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Induction of Calcium Release from Isolated Sarcoplasmic Reticulum by Triphenyltin[1]

Jaw-Jou Kang,[2] I-Ling Chen, and Yu-Wen Cheng

Institute of Toxicology, College of Medicine, National Taiwan University, Taipei, No. 1 Jen-Ai Road, Section 1, Taiwan

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A direct peripheral myopathy has been found in organotin intoxication and suggested to be a significant factor in the development of muscle weakness following exposure. In this study, by using the isolated sarcoplasmic reticulum membrane vesicles, we have shown that triphenyltin dose-dependently induced Ca^{2+} release from the actively and passively loaded sarcoplasmic reticulum vesicles. Triphenyltin induced Ca^{2+} release in ruthenium red-sensitive and insensitive ways with EC_{50} values of 75 and 270 μM, respectively. The Ca^{2+}-ATPase activity and Ca^{2+} uptake of sarcoplasmic reticulum were also inhibited by triphenyltin. Triphenyltin exerted dual effects on the apparent [^{3}H]ryanodine binding. Triphenyltin (0.5-10 μM) dose-dependently potentiated the [^{3}H]ryanodine binding; however, the [^{3}H]ryanodine binding decreased as the concentration of triphenyltin increased. The dissociation of bound [^{3}H]ryanodine was facilitated by triphenyltin. The present study suggested that the internal Ca^{2+} store of skeletal muscle could be depleted by triphenyltin through the inhibition of the Ca^{2+} uptake and the induction of Ca^{2+} release by acting on the Ca^{2+}-ATPase and Ca^{2+} release channel, also known as the ryanodine receptor, of sarcoplasmic reticulum, respectively. These results could partly explain the development of muscle weakness in organotin intoxication; however, their relevance to the development of peripheral myopathy requires further examination.

Key words: Ca^{2+} release, sarcoplasmic reticulum, skeletal muscle, triphenyltin.

The contraction and relaxation of skeletal muscle are regulated by the free [Ca^{2+}] of the myoplasm. The uptake and release of Ca^{2+} from and into the myoplasm are controlled by two molecules located on the sarcoplasmic reticulum (SR), the major internal Ca^{2+} storage site of muscle cell, namely, the Ca^{2+}-ATPase and the Ca^{2+} release channel, also known as the ryanodine receptor, respectively (*1*). The ryanodine receptor forms a tetrameric complex whose electron microscopic structure is identical to that of an individual foot (*2*) and behaves like a Ca^{2+} release channel when incorporated into lipid bilayers (*3, 4*), suggesting that the protein responsible for the rapid Ca^{2+} release from SR might be the ryanodine receptor. The Ca^{2+} release properties and channel activities of ryanodine receptor were affected by several structurally unrelated chemicals (*5*), including heavy metals (*6*), and sulfhydryl (SH) reagents (*7, 8*).

The SH-reactive metals, including Ag^+ (*9*), were shown to induce skeletal muscle contracture and Ca^{2+} release from SR either by acting on the apparent physiological release site in SR (*10*), possibly the ryanodine receptor channel, or by inhibition of the Ca^{2+}-ATPase (*11*). Triphenyltin (*12*) was shown to induce muscle contracture in mouse diaphragm. In this study, we further examined the effect of triphenyltin on SR membrane vesicles isolated from skeletal muscle and found that triphenyltin dose-dependently induced Ca^{2+} release from the SR and affected the ryanodine binding to its receptor. The Ca^{2+}-ATPase activity and Ca^{2+} uptake by the SR were also inhibited by triphenyltin.

MATERIALS AND METHODS

Preparation of Sarcoplasmic Reticulum Fraction—The triad-enriched heavy fraction of sarcoplasmic reticulum (SR) was prepared from rabbit leg and back muscles by differential centrifugation as previously described (*13*). The final sedimentable SR fraction was homogenized and the protein concentration determined by the method of Lowry *et al.* (*14*) using bovine serum albumin as a standard. The B_{max} of [^{3}H]ryanodine binding of the isolated microsomes was normally in the range of 2.8-3.4 pmol/mg protein and the values of specific activity of the Ca^{2+}-ATPase in the absence and presence of Ca^{2+} ionophore, A23197, were 0.48 ± 0.02 and 4.43 ± 0.35 μmol P_i/min/mg protein, respectively. The preparation was quickly frozen in liquid nitrogen and stored at -70°C.

Ca^{2+} Release Assay—The time course of Ca^{2+} release from SR vesicles was investigated with a Ca^{2+}-sensitive probe, the antipyrylazo III, in a dual-wavelength spectro-

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[2] To whom correspondence should be addressed. Tel: +886-2-3123456 (ext 8603), Fax: +886-2-3410217, E-mail: jjkang@ccms.ntu.edu.tw

Abbreviations: DTT, dithiothreitol; MOPS, 3-(*N*-morpholino)propanesulfonic acid; RR, ruthenium red; SH, sulfhydryl; SR, sarcoplasmic reticulum.

photometer (SLM, Aminco DW-2000) with no addition of precipitating agent using a method modified from that of Palade (*15*) as previously described (*13*). The Ca^{2+} release was induced from the actively loaded SR vesicles by addition of metal ions, including Ag^{+} and triphenyltin. The amount of Ca^{2+} released was measured at 10 min after the addition of metal ions and calculated according to the absorbance-concentration curve derived from the titration of buffer containing 100 μM antipyrylazo III with the addition of a known concentration of Ca^{2+}. The percentages of Ca^{2+} release induced at various concentrations of triphenyltin were calculated by comparison with the maximal release induced by Ag^{+} (10 μM) and triphenyltin (500 μM).

$^{45}Ca^{2+}$ Efflux and Uptake Measurement—The Ca^{2+} efflux from passively loaded SR vesicles was measured with a radioactive isotope using the Millipore filtration method described by Kasai and Nunogaki (*16*). The SR (1 mg/ml) were loaded with Ca^{2+} overnight on ice in the loading buffer containing 150 mM KCl, 20 mM MOPS, and 1 mM $CaCl_2$ (pH 6.8) with the addition of 1.5 μCi/ml $^{45}Ca^{2+}$ (Amersham, 2.03 mCi/ml). To measure the Ca^{2+} efflux, 100 μl of loaded SR was withdrawn and added to 0.9 ml of releasing buffer containing 150 mM KCl, 20 mM MOPS, and 1 mM EGTA (pH 6.8) with the addition of metal ions at the indicated concentration. To stop the efflux, 4 ml of stopping buffer containing 150 mM KCl, 20 mM MOPS, 5 mM $MgCl_2$, and 5 μM ruthenium red (pH 6.8) was added at the time indicated. The stopped solutions were quickly filtered through cellulose filter (Millipore, DAWP 0.65 μm) and washed once with 5 ml of stopping buffer. The filters were then air-dried and the radioactivity was counted in a liquid scintillation counter (Beckman, 2200CA). The radioactivity remaining on the filter represents the $^{45}Ca^{2+}$ within the SR vesicles. The control SR vesicles were taken through the same procedure except that no metal ions were added in the release-inducing buffer.

The uptake of $^{45}Ca^{2+}$ by SR vesicles (1 mg/ml) was measured in the loading buffer as above with 5 μM ruthenium red and metal ions at the indicated concentration. The uptake was started by the addition of 5 mM Mg·ATP and incubation was continued for 1 min at room temperature. At the end of incubation, 100 μl of loaded SR was withdrawn and filtered through the Millipore filter, then washed once with 5 ml of stopping buffer and the radioactivity was counted.

[^{3}H]Ryanodine Binding and Dissociation—The apparent (equilibrium) ryanodine binding was measured according to Pessah *et al.* (*17*) with some modification. SR vesicles (500 μg/ml) were incubated at 37°C for 2 h in a medium containing 250 mM KCl, 15 mM NaCl, 50 μM $CaCl_2$, 10 nM [^{3}H]ryanodine (NEM, 86.7 Ci·mmol^{-1}), 20 mM Tris-HCl (pH 7.1), and the test compounds at the concentrations indicated in each experiment. Non-specific binding was measured in the presence of 1 μM cold ryanodine (Calbiochem). At the end of incubation, 200 μl of each reaction mixture was withdrawn, added to 5 ml of ice-cold buffer to quench the reaction, rapidly filtered through a Whatman GF/B glass filter and rinsed once with 5 ml of ice-cold buffer. The data shown are the average values of triplicate determinations of at least three different preparations.

For the dissociation experiment, the SR vesicles were incubated with 10 nM [^{3}H]ryanodine as above for 2 h and 10 μM cold ryanodine or metal ions at the concentrations indicated were then added. After incubation for another hour, the final solutions were filtered and radioactivity was counted as described above.

Ca^{2+}-ATPase Measurement—ATPase activity was determined by means of a coupled-enzyme spectrophotometric ADP-release assay (*18*) by measuring the oxidation of NADH at 340 nm in 20 mM MOPS (pH 6.8) containing 0.3 mg/ml NADH, 5 mM $MgCl_2$, 0.2 mM EGTA, 0.45 mM phosphoenolpyruvate, 5 U/ml pyruvate kinase, 10 U/ml lactate dehydrogenase, and 4 μM Ca^{2+} ionophore, A23187. The ATPase activity was measured by incubating 5-10 μg of protein with or without the test compounds in a 1 ml assay mixture at 37°C for 5 min. The reaction was started by the addition of 1 mM ATP. Ca^{2+}-ATPase was calculated as the difference of activities measured with and without the addition of 0.2 mM $CaCl_2$.

RESULTS

Triphenyltin-Induced Ca^{2+} Release from Sarcoplasmic Reticulum—The effect of triphenyltin on the Ca^{2+} release was studied using sarcoplasmic reticulum (SR) membrane vesicles isolated from rabbit skeletal muscle and using the metallochromic Ca^{2+} indicator dye, antipyrylazo III, to monitor the Ca^{2+} uptake and release. As shown in Fig. 1, 2 mM caffeine induced a rapid Ca^{2+} release from the actively loaded SR, as indicated by the sharp increase in the absorbance (trace 1) and the released Ca^{2+} was slowly taken up by the SR. Addition of 2 μM A23187, a Ca^{2+} ionophore, induced an irreversible Ca^{2+} release from the SR. As seen by others (*10*), addition of 5 μM Ag^{+} (trace 2) induced a rapid release of the actively loaded Ca^{2+} and the released Ca^{2+} was not taken up again by the SR. As seen with Ag^{+}, triphenyltin (traces 3-5) induced a slow and irreversible Ca^{2+} release from SR vesicles. Ca^{2+} release induced by 50 μM triphenyltin was completely inhibited by pretreatment with 2 μM ruthenium red (RR; trace 7), a specific Ca^{2+} release blocker, or 1 mM dithiothreitol (DTT; trace 8), a thiol-reducing agent, suggesting that triphenyltin triggers Ca^{2+} release *via* an oxidation reaction with the Ca^{2+} release channel, also known as the ryanodine receptor, of the SR. However, a slow release of Ca^{2+} was observed at 100 μM triphenyltin in the presence of RR (trace 6), suggesting a second site of action. The Ca^{2+} release induced by triphenyltin and triphenyltin in the presence of Ca^{2+} release blocker (2 μM RR) was dose-dependent with EC_{50} values of 75 and 270 μM and maximum release at 200 and 750 μM, respectively (Fig. 2A). The inability of SR to take up the released Ca^{2+} suggests that either the release channel was locked in an open state or the uptake of Ca^{2+} by Ca^{2+}-ATPase was inhibited. Direct measurement of the Ca^{2+}-ATPase activity of SR has shown that triphenyltin dose-dependently inhibited the ATPase activity with an IC_{50} value of 3 μM (Fig. 2B).

Triphenyltin Promotes the $^{45}Ca^{2+}$ Efflux and Inhibits $^{45}Ca^{2+}$ Uptake by SR—The effects of triphenyltin on Ca^{2+} efflux and uptake were examined by a radioactive isotope and filtration method. Ca^{2+} efflux from SR vesicles passively loaded with $^{45}Ca^{2+}$ was measured by dilution of the vesicles into Ca^{2+} free media containing 1 mM EGTA. As can be seen in Fig. 3, the efflux of loaded $^{45}Ca^{2+}$ was

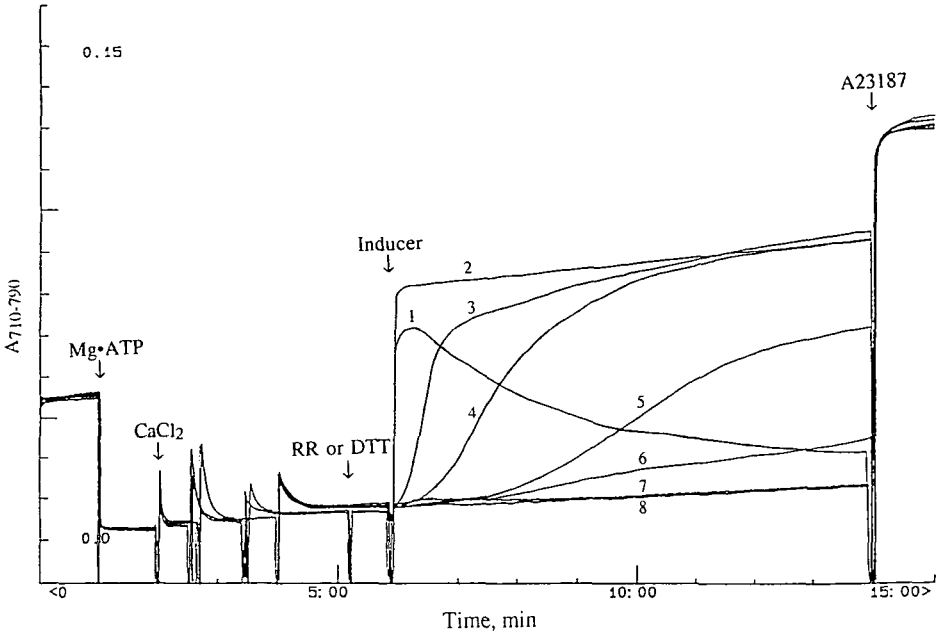

Fig. 1. **Induction of Ca^{2+} release by Ag^{+} and TPT from SR membrane vesicles.** SR vesicles (0.5 mg/ml) were loaded with Ca^{2+} and the Ca^{2+} concentration was monitored in terms of the absorbance difference at 710 and 790 nm according to the procedure outlined in "MATERIALS AND METHODS." Trace 1, Ca^{2+} release induced by 2 mM caffeine; trace 2, Ca^{2+} release induced by 5 μM Ag^{+}; traces 3–5, Ca^{2+} release induced by 200, 100, and 50 μM triphenyltin, respectively; trace 6, Ca^{2+} release induced by 100 μM triphenyltin with prior addition of 2 μM RR. Traces 7 and 8 are Ca^{2+} release induced by 50 μM triphenyltin with prior addition of 2 μM RR and 1 mM DTT, respectively.

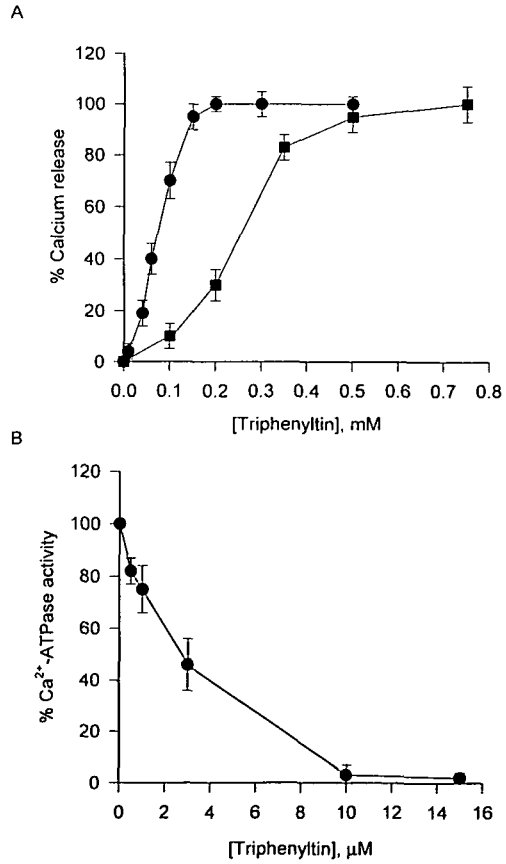

Fig. 2. **Dose-response of Ca^{2+} release induction and Ca^{2+}-ATPase inhibition by triphenyltin.** In A, the dose-response of Ca^{2+} release induced by triphenyltin without (●) or with (■) prior addition of 2 μM RR was measured as in Fig. 1. In B, the inhibitory effect of triphenyltin on Ca^{2+}-ATPase was measured according to the procedures outlined in "MATERIALS AND METHODS." The data are expressed as mean ± SEM (n=6).

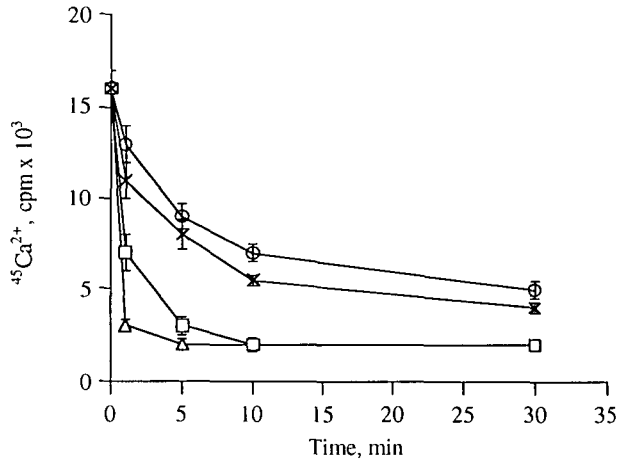

Fig. 3. **Ca^{2+} efflux induced by Ag^{+} and TPT.** The effect of 5 μM Ag^{+} (△), 100 μM triphenyltin (□), or 100 μM triphenyltin with 1 mM DTT (×) on the Ca^{2+} efflux from SR vesicles passively loaded with $^{45}Ca^{2+}$ was measured by dilution of the vesicles into Ca^{2+}-free media containing 1 mM EGTA and filtration as outlined in "MATERIALS AND METHODS." The control Ca^{2+} efflux (○) was measured with no addition of metal ions. The data are expressed as mean ± SEM (n=5).

accelerated in the presence of Ag^{+} (△), amounting to 81% compared to the 19% efflux of the control (○) within 1 min. Triphenyltin (□; 100 μM) accelerated the efflux of passively loaded $^{45}Ca^{2+}$ but at a slower rate, 56 and 81% at 1 and 5 min, respectively. The effect of triphenyltin was inhibited in the presence of 1 mM DTT (×).

The active Ca^{2+} uptake by SR was measured in the presence of 5 μM RR to block the Ca^{2+} release channel. The $^{45}Ca^{2+}$ uptake was inhibited by 82% in the presence of 10 μM triphenyltin (Table I). The inhibitory effect of triphenyltin was also blocked by 1 mM DTT.

Effect of Organotin on Apparent [^{3}H]Ryanodine Binding—The plant alkaloid, ryanodine, has been shown to bind

TABLE I. **Effects of triphenyltin on $^{45}Ca^{2+}$ uptake by SR.** The active Ca^{2+} uptake of SR by Ca^{2+}-ATPase in the presence of 10 μM triphenyltin or 10 μM triphenyltin plus 1 mM DTT was measured in the uptake solution containing 5 mM MgATP and 5 μM of the Ca^{2+} release channel blocker, ruthenium red.

	$^{45}Ca^{2+}$, cpm$\times 10^4$
Control	8.5 ± 1.1[a]
TPT	1.5 ± 0.21
TPT+DTT	7.2 ± 0.7

[a]The data are expressed as mean$\pm$SEM ($n=6$).

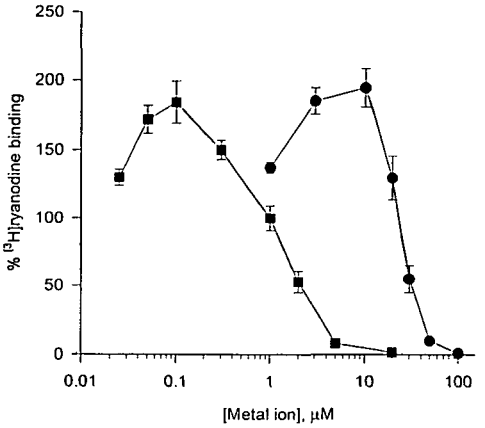

Fig. 4. **Effects of Ag^+ and TPT on apparent [^{3}H]ryanodine binding.** The apparent [^{3}H]ryanodine binding was measured after 2 h incubation of SR vesicles with 10 nM [^{3}H]ryanodine and Ag^+ (■) or triphenyltin (●) at the concentrations indicated according to the procedures outlined in "MATERIALS AND METHODS." The data are expressed as mean$\pm$SEM ($n\geq6$).

specifically to the Ca^{2+} release channel of SR (*19*) and the ligand binding was used as a probe for the channel activity (*20*). At Ca^{2+} release-inducing concentrations (1-10 μM), Ag^+ inhibited the apparent [^{3}H]ryanodine binding (Fig. 4); however, the apparent binding was potentiated at lower concentrations of Ag^+ (0.02-0.1 μM). Triphenyltin, like Ag^+, exerted dual effects on the apparent [^{3}H]ryanodine binding. Triphenyltin (0.5-10 μM) dose-dependently potentiated the [^{3}H]ryanodine binding, but the [^{3}H]ryanodine binding decreased as the concentration of triphenyltin was increased and the [^{3}H]ryanodine binding was completely blocked at higher concentrations than 50 μM.

Effect of Ag^+ and Triphenyltin on Dissociation of Bound [^{3}H]Ryanodine—The Ag^+ has been shown to increase the dissociation of [^{3}H]ryanodine from its receptor (*17*). The effects of triphenyltin on the dissociation of [^{3}H]ryanodine were examined and the data are summarized in Table II. The apparent [^{3}H]ryanodine binding dropped slowly from 2.6 ± 0.3 to 1.8 ± 0.31 pmol/mg protein within 1 h in the presence of 10 μM cold ryanodine. The bound [^{3}H]ryanodine dissociated from SR rapidly in the presence of Ag^+ (10 μM) and triphenyltin (100 μM) and the apparent binding decreased significantly to 0.2 ± 0.05 and 0.33 ± 0.12 pmol/mg protein, respectively. The effects of Ag^+ and triphenyltin were inhibited by DTT (data not shown).

TABLE II. **The effects of Ag^+ and triphenyltin on the dissociation of bound [^{3}H]ryanodine from SR.** After equilibration of the SR with 10 nM [^{3}H]ryanodine for 2 h (2.6 ± 0.3 pmol/mg protein), 10 μM ryanodine, 10 μM Ag^+, or 100 μM TPT was added, and incubation was continued for another hour to determine the effects of these ions on the dissociation of the bound [^{3}H]ryanodine from SR.

	[^{3}H]Ryanodine binding (pmol/mg protein)
10 μM ryanodine	1.8 ± 0.31[a]
Ag^+	0.2 ± 0.05*[,b]
TPT	0.33 ± 0.12*

[a]The data are expressed as mean$\pm$SEM ($n=3$). [b]Values marked * are significantly different from the control value, $p<0.05$.

DISCUSSION

Sulfhydryl- (SH-) reactive agents, including heavy metals (*6*), anthraquinones (*21*), and dithiodipyridine (*22*) have been shown to induce Ca^{2+} release from SR through oxidation of endogenous SH groups on or associated with the Ca^{2+} release channel (*23*), the ryanodine receptor. By using isolated SR membrane vesicles, we have shown that triphenyltin dose-dependently induced Ca^{2+} release from the actively loaded SR vesicles. The release could be blocked by pretreatment with ruthenium red (RR) and dithiothreitol (DTT), suggesting that the release was due to the oxidation of the SH moiety of the ryanodine receptor. The activation of the Ca^{2+} release channel of SR by triphenyltin was further supported by the induction of $^{45}Ca^{2+}$ efflux from the passively loaded SR by triphenyltin. Although triphenyltin could induce Ca^{2+} release and Ca^{2+} efflux from SR, the rate of Ca^{2+} release was much slower than that seen with Ag^+ in both release (Fig. 1) and efflux (Fig. 3) measurement. Two distinct forms of Ca^{2+} release from SR in response to addition of triphenyltin were observed. Triphenyltin, at higher concentrations than 100 μM, induced an RR-insensitive release, as seen with other SH-reactive heavy metals (*6*). Previously Ag^+ was also shown to induce Ca^{2+} release from SR by inhibition of the Ca^{2+}-ATPase (*11*). We have shown that triphenyltin also dose-dependently inhibited the Ca^{2+}-ATPase activity and the active uptake of $^{45}Ca^{2+}$ by SR. However, the concentrations at which triphenyltin inhibits the Ca^{2+}-ATPase are much lower than those that induce Ca^{2+} release. These results suggest that the ruthenium red-insensitive release induced by triphenyltin at higher concentrations may not be related to the interaction of this reagent with the ryanodine receptor and Ca^{2+}-ATPase.

It has been suggested that ryanodine binding to SR vesicles could be used as a probe for the channel activity (*20*). The binding of ryanodine to SR vesicles was enhanced by several Ca^{2+} release inducers (*17*) and paralleled closely the transport characteristics of Ca^{2+}-induced Ca^{2+} release (*24*). In this study, we have found that triphenyltin exerted a biphasic effect on apparent ryanodine binding, that is, enhancing at lower concentrations, but inhibitory at higher concentrations. Abramson *et al.* (*21*) observed that doxorubicin, an SH-reactive anthraquinone, induces Ca^{2+} release and stimulates ryanodine binding; however, Ag^+ and aryldisulfides induce Ca^{2+} release, but cause a rapid displacement of bound ryanodine from its receptor (*17*), suggesting that although both induce Ca^{2+} release, anthraquinones and Ag^+ might interact with a different SH group (*23*). It is possible that triphenyltin might be able to react with more than one SH group. At the high concentration of 10 μM or more, triphenyltin reacted with one SH group, possibly the one that reacted with Cu^{2+}/cysteine (*25*),

reactive disulfides (*22*) or heavy metals (*10, 26*), to trigger Ca^{2+} release from SR and displacement of bound ryanodine. On the other hand, at concentrations lower than 10 μM, triphenyltin reacted with an SH group which caused only the potentiation of apparent ryanodine binding without inducing Ca^{2+} release. The ability of triphenyltin to facilitate the dissociation of bound ryanodine at high concentrations further supports this view. Similar results were also seen with Ag^+; addition of Ag^+ (0.01-0.1 μM) dose-dependent increased the apparent [^{3}H]ryanodine binding but inhibited the binding at higher concentrations (0.1-10 μM), as with triphenyltin (Fig. 4).

Alkyltin compounds are used as stabilizers and antifouling agents, and bioconcentration has been demonstrated in crabs, oysters, and salmon. Organotins have been shown to be neurotoxic (*27, 28*) and immunotoxic agents (*29*). A direct peripheral myopathy has also been found in organotin intoxication and suggested to be a significant factor in the development of muscle weakness following exposure (*30, 31*). Our results showed that triphenyltin could induce Ca^{2+} release from the SR and inhibit the Ca^{2+} uptake by SR, which might result in depletion of the internal Ca^{2+} store of the skeletal muscle. The results from this study could partly explain the development of muscle weakness; however, the relevance of this effect exerted by triphenyltin to the development of peripheral myopathy in organotin intoxication still needs further investigation.

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