NIH Public Access
Author Manuscript
Neurotoxicology. Author manuscript; available in PMC 2008 July 1.

Published in final edited form as:
Neurotoxicology. 2007 July ; 28(4): 770–779.

RYANODINE RECEPTOR TYPE 1 (RYR1) POSSESSING MALIGNANT HYPERTHERMIA MUTATION R615C EXHIBITS HEIGHTENED SENSITIVITY TO DYSREGULATION BY NONCOPLANAR 2,2',3,5',6-PENTACHLOROBIPHENYL (PCB 95)

Tram Anh Ta and **Isaac N. Pessah**
Center for Children's Environmental Health and Department of Molecular Biosciences, School of Veterinary Medicine, University of California, Davis, CA 95616 USA

Abstract

Malignant hyperthermia (MH) susceptibility is conferred by inheriting one of >60 missense mutations within the highly regulated microsomal Ca^{2+} channel known as ryanodine receptor type 1 (RyR1). Although MH susceptible patients lack overt clinical signs, a potentially lethal MH syndrome can be triggered by exposure to halogenated alkane anesthetics. This study compares how non-coplanar 2,2',3,5',6-pentachlorobiphenyl (PCB 95), a congener identified in environmental and human samples, alters the binding properties of [^{3}H]ryanodine to ryanodine receptor type 1 (RyR1) *in vitro*. Junctional sarcoplasmic reticulum (SR) was isolated from skeletal muscle dissected from wild type pigs ($_{Wt}$RyR1) and pigs homozygous for MH mutation R615C ($_{MH}$RyR1), a mutation also found in humans. Although the level of $_{Wt}$RyR1 and $_{MH}$RyR1 expression is the same, $_{MH}$RyR1 shows heightened sensitivity to activation and altered regulation by physiological cations. We report here that $_{MH}$RyR1 shows more pronounced activation by Ca^{2+}, and is less sensitive to channel inhibition by Ca^{2+} and Mg^{2+}, compared to $_{Wt}$RyR1. In a buffer containing 100nM free Ca^{2+}, conditions typically found in resting cells, PCB 95 (50−1000nM) enhances the activity of $_{MH}$RyR1 whereas it has no detectable effect on $_{Wt}$RyR1. PCB 95 (2μM) decreases channel inhibition by Mg^{2+} to a greater extent in $_{MH}$RyR1 (IC_{50} increased 9-fold) compared to $_{Wt}$RyR1 (IC_{50} increased by 2.5-fold). PCB95 reduces inhibition by Ca^{2+} 2-fold more with $_{MH}$RyR1 than $_{Wt}$RyR1. Our data suggest that non-coplanar PCBs are more potent and efficacious toward $_{MH}$RyR1, and have more profound effects on its cation regulation. Considering the important roles of Ca^{2+} and Mg^{2+} in regulating Ca^{2+} signals involving RyR channels, these data provide the first mechanistic evidence that a genetic mutation known to confer susceptibility to pharmacological agents also enhances sensitivity to an environmental contaminant.

Keywords

Ryanodine receptor; calcium; malignant hyperthermia; polychlorinated biphenyls; gene-environment interaction.

Corresponding Author: Isaac N. Pessah, Ph.D. Department of Molecular Biosciences School of Veterinary Medicine, University of California, Davis One Shields Avenue Davis, CA 95616 Telephone: (530) 752−6966 Fax: (530) 752−4698 E-mail: inpessah@ucdavis.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Introduction

Ryanodine receptors (RyRs) are large molecular weight proteins that assemble as tetramers and function as high conductance calcium channels that release Ca^{2+} that is stored within the sarcoplasmic/endoplasmic reticulum (SR/ER) of most cells. The large cytoplasmic domain of RyRs physically and/or functionally associate with plasma membrane proteins affording mechanisms that tightly couple extracellular stimuli into localized intracellular calcium signals that amplify and integrate cellular signaling events such as excitation-contraction (E-C), excitation-transcriptional, and excitation-secretion coupling (Meissner 2002; Fill and Copello, 2002; Wamhoff et al., 2006). Moreover, RyRs have been shown to regulate energy metabolism by mitochondria (Hajnoczky et al., 2002). There are three genetic isoforms of RyRs: RyR1, which is responsible for skeletal muscle E-C coupling; RyR2, which is responsible for cardiac muscle E-C coupling; and RyR3, originally characterized in the central nervous system (Pessah et al., 1985; Takeshima et al., 1989; Marks et al., 1989; Zorzato et al., 1990; Nakai et al., 1990; Otsu et al., 1990; Giannini et al., 1995; Hakamata et al., 1992). In recent years, it has become clear that all three RyR isoforms are widely expressed in both excitable and non-excitable cells.

Dysfunction of RyR1 and its closely related cardiac isoform, RyR2, are known to contribute to heritable diseases of muscle. Missense mutations within three “hot spots” of the *ryr1* gene have been linked to malignant hyperthermia (MH) susceptibility, central core disease (CCD), and multi-minicore disease (MmD) (MacLennan et al., 1990, Du et al., 2004; Avila et al., 2001). Interestingly, analogous mutations within the *ryr2* gene have been linked to cardiac arrhythmogenesis, including arrhythmogenic right ventricular dysplasia type 2 (ARVD2), catecholaminergic polymorphic ventricular tachycardia (CPVT) and familial polymorphic ventricular tachycardia (FPVT) (Bers and Perez-Reyes, 1999; Marks et al., 2002; Wang et al., 2004).

MH is an inherited, life-threatening disorder of skeletal muscle in human and animals possessing point mutations within RyR1. The prevalence of the MH genotype in humans is 1 out of 20,000 anesthetized adults (MacLennan and Phillips, 1992). It should be noted though that the incidence of MH mutations in the human population is likely to be much higher since only a fraction of the population undergoes general anesthesia with triggering agents and susceptible individuals do not always trigger with a single anesthetic. Patients expressing RyR1 with an MH mutation ($_{MH}$RyR1) show no overt clinical signs of disease. However when exposed to halogenated inhalation anesthetics and/or depolarizing blockers of skeletal muscle contraction, individuals can undergo a fulminant MH episode characterized by a striking increase in end tidal carbon dioxide, intense production of heat, tachycardia, unstable hemodynamics, lactic acidosis, and skeletal muscle rigidity (Gronert GA, 1980; Gronert and Antognini, 1994). Thus, MH is a true pharmacogenic disorder that remains sub-clinical until triggered by a chemical or physical stressor. If symptoms of a clinical MH episode are not promptly treated with administration of the muscle relaxant dantrolene, the fulminant episode is lethal. Dantrolene is therefore the drug of choice to abrogate clinical MH. The mechanism by which dantrolene exerts its relaxing action on muscle contractility is by preventing RyR1 channels from assuming long-lived open states (Paul-Pletzer et al., 2001; Kobayashi et al., 2005). The fulminant MH syndrome results from the uncontrollable release of SR-stored Ca^{2+} through $_{MH}$RyR1 (MacLennan and Phillips, 1992). A number of studies have shown that $_{MH}$RyR1 is hypersensitive to Ca^{2+} channel activation and/or hyposensitive to channel inhibition (Lopez et al., 2005; Yang et al., 2003; Louis et al., 2001; Ording et al., 1997; Sei et al., 2002; Wappler et al., 1999 & 2003; Wehner et al., 2002).

Although MH susceptible patients can be successfully identified using the *In Vitro Contracture Test* (IVCT) (European Malignant Hyperthermia Group, 1984), the underlying mechanisms

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

that lead to a fulminant MH episode and its severity in patients are still poorly understood (Loke and MacLennan, 1998). Although >60 mutations have been linked to MH susceptibility, only about 50% of MH episodes have been linked to RyR1 mutations. Furthermore, patients with a known MH mutation may not go into a fulminant MH episode on the first or even subsequent exposures to triggering agents (e.g. anesthetics), and they exhibit different degrees of severity in response to the same triggering agent among patients in the same susceptible family (Loke and MacLennan, 1998). This variability in triggering the clinical MH phenotype could reflect the influence of yet unidentified physiological and/or environmental modifiers that allosterically sensitize the RyR1 channel complex toward activation. To examine this hypothesis, we tested if a representative non-coplanar polychlorinated biphenyl, 2,2',3,5',6-pentachlorobophenyl (PCB 95), differentially influences $_{Wt}$RyR1 and $_{MH}$RyR1 function *in vitro*.

Previous studies from our laboratory have shown that ortho-substituted PCB congeners potently affect the mobilization of Ca^{2+} from skeletal SR and brain ER by shifting RyR1 sensitivity to Ca^{2+} and Mg^{2+} through a highly selective and structurally specific interaction with the FKBP12 (FK506-binding protein)/RyR1 complex (Wong and Pessah, 1996 & 1997). The most active PCB congeners toward RyR1 include many of those found at highest concentrations in human tissues including PCB 153 (2,2',4,4',5,5'-hexachloro), 170 (2,2',3,3',4,4',5-heptachloro) and 180 (2,2',3,4,4',5,5'-heptachloro) (Pessah etal., 2006). In the present study, we use PCB 95, one of the most potent RyR1 sensitizers identified to date (Pessah et al., 2006), to test the hypothesis that $_{MH}$RyR1 is more highly susceptible than its wild type counterpart to dysregulation by non-coplanar PCBs. In addition to its potent RyR1 activity, PCB 95 has been selected for these initial studies since it has been recently reported to constitute 3−4% of the total PCBs found in contaminated San Francisco Bay sediments (Hwang et al., 2006), and has also been detected in several human tissues when analytically resolved (Covaci et al., 2002; Chu et al., 2003; DeCaprio et al., 2005; Jursa et al., 2006). Thus PCB 95 may be more frequently found in human and animal tissues then previously thought since it is often not resolved from PCB 66 (2,3',4,4'-tetrachloro) by GC. For example 4.2% of the total PCB burden in Fox River fish was PCB59+66 (Kostyniak et al., 2005). In a recent study of serum samples drawn from a Native American population, PCB 95 contributed an average of 1% of total serum PCBs detected (DeCaprio et al., 2005). Furthermore, analysis of age-dependent congener profile revealed that PCB 95 were higher (> 2% of total PCBs measured) among the youngest 5% of the study population (age range 18−24) (DeCaprio et al., 2005).

The human $_{MH}$RyR1 mutation R614C occurs in about 2% of MH susceptible patients, and the homologous mutation (R615C RyR1) occurs in the “Pietrain” pig line. The Pietrain is homozygous for the R615C RyR1 mutation, and was selected for growth and leanness traits; *i.e.*, faster-growing pigs at heavier slaughter weights while providing high quality leaner meat (Band et al., 2005). In the present study, junctional SR membrane vesicles are isolated from skeletal muscle dissected from R615C RyR1 homozygote MH susceptible Pietrain pigs and their wild type breed-matched counterparts. We report for the first time that $_{MH}$RyR1 exhibits significantly higher sensitivity to a non-coplanar PCB than does wild type RyR1.

Materials and Methods

Materials

—[^{3}H]Ryanodine ([^{3}H]Ry; 60−90 Ci/mmol; >99% pure) was purchased from Perkin-Elmer New England Nuclear (Wilmington, DE). PCB 95 (>99% pure) was purchased from Ultra Scientific (North Kensington, RI). Chemiluminescence chemicals were purchased from PerkinElmer Life Science Products. Unlabeled ryanodine (>99% by HPLC) and leupeptin were purchased from Calbiochem (San Diego, CA). Monoclonal 34C–(RyR1)-1° antibody was obtained from Developmental Studies Hybridoma Bank (Iowa City, IA). Polyvinylidene

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

difluoride microporous membranes were purchased from Millipore (Bedford, MA). Phenylmethylsulfonylfluoride (PMSF), imidazole, and A-5906–(RyR1)-2° antibody for RyR1 were purchased from Sigma (Saint Louis, MO). All other reagents were of the highest purity commercially available.

Membrane Preparations

— Porcine skeletal muscle junctional SR (JSR) membrane vesicles were isolated from flash-frozen longissimus dorsi muscle from 3 "Pietrain" (R615C-RyR1 homozygous) MH susceptible pigs and 3 "Near Pietrain" MH normal (control) pigs that are wildtype at the *ryr1* locus (Band et al., 2005). The pigs were raised at the University of Minnesota as a standing herd and their genotype assessed by PCR. Pigs were slaughtered by approved methods, and skeletal muscle dissected and frozen immediately. Frozen skeletal muscle was a generous gift of Dr. Bradley R. Fruen, University of Minnesota, and shipped frozen to UC Davis. The junctional SR (JSR) membrane vesicles were prepared from the frozen muscle by a modification of the method of Saito et al., (Saito et al., 1984). Briefly, 110 g of muscle was homogenized in 5-fold (w/v) ice-cold buffer consisting of 5mM imidazole, 300 mM sucrose, 100 μg/ml PMSF, and 10 μg/ml leupeptin, pH 7.4. The homogenate was centrifuged at 8,000 × g for 10 min. The resulting supernatant was collected and centrifuged at 110,000 × g for 1 hr. The crude microsomal pellets were suspended in homogenization buffer with a glass Dounce homogenizer and heavy SR vesicles from this solution further purified by centrifugation at 70,000 x g on a sucrose gradient for 15 hr at 4°C. Junctional SR vesicles enriched in RyR1 Ca^{2+} channel complexes were collected from the 38−45% sucrose interface and centrifuged at 110, 000 x g for 1 hr. The pellet was collected and suspended in storage buffer containing 10% sucrose, aliquoted in microfuge tubes, and rapidly frozen in liquid nitrogen and stored at –80°C until needed. Protein concentrations were measured by the method of Lowry using BSA as a standard (Lowry et al., 1951). Porcine JSR membranes were shown enriched in functional RyR1 channels and active Ca^{2+} transport properties (Fill et al., 1990; Mickelson et al., 1988; Ta and Pessah, in preparation).

Western Blot Analysis

—Proteins were denatured in 1:1 in a solution containing 10% mercaptoethanol and 10% sucrose-HEPES buffer for 15 min at 80 °C before being loaded onto a 3−10% gradient sodium dodecyl sulfate-polyacrylamide gel and subjected to electrophoresis at 200 V for 45 min at 4° C. The size-separated proteins were then transferred slowly onto polyvinylidene difluoride microporous membranes using an electroblotter for 16 hr at 30 V followed by a rapid transfer for 1 hr at 150 V. The transferred proteins were then incubated in TTBS buffer (25 mM Tris base, 137 mM NaCl, 0.05% Tween 20, pH 7.4) containing 5% nonfat dry milk at ambient temperature for 30 min. The blot was then probed with 34C primary antibody (1:200 dilution) diluted 200 times in TTBS buffer plus 1% bovine serum albumin at 24 °C. After 1 hr, the blot was rinsed in TTBS buffer three times and then incubated with the secondary antibody (goat anti-rabbit IgG at a 1:20,000 dilution) for 1 hr at 24 °C. After a final rinse step with TTBS, enhanced chemiluminescence techniques were used to visualize the immunoblots.

Measurement of [^{3}H]Ry Binding

—Since [^{3}H]Ry binds with high affinity to an open state of the RyR channel, occupancy of [^{3}H]Ry-binding sites provides a convenient biochemical indicator of modulation of the open probability of the channel (i.e. channel activity) by exogenously added ligands (Pessah et al., 1985, 1987 and 1991). Equilibrium measurements of specific high-affinity [^{3}H]Ry binding and incubation conditions were determined according to the method developed by Pessah et al. (Pessah et al., 1985; Pessah et al., 1987; Needleman and Hamilton, 1997). To determine maximum binding level of $_{Wt}$RyR and $_{MH}$RyR, an unlabeled ryanodine titration binding

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

experiment shown in Fig. 1A was performed in high salt buffer containing 1 M KCl, 15 mM NaCl, 20 mM HEPES, 100 μM $CaCl_2$, 1 nM [^{3}H]Ry with various concentration of unlabeled ryanodine, incubated for 3 hr at 37°C. The influence of PCB 95 concentration on [^{3}H]Ry binding to RyR1 (Fig. 2) was determined by incubating the SR vesicles in physiological intracellular salt binding buffer (140 mM KCl, 15 mM NaCl, 20 mM HEPES, 100 nM $CaCl_2$, 1 nM [^{3}H]Ry at pH 7.4) and was incubated for 20 hr at 24°C. Similarly, the effect of PCB 95 on the modulation of ryanodine binding by Ca^{2+} and Mg^{2+} was determined by obtaining the Ca^{2+} activation curves (Fig. 3) and Mg^{2+} inhibition curves (Fig.4) in the presence of various PCB 95 concentrations added from a 100x stock in dimethylsulfoxide before initiating the binding by addition of radioligand. Free concentration of Ca^{2+} (50nM to 50μM) was adjusted by titrating EGTA based on calculations determined using the Bound and Determined (B.A.D.) software program (Brooks and Storey, 1992). Nonspecific binding was assessed by incubating SR vesicles with 1000-fold excess unlabeled ryanodine. The binding reactions were filtered through GF/B glass-fiber filters. The filters were washed twice with 3 ml of ice-cold harvest buffer containing 20 mM Tris, 250 mM KCl, 15 mM NaCl, 50 μM $CaCl_2$, pH 7.1 and were soaked in 5 ml of scintillation cocktail overnight. The radioactivity was measured using a scintillation counter (Beckman, model LS 6000IC). Specific [^{3}H]Ry binding was determined by subtracting nonspecific binding from total binding and was greater than 90% under all buffer conditions, and unaffected by PCB 95.

Data Analysis

— The ORIGIN™ computer software (Microcal Software, Inc., Northampton, MA) was employed to analyze [^{3}H]Ry binding data. Non-linear regression for one-site binding (Fig.1A) and Scatchard analysis (Fig. 1B) were utilized to determine maximum receptor activation (B_{max}) and dissociation constant (K_d) for $_{Wt}$RyR1 and $_{MH}$RyR1. Analysis of the effect of PCB 95 on [^{3}H]Ry receptor binding parameters was performed using non-linear curve fitting of data across the entire dose-response curve using a sigmoidal equation to calculate the concentration needed to obtain 50% of the maximum receptor activity (EC_{50} or IC_{50}). One-way ANOVA was utilized to compare B_{max}, K_d, protein expression, EC_{50} or IC_{50} values for $_{Wt}$RyR1 and $_{MH}$RyR1 to test if a population difference between the two groups were significant and to report P values. Experiments were performed in triplicates and the data are presented as mean ± standard error of the replicated experiments on muscle prepared from three animals per genotype.

Results

In order to determine if junctional microsomal membrane vesicles isolated from skeletal muscle possessing RyR1 missense mutation R615C were quantitatively different compared to preparations isolated in an identical manner from wild type animals, [^{3}H]Ry receptor binding affinity and density were compared. Fig. 1A shows equilibrium binding curves measured with 25 μg of junctional microsomal protein from either wild type or MH susceptible preparations. These determinations were made in the presence of high salt which was shown to maximize the binding of [^{3}H]Ry to the high affinity site on RyR1 (1 M KCl, 100 μM Ca^{2+}, 20 mM HEPES, 15 mM NaCl) (Pessah et al., 1987;Pessah and Zimanyi 1991a). [^{3}H]Ry was titrated from 0.25 to 129 nM and the reactions were quenched by rapid filtration after 3 hr incubation at 37°C. Scatchard plot analysis showed that both $_{Wt}$RyR1 and $_{MH}$RyR1 preparations possessed the similar [^{3}H]Ry binding-site density and affinity (B_{max} of 3.5 ± 0.2 and 3.0 ± 0.1 pmol/mg protein ($P < 0.288$), K_d of 2.9 ± 0.3 and 2.1 ± 0.2 nM ($P < 0.039$), respectively, n = 6, Fig. 1B). The level of $_{Wt}$RyR1 and $_{MH}$RyR1 expression in the two preparations was confirmed by western blot analysis (Fig.1C) and densitometric analysis of western blots did not reveal any significant difference in the level of protein expression between the two preparations ($P <0.85$, Fig. 1D).

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Previous studies have shown that MH missense mutations resulted in hypersensitivity of RyR1 channels to channel activation by calcium, 4-chloro-*m*-cresol, and caffeine (Ording et al, 1997; Sei et al., 2002; Wappler et al., 1999 & 2003; Wehner et al., 2002; Yang 2003). In addition, a recent study by Lopez et al. showed that expression of several human MH mutations in myotubes result in persistent elevation of resting cytoplasmic Ca^{2+} (Lopez et al., 2005). In the present study, we compared the effect of PCB 95 on $_{MH}$RyR1 function with that of $_{Wt}$RyR1 under conditions that mimic the cytoplasmic Ca^{2+} concentration in resting cells. These experiments were performed under more physiological salt conditions (140 mM KCl, 15 mM NaCl, 20 mM HEPES, 1 nM [^{3}H]Ry, 100 nM Ca^{2+}, pH 7.4), and incubation was allowed to proceed for 20 hr at 24°C. Under this assay condition, PCB 95 increased the amount of [^{3}H] Ry bound in a PCB 95 dose-dependent manner, and reflects the activating actions of PCB 95 on overall channel activity (Wong and Pessah, 1996). The EC_{50} for PCB 95 activation of $_{Wt}$RyR1 was >3-fold higher than that of $_{MH}$RyR1 (EC_{50}s of 3.6 ± 0.2 μM *vs*. 1.1 ± 0.1 μM, respectively, n = 6; Fig. 2). Furthermore,Fig. 2, *inset*, highlights the more profound effect of lower concentrations (≤ 1000 nM) of PCB 95 on activation of $_{MH}$RyR1, concentrations that had little detectable effect on $_{Wt}$RyR1.

RyR1 endogenous modulators such as Ca^{2+} and Mg^{2+} play an important role in regulation of the channel function to ensure the proper cellular signaling (Meissner, 2002). We therefore examined if these physiological cations differentially regulated $_{Wt}$RyR1 and $_{MH}$RyR1 in the presence and absence of 250 nM PCB 95. Fig. 3A shows the dose-response for Ca^{2+} activation of [^{3}H]Ry binding and Fig. 3B shows the Logit-log analysis for [^{3}H]Ry binding in the presence and absence of 250 nM PCB 95. In the absence of 250 nM PCB 95, Fig. 3A shows that EC_{50s} for $_{Wt}$RyR1 and $_{MH}$RyR1 are significantly different (170.8 ± 2.5 and 217.0 ± 5.9 nM, respectively, P-value < 0.05) and Logit-log analysis in Fig. 3B shows that $_{MH}$RyR1 exhibited a 3-fold steeper Logit slope compared with that of $_{Wt}$RyR1 (1.5 ± 0.8 and 0.5 ± 0.2, respectively, n = 6, Table 1A). Similarly, in the presence of 250 nM PCB 95, EC_{50s} for $_{Wt}$RyR1 and $_{MH}$RyR1 are also significantly different (148.6 ± 7.6 and 171.5 ± 0.7 nM, respectively, P-value <0.05) and the Logit slopes for both $_{MH}$RyR1 and $_{Wt}$RyR1 are steeper compared to their respective controls lacking PCB 95 (3.3 ± 1.1 and 2.5 ± 0.9, respectively, n = 6, Table 1A).

It is important to note that specifically at 1 μM $[Ca^{2+}]_{free}$ (shown in Fig. 3A), $_{MH}$RyR1 attained a 4-fold higher absolute amount of optimal [^{3}H]Ry binding compared to $_{Wt}$RyR1 (0.25 ± 0.01 *vs*. 0.065 ± 0.011 pmol/mg protein). Interestingly, the presence of PCB 95 increased the binding level of $_{Wt}$RyR1 preparations to that observed with $_{MH}$RyR1 preparations free of PCB95, whereas PCB 95 further increased optimal binding of $_{MH}$RyR1 2.5-fold (to 0.62 ± 0.01 pmol/ mg protein). Thus PCB 95 increased the level of [^{3}H]Ry binding in $_{MH}$RyR1 preparations by nearly 10-fold compared to $_{Wt}$RyR1 preparations free of PCB 95 at1 μM $[Ca^{2+}]_{free}$, reflecting its much higher potency toward $_{MH}$RyR1. Fig. 3D summarizes the EC_{50}s for Ca^{2+} activation of [^{3}H]Ry binding in the presence of several PCB 95 concentrations. As PCB 95 concentration is increased, both $_{MH}$RyR1 and $_{Wt}$RyR1 became more sensitive to activation by Ca^{2+}; however, the impact on $_{MH}$RyR1 was more significant than that of $_{Wt}$RyR1. Although the EC_{50}s for $_{Wt}$RyR1 and $_{MH}$RyR1 were significantly different from one another in the absence of PCB 95, they diverge significantly as PCB95 concentration is increased (* P < 0.05 for PCB 95 < 250 nM, and **P < 0.001 for PCB 95 > 250 nM).

We next examined the effect of PCB 95 on the ability of Mg^{2+} to inhibit [^{3}H]Ry binding to $_{Wt}$RyR1 and $_{MH}$RyR1 in buffer containing optimal (100 μM) Ca^{2+}, mimicking the local Ca^{2+} concentration attained at the cytoplasmic face of RyRs during physiological activation (Perez et al., 1999; Niggli E, 1999; Jaggar et al., 2000). As shown in Fig. 4A, the presence of 2 μM PCB 95 significantly increases the amount of [^{3}H]Ry specific binding to both $_{MH}$RyR1 and $_{Wt}$RyR1 preparations. Furthermore, in the presence of PCB 95, RyRs became hyposensitive to inhibition by Mg^{2+}. The Logit slope for Mg^{2+} inhibition of $_{MH}$RyR1 was

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

much steeper compared to that of $_{Wt}$RyR1 (−0.76 ± 0.96 *vs*. −0.21 ± 0.05, Table 1B). However, in the presence of 2 μM PCB 95, the Logit slopes for both $_{MH}$RyR1 and $_{Wt}$RyR1 were similar (−0.754 ± 0.15 and −0.89 ± 0.091, respectively, Table 1B.) Importantly, the action of PCB 95 on Mg^{2+} inhibition is significantly more profound on $_{MH}$RyR1 than on $_{Wt}$RyR1 (Fig. 4C). In the presence of 2 μM PCB 95, the IC_{50}s for inhibition of $_{Wt}$RyR1 and $_{MH}$RyR1 increased to 737 ± 59 μM (n = 6) and 2659 ± 112 μM (n = 6), respectively, Table 1B. Fig. 4D summarizes the IC_{50}s for magnesium inhibition in the presence of several PCB 95 concentrations. As PCB 95 concentration increased, RyRs became increasingly hyposensitive to Mg^{2+} inhibition for both $_{MH}$RyR1 and $_{Wt}$RyR1; however, the impact was significantly greater on $_{MH}$RyR1 (** $P < 0.001$) than that of $_{Wt}$RyR1.

Ca^{2+} above 100μM is also known to inhibit RyR1 in a dose-dependent manner (Pessah et al., 1985; 1987). We therefore studied the effect of PCB 95 on the inhibitory potency of Ca^{2+} using equilibrium [^{3}H]Ry binding analysis under the same buffer conditions described above (graphs are not shown). In the absence of PCB 95, Ca^{2+} inhibited both $_{Wt}$RyR1 and $_{MH}$RyR1 with similar potencies (IC_{50}s are 110.7 ± 10.6 μM and 133.9 ± 26.5 μM, Table 1C). In the presence of 2 μM PCB 95, however, the inhibitory potencies for $_{Wt}$RyR1 were significantly greater ($P < 0.001$) from that of $_{MH}$RyR1 (1.2 ± 0.1 mM and 2.6 ± 0.2 mM, respectively).

Discussion

MH susceptible individuals remain sub-clinical until triggered by exposure to general anesthetic agents such as halothane, enflurane, isoflurane, desflurane, and sevoflurane, or a depolarizing muscle blocker such as succinylcholine (Gronert and Antognini, 1994). Considering that MH susceptibility is conferred by any one of more than 60 point mutations in RyR1 *and* requires an environmental trigger to elicit a clinical episode, we tested if $_{MH}$RyR1 has a particularly heightened sensitivity to a non-coplanar PCB of environmental relevance, PCB 95. The rationale for testing this hypothesis is based on the fact that MH susceptible humans may not trigger on the first or even after several exposures to triggering agents (e.g. such as inhalation anesthetics), and they exhibit different degrees of severity in response to the same triggering agent among patients in the same susceptible family (Loke and MacLennan, 1998). This variability in phenotype could reflect the influence of exposures to one or more environmental modifiers that allosterically sensitize the RyR1 channel complex toward become more active (*i.e.*, hyperactive). We first chose to examine the possible influence of non-coplanar PCB 95 on $_{MH}$RyR1 since: (1) its mechanism of interaction with $_{Wt}$RyR1 from skeletal and cardiac muscle (Wong and Pessah, 1996; 1997) and brain (Wong et al., 1997) are well characterized, (2) its actions on primary neurons and PC12 cells suggest RyR-mediated mechanisms are involved in its cellular toxicity (Wong et al 2001; Gafni et al., 2004), (3) the availability of breed-matched muscle tissue from MH susceptible and MH normal pigs, and (4) PCB 95, when resolved by gas chromatography, contributes 4−5% of the PCB burden in San Francisco Bay sediments (Hwang et al., 2006), and is commonly detected in human serum (DeCaprio et al., 2005; Jursa et al., 2006), adipose (Covaci et al., 2002), and liver, kidney, muscle, and brain (Chu et al., 2003).

In the present study, we compared the influences of PCB 95, a non-coplanar PCB, on the regulation of $_{MH}$RyR1 and $_{Wt}$RyR1 using [^{3}H]Ry binding analysis, a reliable indicator of channel conformation, and a sensitive and reliable reporter of how physiological (Pessah et al., 1987; Pessah and Zimanyi, 1991b; Fessenden et al., 2000; Feng et al., 2002) and xenobiotic modifiers influence the channel's activity (Buck et al., 1992; Buck et al., 1999; Chen et al., 1999; Feng et al., 1999; Chen et al., 2003). Consistent with previous studies (Mickelson et al., 1988), we found no significant difference in RyR1 protein expression between junctional SR preparations isolated form MH susceptible and wild type muscle, indicating that the mutation does not cause alterations in RyR1 protein transcription, translation and targeting. Therefore,

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

measurable alteration in aspects of channel regulation of $_{MH}$RyR1 compared to $_{Wt}$RyR1 must be due primarily to abnormal regulation of the channel complex possessing the R615C mutation (Yang et al., 2003). In the present study, we found PCB 95 differentially altered the properties $_{MH}$RyR1 and $_{Wt}$RyR1, especially regulation conferred by physiologically important cations.

$_{MH}$RyR1 is more profoundly influenced by PCB 95 than $_{Wt}$RyR1 in several respects. PCB 95 dose response curves shown in Fig. 2 reveal that $_{MH}$RyR1 possessing point mutation R615C were significantly more sensitive to activation by PCB 95. In the presence of low PCB 95 concentration (<250 nM), EC_{50} values for activation by Ca^{2+} for $_{Wt}$RyR1 and $_{MH}$RyR1 were not different from one another, suggesting that higher PCB 95 concentrations can produce the same level of allosteric activation of $_{Wt}$RyR1 seen with $_{MH}$RyR1, and that indeed the sensitizing action of PCB 95 on both genotypes were saturable. However, PCB 95 (<250 nM) significantly enhanced [^{3}H]Ry binding to $_{MH}$RyR1 but not $_{Wt}$RyR1, reflecting the higher sensitivity of $_{MH}$RyR1 channels to activation by PCB 95. Voltage clamp studies of single channels reconstituted in bilayer lipid membranes currently underway in our lab have verified that $_{MH}$RyR1 respond with a significantly higher open probability than $_{Wt}$RyR1 when exposed to nanomolar PCB 95 (Pessah et al., in preparation). In the present study, as the concentration of PCB 95 was increased, EC_{50}s for activation of [^{3}H]Ry binding with $_{Wt}$RyR1 and $_{MH}$RyR1 were affected; however, the shift in the dose-response curve was more profound with $_{MH}$RyR1. Therefore, PCB 95 enhanced the sensitivity for achieving a Ca^{2+}-induced open conformation of the channel, measured as an increased binding of [^{3}H]Ry. In this respect, the Ca^{2+} sensitivity of $_{MH}$RyR1 was more dramatically shifted than $_{Wt}$RyR1. Interestingly, Gronert et al. reported that physiological or emotional stress, anxiety, excitement, or sudden changes of environmental temperature can also initiate MH in some patients (Gronert et al., 1980). The present results raise a compelling possibility, that exposure to low levels of RyR-active PCBs such as PCB 95 could further sensitize MH susceptible individuals to the adverse effects of general anesthetic agents, depolarizing muscle blockers, or general environmental stresses such as temperature or emotional state to a greater extent than MH normal individuals. Since Ca^{2+} regulation of RyRs is essential for proper function of muscle cells, RyR inhibition by mM of Ca^{2+} and Mg^{2+} act as a protective mechanism to ensure intracellular calcium homeostasis.

The massive increase in cytosolic Ca^{2+} concentration seen in a triggered MH patient is due to inability of cations (Ca^{2+} and Mg^{2+}) to inhibit RyR1 from releasing calcium, especially when intracellular Ca^{2+} concentrations reached an abnormally high level (Lopez et al., 2005; Yang et al., 2003; Louis et al., 2001; Ording et al, 1997; Sei et al., 2002; Wappler et al., 1999 & 2003; Wehner et al., 2002). In the present study, we found that channel inhibition by Ca^{2+} and Mg^{2+} was not affected by low concentration of PCB 95 (<250 nM); however, at a concentration of PCB 95 (≥1 μM), RyRs became hyposensitive to channel inhibition by these cations. IC_{50} for Ca^{2+} inhibition of $_{Wt}$RyR1 increased by 10-fold compared with its control, whereas IC_{50} for $_{MH}$RyR1 increased by 20-fold relative to its respective control (without 2 μM PCB 95). IC_{50} for Mg^{2+} inhibition of $_{Wt}$RyR1 increased by 2.5-fold compared with its control, whereas IC_{50} for $_{MH}$RyR1 increased by 9-fold relative to their respective control (without 2 μM PCB 95). In the presence of 2 μM PCB 95, IC_{50} values for $_{MH}$RyR1 are 2-fold higher for calcium inhibition and more than 3-fold higher for magnesium inhibition compared to those of $_{Wt}$RyR1. Therefore, in the presence of PCB 95, physiological feedback inhibition of the channel open state by Ca^{2+} and Mg^{2+} are greatly affected in both genotypes, but more profoundly influenced in the presence of an MH susceptibility point mutation.

Missense mutations on *ryr1* gene are known to cause about 50% of the clinical MH cases reported (Gronert et al., 2004). However, the fact that *ryr1* mutations are expressed in cells other than muscle raises the possibility that exposure to RyR1-active PCBs and possibly other persistent organic pollutants may elicit a broader toxicological impact in individuals carrying

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

these mutations. RyR1s are expressed within the central nervous system (Ledbetter et al., 1994; Martin et al., 1998) and in specific immune cell such as B lymphocytes (Sei et al., 1999) and dendritic cells (O'Connell et al., 2002; Goth et al., 2006) were they contribute essential aspects of function. In fact, human MH mutations have been recently shown to increase the sensitivity of B lymphocytes to xenobiotic challenges (Ducreux et al., 2004; Sei et al., 2002). The present findings raise the intriguing question if individuals possess RyR mutations would be at higher risk of adverse outcomes resulting from exposure to non-coplanar PCBs and possibly other persistent organic pollutants.

If the higher sensitivity of $_{MH}$RyR1 point mutation R615C to dysregulation by PCB 95 shown here extends to their influence on the integrity of RyR-mediated Ca^{2+} signaling could influence functions in a number of organ systems.

The more pronounced effects of PCB 95 on [^{3}H]Ry receptor binding to $_{MH}$RyR1 compared to that of $_{Wt}$RyR1 is likely to extend to complex mixtures of non-coplanar PCBs known to predominate in several human tissues, including PCBs 153, 170, and 180 (Pessah et al., 2006). The results therefore raise the possibility that individuals who possess MH mutations within RyR1 and have been exposed to non-coplanar PCBs, may be more susceptible their adverse influences, and possibly amplify those of triggering agents. Recent development of knock-in mice that express human $_{MH}$RyR1 mutations (Chelu et al., 2006; Yang et al., 2006) will permit direct testing of this hypothesis *in vivo*.

Acknowledgments

The authors wish to thank Dr. Bradley R. Fruen (Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, Minneapolis) for his generous gift of the porcine muscle tissues. Supported by NIEHS 1PO1 ES11269, 4P42 ES04699 and 3P42ES05707 (INP). TAT was supported by training grant T32 ES07059.

References

Avila G, O'Brien JJ, Dirksen RT. Excitation-contraction uncoupling by a human central core disease mutation in the ryanodine receptor. Proc Natl Acad Sci U S A 2001;98:4215–20. [PubMed: 11274444]

Band G, Guimaraes SEF, Lopes PS, Schierholt AS, Silva KM, Pires AV, Benevenuto AV, Gomide LA. Relationship between the Porcine Stress Syndrome gene and pork quality traits of F2 pigs resulting from divergent crosses. Genet Mol Biol 2005;28:88–91.

Bers DM, Perez-Reyes E. Ca^{2+} channels in cardiac myocytes: structure and function in Ca^{2+} influx and intracellular Ca release. Cardiovasc Res 1999;42:339–60. [PubMed: 10533572]

Brooks SP, Storey KB. Bound and determined: a computer program for making buffers of defined ion concentrations. Anal Biochem 1992;201:119–26. [PubMed: 1621949]

Buck E, Zimanyi I, Abramson JJ, Pessah IN. Ryanodine stabilizes multiple conformational states of the skeletal muscle calcium release channel. J Biol Chem 1992;267:23560–67. [PubMed: 1331089]

Buck ED, Lachnit WL, Pessah IN. Mechanisms of δ-Hexachlorocyclohexane Toxicity: I. Relationship Between Altered Ventricular Myocyte Contractility and Ryanodine Receptor Function. J Pharmacol Exp Therap 1999;289:477–85. [PubMed: 10087040]

Chelu MG, Goonasekera SA, Durham WJ, Tang W, Lueck JD, Riehl J, Pessah IN, Zhang P, Bhattacharjee MB, Dirksen RT, Hamilton SL. Heat- and Anesthesia-Induced Malignant Hyperthermia in a RyR1 Knock-in Mouse. FASEB J 2006;20:329–30. [PubMed: 16284304]

Chen L, Estéve E, Sabatier J-M, Ronjat M, De Waard M, Allen PD, Pessah IN. Maurocalcine and peptide A stabilize distinct subconductance states of ryanodine receptor type 1 (RyR1) revealing a proportional gating mechanism. J Biol Chem 2003;278:16095–106. [PubMed: 12586831]

Chen L, Molinski TF, Pessah IN. Bastadin 10 stabilizes the open conformation of ryanodine-sensitive Ca^{2+} release channel in an FKBP12-dependent manner. J Biol Chem 1999;274:32603–12. [PubMed: 10551814]

Chu S, Covaci A, Schepens P. Levels and chiral signatures of persistent organochlorine pollutants in human tissues from Belgium. Environ Res 2003;93:167–76. [PubMed: 12963401]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Covaci A, de Boer J, Ryan JJ, Voorspoels S, Schepens P. Distribution of organobrominated and organochlorinated contaminants in Belgian human adipose tissue. Environ Res 2002;88:210–18. [PubMed: 12051799]

DeCaprio AP, Johnson GW, Tarbell AM, Carpenter DO, Chiarenzelli JR, Morse GS, Santiago-Rivera AL, Schymura MJ, Akwesasne Task Force on the Environment. Polychlorinated biphenyl (PCB) exposure assessment by multivariate statistical analysis of serum congener profiles in an adult Native American population. Environ Res 2005;98:284–302. [PubMed: 15910784]

Du GG, Khanna VK, Guo X, MacLennan DH. Central core disease mutations R4892W, I4897T and G4898E in the ryanodine receptor isoform 1 reduce the Ca^{2+} sensitivity and amplitude of Ca^{2+}-dependent Ca^{2+} release. Biochem J 2004;382:557–64. [PubMed: 15175001]

Ducreux S, Zorzato F, Muller C, Sewry C, Muntoni F, Quinlivan R, Restagno G, Girard T, Treves S. Effect of ryanodine receptor mutations on interleukin-6 release and intracellular calcium homeostasis in human myotubes from malignant hyperthermia-susceptible individuals and patients affected by central core disease. J Biol Chem 2004;279:43838–46. [PubMed: 15299003]

European Malignant Hyperthermia Group. A protocol for the investigation of malignant hyperpyrexia (MH) susceptibility. The European Malignant Hyperpyrexia Group. Br J Anaesth 1984;56:1267–69. [PubMed: 6487446]

Feng W, Liu G, Xia R, Abramson JJ, Pessah IN. Site-selective modification of hyperrreactive cysteines of ryanodine receptor complex by quinones. Molec Pharmacol 1999;55:821–31. [PubMed: 10220560]

Feng W, Tu J, Yang T, Vernon PS, Allen PD, Worley PF, Pessah IN. Homer regulates gain of Ryanodine Receptor. J Biol Chem 2002;277:44722–30. [PubMed: 12223488]

Fessenden JD, Wang Y, Moore RA, Chen SRW, Allen PD, Pessah IN. Divergent physiological and pharmacological properties of ryanodine receptor types 1 and 3 expressed in a myogenic cell line. Biophys J 2000;79:2509–25. [PubMed: 11053126]

Fill M, Copello JA. Ryanodine receptor calcium release channels. Physiol Rev 2002;82:893–22. [PubMed: 12270947]

Fill M, Coronado R, Mickelson JR, Vilven J, Ma JJ, Jacobson BA, Louis CF. Abnormal ryanodine receptor channels in malignant hyperthermia. Biophys J 1990;57:471–5. [PubMed: 2306496]

Gafni J, Wong PW, Pessah IN. Noncoplanar 2,2',3,5',6-pentachlorobiphenyl (PCB 95) and rapamycin amplify ryanodine receptor signaling and Ca^{2+} entry mediated by ionotrophic glutamate receptors in cerebellar granule neurons. Toxicol Sci 2004;77:72–82. [PubMed: 14600284]

Giannini G, Conti A, Mammarella S, Scrobogna M, Sorrentino V. The ryanodine receptor/calcium channel genes are widely and differentially expressed in murine brain and peripheral tissues. J Cell Biol 1995;128:893–904. [PubMed: 7876312]

Goth SR, Chu R, Gregg PJ, Cherednichenko G, Pessah IN. Uncoupling of ATP-mediated calcium signaling and dysregulation of IL-6 secretion in dendritic cells by nanomolar thimerosal. Env. Health Perspect 2006;114:1083–109. [PubMed: 16835063]

Gronert, GA.; Antognini, JF. Malignant Hyperthermia. In: Miller, RG., editor. Anesthesia. Churchill Livingstone; New York: 1994. p. 1075-93.

Gronert GA. Malignant hyperthermia. Anesthesiology 1980;53:395–423. [PubMed: 6999950]

Hajnoczky G, Csordas G, Yi M. Old players in a new role: mitochondria-associated membranes, VDAC, and ryanodine receptors as contributors to calcium signal propagation from endoplasmic reticulum to the mitochondria. Cell Calcium 2002;32:363–77. [PubMed: 12543096]

Hakamata Y, Nakai J, Takeshima, Imoto K. Primary structure and distribution of a novel ryanodine receptor/calcium release channel from rabbit brain. FEBS Lett 1992;312:229–35. [PubMed: 1330694]

Harrad S, Ren J, Hazrati S, Robson M. Chiral signatures of PCB#s 95 and 149 in indoor air, grass, duplicate diets and human faeces. Chemosphere 2006;63:1368–76. [PubMed: 16289232]

Hwang H-M, Green PG, Young TM. Tidal salt marsh sediment in California, USA: 1. Occurrence and sources of organic contaminants. Chemosphere 2006;64:1383–92. [PubMed: 16442586]

Jaggar JH, Porter VA, Lederer WJ, Nelson MT. Calcium sparks in smooth muscle. Am J Physiol Cell Physiol 2000;278:C235–56. [PubMed: 10666018]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Jursa S, Chovancova J, Petrik J, Loksa J. Dioxin-like and non-dioxin-like PCBs in human serum of Slovak population. Chemosphere Jul;2006 64(4):686–91. [PubMed: 16337987]

Kobayashi S, Bannister ML, Gangopadhyay JP, Hamada T, Parness J, Ikemoto N. Dantrolene stabilizes domain interactions within the ryanodine receptor. J Biol Chem 2005;280:6580–7. [PubMed: 15611117]

Kostyniak PJ, Hansen LG, Widholm JJ, Fitzpatrick RD, Olson JR, Helferich JL, Kim KH, Sable HJ, Seegal RF, Pessah IN, Schantz SL. Formulation and characterization of an experimental PCB mixture designed to mimic human exposure from contaminated fish. Toxicol Sci 2005;88:400–11. [PubMed: 16177234]

Ledbetter MW, Preiner JK, Louis CF, Mickelson JR. Tissue distribution of ryanodine receptor isoforms and alleles determined by reverse transcription polymerase chain reaction. J Biol Chem 1994;269:31544–51. [PubMed: 7989322]

Loke JC, MacLennan DH. Malignant hyperthermia and central core disease: disorders of Ca^{2+} release channels. Am J Med 1998;104:470–86. [PubMed: 9626031]

Loke JC, MacLennan DH. Bayesian modeling of muscle biopsy contracture testing for malignant hyperthermia susceptibility. Anesthesiology 1998;88:589–600. [PubMed: 9523800]

Lopez JR, Linares N, Pessah IN, Allen PD. Enhanced response to caffeine and 4-chloro-m-cresol in malignant hyperthermia-susceptible muscle is related in part to chronically elevated resting $[Ca^{2+}]_i$. Am J Physiol Cell Physiol 2005;288:C606–12. [PubMed: 15537710]

Louis CF, Balog EM, Fruen BR. Malignant hyperthermia: an inherited disorder of skeletal muscle Ca^{2+} regulation. Biosci Rep 2001;21:155–68. [PubMed: 11725864]

Lowry OH, Rosebrough NJ, Farr AL, Randal RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75. [PubMed: 14907713]

MacLennan DH, Duff C, Zorzato F, Fujii J, Phillips M, Korneluk RG, Frodis W, Britt BA, Worton RG. Ryanodine receptor gene is a candidate for predisposition to malignant hyperthermia. Nature 1990;343:559–61. [PubMed: 1967823]

MacLennan DH, Phillips M. Malignant hyperthermia. Science 1992;256:789–94. [PubMed: 1589759]

Marks AR, Priori S, Memmi M, Kontula K, Laitinen PJ. Involvement of the cardiac ryanodine receptor/calcium release channel in catecholaminergic polymorphic ventricular tachycardia. J Cell Physiol 2002;190:1–6. [PubMed: 11807805]

Marks AR, Tempst P, Hwang KS, Taubman MB, Inui M, Chadwick C, Fleischer S, Nadal-Ginard B. Molecular cloning and characterization of the ryanodine receptor/junctional channel complex cDNA from skeletal muscle sarcoplasmic reticulum. Proc Natl Acad Sci USA 1989;86:8683–87. [PubMed: 2813419]

Martin C, Chapman KE, Seckl JR, Ashley RH. Partial cloning and differential expression of ryanodine receptor/calcium-release channel genes in human tissues including the hippocampus and cerebellum. Neuroscience 1998;85:205–16. [PubMed: 9607712]

Meissner G. Regulation of mammalian ryanodine receptors. Front Biosci 2002;7:d2072–80. [PubMed: 12438018]

Mickelson JR, Gallant EM, Litterer LA, Johnson KM, Rempel WE, Louis CF. Abnormal sarcoplasmic reticulum ryanodine receptor in malignant hyperthermia. J Biol Chem 1988;263:9310–15. [PubMed: 3379071]

Nakai J, Imagawa T, Hakamata Y, Shigekawa M, Takeshima H, Numa S. Primary structure and functional expression from cDNA of the cardiac ryanodine receptor/calcium release channel. FEBS Lett 1990;271:169–77. [PubMed: 2226801]

Needleman DH, Hamilton SL. Related Articles. Factors influencing [^{3}H]ryanodine binding to the skeletal muscle Ca2+ release channel. Anal Biochem 1997;248:173–79. [PubMed: 9177737]

Niggli E. Localized intracellular calcium signaling in muscle: calcium sparks and calcium quarks. Annu Rev Physiol 1999;61:311–35. [PubMed: 10099691]

O'Connell PJ, Klyachko VA, Ahern GP. Identification of functional type 1 ryanodine receptors in mouse dendritic cells. FEBS Lett 2002;512:67–70. [PubMed: 11852053]

Ording H, Glahn K, Gardi T, Fagerlund T, Bendixen D. 4-Chloro-m-cresol test--a possible supplementary test for diagnosis of malignant hyperthermia susceptibility. Acta Anaesthesiol Scand 1997;41:967–72. [PubMed: 9311392]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Otsu K, Willard HF, Khanna VK, Zorzato F, Green NM, MacLennan DH. Molecular cloning of cDNA encoding the Ca^{2+} release channel (ryanodine receptor) of rabbit cardiac muscle sarcoplasmic reticulum. J Biol Chem 1990;265:13472–83. [PubMed: 2380170]

Paul-Pletzer K, Palnitkar SS, Jimenez LS, Morimoto H, Parness J. The skeletal muscle ryanodine receptor identified as a molecular target of [3H]azidodantrolene by photoaffinity labeling. Biochem 2001;40:531–42. [PubMed: 11148048]

Perez GJ, Bonev AD, Patlak JB, Nelson MT. Functional coupling of ryanodine receptors to K_{Ca} channels in smooth muscle cells from rat cerebral arteries. J. Gen. Physiol 1999;113:229–38. [PubMed: 9925821]

Pessah IN, Hansen LG, Albertson TE, Garner CE, Ta TA, Do ZN, Kim KH, Wong PW. Structure-Activity Relationship for Noncoplanar Polychlorinated Biphenyl Congeners toward the Ryanodine Receptor-Ca^{2+} Channel Complex Type 1 (RyR1). Chem Res Toxicol 2006;19:92–101. [PubMed: 16411661]

Pessah IN, Stambuk RA, Casida JE. Ca^{2+}-activated ryanodine binding: mechanisms of sensitivity and intensity modulation by Mg^{2+}, caffeine, and adenine nucleotides. Mol Pharmacol 1987;31:232–38. [PubMed: 2436032]

Pessah IN, Waterhouse AL, Casida JE. The calcium-ryanodine receptor complex of skeletal and cardiac muscle. Biochem Biophys Res Commun 1985;128:449–56. [PubMed: 3985981]

Pessah, IN.; Wong, PW. Etiology of PCB Neurotoxicity: From Molecules to Cellular Dysfunction. In: Robertson, L.; Hansen, L., editors. Progress In Polychlorinated Biphenyl Toxicology. Academic Press; NY: 2001. p. 179-184.

Pessah IN, Zimanyi I. Characterization of multiple [^{3}H]ryanodine binding sites on the Ca^{2+} release channel of sarcoplasmic reticulum from skeletal and cardiac muscle: evidence for a sequential mechanism in ryanodine action. Mol Pharmacol 1991a;39:679–89. [PubMed: 1851961]

Saito A, Seiler S, Chu A, Fleischer SJ. Preparation and morphology of sarcoplasmic reticulum terminal cisternae from rabbit skeletal muscle. J Cell Biol 1984;99:875–85. [PubMed: 6147356]

Sei Y, Brandom BW, Bina S, Hosoi E, Gallagher KL, Wyre HW, Pudimat PA, Holman SJ, Venzon DJ, Daly JW, Muldoon S. Patients with malignant hyperthermia demonstrate an altered calcium control mechanism in B lymphocytes. Anesthesiology 2002;97:1052–58. [PubMed: 12411786]

Sei Y, Gallagher KL, Basile AS. Skeletal muscle type ryanodine receptor is involved in calcium signaling in human B lymphocytes. J Biol Chem 1999;274:5995–02. [PubMed: 10026226]

Takeshima H, Nishimura S, Matsumoto T, Ishida H, Kanawa K, Minamoto N, Matsuo N, Ueda M, Hanaoka M, Hirose T, Numa S. Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor. Nature Lond 1989;339:439–45. [PubMed: 2725677]

Wamhoff BR, Bowles DK, Owens GK. Excitation-transcription coupling in arterial smooth muscle. Circ Res 2006;98:868–78. [PubMed: 16614312]

Wang HL, Dai DZ, Gao E, Zhang YP, Lu F. Dispersion of ventricular mRNA of RyR2 and SERCA2 associated with arrhythmogenesis in rats. Acta Pharmacol Sin 2004;25:738–43. [PubMed: 15169625]

Wappler F, Anetseder M, Baur CP, Censier K, Doetsch S, Felleiter P, Fiege M, Fricker R, Halsall PJ, Hartung E, Heffron JJ, Heytens L, Hopkins PM, Klingler W, Lehmann-Horn F, Nivoche Y, Tegazzin V, Tzanova I, Urwyler A, Weisshorn R, Schulte am Esch J. Multicentre evaluation of in vitro contracture testing with bolus administration of 4-chloro-*m*-cresol for diagnosis of malignant hyperthermia susceptibility. Eur J Anaesthesiol 2003;20:528–36. [PubMed: 12884985]

Wappler F, Scholz J, Fiege M, Kolodzie K, Kudlik C, Weisshorn R, Schulte am Esch J. 4-chloro-m-cresol is a trigger of malignant hyperthermia in susceptible swine. Anesthesiology 1999;90:1733–40. [PubMed: 10360873]

Wehner M, Rueffert H, Koenig F, Neuhaus J, Olthoff D. Increased sensitivity to 4-chloro-m-cresol and caffeine in primary myotubes from malignant hyperthermia susceptible individuals carrying the ryanodine receptor 1 Thr2206Met (C6617T) mutation. Clin Genet 2002;62:135–46. [PubMed: 12220451]

Wong PW, Brachney WR, Pessah IN. *Ortho*-substituted polychlorinated biphenyls (PCBs) alter microsomal calcium transport by direct interaction with ryanodine receptors of mammalian brain. J Biol Chem 1997;272:15145–53. [PubMed: 9182535]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Wong PW, Garcia E, Pessah IN. *Ortho*-substituted PCB 95 alters Ca^{2+} signaling and pH in PC12 cells by an immunophilin FKBP12-dependent mechanism. J Neurochem 2001;76:450–63. [PubMed: 11208908]

Wong PW, Pessah IN. Noncoplanar PCB 95 alters microsomal calcium transport by an immunophilin FKBP12-dependent mechanism. Molec Pharmacol 1997;51:692–702.

Wong PW, Pessah IN. Ortho-substituted polychlorinated biphenyls alter calcium regulation by a ryanodine receptor-mediated mechanism: structural specificity toward skeletal- and cardiac-type microsomal calcium release channels. Molec Pharmacol 1996;49:740–51. [PubMed: 8609904]

Yang T, Riehl J, Esteve E, Matthaei KI, Goth SR, Allen PD, Pessah IN, Lopez JR. Functional and Pharmacological characterization of malignant hyperthermia in the R163C-RyR1 knock-in mouse. Anesthesia. 2006in press

Yang T, Ta TA, Pessah IN, Allen PD. Functional defects in six ryanodine receptor isoform-1 (RyR1) mutations associated with malignant hyperthermia and their impact on skeletal excitation-contraction coupling. J Biol Chem 2003;278:25722–30. [PubMed: 12732639]

Zimanyi I, Pessah IN. Comparison of [^{3}H]ryanodine receptors and Ca^{2+} release from rat cardiac and rabbit skeletal sarcoplasmic reticulum. J Pharmacol Exper Ther 1991b;256:938–46. [PubMed: 1848635]

Zorzato F, Fujii J, Otsu K, Phillips M, Green NM, Lai FA, Meissner G, MacLennan DH. Molecular cloning of cDNA encoding human and rabbit forms of the Ca^{2+} release channel (ryanodine receptor) of skeletal muscle sarcoplasmic reticulum. J Biol Chem 1990;265:2244–56. [PubMed: 2298749]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

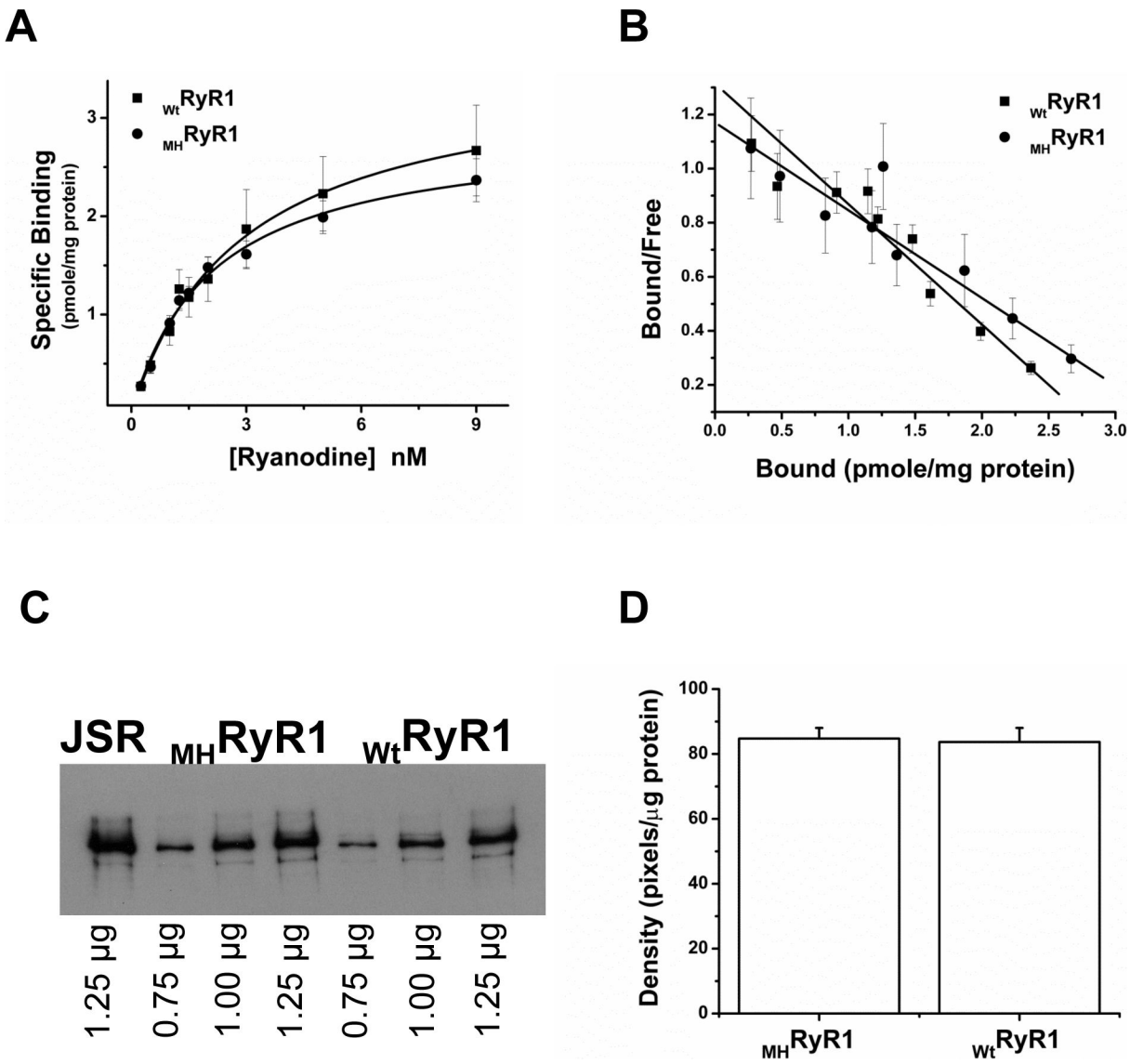

Figure 1.

Determination of protein expression and binding affinity for $_{Wt}$RyR1 and $_{MH}$RyR1.

(A) Non-linear regression for [^{3}H]Ry binding at equilibrium measured in high salt buffer (1 M KCl, 20 mM HEPES, 15 mM NaCl, 1 nM [^{3}H]Ry, 3 hrs incubation at 37°C) in the presence of unlabeled ryanodine at various concentrations was employed to determine B_{max} and K_{-1}. **(B)** Scatchard plots for both $_{Wt}$RyR1 and $_{MH}$RyR1. **(C)** Western Blots of $_{Wt}$RyR and $_{MH}$RyR. JSR lane refers to junctional sarcoplasmic reticulum prep obtained from rabbit and is used as a MW marker (M_r = 565,000). **(D)** Densitometric analysis of Western blots. Data are mean ± standard error of n = 6. Non-linear regression and Scatchard plots were obtained as described in Methods.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

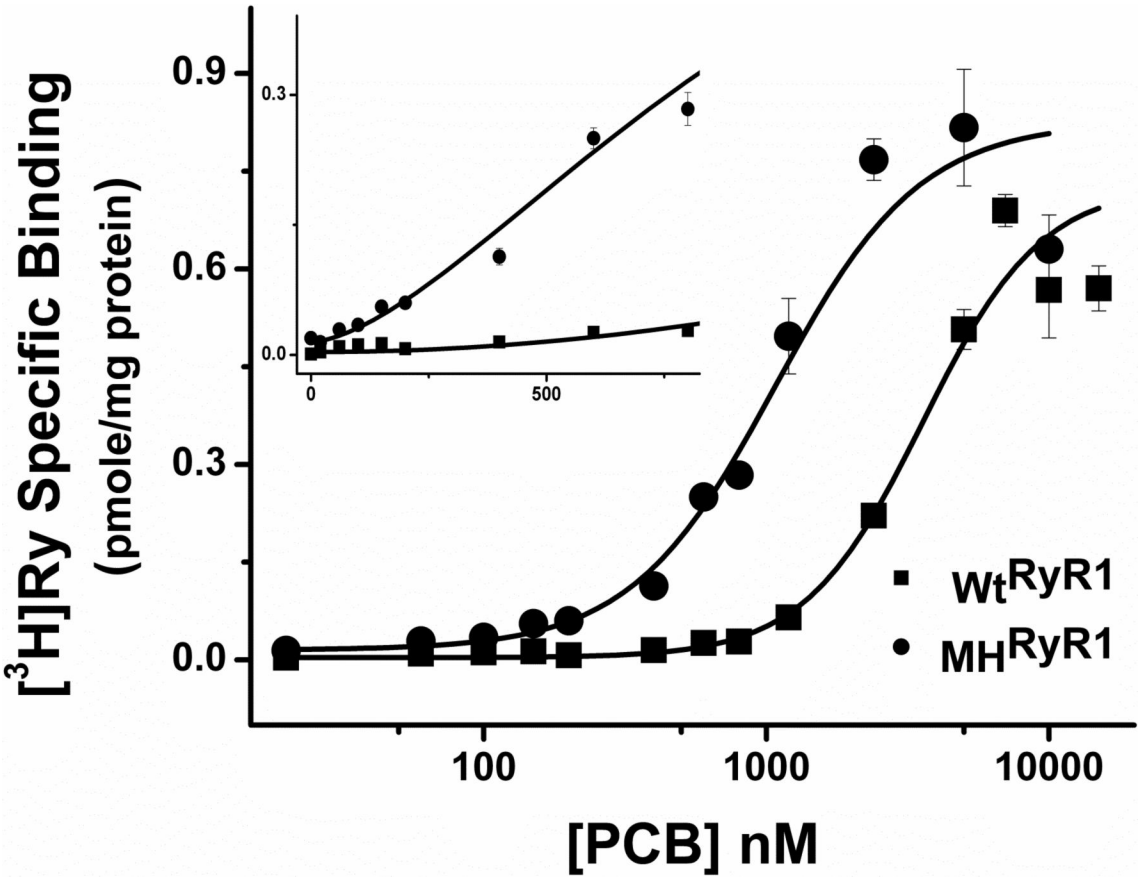

Figure 2.

PCB 95 enhances [^{3}H]Ry binding to porcine $_{Wt}$RyR1 and $_{MH}$RyR1. The influence of PCB 95 on [^{3}H]Ry binding was tested in the presence of 100 nM free Ca^{2+} in the assay buffer (mimicking the intracellular resting state) as described in Methods. *Inset* shows the higher potency of PCB 95 <1μM on $_{MH}$RyR1 compared with $_{Wt}$RyR1. Data are mean ± standard error of n = 6. EC_{50}s were obtained by fitting data to a Sigmoidal curve as described in Methods.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

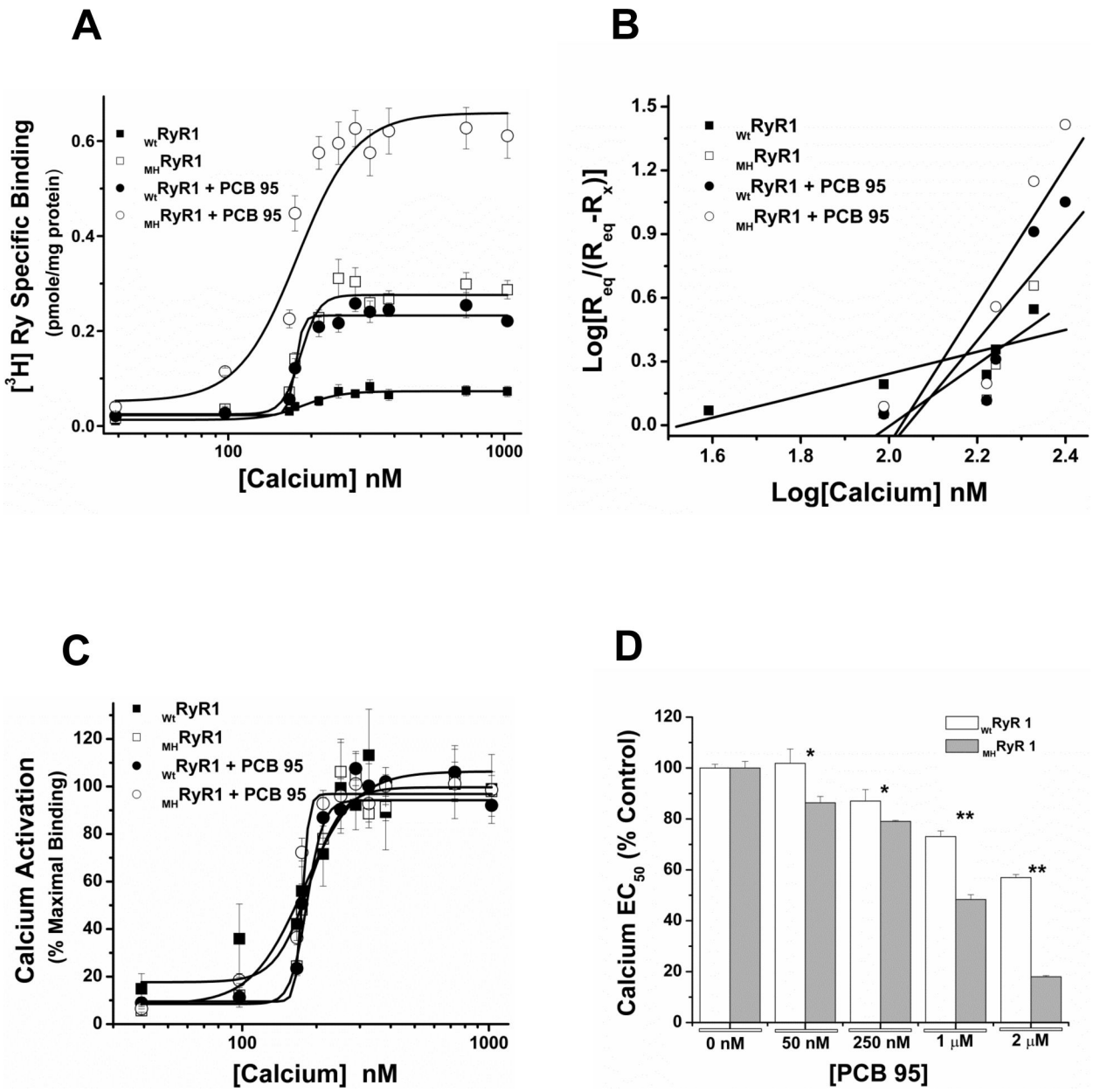

Figure 3.

PCB 95 more significantly sensitizes $_{MH}$RyR1 to Ca^{2+} activation compared to $_{Wt}$RyR1. Calcium activation of [^{3}H]Ry binding to porcine junctional SR vesicles enriched in $_{Wt}$RyR1 and $_{MH}$RyR1 were performed in a common binding buffer (140 mM KCl, 20 mM HEPES, 15 mM NaCl, 100 μM $CaCl_2$), 1 nM [^{3}H]Ry, pH 7.4, 20 hours incubation at 24°C. The free [Ca^{2+}] was adjusted by adding EGTA to the assay buffer based on calculations made with the B.A.D. program. **(A)** [^{3}H]Ry specific binding for $_{Wt}$RyR1 and $_{MH}$RyR1 in the presence of 250 nM PCB 95. **(B)** Logit-log analysis showing the shift in the slope and half-activation constant EC_{50} for Ca^{2+} in the presence of 250 nM PCB 95. **(C)** Normalized Ca^{2+} activation curves for $_{Wt}$RyR1 and $_{MH}$RyR1 for data in (A) highlighting the rightward shifts in the Ca^{2+} activation in the presence of PCB 95. **(D)** Summary of EC_{50} values for Ca^{2+} activation measured in the presence of various concentrations of PCB 95. *Statistical analysis:* ANOVA one-way, P-values: * ≤ 0.05, ** ≤0.001. Data are mean ± standard error of n = 6.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

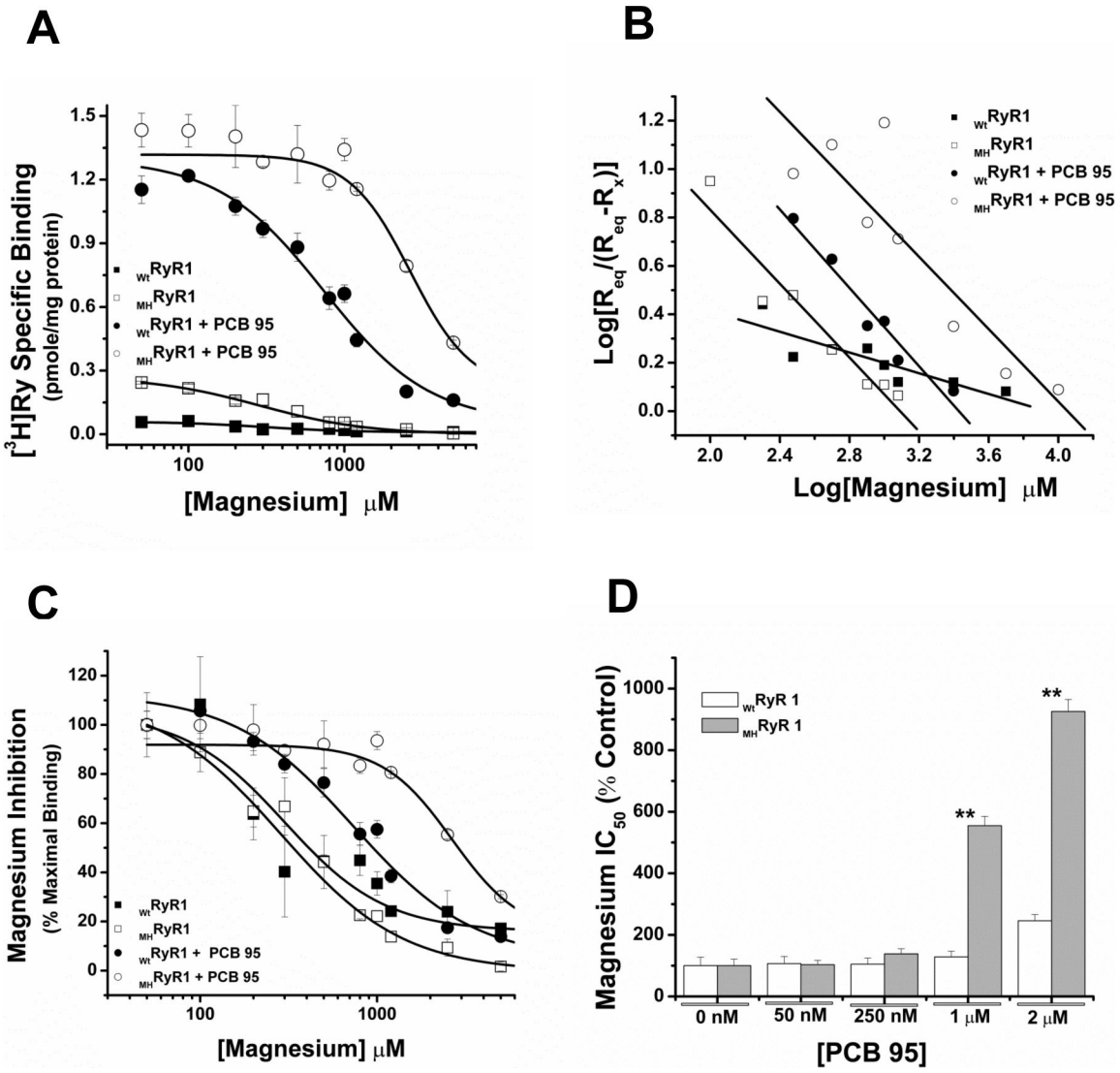

Figure 4.

PCB 95 attenuates the inhibitory effects of Mg^{2+} to a greater extent in $_{MH}$RyR1 than $_{Wt}$RyR1. Mg^{2+} inhibition of [^{3}H]Ry binding was measured in an assay buffer containing 140 mM KCl, 20 mM HEPES, 15 mM NaCl, 100 μM $CaCl_2$, 1 nM [^{3}H]Ry, pH 7.4 and incubated for 20 hours at 24°C. **(A)** [^{3}H]Ry specific binding for $_{Wt}$RyR1 and $_{MH}$RyR1 in the presence and absence of 2 μM PCB 95. **(B)** Logit-log analysis of the data shown in (A). **(C)** Normalized inhibition curves for Mg^{2+} in the presence and absence of 2 μM PCB 95 highlighting the leftward shifts in inhibition for $_{WT}$RyR1 and $_{MH}$RyR1. **(D)** Summary of IC_{50} values for Mg^{2+} in the presence of various concentrations of PCB 95. *Statistical analysis*: ANOVA one-way comparison, ** P-values ≤ 0.001. Data are mean ± standard error of n = 6.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Table 1

(A) EC_{50}s (nM) for activation of $_{Wt}$RyR1 and $_{MH}$RyR1 by Ca^{2+} with and without 250 nM PCB 95. **(B)** and **(C)** IC_{50}s (μM) for inhibition of RyR1s by Mg^{2+} and Ca^{2+}, respectively, with and without 2 μM PCB 95. EC_{50}s and IC_{50}s were obtained by Sigmodal curve fit. Slopes and R^2 values were obtained by for Logit-log analysis.

(A)	**Calcium Activation**	**Logit Analysis**	
	EC_{50} (nM)	**Slope**	**R^2**
$_{Wt}$RyR1	170.8 ± 2.5	0.52 ± 0.18	0.85
$_{MH}$RyR1	216.9 ± 5.9	1.46 ± 0.81	0.79
$_{Wt}$RyR1 + 250 nM PCB95	148.7 ± 7.6	2.52 ± 0.92	0.85
$_{MH}$RyR1 + 250 nM PCB95	171.5 ± 0.7	3.27 ± 1.06	0.87

(B)	**Magnesium Inhibition**	**Logit Analysis**	
	IC_{50} (μM)	**Slope**	**R2**
$_{Wt}$RyR1	300 ± 82	− 0.22± 0.05	0.87
$_{MH}$RyR1	287 ± 62	− 0.76 ± 0.09	0.96
$_{Wt}$RyR1 + 2μM PCB95	737 ± 59	− 0.89 ± 0.09	0.98
$_{MH}$RyR1 + 2 μM PCB95	2659 ± 11	− 0.75 ± 0.15	0.89

(C)	**Calcium Inhibition**	**Logit Analysis**	
	IC_{50} (μM)	**Slope**	**R2**
$_{Wt}$RyR1	110.7 ± 10.6	− 0.16 ± 0.03	0.93
$_{MH}$RyR1	133.9 ± 26.5	− 0.12 ± 0.02	0.99
$_{Wt}$RyR1 + 2μM PCB95	1201.4 ± 111	− 0.79 ± 0.14	0.93
$_{MH}$RyR1 + 2μM PCB95	2560.8 ± 180	− 1.01 ± 0.09	0.98