ACCELERATED COMMUNICATION

Noncoplanar PCB 95 Alters Microsomal Calcium Transport by an Immunophilin FKBP12-Dependent Mechanism

PATTY W. WONG and ISAAC N. PESSAH

Department of Molecular Biosciences, School of Veterinary Medicine, University of California, Davis, California 95616

SUMMARY

Ortho-substituted polychlorinated biphenyls (PCBs) have been shown to alter microsomal Ca^{2+} transport by selective interaction with ryanodine receptors (RyRs) of muscle sarcoplasmic reticulum (SR) and brain endoplasmic reticulum. The mechanism underlying the actions of PCBs on Ca^{2+} transport is further elucidated with skeletal SR enriched in Ry_1R. Disruption of the association between immunophilin FKBP12 and Ry_1R with FK 506 or rapamycin completely eliminates PCB 95-enhanced binding of $[^3H]$ryanodine ($IC_{50} \sim 35~\mu M$) to Ry_1R and PCB 95-induced release of Ca^{2+} from actively loaded SR vesicles ($IC_{50} \sim 11~\mu M$), demonstrating a FKBP12-dependent mechanism. FK 506 selectively eliminates PCB 95-induced Ca^{2+} release from SR because Ry_1R maintains responsiveness to caffeine and Ca^{2+}. PCB 95 and FK 506 are used to examine the relationship between ryanodine-sensitive Ca^{2+} channels and ryanodine-insensitive Ca^{2+} leak pathways present in SR vesicles. Micromolar ryanodine completely blocks ryanodine-sensitive Ca^{2+} efflux but neither eliminates the ryanodine-in-sensitive Ca^{2+} leak unmasked by thapsigargin nor enhances the loading capacity of SR vesicles. PCB 95 alone enhances thapsigargin evoked Ca^{2+} release and therefore diminishes the loading capacity of SR vesicles. However, in the presence of micromolar ryanodine, PCB 95 dose-dependently eliminates the Ca^{2+} leak unmasked by thapsigargin and significantly enhances the loading capacity of SR vesicles. The actions of PCB 95 on SR-loading capacity are additive with those of FK 506. Structural specificity for these novel actions are further demonstrated with coplanar PCB 126, which is inactive toward Ry_1R and lacks the ability to alter the Ca^{2+} leak pathway. The results reveal that FKBP12 relates ryanodine-insensitive Ca^{2+} "leak" and ryanodine-sensitive Ca^{2+} channel efflux pathways of SR by modulating distinct conformations Ry_1R complexes. Noncoplanar PCBs, like PCB 95, alter SR Ca^{2+} buffering by an FKBP12-mediated mechanism. An immunophilin-based mechanism could account for the toxic actions attributed to certain noncoplanar PCB congeners.

Temporal and spatial changes in intracellular Ca^{2+} are known to regulate a large number of cellular functions. Because Ca^{2+} plays a central role in signaling, cells have evolved a very effective array of pumps and exchangers to extrude Ca^{2+} across the plasmalemma or store Ca^{2+} within intracellular organelles. SERCA pumps, for example, effectively accumulate Ca^{2+} within the lumen of ER and SR, thereby creating a major intracellular Ca^{2+} buffer. Macromolecules that contribute to the rise in cytosolic Ca^{2+} include ligand- and voltage-operated Ca^{2+} entry channels within the plasma membrane, Ca^{2+} release channels localized within ER and SR membranes, and the mitochondrial permeability transition pore (1, 2). Two classes of ligand-gated Ca^{2+} release channels are localized within specialized regions of

ER/SR: (i) IP$_3$-sensitive Ca^{2+} release channels (IP$_3$Rs) and (ii) ryanodine-sensitive Ca^{2+} release channels (RyRs). Although these two classes of Ca^{2+} release channels share sequence homology and structural similarity, their differences in pharmacology, conductance properties, distribution among various tissues, and distribution within individual cell types suggest different functional roles in cellular signaling. Microsomal Ca^{2+} release channels exist in association with several accessory proteins. One of the accessory proteins, immunophilin FKBP12 is tightly associated with both IP$_3$R and RyR proteins (3). The association of FKBP12 with Ry_1R channel complex seems to be important in regulating aspects of Ry_1R channel gating behavior because its removal from Ry_1R promotes subconductance states, increases open

---

ABBREVIATIONS: SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}/ATPase; APIII, antipyrylazo III; CICR, Ca^{2+}-induced Ca^{2+} release; DMSO, dimethylsulfoxide; ER, endoplasmic reticulum; FKBP12, FK 506 binding protein of 12 kDa; IP$_3$R, inositol-1,4,5-trisphosphate receptor; PCB, polychlorinated biphenyl; RyR, ryanodine receptor; Ry_1R, skeletal isoform of ryanodine receptor; SR, sarcoplasmic reticulum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

probability, and alters channel sensitivity toward agonists such as caffeine and Ca^{2+} (4). Similarly, the association of FKBP12 with IP_3R seems to stabilize the channel complex and promote optimal cooperativity among subunits (5). Chemical substances that modify the native interaction between FKBP12 and RyRs would be expected to alter microsomal Ca^{2+} transport and influence one or more Ca^{2+}-dependent processes downstream.

Results from *in vivo* studies with animals (6), *in vitro* studies with cell cultures (7), and studies with subcellular membrane preparations (8–10)[1] have revealed that certain *ortho*-substituted PCB congeners possess potent biological activity toward the nervous system. Inhibition of Ca^{2+}-dependent ATPase activity has been suggested to be a major molecular mechanism by which these noncoplanar PCBs induce neurotoxicity (10). Recently, certain PCBs have been demonstrated to mobilize microsomal Ca^{2+} by direct interaction with RyRs localized within muscle SR and neuronal ER, without markedly altering SERCA pumps or IP_3R activities (8).[1] The exact mechanism by which PCB 95 alters microsomal Ca^{2+} transport and the function of RyRs is unclear. One of the most potent and efficacious PCB congeners found to alter RyRs activity, noncoplanar PCB 95 (2,2′,3,5′,6-pentachlorobiphenyl) has also been recently shown to alter neuroplasticity in the rat hippocampal slice preparations (11). PCB 95 administered to rats perinatally results in offspring exhibiting significant depression in locomotor activity and altered performance in the radial arm maze, which assesses spatial learning and memory functions.[2] However, the relationship between the effect of PCB 95 on RyR functions and its effect on neuroplasticity in mammalian brain is unknown.

In the current study, we used skeletal SR enriched in FKBP12/Ry_1R complex to elucidate the mechanism by which PCB 95 alters SR Ca^{2+} transport. A significant new finding was that PCB 95 mobilizes Ca^{2+} through an FKBP12-dependent mechanism. Dissociation of FKBP12 from the Ry_1R channel complex with FK 506 completely negates PCB 95-induced Ca^{2+} release from skeletal SR, even though the channel remains fully responsive to caffeine and Ca^{2+}. FK 506 is found to dramatically enhance the steady state filling capacity of SR vesicles for Ca^{2+}, consistent with the role of FKBP12 in regulating channel and leak states of Ry_1R (5, 12). PCB 95 is shown to significantly enhance ryanodine-sensitive Ca^{2+} release and concomitantly eliminate a ryanodine-insensitive Ca^{2+} "leak" normally present in SR membranes. The present results reveal that *ortho*-substituted PCB 95 alters Ca^{2+} buffering in the microsome by directly interacting with the FKBP12/RyR complex. Considering the important role of FKBP12 in regulating immune and neuronal cell functions, as well as the differential expression of RyR isoforms in distinct regions of the central and peripheral nervous systems of mammals (13), this newly identified mechanism may play an important role in the toxicity of noncoplanar PCBs.

[1] P. W. Wong, W. R. Brackney, and I. N. Pessah. Ortho-substituted PCBs alter microsomal calcium transport by direct interaction with ryanodine receptors of mammalian brain. Submitted for publication.

[2] S. L. Schantz, B. W. Seo, P. W. Wong, and I. N. Pessah. Long-term effects of developmental exposure to 2,2′,3,5′,6-pentachlorobiphenyl (PCB 95) on locomotor activity, spatial learning and memory and brain ryanodine binding. Submitted for publication.

Experimental Procedures

Materials. Neat Ultra-certified PCB congeners (purity, > 99%) were purchased from Ultra Scientific (North Kingstown, RI). [^{3}H]Ryanodine (specific activity, 60–80 Ci/mmol; purity, > 99%) was obtained from New England Nuclear (Wilmington, DE). FK 506 and rapamycin (purity, > 95%) were purchased from Signal Transduction (San Diego, CA). Thapsigargin was obtained from Sigma Chemical (St. Louis, MO). All other chemicals were of the highest grade available commercially.

Membrane preparations. Junctional SR membrane vesicles enriched in the skeletal isoform of ryanodine receptor, Ry_1R, were prepared from fast-twitch skeletal muscle obtained from 3–4-kg male New Zealand White rabbits according to a previously reported method (8).

[^{3}H]Ryanodine binding assays. Measurements on specific binding of [^{3}H]ryanodine to skeletal SR closely followed a previously reported method (8). The ability of FK 506 and rapamycin to modulate high affinity binding of 1 nM [^{3}H]ryanodine to Ry_1R in the presence and absence of PCB 95 was determined by incubating 0–200 μM FK 506 or rapamycin in an assay buffer consisting of 12.5 μg of skeletal SR protein, 140 mM KCl, 15 mM NaCl, 20 mM HEPES, pH 7.4, 10% sucrose, 50 μM $CaCl_2$, 1 mM $MgCl_2$, and 0, 0.6, or 1 μM PCB 95 in a final volume of 250 μl. The reaction mixtures were allowed to equilibrate at 37° for 3.5 hr with constant shaking. Values of IC_{50} and Hill coefficients were calculated by sigmoidal curve fitting of the dose-response curves using ENZFITTER computer software (Elsevier-Biosoft, Cambridge, UK). Experiments were performed in duplicate and repeated at least twice with two different membrane preparations.

Ca^{2+} transport measurements. Net Ca^{2+} flux across the SR membrane vesicles was monitored by metallochromic dye APIII according to a previously reported method (8). Vesicles were actively loaded to capacity by serial additions of 24 nmol of $CaCl_2$ in the presence of ATP and a regenerating system consisting of creatine phosphokinase and phosphocreatine at 37°. The abilities of FK 506 and rapamycin to inhibit PCB 95-induced Ca^{2+} release from loaded SR membrane vesicles were determined by preincubating the vesicles with 0–50 μM rapamycin before Ca^{2+} loading or adding 50 μM FK 506 3 min before the introduction of PCB 95. PCB 95 (1 μM) was introduced to the assay to assess its ability to mobilize Ca^{2+} from the drug-treated vesicles.

Caffeine-induced Ca^{2+} release and CICR responses of SR vesicles were studied in the presence of FK 506 or PCB 95. FK 506 (50 μM) or 6 μl of DMSO (control) was introduced to the actively loaded SR vesicles, and the assay mixture was permitted to incubate for ~3 min at 37°. Then, 1 μM PCB 95 was added and immediately followed by 10 mM caffeine or 84 nmol of Ca^{2+} to assess the responsiveness of the Ca^{2+} release channel to modulators known to have effector sites on the Ry_1R protein. In experiments aimed at assessing CICR, once the release phase was complete, 500 μM ryanodine was introduced into the assay mixture to fully block Ry_1R, thus initiating active reaccumulation of Ca^{2+} into the vesicles.

The ability of selected PCB congeners to modulate thapsigargin-evoked Ca^{2+} efflux from SR was examined in the presence or absence of channel-blocking concentration of ryanodine. Membrane vesicles were loaded to near capacity as described above. Once the loading phase was complete, 375 nM thapsigargin was introduced to inhibit the SERCA pump and therefore block active Ca^{2+} uptake immediately after the addition of 5 μM PCB 95 or PCB 126. The ability of PCBs to modulate the ryanodine-insensitive Ca^{2+} efflux was studied by preincubating the vesicles for 3 min with 0–10 μM PCB 95 or 5 μM PCB 126 in the presence of 500 μM ryanodine to block all ryanodine-sensitive Ca^{2+} channels before initiating Ca^{2+} loading (12). Ryanodine-insensitive Ca^{2+} efflux was then evoked by the addition of 375 nM thapsigargin.

The ability of FK 506 to alter thapsigargin-evoked Ca^{2+} efflux from SR vesicles was also studied. FK 506 (50 μM) or 6 μl of DMSO

(control) was added to actively loaded SR vesicles, and the reaction mixture was allowed to incubate for ~3 min before the addition of 375 nM thapsigargin to induce Ca^{2+} efflux. The ability of 50 μM FK 506 to alter ryanodine-insensitive Ca^{2+} efflux was examined by performing the measurements with ryanodine-pretreated SR vesicles. In these experiments, SR vesicles were pretreated for 3 min with 500 μM ryanodine before loading with Ca^{2+}. Once the loading was complete, 50 μM FK 506 or 6 μl of DMSO (control) was introduced, and the reaction mixture was allowed to incubate for 3 min before the addition of 375 nM thapsigargin to induce Ca^{2+} efflux.

At the end of each experiment, absorbance signals were calibrated by the addition of 1 μg of the Ca^{2+} ionophore A23187 followed by additions of 24 nmol of CaCl$_2$ from a National Bureau of Standards stock. The initial rates of Ca^{2+} efflux and Ca^{2+} reaccumulation under various experimental conditions were determined by linear regression analysis of the first 40–100 sec of the respective data. Values of IC$_{50}$, EC$_{50}$, and Hill coefficient were obtained by sigmoidal curve fitting of respective dose-response curves with the use of EN-ZFITTER computer software. The rates of caffeine-induced Ca^{2+} release, CICR, and thapsigargin-evoked Ca^{2+} efflux in the presence of FK 506, PCBs, and ryanodine were analyzed by one-tailed paired t test ($\alpha = 0.05$). Experiments were repeated with three times with at least two different membrane preparations.

Determination of Ca^{2+}-loading capacity. The influence of PCB 95 and FK 506, singly or in combination, on vesicle-loading capacity was determined with native SR or ryanodine (500 μM)-pretreated SR. The 1 μM PCB 95- and 50 μM FK 506-, singly or in combination, pretreated vesicles were loaded by serial addition of 24 nmol of CaCl$_2$ by permitting the dye signal to return to base-line between Ca^{2+} additions. When capacity was approached, small additions of Ca^{2+} were made until the vesicles ceased to accumulate Ca^{2+}. Ca^{2+} ionophore A23187 (1 μg) was added to each assay to release the accumulated Ca^{2+} from the vesicles. Ca^{2+}-loading capacities under various experimental conditions were determined as the amount of ionophore-releasable Ca^{2+} calculated from the difference between the absorbance signal before and after the addition of A23187. The dye signal was calibrated for linearity with two additions of 24 nmol of CaCl$_2$ at the end of each experiment. Data were analyzed by with one-tailed paired t test ($\alpha = 0.05$). The measurements were repeated at least three times with at least two different membrane preparations.

Results and Discussion

FK 506 and rapamycin completely eliminated PCB 95-induced Ca^{2+} channel activation. Fig. 1 demonstrates that the level of high affinity binding of [^{3}H]ryanodine (1 nM) to skeletal SR preparations is low when assayed in the presence of a physiological concentration of monovalent cations (140 mM K$^+$ and 15 mM Na$^+$). Incubation with 600 nM or 1 μM of the noncoplanar congener PCB 95 enhanced the specific occupancy of [^{3}H]ryanodine to Ry$_1$R in a dose-dependent manner (Fig. 1), as previously reported (8).[1] A new significant finding is that although neither FK 506 nor rapamycin significantly altered the high affinity binding of [^{3}H]ryanodine to Ry$_1$R, both FK 506 (Fig. 1A) and rapamycin (Fig. 1B) inhibited PCB 95-enhanced [^{3}H]ryanodine occupancy in a dose-dependent manner. The IC$_{50}$ values for FK 506 and rapamycin are ~40 μM and ~30 μM, respectively, which parallel the slightly higher binding affinity of rapamycin to FKBP12 than that of FK 506 (14). The values of IC$_{50}$ are independent of the concentrations of PCB 95 (Table 1). At concentrations known to dissociate FKBP12 from Ry$_1$R (4), both FK 506 and rapamycin negated PCB 95-enhanced binding of [^{3}H]ryanodine to Ry$_1$R, which suggests that PCB 95
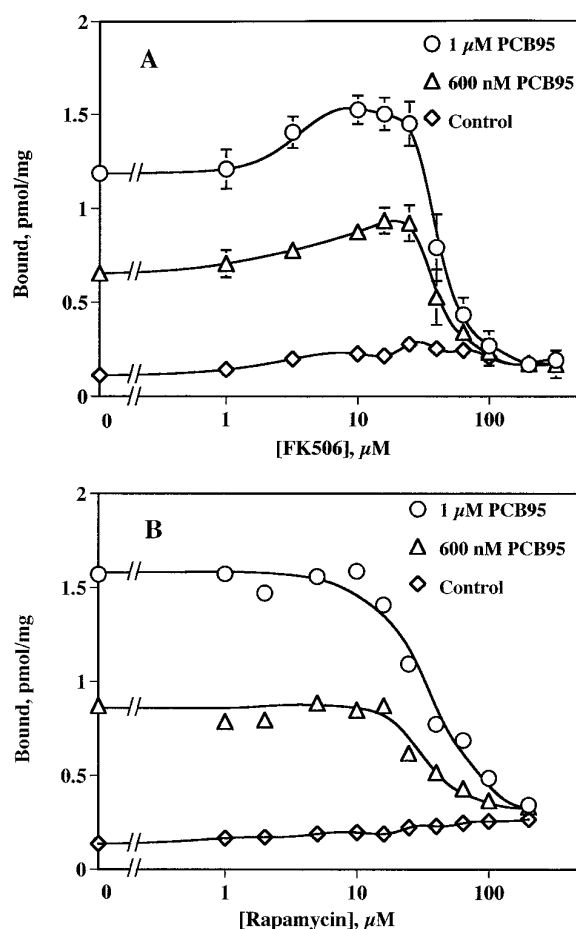

Fig. 1. FK 506 and rapamycin negate PCB 95-enhanced [^{3}H]ryanodine binding to Ry$_1$R in skeletal SR. The binding of [^{3}H]ryanodine to rabbit skeletal muscle SR enriched in Ry$_1$R was measured in buffer consisting of 140 mM KCl, 15 mM NaCl, 20 mM HEPES, pH 7.4, 10% sucrose, 50 μM CaCl$_2$, 1 mM MgCl$_2$, 1 nM [^{3}H]ryanodine, and 12.5 μg of skeletal SR protein in a final volume of 250 μl as described in Experimental Procedures. Measurements were made in the absence (Control; ◇) or presence of 0.6 (△) and 1 (○) μM PCB 95. Both FK 506 (A) and rapamycin (B) inhibited PCB 95-enhanced [^{3}H]ryanodine binding in a dose-dependent manner with values of IC$_{50}$ and Hill coefficient as summarized in Table 1. Each determination was performed in duplicate. A, Mean ± standard error of three determinations. B, Representative of two determinations with similar results.

enhances the SR Ca^{2+} release channel activity in a FKBP12-dependent manner.

Ca^{2+} transport measurements with the metallochromic indicator APIII reveal that the addition of 1 μM PCB 95 induced a net Ca^{2+} efflux from actively loaded SR vesicles (Fig. 2A, trace a). FK 506 (50 μM) introduced ~3 min before the addition of 1 μM PCB 95 completely eliminated the response to PCB 95 (Fig. 2A, trace b). Similarly, preincubation of the vesicles for 3 min with 0–50 μM rapamycin before Ca^{2+} loading caused a dose-dependent inhibition of the PCB 95-induced Ca^{2+} release from SR vesicles (Fig. 2B), with an IC$_{50}$ value of 11 μM and complete inhibition at 50 μM rapamycin (Fig. 2B, inset; Table 1). Fig. 2C shows that 50 μM FK 506 in the transport assay altered neither the slope (0.57 ± 0.03 absorption units/mM and 0.55 ± 0.02 absorption units/mM for FK 506 and DMSO control, respectively; $p > 0.1$) nor the regression coefficient ($r > 0.996$ for both conditions) of the dye calibration curves, which indicates that the drug does not

TABLE 1

FK 506 and rapamycin fully negate PCB 95-enhanced [^{3}H]ryanodine binding to Ry$_1$R and PCB 95-induced Ca^{2+} release from isolated rabbit skeletal SR vesicles

Condition	Rapamycin		FK506	
	IC$_{50}$[b]	Hill coefficient[b]	IC$_{50}$[c]	Hill coefficient[c]
	μM		μM	
[^{3}H]Ryanodine binding[a]				
Control	n.d.	n.d.	n.d.	n.d.
600 nM PCB 95	29.5 ± 0.9	2.6 ± 0.2	42.7 ± 8.0	3.5 ± 0.4
1 μM PCB 95	29.5 ± 6.9	2.7 ± 0.9	41.2 ± 5.5	4.4 ± 0.7
PCB induced Ca^{2+} release[d]				
1 μM PCB 95	11.1 ± 0.1	3.3 ± 0.1	N.D.	N.D.

[a] Binding assays were conducted in duplicate in 140 mM KCl; 15 mM NaCl; 20 mM HEPES, pH 7.4; 10% sucrose; 50 μM CaCl$_2$; 1 mM MgCl$_2$; 1 nM [^{3}H]ryanodine; 0, 0.6, or 1 μM PCB 95; 0–200 μM FK 506 or rapamycin; and 12.5 μg of skeletal SR protein with final volume of 250 μl, as described in Experimental Procedures.
[b] Data represent mean ± range of two experiments.
[c] Data represent mean ± standard error of three experiments.
[d] Rates of PCB 95-induced Ca^{2+} release from SR were determined in a buffer of 18.5 mM K-MOPS, pH 7.0, 92.5 mM KCl, 7.5 mM Na-pyrophosphate, 250 μM AP III, 1 mM Mg-ATP, 20 μg/ml creatine phosphokinase, 5 mM phosphocreatine, 0–50 μM rapamycin, and 50 μg of skeletal SR protein with final volume of 1.2 ml, as described in Experimental Procedures.
n.d., nondetectable; N.D., not determined.

alter the calibration or sensitivity of the APIII dye for Ca^{2+}. Similarly, rapamycin at ≤50 μM failed to interfere with the APIII dye (Fig. 2B, Ca^{2+} calibrations of *traces a–e*).

Marks *et al.* (4) have shown that the high affinity interaction between the Ry$_1$R oligomer and FKBP12 is essential for stabilizing the native full conductance gating behavior of the SR Ca^{2+} release channel, because Ry$_1$R expressed heterologously in the absence of FKBP12 exhibits several channel subconductances when reconstituted in bilayer lipid membranes. Further support of the functional importance of the association of FKBP12 with Ry$_1$R channel complex comes from pharmacological studies with immunosuppressant FK 506 and its analogs. Studies from several laboratories (4, 5) have revealed that 1–50 μM FK 506 or rapamycin is sufficient to dissociate FKBP12 from Ry$_1$R, although it is not clear whether complete dissociation of the immunophilin is achieved (3). Treatment of SR vesicles with FK 506 has been demonstrated to promote dissociation of FKBP12 from the Ry$_1$R complex and therefore alter SR Ca^{2+} transport as well as channel gating behavior (4, 15). A radioligand binding study with [^{3}H]ryanodine demonstrated that incubation of SR vesicles with 50 μM FK 506 increased [^{3}H]ryanodine binding capacity to SR with a 2–3-fold reduction in binding affinity (16), which suggests that the FKBP12-deficient Ry$_1$R channel exhibits subconductance states that bind to [^{3}H]ryanodine with much lower affinity than that of the native channel. These results are consistent with the binding data shown in the current study. Measurements of single channels in bilayer lipid membranes revealed that 10–12 μM FK 506 or rapamycin is sufficient to release FKBP12 and modify channel conductance, actions consistent with a channel modulatory role for the immunophilin (3). In the current study, FK 506 or rapamycin completely eliminated PCB 95-induced Ca^{2+} release and PCB 95-enhanced binding of [^{3}H]ryanodine to Ry$_1$R in the same concentration range required to dissociate FKBP12 from Ry$_1$R, which suggests a strong correlation between the activity of PCB 95 toward Ry$_1$R and the integrity of the FKBP12/Ry$_1$R complex.

FK 506 did not eliminate responses of SR to caffeine or Ca^{2+}. Unlike FK 506, caffeine and Ca^{2+} are thought to interact with Ry$_1$R channel through effector sites located on the Ry$_1$R protein (17). Although FK 506 completely eliminated responses of Ry$_1$R to PCB 95, the drug failed to inhibit

the response of Ry$_1$R to 10 mM caffeine (Fig. 3A, *trace c*). The amount of SR Ca^{2+} released by caffeine is similar regardless of the presence or absence of FK 506 (Fig. 3A, compare plateaus in *traces a* and *b*). Under the experimental conditions used here, the initial rate of caffeine-induced Ca^{2+} release in the presence of 50 μM FK 506 was the same as that of the DMSO control (6 μl) (101% control, $p > 0.1$) (Fig. 3A, compare *traces a* and *c*; Table 2). However, if caffeine is introduced immediately after the addition of 1 μM PCB 95 (just before PCB 95 begins to mobilize SR Ca^{2+}), the initial rate of Ca^{2+} release is significantly enhanced compared with the DMSO control (200% control, $p < 0.05$) (Fig. 3A, compare *traces a* and *b*; Table 2).

Fig. 3B shows the CICR responses of actively loaded SR vesicles to the bolus addition of 84 nmol of Ca^{2+} under various experimental conditions. FK 506 (50 μM) did not significantly alter the initial rate of CICR (92% control, $p > 0.1$) (Fig. 3B, compare *traces a* and *c*; Table 2). In contrast, 1 μM PCB 95 significantly enhanced the initial rate of CICR (251%, $p < 0.01$) (Fig. 3B, compare *traces a* and *b*; Table 2). Interestingly, both FK 506 and PCB 95 markedly enhanced the rate of Ca^{2+} reaccumulation once ryanodine-sensitive Ca^{2+} channels were fully blocked by 500 μM ryanodine or 1 μM ruthenium red (ruthenium red data not shown) (added once the CICR responses begin to plateau) (Fig. 3B, compare the reuptake phase in *traces a–c*). The maximum rates of Ca^{2+} reuptake relative to DMSO control were 446% in the presence of FK 506, and 189% in the presence of PCB 95 (Table 2). PCB 95 (> 1 μM) further enhanced the rate and amount of Ca^{2+} reaccumulation to a level seen with 50 μM FK 506 (data not shown). In each experiment, the vesicles were loaded to near their steady state capacity in the initial loading phase. This is demonstrated by the inability of the control vesicles to reaccumulate the 84 nmol of Ca^{2+} added for CICR once ryanodine-sensitive channels were blocked (Fig. 3B, *trace a*). The addition of 1 μg of ionophore A23187 at the end of the experiment shows that all of the Ca^{2+} reaccumulated by vesicles can be quickly mobilized. Calibration with standard Ca^{2+} solution revealed that the drugs do not interfere with dye calibration. The rates of caffeine-induced Ca^{2+} release, CICR, and Ca^{2+} reuptake under various experimental conditions are summarized in Table 2. As a result, FK 506 (and rapamycin) eliminated the actions of PCB 95 on
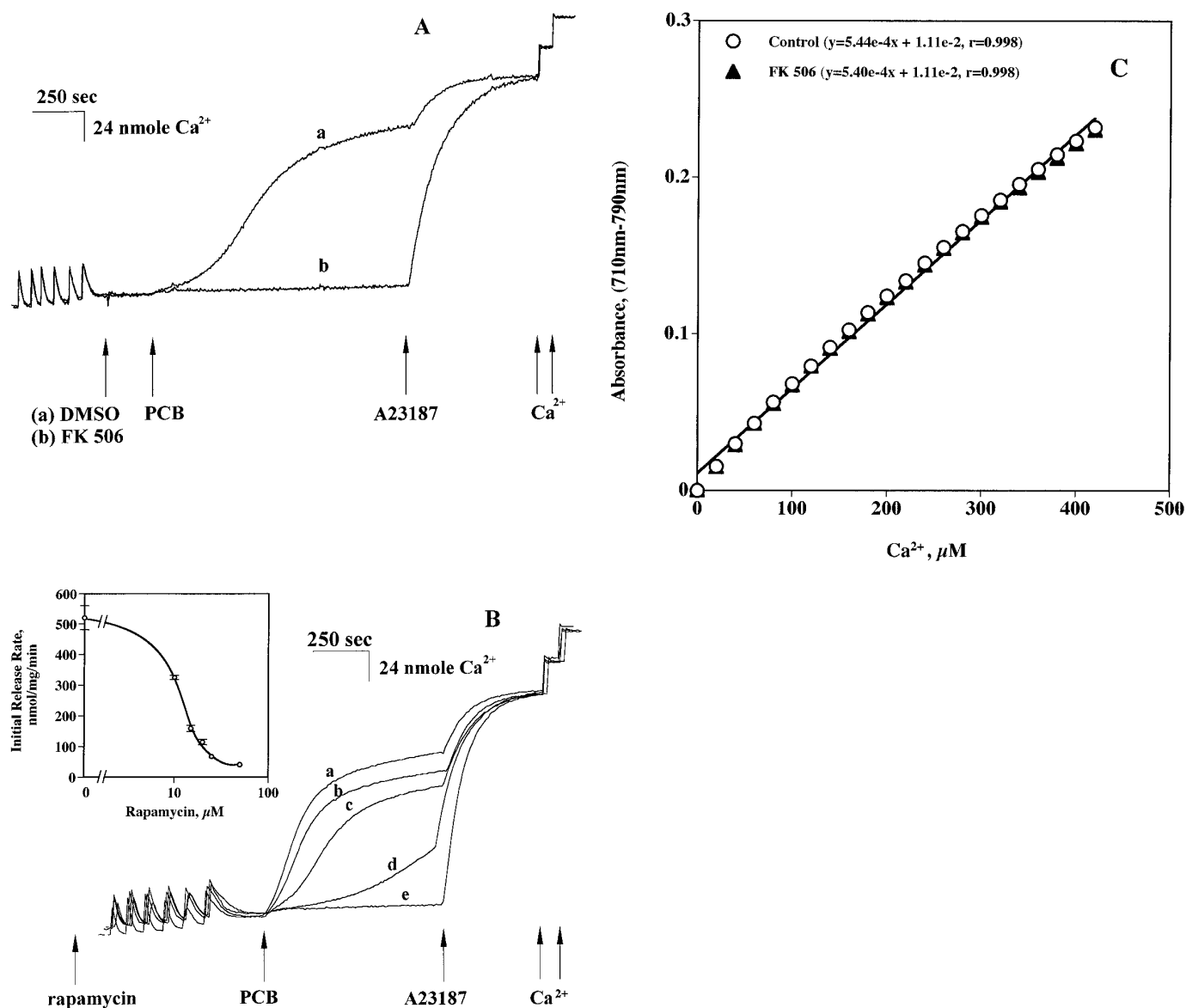
Fig. 2. FK 506 and rapamycin fully block PCB 95-induced Ca^{2+} release from actively loaded skeletal SR vesicles. Ca^{2+} transport across rabbit skeletal muscle SR vesicles was measured in buffer consisting of 18.5 mM K-3-(*N*-morpholino)propanesulfonic acid, pH 7.0, 92.5 mM KCl, 7.5 mM Na-pyrophosphate, 250 μM APIII, 1 mM Mg-ATP, 20 μg/ml creatine phosphokinase, 5 mM phosphocreatine, and 50 μg of skeletal SR protein in a final volume of 1.2 ml as described in Experimental Procedures. Vesicles were actively loaded with 144 nmol of Ca^{2+} during the loading phase. A, *Trace a*, 1 μM PCB 95-induced Ca^{2+} release from SR membrane vesicles. *Trace b*, prior addition of 50 μM FK 506 completely inhibited Ca^{2+} release induced by 1 μM PCB 95. B, Preincubation with rapamycin at 3 min before Ca^{2+} loading inhibited Ca^{2+} release induced by 1 μM PCB 95 in a dose-dependent manner with values of IC_{50} and Hill coefficient as summarized in Table 1. Rapamycin concentrations were 0 (6 μl of DMSO control) for *trace a*; 10 μM, *trace b*; 15 μM, *trace c*; 20 μM, *trace d*; and 50 μM, *trace e*. For the experiment shown, the initial rates of PCB 95-induced Ca^{2+} in the presence of 0, 10, 15, 20, and 50 μM rapamycin were 529, 344, 154, 60, and 34 nmol/mg/min, respectively. *Inset*, dose-response relationship for rapamycin inhibition of PCB 95-induced Ca^{2+} release (mean $\pm$ standard error of three experiments). *x*-axis, concentration of rapamycin in log scale. C, Ca^{2+} calibration curves in the presence of 6 μl of DMSO (*Control*; $\bigcirc$) or 50 μM FK 506 ($\blacktriangle$) in the presence of 1 μg of A23187. FK 506 (50 μM) did not alter the calibration and sensitivity of the APIII dye for Ca^{2+}.

SR Ca^{2+} transport without overtly changing the caffeine- and Ca^{2+}-induced release. However, FK 506 and PCB 95 acted in a similar manner to enhance the Ca^{2+}-buffering capacity of SR.

Taken together, the current results demonstrate that the noncoplanar PCB 95 profoundly modifies Ca^{2+} channel function and Ca^{2+} transport properties of SR membrane vesicles in a manner that requires the integrity of the FKBP12/Ry_1R complex. Because SR vesicles treated with FK 506 or PCB 95 maintain responses to Ry_1R channel activators (caffeine and Ca^{2+}), the integrity of the Ca^{2+} release channel and its

associated effector sites remains intact. Selective loss of PCB 95-induced mobilization of SR Ca^{2+} with FK 506 implies that the PCB 95 binding site is located on FKBP12 or the FKBP12/Ry_1R interface. The differential influence of FK 506 and PCB 95 on CICR and caffeine-induced Ca^{2+} release may reflect a fundamentally different mechanism by which these agents alter a functionally important protein/protein interaction. However, both compounds enhance the rate of Ca^{2+} reaccumulation once ryanodine-sensitive channels are fully blocked by micromolar ryanodine. One functional role proposed for FKBP12 is the regulation of channel and leak
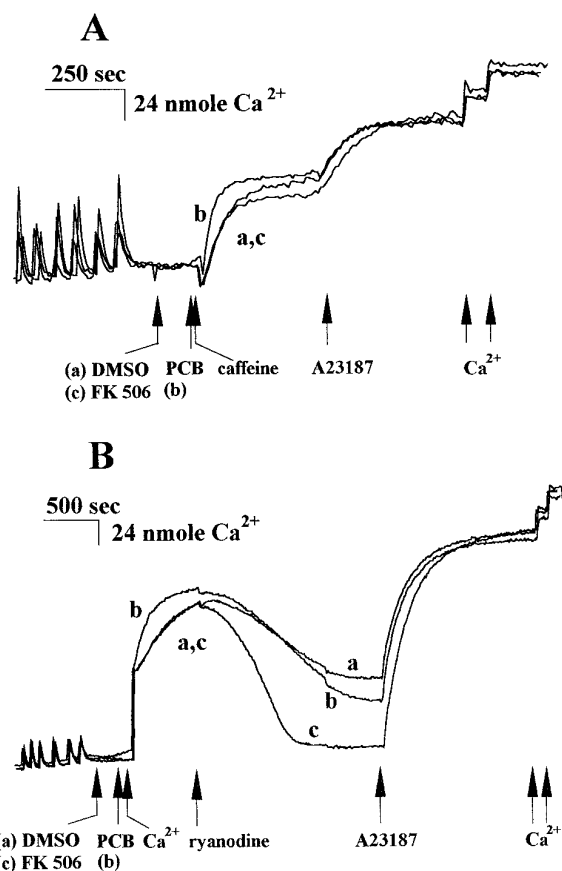
A

B

Fig. 3. FK 506 does not affect responses of Ry_1R to caffeine and Ca^{2+}. Vesicles were actively loaded with 144 nmol of Ca^{2+} during the loading phase as described in Experimental Procedures. A, Ca^{2+} release was initiated by the addition of 10 mM caffeine. *Trace a*, control with 6 μl of DMSO followed by 10 mM caffeine. *Trace b*, 1 μM PCB 95 significantly enhanced the rate of caffeine-induced Ca^{2+} release from SR vesicles. *Trace c*, addition of 50 μM FK 506 before 10 mM caffeine did not alter the rate of caffeine-induced Ca^{2+} release. For the experiment shown, the initial rates of caffeine-induced Ca^{2+} release from SR vesicle in the presence of DMSO, PCB 95, and FK 506 were 19.5, 41.0, and 19.0 nmol/mg/sec, respectively. B, CICR was initiated by the addition of a bolus of 84 nmol of Ca^{2+}. Once the Ca^{2+} release was complete, Ca^{2+} reuptake was initiated by the addition of 500 μM ryanodine. *Trace a*, control with 6 μl of DMSO followed by the addition of 84 nmol of Ca^{2+}. *Trace b*, 1 μM PCB 95 enhanced the rates of CICR and Ca^{2+} reuptake. *Trace c*, 50 μM FK 506 did not alter the rate of CICR from loaded vesicles but significantly enhanced Ca^{2+} reuptake. For the experiment shown, the initial rates of CICR in the presence of DMSO, PCB 95, and FK 506 were 4.5, 11.0, and 4.0 nmol/mg/sec, respectively. The rates of Ca^{2+} reaccumulation in the presence of DMSO, PCB, and FK 506 were 2.1, 2.4, and 6.4, nmol/mg/sec, respectively. The experiments shown are representative of three replicated measurements with similar results. The rates of caffeine-induced Ca^{2+} release, CICR, and Ca^{2+} reuptake after the addition of ryanodine are summarized in Table 2.

conformations of the protein complex (3, 5, 12). The current results suggest that although FK 506 and PCB 95 influence CICR and caffeine-induced Ca^{2+} in distinct ways, both agents significantly enhance Ca^{2+} reaccumulation once an inhibitory concentration of ryanodine is introduced. The enhancement in rate and amount of Ca^{2+} reaccumulated by FK 506 or PCB 95 in the presence of channel blocker could reflect a marked increase in the absolute Ca^{2+}-loading capacity of SR vesicles.

PCB 95 and FK 506 eliminated a ryanodine-insensitive Ca^{2+} leak pathway from SR. Recent studies with

TABLE 2

PCB 95, but not FK 506, enhances responses of SR to caffeine and Ca^{2+}, whereas both PCB 95 and FK 506 enhance the Ca^{2+} reaccumulation in SR vesicles

Condition	Initial Ca^{2+} release rate	Associated p^b	% Control
	nmol/mg/sec		
Caffeine-induced Ca^{2+} release (10 mM caffeine)^a			
Control	16 ± 2		100
50 μM FK 506	16 ± 1	0.427	101
1 μM PCB 95	32 ± 5	0.035	200
CICR (84 nmol of Ca^{2+})^a			
Control	4.7 ± 0.5		100
50 μM FK 506	4.3 ± 0.3	0.131	92
1 μM PCB 95	11.7 ± 0.3	0.004	251

Condition	Reuptake rate	Associated p^b	% Control
	nmol/mg/sec		
Ca^{2+} reaccumulation (500 μM ryanodine)^a			
Control	1.1 ± 0.6		100
50 μM FK 506	4.7 ± 0.9	0.004	446
1 μM PCB 95	2.0 ± 0.3	0.057	189

Data represent mean $\pm$ standard error of three experiments.
^{a} Rates of caffeine-induced Ca^{2+} release, CICR, and Ca^{2+} reaccumulation were determined in a buffer of 18.5 mM K-MOPS, pH 7.0, 92.5 mM KCl, 7.5 mM Na-pyrophosphate, 250 μM APIII, 1 mM Mg-ATP, 20 μg/ml creatine phosphokinase, 5 mM phosphocreatine, and 50 μg of skeletal SR protein with final volume of 1.2 ml, as described in Experimental Procedures.
^{b} Significant difference from the paired control if $p < 0.05$ (one-tailed paired t test, $\alpha = 0.05$).

brominated macrocyclic bastadins isolated from the marine sponge *Ianthella basta* have indicated that bastadin 5 enhances SR-loading capacity by modulating the $FKBP12/Ry_1R$ complex and converting a ryanodine-insensitive efflux pathway (leak) into a ryanodine-sensitive efflux pathway (channel) that recognizes ryanodine with high affinity (12). PCB 95 has been shown to modulate [^{3}H]ryanodine binding sites of Ry_1R in a manner very similar to bastadin 5 (8, 16). Both PCB 95 (8) and bastadin 5 (16) increase the affinity and capacity of high affinity [^{3}H]ryanodine binding to Ry_1R, as well as significantly altering modulation of Ry_1R by Ca^{2+} and Mg^{2+}.

To test the hypothesis that PCB 95, like bastadin 5, alters SR Ca^{2+}-loading capacity by converting a ryanodine-insensitive Ca^{2+} efflux pathway (leak) normally present in SR into a ryanodine-sensitive efflux (channel), the SERCA pump inhibitor thapsigargin was used. In the absence of ryanodine, the addition of thapsigargin blocks SERCA pump activity, which would be expected to evoke Ca^{2+} efflux from actively loaded SR vesicles through both ryanodine-sensitive and -insensitive pathways. In contrast, pump blockade on actively loaded SR vesicles pretreated with high micromolar ryanodine should only unmask Ca^{2+} efflux through a ryanodine-insensitive pathway. Fig. 4A demonstrates that after completion of active Ca^{2+} loading under the control condition, the addition of thapsigargin evokes release of accumulated Ca^{2+} even though extravesicular Ca^{2+} level is initially below threshold to activate CICR (Fig. 4A, *trace a*). Pretreatment of SR vesicles with 500 μM ryanodine or 2 μM ruthenium red has been shown to completely block caffeine-induced Ca^{2+} release or CICR under conditions identical to those used here (12). Fig. 4B shows that the addition of thapsigargin after completion of Ca^{2+} loading to SR vesicles pretreated with 500 μM ryanodine unmasks a ryanodine-insensitive Ca^{2+} efflux pathway (Fig. 4B, *trace a*), consistent with previous findings (12). The magnitude of the ryanodine-insensitive
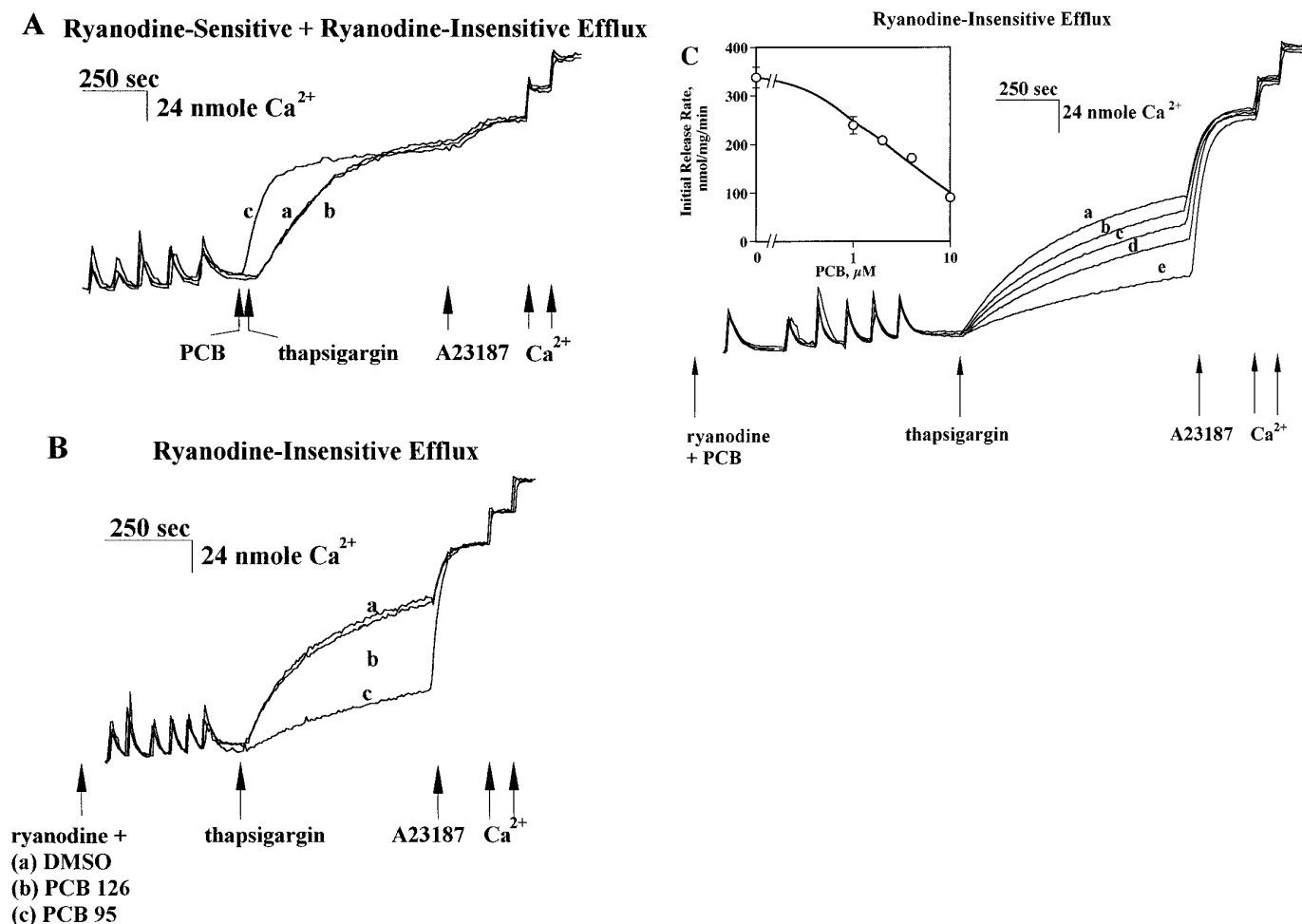
A   Ryanodine-Sensitive + Ryanodine-Insensitive Efflux

B   Ryanodine-Insensitive Efflux

C   Ryanodine-Insensitive Efflux

Fig. 4. Noncoplanar PCB 95, but not PCB 126, eliminates a ryanodine-insensitive Ca²⁺ efflux pathway found in native SR. A, Vesicles were actively loaded with 120 nmol of Ca²⁺ as described in Experimental Procedures. *Trace a*, control rate of Ca²⁺ efflux induced by 375 nM thapsigargin. The addition of 5 μM PCB 95 (*trace c*) but not 5 μM PCB 126 (*trace b*) significantly enhanced thapsigargin-induced Ca²⁺ efflux. For the experiment shown, the initial rates of thapsigargin-evoked Ca²⁺ efflux in the presence of DMSO, PCB 126, and PCB 95 were 365, 343, and 1044 nmol/mg/min, respectively. B, Vesicles were pretreated at 3 min with 500 μM ryanodine and actively loaded with 144 nmol of Ca²⁺ during the loading phase. Additions of DMSO (control; *trace a*), 5 μM PCB 126 (*trace b*), or 5 μM PCB 95 (*trace c*) were made to individual cuvettes before the loading phase where indicated. Ca²⁺ efflux was initiated by 375 nM thapsigargin. Note that only PCB 95 significantly reduced the ryanodine-insensitive component of Ca²⁺ release. For the experiment shown, in the presence of 500 μM ryanodine, the initial rates of thapsigargin-evoked Ca²⁺ release in the presence of DMSO, PCB 126, and PCB 95 were 353, 351, and 77 nmol/mg/min, respectively. C, Ryanodine-pretreated (500 μM) vesicles were exposed to 6 μl of DMSO (*trace a*) or 1 (*trace b*), 2 (*trace c*), 4 (*trace d*), or 10 (*trace e*) μM PCB 95 just before active loading with 144 nmol of Ca²⁺. PCB 95 eliminated the ryanodine-insensitive Ca²⁺ efflux with values of IC₅₀ and Hill coefficient of 3.5 ± 0.2 μM and 0.8 ± < 0.1, respectively. For the experiment shown, in the presence of 500 μM ryanodine, the initial rates of thapsigargin-evoked Ca²⁺ efflux in the presence 0, 1, 2, 4, and 10 μM PCB 95 were 333, 245, 209, 168, and 87 nmol/mg/min, respectively. *Inset*, mean ± standard error for three replicated experiments. *x*-axis, concentration of PCB 95 in log scale. The experiments were the representative of three measurements with identical results.

component of Ca²⁺ efflux has been shown to be directly related to the filling state of the vesicles and is significant only when vesicles possess a physiological Ca²⁺ gradient across the membrane. Ryanodine-insensitive Ca²⁺ efflux becomes apparent when > 0.5 μmol of Ca²⁺/mg of protein is loaded into the SR lumen (12) and becomes appreciable with 2.9 μmol/mg of protein luminal Ca²⁺ (Fig. 4B, *trace a*). Coplanar PCB 126 (3,3′,4,4′,5-pentachlorobiphenyl; 5 μM), a PCB congener lacking activity toward RyRs and SR/ER Ca²⁺ transport (8),[1] does not alter thapsigargin-evoked Ca²⁺ efflux regardless of whether the vesicles are pretreated with micromolar ryanodine (95% and 92% of the respective control in the absence and presence of ryanodine, respectively; $p >$ 0.05) (Fig. 4, A and B, compare *traces a* and *b*). In marked contrast, 5 μM PCB 95 dramatically (386% of control, $p <$

0.025) enhanced the initial rate of Ca²⁺ efflux evoked by the addition of 375 nM thapsigargin (Fig. 4A, compare *traces a* and *c*). Importantly, in the presence of channel-blocking concentration of ryanodine, the ryanodine-insensitive component of Ca²⁺ efflux unmasked by addition of thapsigargin is greatly reduced by the presence of PCB 95 in a dose-dependent manner (Fig. 4B, compare *traces a* and *c*; Fig. 4C). The IC₅₀ and Hill coefficient for the elimination of ryanodine-insensitive Ca²⁺ leak by PCB 95 are 3.5 ± 0.2 μM and 0.81 ± 0.05, respectively (Fig. 4C, *inset*).

These results suggest that through its actions on the FKBP12/Ry₁R complex, PCB 95 relates ryanodine-insensitive and -sensitive Ca²⁺ efflux pathways in native SR. Micromolar ryanodine eliminates CICR and caffeine-induced Ca²⁺ release but not a major ryanodine-insensitive Ca²⁺

leak unmasked by SERCA pump blockade (16, 18). In contrast, PCB 95 (i) enhances net Ca^{2+} efflux from SR with the presence or absence of pump activity and (ii) eliminates the ryanodine-insensitive component of Ca^{2+} efflux (leak) unmasked by thapsigargin. The most direct interpretation of these results is that PCB 95 enhances the proportion of channel to leak states of Ry_1R on the SR membrane. Coplanar PCB 126 alters neither the ryanodine-sensitive Ca^{2+} efflux nor the ryanodine-insensitive Ca^{2+} leak unmasked by thapsigargin, demonstrating the structural specificity of the noncoplanar PCB 95 for eliciting the unique actions on SR Ca^{2+} transport. This hypothesis is further supported by the enhancement in high affinity binding capacity of SR for [3H]ryanodine in the presence of PCB 95 but not PCB 126.

To determine the role of FKBP12 in regulating ryanodine-sensitive and -insensitive Ca^{2+} efflux pathways of SR, the action of FK 506 on thapsigargin-evoked Ca^{2+} efflux from actively loaded SR membrane vesicles was examined. The addition of 50 μM FK 506 to Ca^{2+}-loaded SR depressed the initial rate of thapsigargin-evoked Ca^{2+} release to 56% of

A **Ryanodine-Sensitive + Ryanodine-Insensitive Efflux**

500 sec

24 nmole Ca^{2+}

a

b

(a) DMSO   thapsigargin   A23187 Ca^{2+}
(b) FK 506

B **Ryanodine-Insensitive Efflux**

500 sec

24 nmole Ca^{2+}

a

b

ryanodine   (a) DMSO   thapsigargin   A23187 Ca^{2+}
            (b) FK 506

Fig. 5. FK 506 eliminates a ryanodine-insensitive Ca^{2+} efflux pathway found in native SR. A, Vesicles were actively loaded with 120 nmol of Ca^{2+} during the loading phase. Compared with 6 μl of DMSO (control; *trace a*), 50 μM FK 506 (*trace b*) significantly reduced net Ca^{2+} efflux induced by the addition of 375 nM thapsigargin. B, Ryanodine-pretreated vesicles were actively loaded with 120 nmol of Ca^{2+} during the loading phase. *Trace a* (DMSO control), ryanodine-insensitive component of Ca^{2+} efflux unmasked by the addition of 375 nM thapsigargin. *Trace b*, 50 μM FK 506 significantly reduced the ryanodine-insensitive component of Ca^{2+} release unmasked by thapsigargin. For the experiment shown, the initial rates of thapsigargin-evoked Ca^{2+} efflux in the presence of DMSO, FK 506, ryanodine singly, and ryanodine and FK 506 in combination were 411, 246, 202, and 90 nmol/mg/min, respectively. The experiments shown were representative of three replicated measurements with identical results.

control ($p < 0.01$) (Fig. 5A, compare *traces a* and *b*). The underlying mechanism by which FK 506 reduces the initial rate of Ca^{2+} release from SR may stem from the ability of the drug to promote subconductances of the individual channels on dissociation of the FKBP12/Ry_1R complex (3).
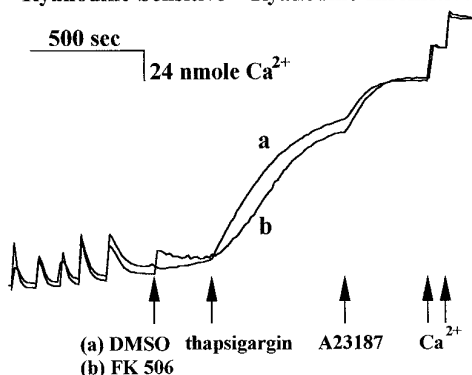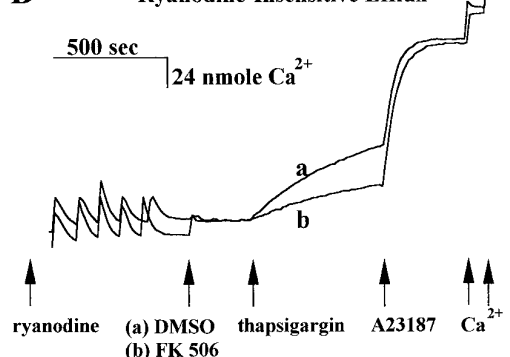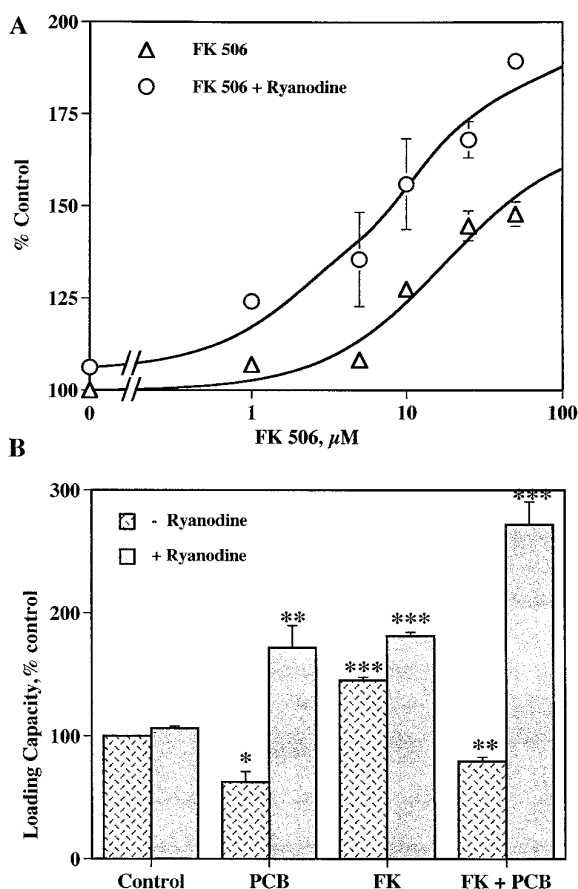
The initial rate of ryanodine-insensitive Ca^{2+} efflux is measured with vesicles preincubated with 500 μM ryanodine (Fig. 5B). On the addition of thapsigargin, the initial rate of Ca^{2+} release is 190 ± 9 nmol/mg/min (50% of the nonryanodine-pretreated control) (Fig. 5, A and B, *trace a*). A significant finding is that the introduction of 50 μM FK 506 on completion of the loading phase significantly reduces the rate of ryanodine-insensitive Ca^{2+} efflux in a manner similar to that seen for PCB 95 (Figs. 4C; Fig. 5B, compare *traces a* and *b*). The addition of A23187 and Ca^{2+} standard at the end of each experiment demonstrates that all the vesicles were loaded equally, and the drugs at the concentrations used do not interfere with the APIII dye for Ca^{2+}. Measurements of SERCA pump activity using an ATPase assay with a coupled enzyme system revealed that neither 50 μM FK 506 (data not shown) nor 10 μM PCB 95 (8) caused any significant change in SERCA pump activity. Therefore, FK 506 and PCB 95 reduce the initial rate of ryanodine-insensitive Ca^{2+} efflux by eliminating the Ca^{2+} leak pathway. As a result, FK 506 and PCB 95 relate the ryanodine-insensitive Ca^{2+} leak pathway to the ryanodine-sensitive Ca^{2+} channel pathway.

By disrupting the association of the FKBP12/Ry_1R channel complex, FK 506 converts ryanodine-insensitive Ca^{2+} leak into ryanodine-sensitive Ca^{2+} channel pathway. The FKBP12-deficient Ry_1R channel exhibits several subconductance states and binds to [3H]ryanodine with low affinity. This hypothesis is further supported by the increase in binding capacity of SR for [3H]ryanodine with reduced binding affinity in the presence of 50 μM FK 506 (16). Therefore, by converting Ca^{2+} leak to subconducting channels, FK 506 reduces the thapsigargin-evoked Ca^{2+} efflux regardless of the presence or absence of channel-blocking concentration of ryanodine.

Bastadin 5 has been shown to enhance Ry_1R channel activity by increasing the open dwell time without altering the unitary conductance of the channel in bilayer lipid membranes measurements (16). PCB 95 has been shown to activate Ry_1R channel, SR Ca^{2+} transport, and thapsigargin-evoked Ca^{2+} efflux in a manner very similar to bastadin 5. PCB 95 enhances the proportion of channel to leak states of the Ry_1R protein complex probably through a similar mechanism as bastadin 5. Like bastadin 5, PCB 95 enhances high affinity [3H]ryanodine binding to SR by increasing both the binding affinity and maximal capacity (8, 16). These results suggest that PCB 95 and bastadin 5 eliminate ryanodine-insensitive Ca^{2+} efflux by converting Ca^{2+} leak into a full conducting channel through a molecular mechanism distinct from FK 506. The enhancement in thapsigargin-evoked Ca^{2+} efflux with PCB 95 in the absence of channel blocker further supports the hypothesis.

FK 506 enhanced SR-loading capacity in a manner additive with PCB 95. To study the relationship of Ca^{2+} efflux pathways and loading capacity of SR vesicles, the loading capacity of SR vesicles pretreated under various experimental conditions was measured. Fig. 6 demonstrates that the loss of ryanodine-insensitive (but not ryanodine-sensitive) Ca^{2+} efflux pathway is correlated with a signifi-

Fig. 6. FK 506 and PCB 95 enhance loading capacity of SR in an additive manner. Vesicles were loaded to capacity by the serial addition of CaCl$_2$ as described in Experimental Procedures. A, FK 506 ($\triangle$) increased loading capacity of SR vesicles in a dose-dependent manner, with an EC$_{50}$ and Hill coefficient of 17.6 $\pm$ 3.5 μM and 1.1 $\pm$ 0.3, respectively. In the presence of 500 μM ryanodine (added 3 min before initiation of the experiment), FK 506 ($\bigcirc$) further increased loading capacity of SR with an EC$_{50}$ and Hill coefficient of 8.3 $\pm$ 1.7 μM and 0.9 $\pm$ 0.2, respectively. B, Preincubation of vesicles with 1 μM PCB 95, 50 μM FK 506, or 500 μM ryanodine, singly or in combination, significantly altered loading capacity of SR in a predictable manner (see text for details). Data are mean $\pm$ standard error of three measurements. (∗, 0.025 $<$ p $<$ 0.05; ∗∗, p $<$ 0.025; ∗∗∗ p $<$ 0.01; one-tailed paired t test).

cant enhancement in the steady state loading capacity of SR vesicles. SR vesicles are sequentially loaded with Ca^{2+} until steady state capacity (no net uptake or release of Ca^{2+}) is reached. Although variation in loading capacity has been observed between SR prepared from different animals (range, 2.8–4.0 μmol/mg), vesicles from the same preparation load in a consistent manner with ≤2% variation among replicated determinations. The presence or absence of a channel-blocking concentration of ryanodine has a negligible influence on the Ca^{2+}-loading capacity of the vesicles (106 $\pm$ 2% of control; Fig. 6, A and B, *Control*). Pretreatment of SR with FK 506 increases loading capacity of the vesicles in a dose-dependent manner with a maximal capacity and an EC$_{50}$ value of 170% of control and 18 μM, respectively (Fig. 6A). Pretreatment of SR with FK 506 and 500 μM ryanodine in combination further enhances Ca^{2+}-loading capacity of the vesicles with maximal capacity to nearly 200% of control, and the apparent potency of FK 506 increases $>$ 2-fold to 8 μM (Fig. 6A). In the presence of 500 μM ryanodine to block

ryanodine-sensitive Ca^{2+} efflux, 1 μM PCB 95 significantly enhances the Ca^{2+}-loading capacity of SR to 171% of control (Fig. 6B, *PCB*). Similarly, in the presence of 500 μM ryanodine, 50 μM FK 506 increases loading capacity to 189% of control. In contrast, the combination of 1 μM PCB 95 and 50 μM FK 506 produces an additive effect with enhancement in Ca^{2+}-loading capacity to 272% of control, which suggests that, in combination, these compounds disrupt the FKBP12/Ry$_1$R complex more profoundly than the use of either alone.

In the absence of ryanodine, pretreatment of SR vesicles with 1 μM PCB 95 significantly reduces Ca^{2+}-loading capacity to 62% of control, which is consistent with an increase in the number of high-conductance channel states in the SR membrane. In contrast, pretreatment of SR with 50 μM FK 506 alone significantly enhances loading capacity to 148% of control, which is consistent with an increase in the number of subconducting channel states in the SR membrane. The combination of PCB 95 and 50 μM FK 506 results in an intermediate changes in SR-loading capacity (to 79% of control), which is consistent with an increase in both full and subconducting channel states in the SR membrane. These interpretations of the observed changes in SR-loading capacity are fully consistent with the ability of each compound to eliminate a ryanodine-insensitive Ca^{2+} leak pathway from SR measured in the release experiments (Figs. 4 and 5).

Recently, Marks *et al.* (4) reported that pretreatment of intact rat soleus muscle with rapamycin increases the magnitude of response to caffeine. The increase in sensitivity of the FKBP12-deficient Ry$_1$R channel to caffeine, and therefore the increase in the leakage of the SR, has been suggested to be the underlying mechanism. The current findings with actively loaded SR membranes, although generally consistent with the findings in rat soleus, extend the mechanistic interpretation of the findings by Marks *et al.* (3). The ability of FK 506 and rapamycin to interact with and dissociate FKBP12 from the Ry$_1$R channel complex results in two important and related changes in SR Ca^{2+} transport: (i) an increase in loading capacity (i.e., enhanced Ca^{2+} buffering) as a direct result of elimination of ryanodine-insensitive Ca^{2+} leak states of Ry$_1$R and (ii) an increase in the number of caffeine-responsive, albeit subconducting, Ca^{2+} channels. This mechanism could fully account for a more robust response on caffeine stimulation in intact muscle fibers because the elevated Ca^{2+} capacity of SR would be expected to result in a more robust response to caffeine. The current results demonstrate that FK 506 does not alter the sensitivity of channels to caffeine *per se* (i.e., influences initial rates of caffeine- or Ca^{2+}-induced Ca^{2+} release under defined loading conditions) but instead increases SR-loading capacity. An increase in extracellular Ca^{2+} is also well known to result in SR Ca^{2+} overload, which enhances the excitability of Ca^{2+} release channels in cardiac muscle cells in culture (1). The current results also reconcile why singly FK 506 or rapamycin fails to significantly alter the high affinity binding properties of [^{3}H]ryanodine but clearly influence the actions of PCB 95. In support of the current findings and interpretations, Lamb and Stephenson (19) recently showed using mechanically skinned skeletal muscle fibers that low (1 μM) concentrations of FK 506 or rapamycin can potentiate SR Ca^{2+} release induced by depolarization or caffeine. These results would be expected if the drugs at a low concentration enhance the loading capacity of the SR lumen due to loss of a

Ca^{2+} leak pathway (as shown in Fig. 6). It was further demonstrated that a higher concentration (20 μM) of FK 506 or rapamycin causes irreversible loss of depolarization-induced Ca^{2+} release without preventing direct actions of caffeine. The latter actions are both use dependent (i.e., dependent on the frequency of depolarization) and dependent on the concentration of immunosuppressive drug. These results are consistent with a concomitant enhancement in SR filling and an increase in the number of subconducting channels on the SR membrane that apparently remain responsive to caffeine but not depolarization.

According to Marks *et al*. (4), FK 506 dissociates FKBP12 from Ry_1R and therefore increases the sensitivity of the FKBP12-deficient channel to threshold concentrations of caffeine and Ca^{2+} in single-channel measurements in bilayer lipid membranes (by increasing mean open time and open probability). Fleischer *et al*. (15) also reported that by dissociating FKBP12 from Ry_1R with FK 506, the sensitivity of SR Ca^{2+} release to caffeine increases. A concentration of caffeine (1.0 mM) that fails to release Ca^{2+} from native SR causes release from FK 506-pretreated SR vesicles. We report that with the optimal concentrations of caffeine (10 mM) and Ca^{2+} (70 μM), the initial rates of Ca^{2+} release seen with FKBP12-deficient SR vesicles are similar to that of native SR. This observation most likely stems from an increase in the number of ryanodine-sensitive Ca^{2+} channels with subconductance behavior.

In conclusion, the current results demonstrate a novel mechanism by which PCB 95 alters microsomal Ca^{2+} regulation in muscle. Dissociation of FKBP12 from the Ry_1R complex by FK 506 (and rapamycin) completely negates the activity of PCB 95 as measured by high affinity binding of [^{3}H]ryanodine and SR Ca^{2+} transport under active loading conditions, which suggests that PCB 95 alters microsomal Ca^{2+} transport through an FKBP12-dependent mechanism. Importantly, the actions of PCB 95, FK 506, and rapamycin on SR Ca^{2+} regulation seem to stem from the ability of these compounds to alter the relationship between ryanodine-insensitive Ca^{2+} leak and the ryanodine-sensitive Ca^{2+} channel states of Ry_1R, which significantly alters Ca^{2+} buffering and release properties of SR. By virtue of their unique and specific activity, certain *ortho*-substituted PCBs are potent and invaluable new probes with which to understand how FKBP12 regulates microsomal Ca^{2+} buffering under physiological and pathophysiological conditions. Furthermore, the newly identify mechanism may underlie the seemingly diverse toxicity that has recently been attributed to noncoplanar PCBs.

References

1. Bootman, M. D., and M. J. Berridge. The elemental principles of calcium signaling. *Cell* **83**:675–678 (1995).

2. Bernardi, P., and V. Petronilli. The permeability transition pore as a mitochondrial calcium release channel: a critical appraisal. *J. Bioeng. Biomembr.* **28**:131–138 (1996).

3. Marks, A. R. Cellular functions of immunophilins. *Physiol. Rev.* **76**:631–649 (1996).

4. Brillantes, A. B., K. Ondrias, A. Scott, E. Kobrinsky, E. Ondriasova, M. C. Moschella, T. Jayaraman, M. Landers, B. E. Ehrlich, and A. R. Marks. Stabilization of calcium release channel (ryanodine receptor) function by FK506-binding protein. *Cell* **77**:513–523 (1994).

5. Cameron, A. M., J. P. Steiner, A. J. Roskams, S. M. Ali, G. V. Ronnett, and S. H. Snyder. Calcineurin associated with the inositol 1,4,5-trisphosphate receptor-FKBP12 complex modulates Ca^{2+} flux. *Cell* **83**:463–472 (1995).

6. Schantz, S. L., J. Moshtaghian, and D. K. Ness. Spatial learning deficits in adult rats exposed to ortho-substituted PCB congeners during gestation and lactation. *Fundam. Appl. Toxicol.* **26**:117–126 (1995).

7. Shain, W., B. Bush, and R. Seegal. Neurotoxicity of polychlorinated biphenyls: structure-activity relationship of individual congeners. *Toxicol. Appl. Pharmacol.* **111**:33–42 (1991).

8. Wong, P. W., and I. N. Pessah. *Ortho*-substituted polychlorinated biphenyls alter calcium regulation by a ryanodine receptor-mediated mechanism: structural specificity toward skeletal- and cardiac-type microsomal calcium release channels. *Mol. Pharmacol.* **49**:740–751 (1996).

9. Kodavanti, P. R., T. R. Ward, J. D. McKinney, and H. A. Tilson. Increased [^{3}H]phorbol ester binding in rat cerebellar granule cells by polychlorinated biphenyl mixtures and congeners: structure-activity relationships. *Toxicol. Appl. Pharmacol.* **130**:140–148 (1995).

10. Kodavanti, P. R., D. S. Shin, H. A. Tilson, and G. J. Harry. Comparative effects of two polychlorinated biphenyl congeners on calcium homeostasis in rat cerebellar granule cells. *Toxicol. Appl. Pharmacol.* **123**:97–106 (1993).

11. Wong, P. W., R. M. Joy, T. E. Albertson, S. L. Schantz, and I. N. Pessah. Ortho-substituted 2,2′,3,5′,6-pentachlorobiphenyl (PCB 95) alters rat hippocampal ryanodine receptors and neuroplasticity in vitro: evidence for altered hippocampal function. *Neurotoxicology* (1997).

12. Pessah, I. N., T. F. Molinski, T. D. Meloy, P. W. Wong, E. D. Buck, and M. M. Mack. Bastadins relate ryanodine-sensitive and ryanodine-insensitive Ca^{2+} efflux pathways in skeletal SR and BC_{3H1} cells. *Am. J. Physiol.* **272**:C601–C614 (1996).

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

14. Lane, W. S., A. Galat, M. W. Harding, and S. L. Schreiber. Complete amino acid sequence of the FK506 and rapamycin binding protein, FKBP, isolated from calf thymus. *J. Prot. Chem.* **10**:151–160 (1991).

15. Timerman, A. P., E. Ogunbumni, E. Freund, G. Wiederrecht, A. R. Marks, and S. Fleischer. The calcium release channel of sarcoplasmic reticulum is modulated by FK-506-binding protein: dissociation and reconstitution of FKBP-12 to the calcium release channel of skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* **268**:22992–22999 (1993).

16. Mack, M. M., T. F. Molinski, E. D. Buck, and I. N. Pessah. Novel modulators of skeletal muscle FKBP12/calcium channel complex from *Ianthella basta*: role of FKBP12 in channel gating. *J. Biol. Chem.* **269**:23236–232349 (1994).

17. Zimanyi, I., and I. N. Pessah. Pharmacology of ryanodine-sensitive Ca^{2+}-release channels, in *Handbook of Membrane Channels: Molecular and Cellular Physiology* (C. Peracchia, ed.). Academic Press, San Diego, 475–494 (1994).

18. Sagara, Y., F. Fernandez-Belda, L. de Meis, and G. Inesi. Characterization of the inhibition of intracellular Ca^{2+} transport ATPases by thapsigargin. *J. Biol. Chem.* **267**:12606–126013 (1992).

19. Lamb, G. D., and D. G. Stephenson. Effects of FK506 and rapamycin on excitation-contraction coupling in skeletal muscle fibres of the rat. *J. Physiol.* **494**:569–576 (1996).

Send reprint requests to: Dr. Isaac N. Pessah, Department of Molecular Biosciences, School of Veterinary Medicine, University of California, Davis, CA 95616. E-mail: inpessah@ucdavis.edu