0026-895X/87/030232-07$02.00/0

MOLECULAR PHARMACOLOGY, 31:232-238

Ca^{2+}-Activated Ryanodine Binding: Mechanisms of Sensitivity and Intensity Modulation by Mg^{2+}, Caffeine, and Adenine Nucleotides

ISAAC N. PESSAH, ROXANNE A. STAMBUK, and JOHN E. CASIDA

Pesticide Chemistry and Toxicology Laboratory, Department of Entomological Sciences, University of California, Berkeley, California 94720

Received September 15, 1986; Accepted December 31, 1986

SUMMARY

The Ca^{2+}-ryanodine receptor complex is a functional unit at the terminal cisternae (TC) of the sarcoplasmic reticulum (SR) whose proteins comprise the Ca^{2+} release channels which may be involved in excitation-contraction coupling. Ca^{2+}, Mg^{2+}, caffeine, and adenine nucleotides, but not inositol 1,4,5-trisphosphate, may exert their inotropic effects on skeletal muscle SR by direct allosteric modulation of the [3H]ryanodine-binding site. Micromolar Ca^{2+} is primarily responsible for activating [^{3}H]ryanodine binding by regulating receptor site density, affinity, and cooperativity. Mg^{2+} reduces the sensitivity to Ca^{2+} activation by directly competing with Ca^{2+} for the activator site. However, inhibition by Mg^{2+} is overcome in the presence of β,γ-methyleneadenosine 5′-triphosphate (AMP-PCP; 1 mM) or caffeine (20 mM). Caffeine dramatically increases the affinity of the Ca^{2+} activator site for Ca^{2+}, whereas AMP-PCP or cAMP enhances the gating efficiency or the lifetime of the open state of the TC SR channel. A kinetic model is proposed for four functional domains of the Ca^{2+}-ryanodine receptor complex: 1) the Ca^{2+}-regulatory domain which binds Ca^{2+} with μM affinity is primarily responsible for gating the Ca^{2+} channel of the TC SR in a cooperative manner, and is inhibited by mM Mg^{2+} by direct competition for the activator site which appears to contain critical sulfhydryl groups; 2) a Ca^{2+}-activated alkaloid binding domain in close proximity to the channel which binds ryanodine with nM affinity and rapidly occludes upon complex formation; 3) a domain which binds caffine with low (greater than mM) affinity and directly influences the sensitivity of the Ca^{2+}-regulatory site; and 4) a domain which binds adenine nucleotides with intermediate affinity (less than mM), does not require phosphorylation, and intensifies the Ca^{2+} signal which triggers opening of the Ca^{2+}-release channel.

Muscle contraction involves depolarization of the transverse tubule membrane resulting in release of Ca^{2+} from the TC of the SR (1). This EC coupling may take place at putative ligand-gated Ca^{2+} release channels localized at the triad junction of the TC SR (1-3). Candidate chemical links in EC coupling include Ca^{2+} (2-8), adenine nucleotides (9), and IP_3 (10, 11). Ca^{2+} release from the SR of skeletal and cardiac intact and skinned muscle fibers and isolated SR membrane vesicles can be induced by a number of pharmacological probes including caffeine (3, 12-16) and ryanodine (17-24). It is not clear whether these diverse compounds exert their inotropic effects by influencing unrelated and biochemically distinct sites or by acting on a single channel gating mechanism.

[^{3}H]Ryanodine binds with nM affinity at Ca^{2+}-regulated sites in skeletal muscle TC SR membrane vesicles (25-27) and cardiac "heavy" SR preparations (25). Radioligand binding occurs only with the Ca^{2+}-activated open state of the TC SR channel which may be involved in release of the Ca^{2+} necessary to activate the contractile elements (hence the term Ca^{2+}-ryanodine receptor complex) (27). Formation of this complex results in rapid occlusion of the ryanodine binding domain (27), possibly preventing complete inactivation of the Ca^{2+} release channel (26, 28, 29).

This study examines the interactions of Ca^{2+}, Mg^{2+}, caffeine, adenine nucleotides, and IP_3 with the Ca^{2+}-ryanodine receptor complex in a physiologically complete assay medium. It recognizes four distinct effector binding domains, leading to a kinetic model of the regulation of the Ca^{2+}-activated channel of the TC SR of skeletal muscle.

Experimental Procedures

Materials [9,21-$^3H_3$]Ryanodine (>99% radiochemical purity, 60 Ci/mmol) was prepared in our laboratory (30). D-*myo*-Inositol trisphosphate (consisting predominantly of IP_3), caffeine, cAMP, AMP-PCP (the nonhydrolyzable ATP analog), and DTNB were obtained from Sigma Chemical Co. (St. Louis, MO).

This study was supported in part by National Institutes of Health Grant 5P01 ES00049.

ABBREVIATIONS: TC, terminal cisternae; AMP-PCP, β,γ-methyleneadenosine 5′-triphosphate; B_{hs}, binding site occupancy at near-saturating ligand level; DTNB, 5,5′-dithiobis(2-nitro)benzoate; EC, excitation-contraction; EGTA, ethylene glycol bis(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid; IP_3, inositol 1,4,5-trisphosphate as the major component in D-*myo*-inositol trisphosphate; SR, sarcoplasmic reticulum.

Isolation of skeletal muscle membranes. The heavy SR fraction from rabbit fast skeletal muscle homogenates (27) was diluted to 5 mg of protein/ml (31), divided into 1-ml aliquots, rapidly frozen in liquid nitrogen, stored at −70°, and thawed as needed, discarding unused portions. These membranes were shown to be enriched in morphological (displaying intact junctional processes) (27), biochemical (enriched with calsequestrin and junctional proteins) (29), and physiological (exhibiting Ca^{2+}-induced Ca^{2+} release) (29) markers of the TC SR.

Measurement of [^{3}H]ryanodine equilibrium binding. The physiologically complete assay medium contained 50 μg of SR protein and 5 nM [^{3}H]ryanodine in 115 mM KCl, 15 mM NaCl, 1 mM free Mg^{2+} (2 or 1 mM $MgCl_2$ in the presence or absence of AMP-PCP, respectively, or completely omitted in some experiments), 10% sucrose, and 40 mM Tris-maleate at pH 7.1. Free Ca^{2+} concentrations from 0.1 to 40 μM were obtained by titrating with the chelating agent EGTA (20–900 μM) in the assay medium containing 60 μM $CaCl_2$ based on an apparent affinity constant of 3.9×10^6 M^{-1} at pH 7.1 (32). For free Ca^{2+} concentrations of 60–300 μM, 10 μl of a 100 × concentrated stock solution of $CaCl_2$ was added directly into the assay medium.

The influence of caffeine (20 mM), adenine nucleotides (1 mM), and IP_3 (40 μM) on the Ca^{2+} activation of [^{3}H]ryanodine binding was measured under competitive conditions (TC SR membranes added last to initiate the reaction) by allowing samples to equilibrate 180 min at 37°. Specific binding was determined by filtration assay (27). [^{3}H] Ryanodine saturation experiments were performed at equilibrium (180 min incubation) in the presence of 60, 40, 10, or 2 μM free Ca^{2+} by titrating unlabeled ryanodine with [^{3}H]ryanodine to give final ligand concentrations ranging from 0.3 to 65 nM in the presence or absence of added test compounds.

Measurement of association/dissociation kinetics. The effect of Ca^{2+}, Mg^{2+}, caffeine, and AMP-PCP (or cAMP), singly or in combination, on the association kinetics of [^{3}H]ryanodine (5 nM) binding was measured by quenching the reaction by rapid filtration at times ranging from 1 to 120 min after the addition of TC SR membranes. Dissociation of [^{3}H]ryanodine from the receptor equilibrium complex was determined by equilibrating 5 nM [^{3}H]ryanodine with membranes for 2 hr at 37°, adding unlabeled ryanodine, Ag^+, or DTNB (10 μM final concentration) to a portion of the incubation mixture, and determining residual specific binding at subsequent times ranging from 30 sec to 8 hr.

Analysis of binding data. The effect of Ca^{2+} concentration on [^{3}H]ryanodine binding in the presence or absence of test compounds was analyzed by the method of Hill (33). A plot of log $(B/B_{ns}-B)$ versus log [Ca^{2+}] with values from 10% to 90% of maximum receptor occupancy at the near-saturating (~80% of B_{max}) concentration of 5 nM [^{3}H]ryanodine (B_{ns}) resulted in a straight line whose intercept with the abscissa is a measure of the apparent affinity of the activator site for Ca^{2+} (K_d/Ca^{2+}) and whose slope (n_H) reflects the degree of cooperativity for Ca^{2+} activation of [^{3}H]ryanodine-binding sites. [^{3}H]Ryanodine saturation experiments were analyzed by linear regression of Hill plots as described above to obtain K_d and n_H values, whereas B_{max} was estimated directly from binding isotherms. The pseudo-first order association rate constant for [^{3}H]ryanodine binding (K_{+1}) was calculated as described by Bennett (34) based on the dissociation rate constant k_{-1} determined in this study using unlabeled ryanodine. All data are means of triplicate determinations with repetition at least three times on different days unless noted otherwise. The statistical significance of differences in binding constants (K_d and n_H) under various assay conditions was assessed by directly comparing the two linear regression models from which they were derived (35).

Results

Mechanism of Ca^{2+} activation of the [^{3}H]ryanodine-binding site. Ca^{2+} activation of TC SR membrane vesicles is essential for specific binding of [^{3}H]ryanodine to its receptor site. The amount of radioligand bound at equilibrium is directly related to the free Ca^{2+} concentration in the assay medium (Fig. 1). Ca^{2+}, in the presence of physiological levels of Na^+, K^+, Mg^{2+}, and adenine nucleotide, activates [^{3}H]ryanodine binding by regulating the density (B_{max}), the apparent affinity (K_d), and the apparent cooperativity (n_H) exhibited by the alkaloid-binding site (Fig. 2). Reducing the free Ca^{2+} from an optimal 60 μM to 2 μM results in a 2.4-fold decrease in B_{max} (from 4.4 to 1.8 pmol/mg of protein; significant at $p < 0.001$), a 4.7-fold decrease in affinity (K_d from 1.6 to 7.5 nM; significant at $p < 0.001$), and a 1.2-fold decrease in n_H (from 1.6 to 1.3; significant at $P < 0.05$) (Fig. 2).

Effect of Mg^{2+} on Ca^{2+} activation of [^{3}H]ryanodine binding. In the absence of Mg^{2+}, specific ligand binding is evident below 1 μM Ca^{2+}, whereas in the presence of physiological Mg^{2+} (1 mM), more than 2 μM Ca^{2+} is needed to detect specific [^{3}H]ryanodine binding (Fig. 1). At a near-saturating level (5 nM) of [^{3}H]ryanodine, Mg^{2+} at 1 mM reduces B_{ns} by 42% and significantly ($p < 0.001$) reduces the apparent affinity of the activator site for Ca^{2+} 2.9-fold without significantly altering n_H for Ca^{2+} activation (Table 1). In [^{3}H]ryanodine saturation experiments in the presence of optimal (60 μM) Ca^{2+}, Mg^{2+} at 1 mM significantly ($p < 0.05$) increases the value of K_d and decreases the B_{max} with a concomitant loss in cooperativity at the ryanodine binding site (Fig. 3, Table 2).

Modulation of Ca^{2+}-activated [^{3}H]ryanodine binding by caffeine and adenine nucleotides. Caffeine at 20 mM in the presence of 1 mM Mg^{2+} decreases the threshold for Ca^{2+} activation of [^{3}H]ryanodine binding by ~20-fold (Fig. 1) and increases the apparent affinity of the activator site for Ca^{2+} by 17- to 27-fold (range of three experiments) (Table 1). The maximum receptor occupancy with near-saturating [^{3}H]ryanodine (5 nM) increases in the presence of caffeine and Mg^{2+} from 1.5 to 2.1 pmol/mg of protein but does not reach the level observed in assay buffer alone (2.6 pmol/mg of protein) (Table 1). These effects of caffeine on Ca^{2+} activation are accompanied by complete loss of cooperativity (n_H from 1.2 to 0.9) (Table 1), although Hill plots show marked deviation from linearity below 40% and above 80% of B_{ns}. At optimal Ca^{2+} caffeine increases the alkaloid-binding site affinity by 2-fold (K_d from 4.5 to 2.3 nM) and the B_{max} density by 27% (B_{max} from 1.9 to 2.6 pmol/mg of protein; significant at $p < 0.001$), although not to the density observed in the absence of Mg^{2+} (3.1 pmol/mg of protein) (Table 2). Despite the presence of Mg^{2+}, caffeine restores positive cooperativity at the [^{3}H]ryanodine-binding site (n_H from 1.1 to 1.7) (Fig. 3).

Unlike caffeine, AMP-PCP at 1 mM in the presence of Mg^{2+} at 1 mM increases the apparent affinity of the activator site for Ca^{2+} only 2.4-fold and results in a 2.4-fold increase in B_{ns} (From 1.5 to 3.6 pmol/mg of protein), a level above that observed in the absence of Mg^{2+} (2.6 pmol/mg of protein) (Table 1). AMP-PCP elicits a complete loss of positive cooperativity in Ca^{2+} activation observed in its absence (Table 1, Fig. 1). This adenine nucleotide in the presence of 1 mM Mg^{2+} nearly triples the alkaloid-binding site affinity (K_d from 4.5 to 1.6 nM) while increasing the B_{max} 2.5-fold (1.9 versus 4.7 pmol/mg of protein) (Table 2). Like caffeine, AMP-PCP elicits strong positive cooperativity at the [^{3}H]ryanodine-binding site (Table 2, Fig. 3). Other adenine nucleotides (e.g., cAMP; Table 1) exhibit the same properties in modulating the [^{3}H]ryanodine-binding site with a potency order of AMP-PCP > cAMP > ADP~adenosine > AMP.

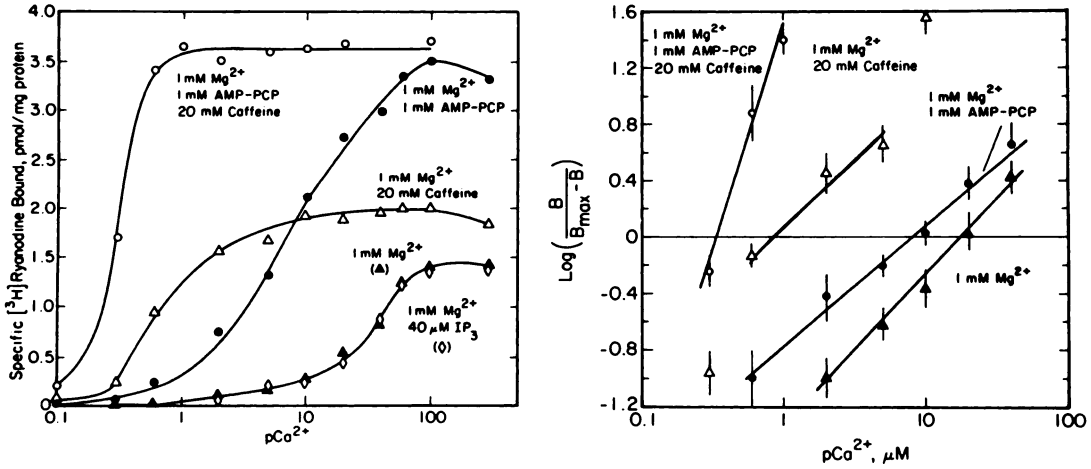


Fig. 1. Effects of Mg^{2+}, caffeine, and adenine nucleotides singly or in combination on Ca^{2+}-activated [^{3}H]ryanodine (5 nM) binding as binding isotherms and their respective Hill plots, where B_{ns} is utilized as B_{max} (see Experimental Procedures). These curves illustrate data used to determine apparent affinities and cooperativity of Ca^{2+} for the activator site summarized in Table 1.

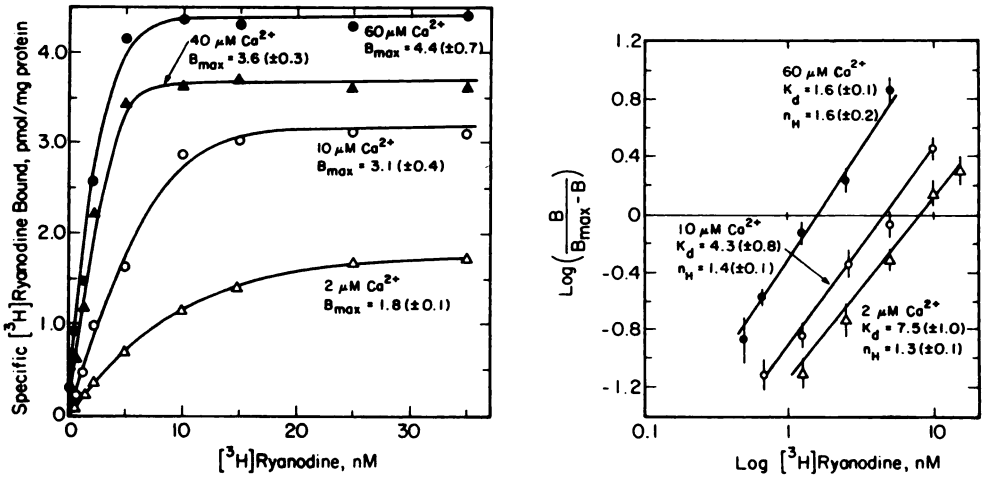


Fig. 2. [^{3}H]ryanodine saturation isotherms and their respective Hill plots assayed in the presence of 1 mM free Mg^{2+} and 1 mM AMP-PCP at four levels of free Ca^{2+}. Hill plots show mean and standard deviation of three experiments.

TABLE 1

Effects of Mg^{2+}, caffeine, and adenine nucleotides on the apparent affinity (K_d/Ca^{2+}) and cooperativity (n_H) for Ca^{2+} activation and the number of [^{3}H]ryanodine-binding sites occupied (B_{ns})

TC SR membranes (50 μg of protein) were incubated 180 min at 37° in assay buffer (see Experimental Procedures) containing 5 nM [^{3}H]ryanodine and 0.1–300 μM free Ca^{2+}. Each Ca^{2+} titration contained the indicated concentrations of free Mg^{2+}, caffeine, adenine nucleotides, and/or IP_3. K_d/Ca^{2+} and n_H were obtained directly from linear regression of Hill plots. Data are means and standard deviations of at least three experiments.

Additions	K_d/Ca^{2+}	n_H	B_{ns}
	μM		pmol/mg of protein
None	6.8 ± 1.7	1.3 ± 0.1	2.6 ± 0.2
Mg^{2+} (1 mM)			
Alone	19.8 ± 4.7	1.2 ± 0.2	1.5 ± 0.3
IP_3 (40 μM)	21.4 ± 1	1.4 ± 0.1	1.8 ± 0.1
AMP-PCP (1 mM)	8.3 ± 4.1	1.0 ± 0.1	3.6 ± 0.4
cAMP (1 mM)	7.5 ± 2.1	1.2 ± 0.2	2.8 ± 0.5
Mg^{2+} (1 mM) + caffeine (20 mM)			
Alone	0.9 ± 0.1	0.9 ± 0.3	2.1 ± 0.8
AMP-PCP (1 mM)	0.3 ± 0.1	3.7 ± 0.3	3.5 ± 0.3
cAMP (1 mM)	0.5 ± 0.1	3.0 ± 0.5	2.9 ± 0.1

The magnitude of stimulation of [^{3}H]ryanodine binding by caffeine and AMP-PCP is a function of their concentration in the range in which they are physiologically active. Caffeine up to 30 mM is much more effective than AMP-PCP up to 3 mM when assayed at 2 μM Ca^{2+} (increase from 0.04 to 0.39 and 0.15 pmol/mg of protein, respectively), whereas at 100 μM Ca^{2+} AMP-PCP is more stimulatory than caffeine (increase from 1.7 to 3.9 and 2.3 pmol/mg of protein, respectively) (Table 3). Caffeine and AMP-PCP in combination stimulate 68-fold (to 2.7 pmol/mg of protein) when assayed in 2 μM Ca^{2+}. Synergism is not observed, however, at optimal Ca^{2+} (60–100 μM) where the stimulation of 5 nM [^{3}H]ryanodine binding is the same as that induced by AMP-PCP alone (to 3.9 pmol/mg of protein under both conditions) (Table 3) and no significant change in K_d or B_{max} is detected (Table 2). However, caffeine and AMP-PCP in tandem, even in the presence of Mg^{2+}, cause a dramatic 62-fold increase in the affinity for Ca^{2+} (K_d/Ca^{2+}) (from 19.8 to 0.32 μM) with a concomitant increase in the apparent cooperativity of Ca^{2+} activation (n_H from 1.2 to 3.7) (Table 1).

Effect of IP_3 on [^{3}H]ryanodine binding. IP_3 at 40 μM does not alter the sensitivity of Ca^{2+} activation or the occupancy of [^{3}H]ryanodine receptor sites under the present assay conditions (Fig. 1, Table 1). There is also no observed change in [^{3}H] ryanodine binding with IP_3 at 40 μM in the presence of 1 mM

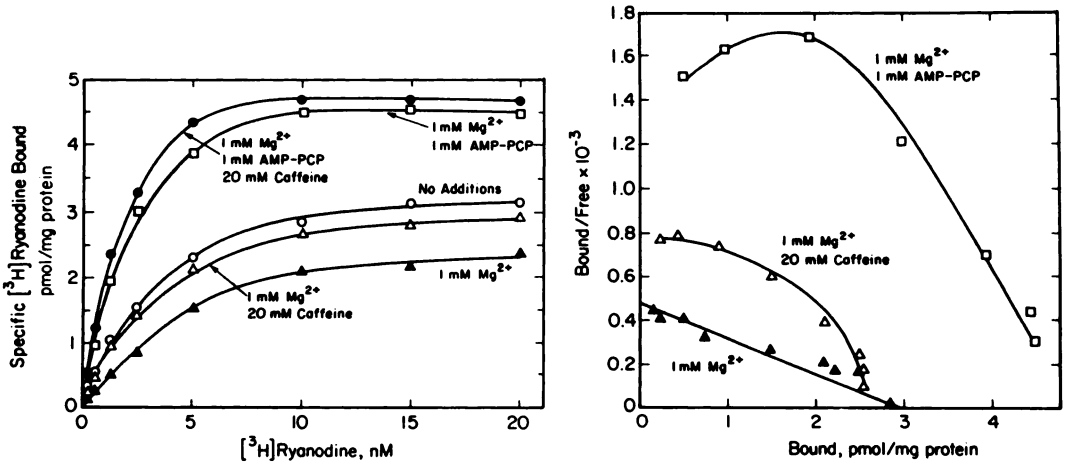


Fig. 3. Modulation of [^{3}H]ryanodine saturation isotherms by Mg^{2+}, AMP-PCP, and caffeine singly or in tandem at optimal (60 μM) Ca^{2+}. Scatchard plots demonstrate the appearance of positive cooperativity at the alkaloid-binding site in the presence of Mg^{2+} and AMP-PCP or caffeine. Equilibrium constants are summarized in Table 2.

TABLE 2

Effect of Mg^{2+}, caffeine, and AMP-PCP on affinity (K_d), degree of cooperativity (n_H), and receptor density (B_{max}) of [^{3}H]ryanodine-binding sites

K_d and n_H were calculated from Hill plots and summarized as the means and standard deviations of at least three experiments. B_{max} values and standard deviations were obtained directly from binding isotherms.

Additions	K_d	n_H	B_{max}
	nM		*pmol/mg of protein*
None	2.2 ± 0.2	1.3 ± < 0.1	3.1 ± 0.2
Mg^{2+} (1 mM)			
Alone	4.5 ± 0.5	1.1 ± 0.2	1.9 ± 0.3
AMP-PCP (1 mM)	1.6 ± 0.1	1.6 ± 0.2	4.7 ± 0.7
cAMP (1 mM)	1.9 ± 0.3	1.7 ± 0.3	3.6 ± 0.4
Caffeine (20 mM)	2.3 ± 0.3	1.7 ± 0.3	2.6 ± 0.4
AMP-PCP (1 mM) + caffeine (20 mM)	1.7 ± 0.1	1.5 ± 0.1	4.6 ± 0.8
cAMP (1 mM) + caffeine (20 mM)	2.6 ± 0.3	1.3 ± 0.2	3.9 ± 0.6

TABLE 3

Effects of caffeine and AMP-PCP singly and in combination on [^{3}H]ryanodine binding at suboptimal (2 μM) and optimal (100 μM) free Ca^{2+} levels

Additions	[^{3}H]Ryanodine binding[a]	
	2 μM Ca^{2+}	100 μM Ca^{2+}
mM	*pmol/mg of protein*	
None	0.04 ± 0.02	1.7 ± 0.2
Caffeine		
1	0.05 ± >0.01	1.6 ± 0.1
3	0.06 ± >0.01	2.2 ± 0.1
10	0.14 ± 0.02	2.1 ± 0.2
30	0.39 ± 0.06	2.3 ± 0.2
AMP-PCP		
0.1	0.03 ± 0.01	1.3 ± 0.1
0.3	0.04 ± <0.01	2.6 ± 0.1
1.0	0.04 ± <0.01	3.3 ± 0.3
3.0	0.15 ± <0.01	3.9 ± 0.3
Caffeine + AMP-PCP		
1 + 0.1	0.04 ± 0.01	1.6 ± 0.1
3 + 0.3	0.08 ± <0.01	2.7 ± 0.2
10 + 1.0	1.3 ± 0.2	3.3 ± 0.1
30 + 3.0	2.7 ± 0.1	3.9 ± 0.3

[a] Binding was determined in assay buffer with 1 mM Mg^{2+} and 80 min incubation.

AMP-PCP, cAMP, or caffeine at Ca^{2+} levels ranging from 2 to 300 μM.

Effect of modulators on [^{3}H]ryanodine binding kinetics. The pseudo-first order rate constant for association (k_{+1}) of [^{3}H]ryanodine with its binding site in assay medium containing 100 μM Ca^{2+} but lacking Mg^{2+} is 5.8 × 10^6 M^{-1} min^{-1} ($t_{1/2}$ = 23.1 min), whereas the presence of 1 mM Mg^{2+} reduces the k_{+1} to 3.6 × 10^6 M^{-1} min^{-1} ($t_{1/2}$ = 36.5 min) (Table 4, Fig. 4). Caffeine at 20 mM only partially restores k_{+1} (to 5.0 × 10^6 M^{-1} min^{-1}) when assayed at optimal Ca^{2+} but elicits a dramatic increase in k_{+1} in 10 μM Ca^{2+}, i.e., from immeasurable levels of specific [^{3}H]ryanodine binding in the presence of 1 mM Mg^{2+} to a k_{+1} = 4.4 × 10^6 M^{-1} min^{-1} ($t_{1/2}$ = 30.1 min) with addition of 20 mM caffeine. In the presence of 1 mM AMP-PCP or cAMP, the effects of Mg^{2+} on k_{+1} are restored to a rate faster than or not significantly different from that measured in the absence of Mg^{2+} (k_{+1} = 7.4 × 10^6 and 6.2 × 10^6 M^{-1} min^{-1},

TABLE 4

Effects of Mg^{2+}, caffeine, and adenine nucleotides on the association rate of [^{3}H]ryanodine at suboptimal (10 μM) and optimal (100 μM) Ca^{2+} levels

Additions	k_{obs} (× 10^{-2})[a]	k_{+1} (× 10^6)[b]	t_w[c]
	min^{-1}	M^{-1} min^{-1}	*min*
10 μM Ca^{2+}			
Alone	1.4 ± 0.06	2.6	49.5
Mg^{2+} (1 mM)	<1.0		
Mg^{2+} (1 mM) + caffeine (20 mM)	2.3 ± 0.1	4.4	30.1
100 μM Ca^{2+}			
Alone	3.0 ± 0.8	5.8	23.1
Mg^{2+} (1 mM)	1.9 ± 0.3	3.6	36.5
Mg^{2+} (1 mM) + AMP-PCP (1 mM)	3.8	7.4	18.2
Mg^{2+} (1 mM) + cAMP (1 mM)	3.2 ± 0.2	6.2	21.6
Mg^{2+} (1 mM) + caffeine (20 mM)	2.6 ± 0.4	5.0	26.7
Mg^{2+} (1 mM) + AMP-PCP or cAMP (1 mM) + caffeine (20 mM)	3.7 ± 0.4	7.2	18.7

[a] Data are means and standard deviations of at least three experiments or the means of duplicate experiments.

[b] Pseudo-first order rate constant based on k_{-1} = 8.0 (± 0.9) × 10^{-4} (see Fig. 4).

[c] Half-time to reach equilibrium.

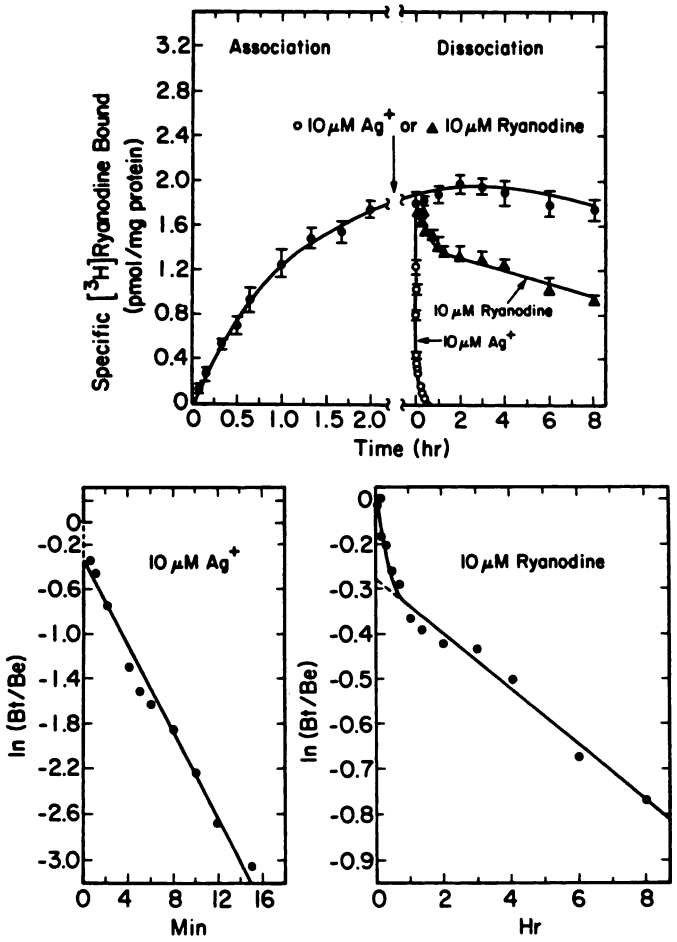


Fig. 4. Association of [^{3}H]ryanodine and subsequent dissociation brought about by addition (↓) of unlabeled ryanodine (10 μM) or Ag^+ (10 μM). Equilibrium was established in assay buffer containing 100 μM Ca^{2+} and 1 mM Mg^{2+}. Natural logarithmic transformation yields k_{-1} for added ryanodine and Ag^+ of 8.0 (±0.9) × 10^{-4} and 0.15 ± 0.02 min^{-1} for 75% (±2%) of all binding sites, respectively (Table 5). The fast component for added ryanodine yields k_{-1} of 0.006 ± 10%, whereas that for added Ag^+ proceeds too rapidly for accurate determination by conventional filtration.

respectively). Caffeine and adenine nucleotides in tandem yield a k_{+1} of 7.2 M^{-1} min^{-1} ($t_{1/2}$ = 18.7 min), even in the presence of 1 mM Mg^{2+} (Table 4).

Dissociation of the Ca^{2+}-[^{3}H]ryanodine receptor complex is bi- or polyphasic with an extremely slow component (representing 75 ± 2% of all binding sites) when a 2000-fold level of unlabeled ryanodine is added to the equilibrium complex (k_{-1} = 0.00080 min^{-1}; $t_{1/2}$ = 14.4 hr) (Fig. 4, Table 5). The calculated K_d value based on kinetic rate constants is ~20 times lower than the apparent K_d value obtained from the equilibrium binding curves (0.22 versus 4.5 nM, respectively) (Tables 2 and 5). In contrast, addition of Ag^+ at 10 μM to the equilibrium complex results in rapid dissociation of [^{3}H]ryanodine (k_{-1} = 0.151 min^{-1}), yielding a calculated K_d value 9 times higher than that obtained from equilibrium experiments (41 versus 4.5 nM, respectively), whereas addition of 10 μM DTNB results in a k_{-1} of 0.017 min^{-1}, yielding a calculated K_d not significantly different from equilibrium experiments (5.2 versus 4.5 nM, respectively).

Discussion

Putative ligand-gated Ca^{2+} channels at the SR are involved in releasing the Ca^{2+} required to activate the contractile elements in the sarcoplasm and hence may constitute a fundamental unit of the EC coupling process. Ca^{2+}-induced Ca^{2+} release from the SR is implicated with EC coupling in both skeletal and cardiac muscle, but its physiological role remains to be firmly established especially in skeletal muscle (1–8). There is a close parallel between regulation of the [^{3}H]ryanodine-binding sites found in muscle membrane preparations and of the Ca^{2+}-induced Ca^{2+} release channels of skinned and intact muscle fibers and SR vesicle preparations including: localization at the TC SR; activation and inactivation by Ca^{2+}; modulation by Mg^{2+}, adenine nucleotides, and caffeine; and inhibition by ruthenium red (25–28). Furthermore, nM levels of ryanodine promote $^{45}Ca^{2+}$ efflux (or inhibit its reuptake) from actively or passively loaded vesicles of skeletal or cardiac origin and directly interfere with the inactivation of Ca^{2+}-induced release of Ca^{2+} from actively loaded skeletal vesicles, suggesting that the alkaloid's primary lesion is to interfere with the complete inactivation of the channels involved (26, 28, 29).

The mechanism by which Ca^{2+}, Mg^{2+}, caffeine, and adenine nucleotides influence the Ca^{2+} activation site and the alkaloid (*Ry*)-binding site serves as a basis for a proposed model for the regulation of channel (*Ch*) gating (Scheme 1).

The following discussion sequentially considers each step in this model. Ca^{2+}, in the presence of physiological levels of Mg^{2+} and adenine nucleotide, is essential for activating (or unmasking) the [^{3}H]ryanodine-binding sites to a state which will recognize the alkaloid and permit its binding by regulating their number, affinity, and degree of cooperativity in binding the ligand (Fig. 2; Refs. 25 and 27). Ag^+ induces Ca^{2+} release from heavy SR vesicles apparently by interacting with critical sulfhydryl moieties at the Ca^{2+} trigger site (36). Results with [^{3}H]ryanodine support this hypothesis. Dissociation of the Ca^{2+}-[^{3}H]ryanodine receptor equilibrium complex is markedly influenced by Ag^+ probably acting as a nonspecific sulfhydryl-derivatizing reagent; DTNB (Table 5) and other aryldisulfides[1] have a similar but apparently more specific effect on thiols at the Ca^{2+} activator site. Whereas this equilibrium complex is only slowly reversed by excess unlabeled ryanodine (suggesting an occluded alkaloid-binding domain), derivatization of specific thiols with DTNB, possibly at the more accessible Ca^{2+}-regulatory site, results in rapid dissociation of the equilibrium complex and agreement between the apparent K_d (from equilibrium binding curves) and the calculated K_d from kinetic constants (Table 5). These results suggest that Ag^+-induced Ca^{2+}

TABLE 5

Dissociation of the Ca^{2+}-[^{3}H]ryanodine receptor equilibrium complex by 10 μM unlabeled ryanodine, Ag^+, or DTNB

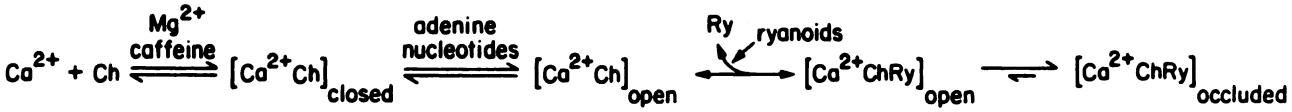
Dissociating agent (10 μM)	k_{-1}[a]	K_d[b]	K_d apparent/K_d calculated[c]
	min^{-1}	nM	
Ryanodine	8.0 (±0.9) ×10^{-4}	0.22	20
Ag^+	1.5 (±0.2) ×10^{-1}	41	0.1
DTNB	1.7 (±0.1) ×10^{-2}	5.2	0.9

[a] The dissociation rate constant determined by addition of 10 μM compound to the equilibrium complex containing 5 nM [^{3}H]ryanodine, 1 mM Mg^{2+}, and 100 μM Ca^{2+} in the assay buffer.

[b] The calculated dissociation constant based on k_{+1} = 3.6 × 10^6 M^{-1} min^{-1} (see Table 4).

[c] Ratio of the apparent K_d estimated from equilibrium experiment (4.5 nM) and the calculated K_d from kinetic constants.

[1] I. N. Pessah, unpublished results.



Scheme 1.

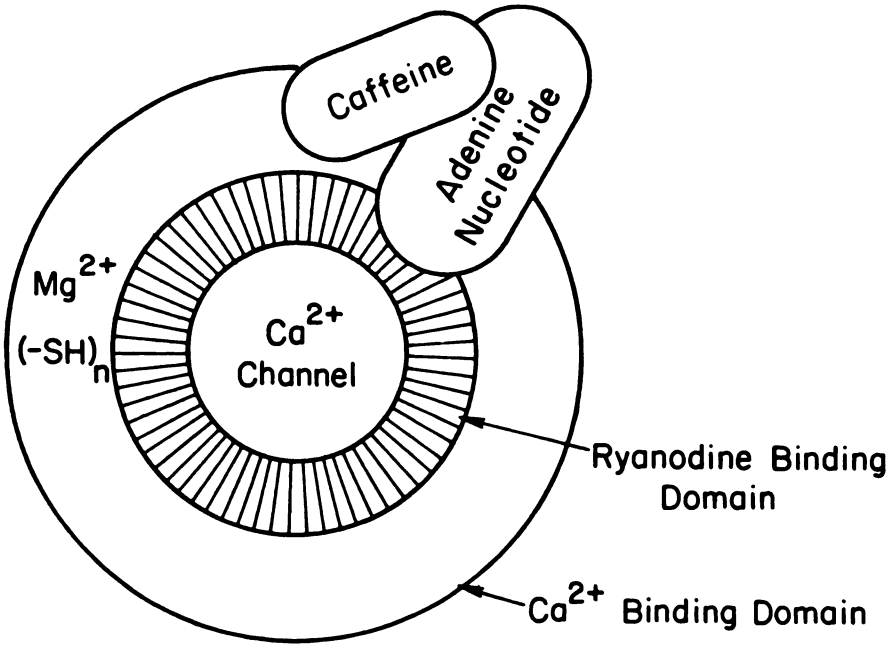


Fig. 5. Schematic diagram of Ca^{2+}-ryanodine receptor complex in its activated state, i.e., the Ca^{2+} channel is open and the [^{3}H]ryanodine-binding site is accessible. This model conceptualizes possible relationships of protein components interacting with modulators of [^{3}H]ryanodine binding and hence channel opening and closing. The ryanodine binding domain is placed proximal to the channel and undergoes rapid occlusion of the alkaloid to a form inaccessible for exchange with ryanoids. The Ca^{2+} binding domain is positioned peripherally since Ag^+- and DTNB-sensitive sulfhydryl groups at the Ca^{2+} binding domain remain accessible upon formation of the Ca^{2+}-ryanodine receptor complex. Two additional domains allosterically modulate [^{3}H]ryanodine binding. The first binds caffeine, thereby having a direct influence on the sensitivity of the Ca^{2+} activator site to Ca^{2+}, whereas the second binds adenine nucleotides, influencing the efficiency of channel opening. These modulatory domains possibly overlap as indicated by the significant synergistic effect at suboptimal Ca^{2+}.

release proceeds via an open state of the TC SR channel not recognized by ryanodine.

Mg^{2+} at physiological levels inhibits [^{3}H]ryanodine equilibrium binding by decreasing both the affinity (K_d) and the number of alkaloid-binding sites (B_{na} and B_{max}) by altering the apparent affinity of the activator site for Ca^{2+} (K_d/Ca^{2+}). It also significantly retards the association kinetics of ligand binding. These properties indicate that Mg^{2+} inhibits the Ca^{2+}-gated open state of the TC SR channels (i.e., the state which exposes the ryanodine binding domain) by direct competition between Mg^{2+} and Ca^{2+} for the activator site, in agreement with findings with skinned cardiac fibers (37). Mg^{2+} may act to reduce the sensitivity of TC SR channels to the Ca^{2+} signal, especially when the localized levels of ATP drop.

Caffeine stimulates [^{3}H]ryanodine binding primarily by increasing the affinity of the activator site for Ca^{2+} which, even in the presence of physiological levels of Mg^{2+}, nearly restores the [^{3}H]ryanodine-binding site density and fully restores the affinity to the level observed in the absence of Mg^{2+}. In fact, caffeine does not significantly influence the formation of the Ca^{2+}-ryanodine receptor complex in the absence of Mg^{2+} and in the presence of optimal Ca^{2+} (27). Caffeine therefore appears to act on a domain which allosterically modulates the sensitivity of the Ca^{2+} activator site for Ca^{2+}, reversing Mg^{2+} inhibition by selectively increasing the apparent affinity of the Ca^{2+} binding site for Ca^{2+}. This action of caffeine on formation of the Ca^{2+}-ryanodine receptor complex parallels its effect on Ca^{2+}-induced release of Ca^{2+} from skinned muscle fibers and membrane vesicles (12–15), providing further evidence that [^{3}H]ryanodine binding reflects the functional Ca^{2+}-release channel at the TC SR.

AMP-PCP and cAMP also modulate the Ca^{2+}-ryanodine receptor complex apparently by noncovalent (i.e., not involving phosphorylation) interaction with a site allosteric to the alkaloid binding domain. ATP levels in the sarcoplasm may therefore play an important physiological role in modulating the Ca^{2+} release channel. Adenine nucleotides stimulate [^{3}H]ryanodine binding by a mechanism different from that of caffeine in causing a dramatic increase in B_{na} to a level above that observed in the absence of Mg^{2+} while having much less effect on the affinity of the activator site for Ca^{2+}. These results suggest that adenine nucleotides exert their stimulation at a site distinct from the caffeine (and Mg^{2+}) modulatory domains. Unlike caffeine, AMP-PCP (or cAMP) completely restores the association rate of complex formation to a level observed in the absence of Mg^{2+}. We propose that, unlike caffeine and Mg^{2+}, which influence the alkaloid binding domain indirectly by modulating the Ca^{2+} activator site, adenine nucleotides influence the kinetics of channel opening once Ca^{2+} is bound to the activator site either by increasing the efficiency of the gating mechanism or by increasing the duration of the open state of the channel. However, the expression of these modulatory domains on the [^{3}H]ryanodine-binding site are strongly influenced by each other, especially at suboptimal free Ca^{2+} and/or nonsaturating alkaloid levels as demonstrated by the dramatic rise in the positive cooperativity in Ca^{2+} activation of [^{3}H] ryanodine binding (Table 1). Under these conditions, pronounced synergism between caffeine and adenine nucleotides suggests that these domains are in close proximity to or partially overlap with one another.

Our findings with [^{3}H]ryanodine and skeletal muscle TC SR membrane preparations demonstrate the highly regulated nature of the Ca^{2+}-ryanodine receptor complex whose properties closely parallel those of the Ca^{2+}-induced Ca^{2+} release channel

of physiological significance in cardiac muscle. We recognize that at least four functional domains comprise the Ca^{2+}-ryanodine receptor complex (Fig. 5): 1) a regulatory domain responsible for gating the Ca^{2+} channel which binds Ca^{2+} (and Mg^{2+}) and contains critical sulfhydryl moieties; 2) an alkaloid binding domain in close proximity to the channel which binds ryanodine only in its Ca^{2+}-activated state and rapidly occludes upon complex formation; 3) a domain which binds caffeine and directly influences the sensitivity of the Ca^{2+} regulatory site to Ca^{2+}; and 4) a domain which binds adenine nucleotides and influences the gating efficiency or lifetime of the open channel. Cooperative regulation of the Ca^{2+}-ryanodine receptor complex and its elution as a high molecular weight oligomer (27) may reflect a functional aggregation of TC SR proteins constituting the channels involved in Ca^{2+} release.

Acknowledgments

Helpful suggestions were provided by Alexandre Fabiato of the Department of Physiology, Medical College of Virginia, Richmond, Virginia. The skillful technical assistance of Alexander Francini is gratefully acknowledged.

References

1. Martonosi, A. N. Mechanisms of Ca^{2+} release from sarcoplasmic reticulum of skeletal muscle. *Physiol. Rev.* **64**:1240–1320 (1984).
2. Fabiato, A., and F. Fabiato. Calcium release from the sarcoplasmic reticulum. *Circ. Res.* **40**:119–129 (1977).
3. Endo, M., Y. Kakuta, and T. Kitazawa. A further study of the Ca-induced Ca release mechanism, in *The Regulation of Muscle Contraction: Excitation Contraction Coupling* (A. D. Grinnel and M. A. B. Brazier, eds.). Academic Press, New York, 181–194 (1981).
4. Ford, L. E., and R. J. Podolsky. Regenerative calcium release within muscle cells. *Science (Wash. D. C.)* **167**:58–69 (1970).
5. Endo, M., and Y. Nakajima. Release of calcium induced by "depolarisation" of the sarcoplasmic reticulum membrane. *Nature New Biol.* **246**:216–218 (1978).
6. Miyamoto, H., and E. Racker. Mechanism of calcium release from skeletal sarcoplasmic reticulum. *J. Membr. Biol.* **66**:193–201 (1982).
7. Meissner, G. Adenine nucleotide stimulation of Ca^{2+}-induced Ca^{2+} release in sarcoplasmic reticulum. *J. Biol. Chem.* **259**:2365–2374 (1984).
8. Chamberlain, B. K., P. Volpe, and S. Fleischer. Calcium-induced calcium release from purified cardiac sarcoplasmic reticulum vesicles. *J. Biol. Chem.* **259**: 7540–7546 (1984).
9. Smith, J. S., R. Coronado, and G. Meissner. Sarcoplastic reticulum contains adenine nucleotide-activated channels. *Nature (Lond.)* **316**:446–449 (1985).
10. Vergara, J., R. Y. Tsien, and H. Delay. Inositol 1,4,5-trisphosphate: a possible chemical link in excitation-contraction coupling in muscle. *Proc. Natl. Acad. Sci. USA* **82**:6352–6356 (1985).
11. Volpe, P., G. Salviati, F. D. Virgilio, and T. Pozzan. Inositol 1,4,5-trisphosphate induces calcium release from sarcoplasmic reticulum of skeletal muscle. *Nature (Lond.)* **316**:347–349 (1985).
12. Endo, M. Calcium release from the sarcoplasmic reticulum. *Physiol. Rev.* **57**:71–108 (1977).
13. Kim, D. H., S. T. Ohnishi, and N. Ikemoto. Kinetic studies of calcium release from sarcoplasmic reticulum *in vitro*. *J. Biol. Chem.* **258**:9662–9668 (1983).
14. Kirino, Y., M. Osakabe, and H. Shimizu. Ca^{2+}-induced Ca^{2+} release from fragmented sarcoplasmic reticulum: Ca^{2+}-dependent passive Ca^{2+} efflux. *J. Biochem.* **94**:1111–1118 (1983).
15. Nagasaki, K., and M. Kasai. Channel selectivity and gating specificity of calcium-induced calcium release channel in isolated sarcoplasmic reticulum. *J. Biochem.* **96**:1769–1775 (1984).
16. Fabiato, A. Calcium-induced release of calcium from the sarcoplasmic reticulum. *J. Gen. Physiol.* **85**:189–320 (1985).
17. Jenden, D. J., and A. S. Fairhurst. The pharmacology of ryanodine. *Pharmacol. Rev.* **21**:1–25 (1969).
18. Fairhurst, A. S., and W. Hasselbach. Calcium efflux from a heavy sarcotubular fraction: effects of ryanodine, caffeine and magnesium. *Eur. J. Biochem.* **13**:504–509 (1970).
19. Fairhurst, A. S. Effect of ryanodine on skeletal muscle reticulum calcium adenosine triphosphatase (CaATPase). *Biochem. Pharmacol.* **22**:2815–2827 (1973).
20. Sutko, J. L., J. T. Willerson, G. H. Templeton, L. R. Jones, and H. R. Besch, Jr. Ryanodine: its alterations of cat papillary muscle contractile state and responsiveness to inotropic interventions and a suggested mechanism of action. *J. Pharmacol. Exp. Ther.* **209**:37–47 (1979).
21. Sutko, J. L., and J. L. Kenyon. Ryanodine modification of cardiac muscle responses to potassium-free solutions: evidence for inhibition of sarcoplasmic reticulum calcium release. *J. Gen. Physiol.* **82**:385–404 (1983).
22. Hilgemann, D. W., M. J. Delay, and G. A. Langer. Activation-dependent cumulative depletions of extracellular free calcium in guinea pig atrium measured with antipyrylazo III and tetramethyl-murexide. *Circ. Res.* **53**:779–793 (1983).
23. Seiler, S., A. D. Wegener, D. D. Whang, D. R. Hathaway, and L. R. Jones. High molecular weight proteins in cardiac and skeletal muscle junctional sarcoplasmic reticulum vesicles bind calmodulin, are phosphorylated, and are degraded by Ca^{2+}-activated protease. *J. Biol. Chem.* **259**:8550–8557 (1984).
24. Fabiato, A. Effects of ryanodine in skinned cardiac cells. *Fed. Proc.* **44**:2970–2976 (1985).
25. Pessah, I. N., A. L. Waterhouse, and J. E. Casida. The calcium-ryanodine receptor complex of skeletal and cardiac muscle. *Biochem. Biophys. Res. Commun.* **128**:449–456 (1985).
26. Fleischer, S., E. M. Ogunbunmi, M. C. Dixon, and E. A. M. Fleer. Localization of Ca^{2+} release channels with ryanodine in junctional terminal cisternae of sarcoplasmic reticulum of fast skeletal muscle. *Proc. Natl. Acad. Sci. USA* **82**:7256–7259 (1985).
27. Pessah, I. N., A. O. Francini, D. J. Scales, A. L. Waterhouse, and J. E. Casida. Calcium-ryanodine receptor complex: solubilization and partial characterization from skeletal muscle junctional sarcoplasmic reticulum vesicles. *J. Biol. Chem.* **261**:8643–8648 (1986).
28. Meissner, G. Ryanodine activation and inhibition of the Ca^{2+} release channel of sarcoplasmic reticulum. *J. Biol. Chem.* **261**:6300–6306 (1986).
29. Pessah, I. N., K. W. Anderson, and J. E. Casida. Solubilization and separation of Ca^{2+}-ATPase from the Ca^{2+}-ryanodine receptor complex. *Biochem. Biophys. Res. Commun.* **139**:235–243 (1986).
30. Waterhouse, A. L., I. Holden, and J. E. Casida. 9,21-Didehydroryanodine: a new principal toxic constituent of the botanical insecticide *Ryania*. *J. Chem. Soc.Chem.Commun.* 1265–1266 (1984).
31. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
32. Fabiato, A. Myoplasmic free calcium concentration reached during the twitch of an intact isolated cardiac cell and during calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned cardiac cell from the adult rat or rabbit ventricle. *J. Gen. Physiol.* **78**:457–497 (1981).
33. Hill, A. W. The possible effects of the aggregation of the molecules of hemoglobin on its dissociation curves. *J. Physiol. (Lond.)* **40**:iv–vii (1910).
34. Bennett, J. P., Jr. Methods in binding studies, in *Neurotransmitter Receptor Binding* (H. I. Yamamura, S. J. Enna, and M. J. Kuhar, eds.). Raven Press, New York, 57–90 (1978).
35. Kleinbaum, D. G., and L. L. Kupper. *Applied Regression Analysis and Other Multivariate Methods.* Duxbury Press, North Scituate, MA (1978).
36. Salama, G., and J. Abramson. Silver ions trigger Ca^{2+} release by acting at the apparent physiological release site in sarcoplasmic reticulum. *J. Biol. Chem.* **259**:13363–13369 (1984).
37. Endo, M. Mechanism of calcium-induced calcium release in the SR membrane, in *The Mechanism of Gated Calcium Transport Across Biological Membranes* (S. T. Ohnishi and M. Endo, eds.). Academic Press, New York, 257–264 (1981).

Send reprint requests to: Dr. Isaac N. Pessah, Pesticide Chemistry and Toxicology Laboratory, Department of Entomological Sciences, University of California, Berkeley, CA 94720.