 1.8 ± 0.5 (3) | 100 |
| Bastadin | 20 (22, 18) | 118 |
| FK506 | ≥25 | 60 |
| 4-CEP | 54 (60, 39) | 81 |
| AMP-PCP | 800 | 100 |
| KCl | 240000 (210000, 270000) | 100 |

- \[^{3}H\]ryanodine binding to mouse parotid acinar cell microsomal membranes
- \[^{3}H\]Ryanodine (6 nM) was incubated with 0.4 mg/ml membranes in standard assay medium for 22 h at 23 °C, and specific binding \[^{3}H\]ryanodine was measured as described under "Experimental Procedures." Values represent the mean ± S.E. of their mean of (N) number of paired experiments performed in duplicate. *, mean treatment differences from control were tested for significance using the paired t statistic for paired observations at p < 0.05.

### TABLE III

| Treatment | Specific bound \[^{3}H\]ryanodine | Control |
|-----------|-----------------------------------|---------|
| Control | 162 ± 17.3 (4) | 100 |
| + 10 mM caffeine | 191 ± 18.9 (4) | 118 |
| + 1 mM MgCl\textsubscript{2} | 97 ± 10.8 (4) | 60 |
| + 10 mM caffeine | 131 ± 13.2 (4) | 81 |
| Control | 172 ± 19.9 (3) | 100 |
| + 1 mM AMP-PCP | 265 ± 29.5 (3) | 156 |
| + 1 mM MgCl\textsubscript{2} | 103 ± 12.3 (3) | 60 |
| + 1 mM AMP-PCP | 237 ± 26.7 (3) | 138 |

**Effect of caffeine and AMP-PCP on MgCl\textsubscript{2} inhibition of \[^{3}H\]ryanodine binding to mouse parotid acinar cell microsomal membranes**

\[^{3}H\]Ryanodine binding to mouse parotid acinar cell microsomal membranes was also found to be affected by MgCl\textsubscript{2} at different concentrations (62). Bidirectional effects of MgCl\textsubscript{2} on \[^{3}H\]ryanodine binding have been described, with different preferences in excitable (62) and nonexcitable cells (63). We found that 4-CEP produced a concentration-dependent increase in \[^{3}H\]ryanodine binding to acinar cell membranes, suggesting that a change in the open state of the RyR had occurred, presumably through an FK506-induced release of a FKBP-12/bastadin mixture. FK506 is the primary target for the immunosuppressor FK506 (65, 66), that, in binding to FKBP-12, dissociates the immunophilin from skeletal muscle (14). We found that FK506 produced a concentration-dependent increase in \[^{3}H\]ryanodine binding to acinar cell membranes, suggesting that a change in the open state of the RyR had occurred, presumably through an FK506-induced release of a FKBP-12/bastadin mixture.


\[ \text{Figure 7. Rosenthal (Scatchard) plot of equilibrium binding of 0.3–30 nM \(^{3}H\)ryanodine to acinar cell microsomal membranes in the presence of modulators. Binding was performed in control assay media (pH 7.4) containing 100 mM Ca\(^{2+}\), 0.5 mM KCl, and 20 mM HEPES (\(r = 0.997\), with \(2 \times 10^{-5}\) M bastadin, \(r = 0.995\), \(7 \times 10^{-5}\) M 4-CEP (\(r = 0.990\)), \(10^{-3}\) M MgCl\(_2\) (\(r = 0.979\), or \(2 \times 10^{-5}\) M ruthenium red (\(r = 0.897\)). Data represent the means of two or more experiments for control, bastadin, and 4-CEP and of a single experiment for MgCl\(_2\). Each experiment was performed in duplicate with different microsomal membrane preparations. Binding constants are summarized in Table IV.}

\[ \text{Figure 8. FK506 and bastadin concentration-dependent effects on \(^{3}H\)ryanodine binding to mouse parotid acinar cell microsomal membranes. Equilibrium binding of 6 nM \(^{3}H\)ryanodine was measured in standard assay media at 22 h and 23 °C. Data means for bastadin effects for a single experiment are representative of two experiments performed in duplicate with different microsomal membrane preparations; data means for FK506 represent a single experiment performed in duplicate. \(^{3}H\)Ryanodine binding of controls (100%) for FK506 and bastadin equaled 69.2 and 63.2 fmol/mg of protein, respectively.}

indicating a similar mechanism for FK506 and bastadin toward modulation of \(^{3}H\)ryanodine binding sites. Increased \(^{3}H\)ryanodine binding in the presence of FK506 and bastadin, as well as FK506 antagonism of bastadin-enhanced binding in mouse parotid acinar cell microsomal membranes, provide evidence that an immunophilin modulates receptor binding of exogenous ryanodine. Therefore, an immunophilin may modulate RyR function in mouse parotid acinar cells presumably through regulation of channel gating behavior. It is of interest to note that both the macrocyclic bromotyrosine bastadins and the chloroderivative of either methyl or ethylphenol share a chemical ring structure with a halogen substitution, providing perhaps some part of the modulator role these agents have in enhancing \(^{3}H\)ryanodine binding to the RyR.

To compare the effects of modulators of \(^{3}H\)ryanodine binding in the parotid acinar cell to those of other cell types, including both excitable and non-excitable types, requires critical evaluation of the effects of different assay conditions used by investigators to measure binding of \(^{3}H\)ryanodine. The magnitude of modulator response and potency, ryanodine binding affinity, and maximal binding site number for RyRs are all influenced by the selection of binding media, salt, and concentration, as well as temperature. The assay conditions we used to obtain half-maximal binding capacity and to describe the effects of modulators on \(^{3}H\)ryanodine binding over time and at equilibrium were performed at 5 and 6 nM \(^{3}H\)ryanodine concentration, respectively (\(K_d\) approximated 6 nM derived from equilibrium \(^{3}H\)ryanodine saturation binding experiments). Modulator concentrations approximated their EC\(_{50}\) or IC\(_{50}\) values in all equilibrium \(^{3}H\)ryanodine saturation binding experiments. Within these limits, we used assay conditions that produced the highest level of \(^{3}H\)ryanodine binding, \(i.e.\) 1) a monovalent ion concentration of 0.5 M and 2) a temperature of 23 °C. Salt concentrations of 0.5 and 1 M, although commonly used to obtain optimum binding (51, 67, 68), are nonphysiological. Lowering the salt concentration from 1 M to near physiological levels (200 mM) has been found to result in marked changes in the extent to which MgCl\(_2\), caffeine, and AMP-PCP affect binding properties of receptor for \(^{3}H\)ryanodine (44, 54). The sensitivity of the RyR to activation by Ca\(^{2+}\) has also been reported to be influenced by salt concentration of binding media (6, 54).

The association of \(^{3}H\)ryanodine with its high affinity site is highly dependent on temperature (Q\(_{10}\) > 3) (42, 45). By lowering the assay temperature from 37 to 23 °C, as reported here for acinar cell microsomes or by Carroll et al. (42) in skeletal muscle sarcoplasmic reticulum, the binding occupancy of \(^{3}H\)ryanodine at equilibrium was increased, implying a temperature-dependent conformational change of the receptor occurred that favored \(^{3}H\)ryanodine binding with a decrease in

\[ \text{Table IV}

\text{Apparent dissociation constants (}K_d, \text{ nM) and maximum binding site occupancy (}B_{\text{max}} \text{, fmol/mg of protein) of} \(^{3}H\)\text{ryanodine binding to mouse parotid acinar cell microsomal membranes in the presence of modulators}

| Modulator (mM) | \(K_d\) | \(B_{\text{max}}\) | Specific bound in the absence of modulator at 6 nM \(^{3}H\)ryanodine |
|---------------|--------|------------------|-----------------------------|
| Control       | 6.2 ± 0.55 | 275 ± 22 | 135 ± 22 |
| 4-CEP (7 \times 10^{-5}) | 1.7 (1.6, 1.8) | 220 (213, 228) | ND |
| Bastadin (2 \times 10^{-5}) | 1.6 (1.4, 1.8) | 282 (238, 325) | 143 (132, 154) |
| Ruthenium red (2 \times 10^{-7}) | 8.5 (7.5, 9.5) | 160 (150, 170) | 171 (169, 173) |
| MgCl\(_2\) (10^{-7}) | 10.0 | 228 | 124 |
| +Caffeine (10^{-5}) | 5.9 | 219 | 132 |
| +AMP-PCP (10^{-3}) | 2.3 | 387 | 201 |
temperature. The observation that bastardin (mixture) did not increase \( B_{\text{max}} \) in parotid acinar cells, but did in skeletal muscle (18) under similar binding conditions but different assay temperatures, suggests that the reported effect of bastardin to stabilize the high affinity binding site conformation at 37 °C may be mimicked by lowering the binding assay temperature.

A significant finding of the present study was the expression of the Ry3R isoform in mouse parotid cells. Ry3R has also been identified in other nonexcitable cells including HeLa, LLC-PK1, and mink lung epithelial cells (7, 10). However, this is the first biochemical characterization of Ry3R in the absence of detectable levels of isoforms Ry1R and Ry2R. Thus far, the function of Ry3R in nonexcitable cells is unclear. Demonstration of a Ca\(^{2+}\)-induced Ca\(^{2+}\) release process (CICR) has been hampered by the fact that caffeine, commonly employed as an activator of CICR, has been found to produce little or no effect on [Ca\(^{2+}\)] in some cells, including mouse parotid acini.

In cell types expressing Ry3R, the response to caffeine has been controversial. Caffeine did not release Ca\(^{2+}\) from intracellular stores of transforming growth factor β-treated mink epithelial cells or Jurkat T-cells (10, 19). The reason(s) for the lack of or controversial activity is due to the expression of low levels of RyR in cells known to express RyR is unclear. One explanation given was that caffeine insensitivity may be due to the expression of low levels of RyR. This may be the case for HeLa cells where \([\text{H}^{3}]\)ryanodine binding was 17-fold less than in rabbit brain (7). In mouse parotid cells, however, \([\text{H}^{3}]\)ryanodine binding capacity was comparable with mouse brain (54, 69). Another possibility is that lack of caffeine sensitivity may be related to the species of animal used. Caffeine has been shown to increase [Ca\(^{2+}\)] in rat parotid and pancreatic acinar cells (21, 22) but not in mouse parotid cells. Similarly, endothelial cells from bovine and rabbit were also found to differ in their responses to caffeine. Caffeine did not produce a marked release of Ca\(^{2+}\) from intracellular stores in bovine endothelial cells but was effective in rabbit aortic endothelial cells (70). In summary, the expression and characterization of Ry3R in mouse parotid acini supports the conclusion that this receptor subtype contributes to the changes in intracellular Ca\(^{2+}\) following cell stimulation. Although in mutant mice lacking Ry3R

where results do not suggest an involvement of Ca\(^{2+}\) mobilization via RyR in lymphocyte proliferation, mutant mice did show increased locomotor activity that may indicate abnormal Ca\(^{2+}\) signaling of certain neurons (71). In nonexcitable mouse parotid cells, Ry3R may be involved in the cross-talk that occurs between the Ca\(^{2+}\) - and cAMP pathways, i.e. Ca\(^{2+}\) regulation of cAMP synthesis suggests that a RyR plays a role in muscarinic augmentation of stimulated cAMP accumulation reported previously by Watson et al. (28).
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