Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 67 (2004) 2071–2079
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Selenium compounds modulate the calcium release channel/ryanodine receptor of rabbit skeletal muscle by oxidizing functional thiols

Ruohong Xia[a], Howard E. Ganther[b], Adam Egge[c], Jonathan J. Abramson[c,*]

[a]*Physics Department, East China Normal University, 3663 Zhongshan North Road, Shanghai 200062, China*
[b]*Department of Nutritional Sciences, University of Wisconsin, Madison, WI 53706, USA*
[c]*Physics Department, Portland State University, Portland, OR 97207, USA*

Received 21 October 2003; accepted 10 February 2004

Abstract

Selenium compounds, such as sodium selenite and Ebselen were shown to increase high affinity ryanodine binding to the skeletal muscle type ryanodine receptor (RyR1) at nanomolar concentrations, and inhibit the receptor at low micromolar concentrations. This biphasic response was observed in both concentration and time-dependent assays. Extensive washing did not reverse either the stimulation or suppression of receptor binding, but both were prevented or reversed by addition of reduced glutathione, GSH. Selenium compounds were also shown to induce Ca^{2+} release from the isolated sarcoplasmic reticulum vesicles. Sodium selenite and Ebselen stimulated the skeletal muscle ryanodine receptor by oxidizing 14 of 47 free thiols per monomer on RyR1 (as detected with the alkylating agent 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin) (CPM). Oxidation of the remaining thiols by these selenium compounds resulted in inhibition of the ryanodine receptor.

Keywords: Selenium; Sarcoplasmic reticulum; Ryanodine receptor; Thiol oxidation; Sodium selenite; Ebselen

1. Introduction

The calcium release channel/ryanodine receptor (RyR) is a multi-ligand modulated ion channel, which rapidly releases Ca^{2+} from sarcoplasmic reticulum (SR) to initiate muscle contractions. It has been demonstrated that the skeletal muscle RyR, RyR1, is modulated by such thiol-reactive agents as glutathione, dithiothreitol (DTT), porphyrins, and quinones [1–3]. Oxidation of critical thiols on the RyR1 opens the Ca^{2+} release channel, while reduction of the disulfides formed closes the channel back down [3]. Recent findings support a model in which the local redox environment influences the sensitivity of RyR1 to activation [4–6]. Moreover, oxidants have been shown to exert biphasic control over the Ca^{2+} release channel. At low concentrations, the Ca^{2+} release mechanism is activated, while at higher concentrations it is inhibited [2,7].

Low concentrations of selenium (Se) are essential for the synthesis of selenocysteine-containing enzymes such as glutathione peroxidase and thioredoxin reductase [8,9]. Deficiencies in Se have been linked to White Muscle Disease in animals and Keshan disease, endemic cardiomyopathy, muscular dystrophy, and cardiovascular disease [10,11] in humans. An excess of Se is also toxic to both animals and man. Many Se compounds are potent oxidants. An excess of Se is likely to create an over oxidized environment in cells and cause cell dysfunction and apoptosis [10]. It has been shown that almost one-third of the Se in the body is in muscle [12]. The effects of Se on muscle function have been controversial. Sodium selenite and Ebselen, both used extensively in this study, were reported to exert both negative and positive inotropic effects on cardiac muscle contractions in a concentration-dependent manner, and these effects were not reversed by washing

Abbreviations: SR, sarcoplasmic reticulum; RyR1, ryanodine receptor type 1 from skeletal muscle; CPM, 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin; DTT, dithiothreitol; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); GSSG, oxidized glutathione; PC, phosphatidyl choline; PMSF, phenylmethanesulfonyl fluoride; CHAPS, 3-((3-cholamidopropyl)dimethylammonio)-1-propane-sulfonate; PSe, phenylalanine-4′-seleninic acid; MSe, methylseleninic acid; BSe, benzene seleninic acid; EC_{50}, concentration that activates to 50% of maximum binding; IC_{50}, concentration that inhibits to 50% of maximum binding

* Corresponding author. Tel.: +1-503-725-3014; fax: +1-503-725-9525.
E-mail address: abramsonj@pdx.edu (J.J. Abramson).

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.02.018

[13–15]. Moreover, in adult mongrel dogs the intravenous administration of 2 mg/kg sodium selenite produced an initial increase in cardiac output followed by a decrease in output [16]. In skeletal muscle, sodium selenite has also been shown to be potent in inducing contraction of the mouse diaphragm in a biphasic manner [17], which was not affected by the external Ca^{2+} concentration. These findings suggest that the Se-dependent contraction was induced by the release of Ca^{2+} from internal Ca^{2+} pools, such as the SR. Lin-Shiau et al. [18] observed that pretreatment with reduced glutathione (GSH) blocked the sodium selenite evoked contraction. They, therefore, postulated that the sodium selenite induced contraction was caused by an interaction with sulfhydryl groups on the calcium release channel complex, which somehow triggered the release of Ca^{2+} from the SR [18]. However, selenium's interaction with the Ca^{2+} release channel of SR has not previously been directly studied.

In this manuscript, we demonstrate for the first time that selenium compounds, including sodium selenite and Ebselen, stimulate calcium release from isolated SR vesicles, and increase high affinity ryanodine binding at concentrations from 10 nM to 1 μM. At higher concentrations, they inhibit the ryanodine receptor. This pattern of a biphasic stimulation and inhibition of the ryanodine receptor is similar to that seen with other thiol oxidants [2,7]. Se-dependent stimulation and inhibition of RyR1 were prevented by GSH and the thiol alkylating agent, CPM. The number of free thiols on the RyR1 (∼47) decreased rapidly in a Se concentration-dependent fashion. The Se-dependent oxidation of one class of reactive thiols appears to stimulate the Ca^{2+} release channel, while oxidation of a second class of thiols induced by exposure to higher concentrations of selenium compounds appears to close the receptor down.

2. Materials and methods

2.1. Materials

Synthesis of D,L-4′-seleninylphenylalanine was as described by Ganther [19]. Benzeneseleninic acid was purchased from Aldrich Chemical. [^{3}H]-Ryanodine was purchased from Perk-Elmer Life Sciences. CPM was purchased from Molecular Probes. All other chemicals were purchased from Sigma Chemical Co.

2.2. Isolation of SR membrane vesicles

SR was isolated from back and leg skeletal muscle from New Zealand White rabbits by the method of MacLennan with small modifications [20]. Fifty micromolar dithiothreitol and 0.2 μg/ml leupeptin were added to all buffers except for the final SR resuspension buffer. Samples were stored in liquid N_2.

2.3. Isolation of the purified RyR1

RyR1 was isolated from SR vesicles by a modification of the method of Lai et al. [21]. Thirty milligrams of SR were incubated with 10 ml of buffer containing 1 M NaCl, 150 μM $CaCl_2$, 100 μM EGTA, 25 mM PIPES, 1.6% CHAPS, 3 mg/ml PC, 0.5 mM PMSF, 1 mM DTT, 0.2 μg/ml leupeptin for 2 h on ice. It was then centrifuged at 100,000 × g for 60 min at 4 °C. The supernatant was placed on a 5–20% continuous sucrose gradient containing 1 M NaCl, 40 mM Tris, 0.9% CHAPS, 4 mg/ml PC, 150 μM $CaCl_2$, 100 μM EGTA, 1 μg/ml leupeptin and 500 μM DTT and centrifuged at 25,000 rpm for 16 h in a Beckman SW28 swinging bucket rotor. Fractions were collected and stored in liquid N_2.

2.4. [^{3}H]-Ryanodine binding experiments

Binding of [^{3}H]-ryanodine was carried out according to the method of Pessah et al. [22]. SR vesicles (0.1 mg protein/ml) were incubated with Se compounds at the designated concentrations in 1 ml of assay buffer containing 250 mM KCl, 15 mM NaCl, 20 mM PIPES, 99 μM $CaCl_2$, 50 μM EGTA, 1 nM [^{3}H]-ryanodine, and 14 nM ryanodine, pH 7.1 at 30 °C for 6 h. Each assay was performed in duplicated and repeated at least three times. The free Ca^{2+} concentrations, in the presence of 50 μM EGTA, were calculated by WinMaxc [23]. The binding reaction was quenched by rapid filtration through Whatman GF/B filters mounted on a 24-well Brandel Cell Harvester. Filters were rinsed three times with a wash buffer containing 50 μM Ca^{2+}, were then put into scintillation vials, filled with scintillation fluid, shaken overnight, and counted the following day.

2.5. Measurement of Ca^{2+} fluxes

Ca^{2+} fluxes across SR vesicles were monitored using a dual wavelength spectrophotometer [24] by measuring the differential absorbance changes of antipyrylazo III (APIII) at 720–790 nm. Ca^{2+} uptake into SR vesicles (0.2 mg/ml) was carried out in a buffer containing 100 mM KCl, 20 mM HEPES, 1 mM $MgCl_2$, 20 uM free Ca^{2+}, and 200 uM APIII. Ca^{2+} uptake was initiated with the addition of 0.5–1 mM Mg^{2+}-ATP. Upon completion of Ca^{2+} uptake, at which time the Ca^{2+} concentration had reached a steady state, Ca^{2+} release was initiated by the addition of various concentrations of Ebselen or sodium selenite. In all experiments, the free extravesicular Ca^{2+} concentration was measured as a function of time. The Ca^{2+} efflux rate was determined from the initial slope of the extravesicular Ca^{2+} concentration versus time.

2.6. Washing of SR

0.2 mg/ml SR was incubated in 10 ml of buffer containing 250 mM KCl, 15 mM NaCl, 20 mM PIPES, 99 μM

$CaCl_2$, 50 μM EGTA, pH 7.1 and the designated concentrations of Se for different periods of time. Samples were incubated in sodium selenite for 30 or 120 min, or with Ebselen for 10 or 60 min at room temperature. After the incubation, treated SR samples were centrifuged in a Ti-60 rotor at 35,000 rpm for 30 min at 2 °C. The supernatants were discarded and the pellets were rinsed and re-suspended at 0.1 mg/ml SR. [^{3}H]-ryanodine binding was then measured as a function of time, as determined above, in the absence of added Se.

2.7. CPM labeling

SR was labeled with 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM) as described by Xia and coworkers [4]. SR at 0.1 mg/ml was incubated in buffer containing 250 mM KCl, 15 mM NaCl, 20 mM PIPES, 1 mM $CaCl_2$, 20 nM CPM, pH 7.1 at room temperature with rigorous stirring for 3 min. The reaction was quenched by addition of a 100-fold excess of glutathione (2 μM), and EGTA was added to bring the free Ca^{2+} concentration to 50 μM. Under conditions in which the Ca^{2+} release channel is closed (1 mM Ca^{2+}), CPM binds specifically to hyperreactive thiols. When this reaction is carried out under conditions in which the Ca^{2+} channel is open (50 μM Ca^{2+}), binding of CPM is slow, non-selective [25], and does not block Se-dependent stimulation of ryanodine binding. For those experiments carried out with the isolated RyR1, CPM pretreatment was carried out with 10 μM CPM.

2.8. Protein free thiol measurement

0.25 μg/ml of the isolated RyR1 was incubated with different concentrations of sodium selenite or Ebselen at 25 °C for 60 min in a buffer containing 250 mM KCl, 15 mM NaCl, 20 mM PIPES, pH 7.1. Ten micromolar CPM was added to each assay solution and incubated for 10 min. The fluorescence was then measured at an excitation wavelength of 397 nm and an emission wavelength of 465 nm. A standard calibration curve was generated by adding 10 μM CPM to known concentrations of cysteine.

3. Results

3.1. Selenium compounds exert biphasic modulation of the RyR1

Se compounds, including sodium selenite, Ebselen, phenylalanine-4′-seleninic acid (PSe), methylseleninic acid (MSe), and benzene seleninic acid (BSe) were shown to stimulate ryanodine binding at nanomolar concentrations and to inhibit ryanodine binding at low micromolar concentrations. This biphasic concentration dependence

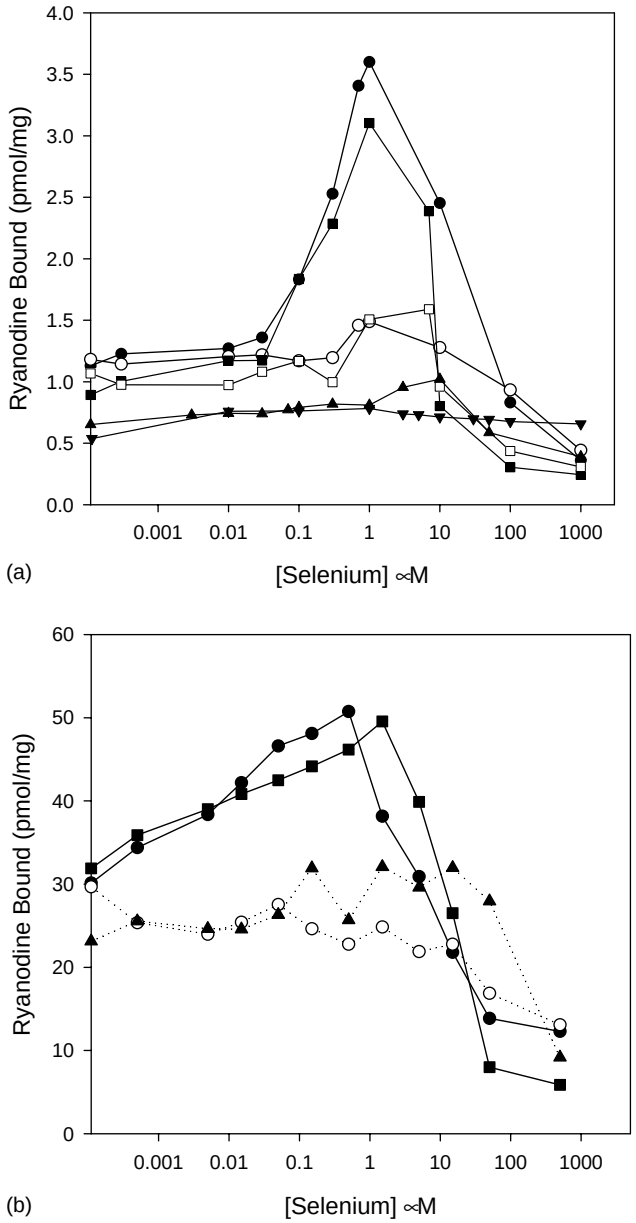

Fig. 1. High affinity ryanodine binding is modulated by Na_2SeO_3 and Ebselen. Ryanodine binding experiments were carried out in a standard ryanodine binding buffer containing 250 mM KCl, 15 mM NaCl, 20 mM PIPES, 99 μM $CaCl_2$, 50 μM EGTA, 1 nM [^{3}H]-ryanodine, and 14 nM ryanodine, pH 7.1 at 30 °C for 6 h at a protein concentration of 0.1 mg/ml, as described in Section 2. In (a), SR vesicles were incubated with various concentrations of Na_2SeO_3 (●) or Ebselen (■) in the absence (●, ■) or following pretreatment with CPM (20 nM) (○, □) or 1 mM GSH (▲, Na_2SeO_3; ▼, Ebselen). In (b) binding measurements were carried out with the purified ryanodine receptor under the same conditions as indicated in (a), except that CPM and GSH treated RyR1 were only shown in the presence of Na_2SeO_3. (●), Na_2SeO_3 as control; (■), Ebselen as control; (○), Na_2SeO_3 in the presence of 10 μM CPM; (▲), Na_2SeO_3 following pretreatment with 1 mM GSH ($n = 6$).

was observed with both isolated SR vesicles (Fig. 1a and Table 1) and with the isolated RyR1 (Fig. 1b). The maximum stimulation of receptor binding occurs with sodium selenite (a 2.8-fold enhancement of binding was

Table 1
Selenium compounds are potent activators and inhibitors of RyR1

	B_{max} (pmol/mg)	K_d (nM)	EC_{50} (μM)	IC_{50} (μM)	Degree of stimulation
Control	2.2 ± 0.4	11.8 ± 0.6	–	–	–
Na_2SeO					
3 μM	3.5 ± 0.3	9.8 ± 0.3	0.3 ± 0.1	31 ± 4	2.82 ± 0.08
50 μM	2.0 ± 0.4	10.9 ± 0.3			
+1 mM GSH	2.3 ± 0.3	11.1 ± 0.4	25 ± 4	100 ± 11	1.37 ± 0.04
Ebselen					
3 μM	3.8 ± 0.5	11.7 ± 0.4	0.22 ± 0.04	9 ± 2	2.50 ± 0.07
50 μM	0.76 ± 0.06	15.3 ± 0.6			
+1 mM GSH	2.0 ± 0.2	12.2 ± 0.5	12 ± 3	80 ± 6	1.12 ± 0.03
PSe					
0.1 μM	4.8 ± 0.5	7.0 ± 0.4	0.05 ± 0.02	30 ± 4	1.83 ± 0.06
50 μM	2.9 ± 0.6	6.4 ± 0.3			
+1 mM GSH[a]	2.3	9.8	30	110	1.26
MSe					
1 μM	3.8 ± 0.4	8.1 ± 0.5	0.29 ± 0.05	0.8 ± 0.1	1.80 ± 0.0 6
50 μM	2.4 ± 0.3	8.8 ± 0.5			
BSe					
0.1 μM	3.25 ± 0.08	15.7 ± 0.5	0.03 ± 0.01	12 ± 2	1.44 ± 0.05
5 μM	1.48 ± 0.04	6.7 ± 0.4			
+1 mM GSH[a]	2.1	10.9	10	45	1.15

EC_{50}, IC_{50}, and degree of stimulation were calculated from ryanodine binding experiments carried out as a function of selenium compound concentration. B_{max} and K_d were calculated from Scatchard plots. Binding experiments were carried out as a function of ryanodine concentration and were repeated at least three times.

[a] $n = 2$, these data sets are the average of two experiments.

observed). The degree of stimulation is defined as the maximum ryanodine binding in the presence of the Se compound divided by control binding (in the absence of Se). BSe had the lowest EC_{50} (30 nM), followed by PSe, MSe, Na_2SeO_3, and Ebselen. Ebselen had the lowest IC_{50}, followed by BSe, PSe, Na_2SeO_3, and MSe.

The biphasic profiles of Se-dependent stimulation at low concentrations and inhibition at higher concentrations were also present in time-dependent ryanodine binding experiments, as shown in Fig. 2. At stimulating concentrations of 1 μM of Na_2SeO_3 or 3 μM Ebselen only a time-dependent enhancement of ryanodine binding was observed. At inhibiting concentrations of 100 μM Na_2SeO_3 or 30 μM Ebselen, a rapid increase of ryanodine binding was followed by a slower decrease of receptor binding.

3.2. The biphasic effects of selenium compounds were prevented by GSH

Both the concentration-dependent stimulation and the inhibition phases of equilibrium ryanodine binding induced by Na_2SeO_3 and Ebselen were eliminated by the presence of 1 mM of the thiol reducing agent GSH. This was observed with either SR vesicles (Fig. 1a or Table 1) or the isolated RyR1 (Fig. 1b). Time-dependent stimulation followed by inhibition of ryanodine binding was also eliminated by 1 mM GSH (data not shown). All of the Se-containing compounds tested had no significant affect on the Ca^{2+} dependence of ryanodine binding. Neither, the EC_{50} or IC_{50} of Ca^{2+}-dependent ryanodine binding was altered by any of the five selenium compounds tested (data not shown). Previous studies have shown that addition of some oxidizing agents such as GSSG have no effect on the Ca^{2+} dependence of ryanodine

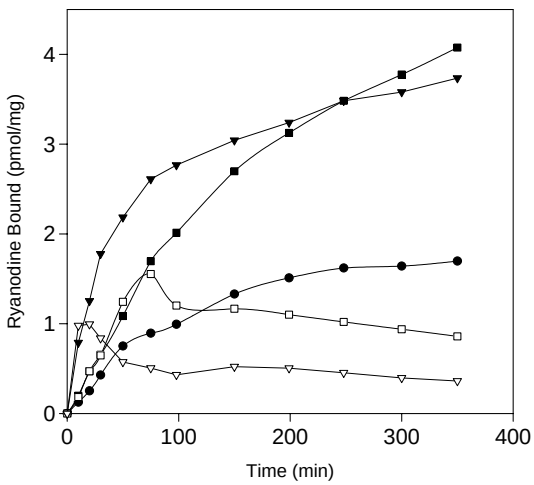

Fig. 2. Time-dependent ryanodine binding is modulated by Na_2SeO_3 and Ebselen. Time-dependent ryanodine binding was carried out in the standard ryanodine buffer as described in Fig. 1, containing 0.1 mg/ml of SR at 30 °C for up to 6 h. The reactions were stopped at the indicated time points. Assays were carried out in the absence of selenium compounds (●), or in the presence of 1 μM Na_2SeO_3 (■), 100 μM Na_2SeO_3 (□), 3 μM Ebselen (▼), or 30 μM Ebselen (▽) ($n = 4$).

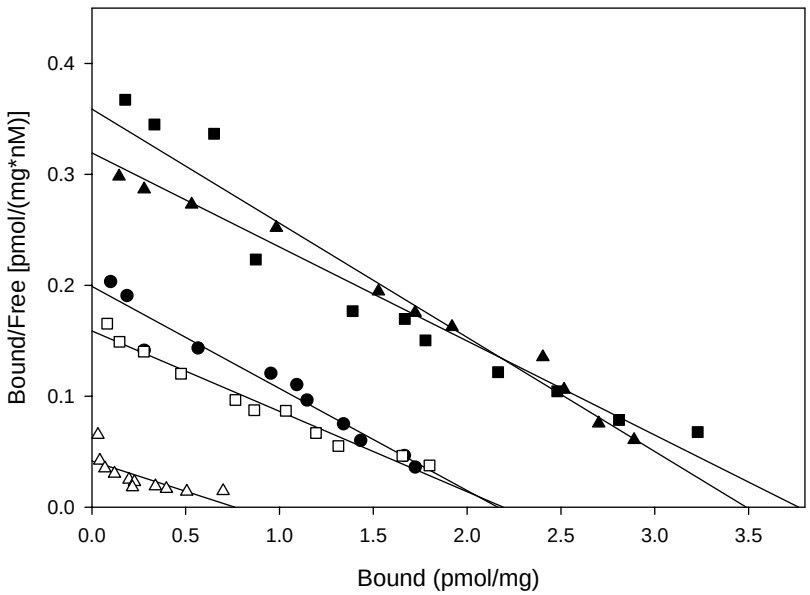

Fig. 3. Scatchard plots in the presence of Na_2SeO_3 or Ebselen. Scatchard plots were derived from concentration-dependent ryanodine binding assays to SR in the absence (●), or presence of 3 μM Na_2SeO_3 (■), 50 μM Na_2SeO_3 (□), 3 μM Ebselen (▲), or 30 μM Ebselen (△). The *x*-intercept is B_{max}, while the inverse slope is K_d. The ryanodine binding conditions are described in Section 2 ($n = 4$).

binding [1,4], while other thiol oxidizing reagents, such as hydrogen peroxide [7] and hypochlorous acid [26] sensitize the receptor to activation at lower Ca^{2+} concentrations.

Scatchard plots (Fig. 3) showed that the administration of stimulating concentrations of either Na_2SeO_3 or Ebselen resulted in an increase in B_{max}, while addition of inhibiting concentrations of these compounds resulted in a decrease in B_{max}. The affinity of the receptor for ryanodine, was not significantly affected by the presence of Na_2SeO_3 or Ebselen.

3.3. Se compounds induced Ca^{2+} efflux from actively loaded SR vesicles

In Fig. 4, the Ca^{2+} release rate induced by Ebselen was measured from SR vesicles actively loaded with Ca^{2+}. The concentration of Ebselen that stimulated the Ca^{2+} release rate to 50% of maximum was 34 μM. The maximum initial release rate induced by Ebselen was 12 nmol/mg/s. Sodium selenite was more effective than Ebselen at inducing rapid release of Ca^{2+} from actively loaded SR vesicles. Addition of 2 μM Na_2SeO_3 induced Ca^{2+} release at

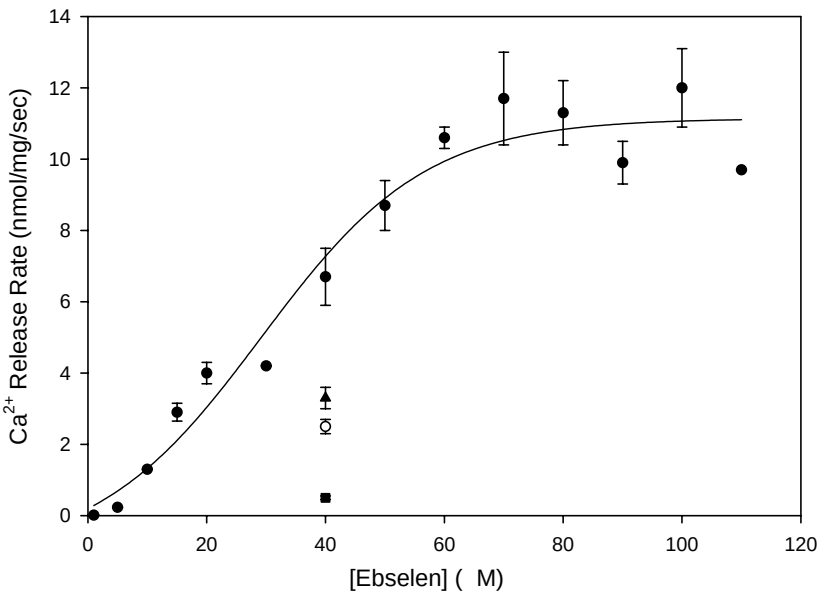

Fig. 4. Ebselen induced Ca^{2+} release from SR vesicles. The initial Ca^{2+} release rate from actively loaded SR vesicles was plotted vs. Ebselen concentration (●), in the presence of 5 μM ruthenium red (○), 2.2 mM Mg^{2+} (▲), or in the presence of 100 μM GSH (■). The results were presented as the mean ± S.E. ($n = 5$). The measurements were carried out as described in Section 2.

an initial rate of 54 nmol/mg/min (not shown). Addition of 100 μM GSH inhibited the release rate induced by addition of 40 μM Ebselen to ~7% of the control. Additions of other RyR1 inhibitors, such as 2.2 mM Mg^{2+} or 5 μM ruthenium red, inhibited the release rates to ~45 or ~33% of the control, respectively.

3.4. Selenium-dependent stimulation of RyR1 was not reversed by washing

To determine if washing reversed selenium-dependent enhancement of ryanodine binding, time-dependent ryanodine binding experiments were carried out following exposure to either Na_2SeO_3 (Fig. 5a) or Ebselen (Fig. 5b). The SR was then spun down and resuspended in a buffer containing no selenium compound. Ryanodine was then added and the time dependence of ryanodine binding was examined. As shown in Fig. 5, controls show a time-dependent increase in binding of ryanodine, but no inactivation of the receptor is observed. In the presence of either 50 μM Na_2SeO_3 or 30 μM Ebselen a rapid activation phase is followed by an inhibition of receptor binding. Removal of free Na_2SeO_3 or Ebselen by centrifugation and washing, following exposure to Na_2SeO_3 for 30 min or Ebselen for 10 min, resulted in a time-dependent stimulation phase somewhat similar to control binding with a complete loss of time-dependent inhibition of receptor binding. However, exposure for longer periods of time (Na_2SeO_3—4 h or Ebselen—1 h) resulted in an overall inhibition of receptor binding, which was not reversed by removal of the free selenium compound. It is clear that both the effects of stimulation and inhibition of Na_2SeO_3 and Ebselen were not removed by washing.

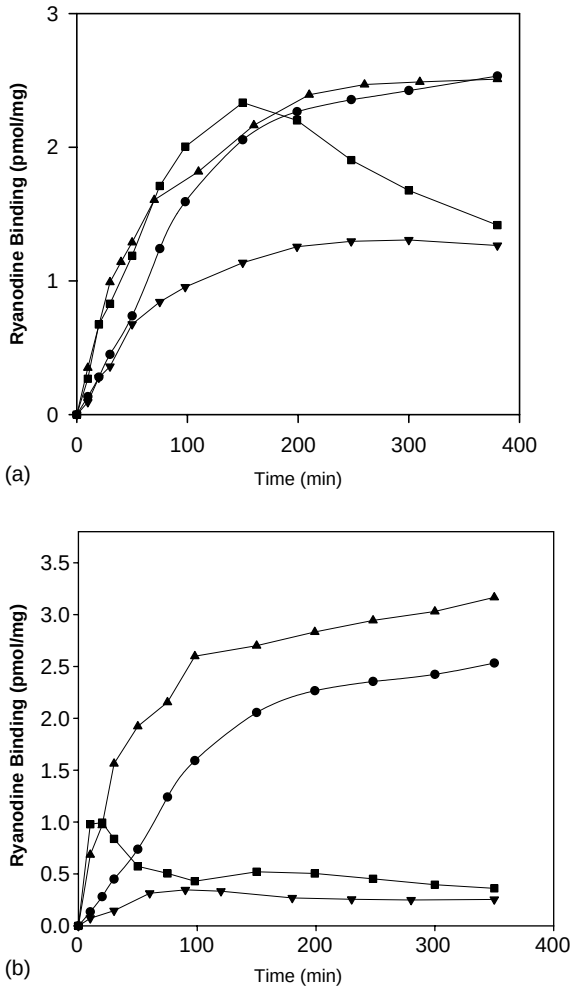

Fig. 5. The effects of Na_2SeO_3 and Ebselen were not reversed by washing. In (a) SR vesicles were treated in the absence (●), or presence of 50 μM Na_2SeO_3 (■), and ryanodine binding was measured as a function of time as in Fig. 2. Samples were exposed to Na_2SeO_3 for either 30 min (▲) or 4 h (▼). They were then pelleted by centrifugation, washed and exposed to [^{3}H]-ryanodine for various period of time. In (b) SR were treated in the absence (●) or presence of 30 μM Ebselen (■). They were then centrifuge, washed, following an incubation time of 10 min (▲), or 1 h (▼) ($n = 3$).

3.5. Selenium compounds oxidized the RyR1 functional thiols

In ryanodine binding experiments, pretreatment of either the SR (Fig. 1a) or the isolated receptor (Fig. 1b) with CPM prevented stimulation of the receptor by Se compounds. Treatment with CPM in the absence of either Na_2SeO_3 or Ebselen had no affect on ryanodine binding. However, as shown in Fig. 6a, treatment of the RyR1 with either Ebselen or Na_2SeO_3 resulted in a concentration-dependent loss of CPM titratable thiols. These results suggest that reactive thiols on the RyR1 are involved in selenium-dependent stimulation of the receptor. In the absence of selenium-containing compounds, the RyR1 contained an average of 47 ± 5 free thiols. This number decreased at low concentrations of either Na_2SeO_3 or Ebselen. At concentrations above 30 μM of either Na_2SeO_3 or Ebselen, almost all CPM detectable free thiols were oxidized and equilibrium ryanodine binding was reduced to negligible levels. The concentration of Na_2SeO_3 that resulted in loss of 50% of the free thiols on RyR1 was 0.7 μM, while the IC_{50} for Ebselen was 14 μM.

4. Discussion

Selenium compounds have been shown to cause an increase in the intracellular Ca^{2+} concentration, to stimulate and inhibit muscle contracture, and to induce cell apoptosis [27,28]. However, the direct effects of selenium compounds on the ryanodine receptor have not previously been studied. In this paper, we demonstrate for the first time that selenium compounds affect Ca^{2+} homeostasis by directly interacting with the calcium release channel in skeletal muscle sarcoplasmic reticulum. The present study reveals that selenium compounds oxidize functional thiol groups of the RyR1, which results in a biphasic modulation of the Ca^{2+} release protein/ryanodine receptor.

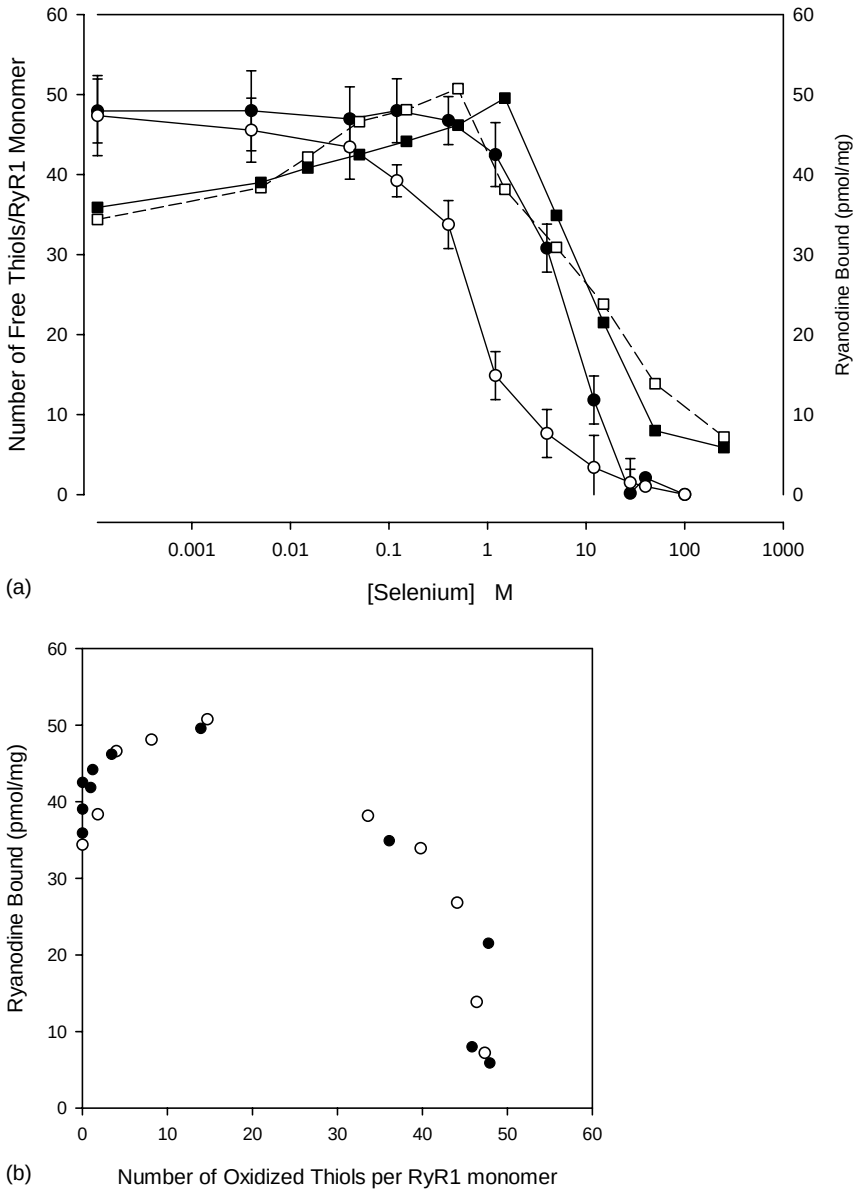

Fig. 6. Na_2SeO_3 and Ebselen modulate ryanodine binding to purified RyR1 by oxidizing free thiols. The number of thiols per monomer of RyR1 was determined by addition of 10 μM CPM to RyR1 (0.25 μg/ml) following pretreatment with varying concentrations of either Na_2SeO_3 (○, □) or Ebselen (●, ■) as described in Section 2. In (a) the number of free thiols/monomer was plotted vs. the concentration of either Na_2SeO_3 (○) or Ebselen (●) (left *y*-axis). The amount of bound ryanodine was also plotted vs. the concentration of Na_2SeO_3 (□), or Ebselen (■) (right *y*-axis). In (b) ryanodine binding was plotted vs. the number of oxidized thiols following treatment with different concentrations of Na_2SeO_3 (○) or Ebselen (●). The data presented in (b) were derived from the data shown in (a).

4.1. Biphasic modulation of RyR1

Many selenium compounds are strong oxidants. Previous studies have shown that selenium compounds induced skeletal muscle contraction in a biphasic manner [17,18]. The mechanism by which these compounds affected cytosolic Ca^{2+} homeostasis and muscle function can be explained by their interaction with the RyR1.

Figs. 1–3 and Table 1 showed that Na_2SeO_3, Ebselen, PSe, MSe and BSe, exert similar biphasic concentration- and time-dependent effects on the RyR1. These compounds were very potent. The EC_{50} for each of these compounds were in the nanomolar concentration range. The most effective stimulator of the RyR1 was BSe with an EC_{50} of 30 ± 10 nM. The biphasic effects indicate that Se compounds interact with the RyR1 at two distinct classes of sites—high affinity site(s), which stimulate the receptor, and low affinity site(s), which closes down the receptor. The increase in ryanodine binding induced by selenium compounds was caused by an increase in the number of available receptor binding sites, B_{max}, not by a change in the affinity of these sites for ryanodine, K_d (Fig. 3 and Table 1). Both Ebselen and Na_2SeO_3 (not shown) triggered Ca^{2+} release from actively loaded SR vesicles (Fig. 4).

In ryanodine binding assays using isolated RyR1 (Fig. 1b), Na_2SeO_3 and Ebselen also displayed a biphasic concentration-dependent stimulation and inhibition of the receptor in a manner similar to experiments carried out with SR vesicles (Fig. 1a). It appears as if the sites responsible for stimulation and inhibition of the RyR1 are localized on the receptor protein or a protein that co-purifies with it (i.e. FKBP12). The previously observed effects of selenium compounds on cardiac muscle [13–16], and skeletal muscle [17] contractility, and the observed rise in intracellular Ca^{2+} concentrations appear to be caused by oxidation of reactive thiols localized on the ryanodine receptor described in this manuscript.

Comparing Fig. 1a and b, it is observed that Ebselen is a more effective inhibitor of binding to the SR, while Na_2SeO_3 is more effective at inhibiting ryanodine binding to the isolated RyR1 at high concentrations. The IC_{50}s for these two selenium compounds are in reversed order: IC_{50} for Na_2SeO_3 (31 μM) is higher than that of Ebselen (9 μM) in Fig 1a, but is lower than that of Ebselen in Fig. 1b (7 μM versus 16 μM). This difference may be due to an altered protein conformation in the isolated RyR1 sample, or it may reflect an alteration in the ability of these compounds to transfer electrons in the native versus the isolated receptor environment.

4.2. Se modulates the RyR1 by oxidizing two groups of the RyR1 functional thiols

The skeletal muscle ryanodine receptor contains 101 cysteine residues per monomer, about half of which readily react with monobromobimane [6] or CPM. Reagents that oxidize sulfhydryl groups have been shown to stimulate RyR1, while thiol reducing agents close down the Ca^{2+} release channel and inhibit ryanodine binding [1,2,4,6,29]. In this paper, it was demonstrated that the number of reactive thiols on the RyR1 decreases as the concentration of selenium compounds increase. In a manner similar to that observed by Sun et al., redox active molecules such at oxidized glutathione, nitric oxide, and increased oxygen tension stimulate the ryanodine receptor by interacting with a class of reversible thiols. However, more extensive oxidation irreversibly inhibits the ryanodine receptor [6].

Selenium compounds tested in this study exerted biphasic control of the RyR1, in a manner similar to other oxidizing agents including naphthoquinines, oxidized glutathione, H_2O_2 [1,2,7], and HOCl [26]. The data presented in this paper strongly suggest that these compounds modulate the RyR1 channel and muscle contractility by oxidizing functional thiols on the RyR1. This is supported by the following observations:

(1) GSH strongly inhibits selenium-dependent modification of the RyR1 (Figs. 1a, b and 2 and Table 1) in both concentration and time-dependent measurements of ryanodine bindings. The EC_{50} and IC_{50} for selenium-dependent stimulation and inhibition of the receptor shifts to much higher concentrations in the presence of 1 mM GSH. GSH either maintains these thiols in a reduced state or it directly interacts with the added selenium compounds (Table 1).
(2) In the presence of GSH, the measured values of B_{max} for selenium-dependent stimulation comes back to that of the control (Table 1), and Ca^{2+} release from SR vesicles induced by Ebselen is inhibited (Fig. 4).
(3) Although Na_2SeO_4 is more oxidized than Na_2SeO_3, it is known not to oxidize thiols, and it has been observed not to affect ryanodine receptor binding (not shown).
(4) CPM, by alkylating free thiols on the RyR1, prevents oxidation of these thiols and prevents stimulation of the receptor by Na_2SeO_3 or Ebselen (Fig. 1).
(5) Centrifuge washing does not reverse the Se-dependent effects since washing cannot reduce disulfide bonds. It appears as if short-term exposure of the SR to either Na_2SeO_3 or Ebselen results in the oxidation of thiols associated with activation of the receptor (14 thiols), while long-term exposure results in the oxidation of a second group of thiols that irreversibly inhibits the receptor (33 thiols) (Fig. 6).

Selenium is a trace element, which is an essential component of selenocysteine-containing enzymes such as glutathione peroxidase and thioredoxin reductase. However, it is toxic to humans at high doses. In skeletal muscle, Se-containing compounds stimulate muscle contractions at low concentrations, and inhibit contractility at higher concentrations. It is proposed on the basis of the work presented in this paper, that the rise in intracellular Ca^{2+} concentration previously observed [17] was caused by the direct interaction between Na_2SeO_3 and the calcium release channel in skeletal muscle SR. This hypothesis is supported by the observations that selenite, selenate and selenomethione are readily taken up or transported across erythrocytes and plasma membranes. Moreover, most forms of selenium restore glutathione peroxidase activity in muscle from Se-depleted animals, which indicates that Se reaches intracellular sites where protein synthesis occurs [30].

Our results demonstrate that, in skeletal muscle, the calcium release channel/RyR1 is highly sensitive to oxidative stress induced by low concentrations of selenium compounds. While low (nanomolar concentrations) stimulate the ryanodine receptor (Ca^{2+} release mechanism), higher concentrations close down the ryanodine receptor. The oxidation of multiple thiols (14), responsible for receptor stimulation, can be prevented by GSH.

Acknowledgments

This work was supported by grants from NIH (R01 AR 48911-01) and the American Heart Association to J.J.A.,

and the National Science Foundation of China (Grant no. 39870235) to R.X.

References

[1] Zable AC, Favero TG, Abramson JJ. Glutathione modulates ryanodine receptor from skeletal muscle sarcoplasmic reticulum. Evidence for redox regulation of the Ca^{2+} release mechanism. J Biol Chem 1996;272:7069–77.

[2] Feng W, Liu G, Abramson JJ, Xia R, Pessah IN. Site-selective modification of hyperreactive cysteines of ryanodine receptor complex by quinones. Mol Pharm 1999;55:821–31.

[3] Zaidi NF, Lagenaur CF, Abramson JJ, Pessah IN, Salama G. Reactive disulfides trigger Ca^{2+} release from sarcoplasmic reticulum via an oxidation reaction. J Biol Chem 1989;264:21725–36.

[4] Xia RH, Stangler T, Abramson JJ. Skeletal muscle ryanodine receptor is a redox sensor with a well defined redox potential that is sensitive to channel modulators. J Biol Chem 2000;275:36556–61.

[5] Feng W, Liu G, Allen PD, Pessah IN. Transmembrane redox sensor of ryanodine receptor complex. J Biol Chem 2000;275:35902–7.

[6] Sun J, Xu L, Eu JP, Stamler JS, Meissner G. Classes of thiols that influence the activity of skeletal muscle calcium release channel. J Biol Chem 2001;276:15625–30.

[7] Favero TG, Zable AC, Abramson JJ. Hydrogen peroxide stimulates the Ca^{2+} release channel from skeletal muscle sarcoplasmic reticulum. J Biol Chem 1995;270:25557–63.

[8] Koller LD, Exon JH. The two faces of selenium—deficiency and toxicity—are similar in animals and man. Can J Vet Res 1986;50:297–306.

[9] Xia YM, Hill KE, Burk RF. Effects of selenium deficiency on hydroperoxide-induced glutathione release from isolated perfused rat heart. J Nutr 1985;115:733–42.

[10] Foster LH, Sumar S. Selenium in health and disease: a review. Crit Rev Food Sci Nutr 1997;37:211–28.

[11] Smyth JB, Wang JH, Barlow RM, Humphreys DJ, Robins M, Stodulski JB. Effects of concurrent oral administration of monensin on the toxicity of increasing doses of selenium in lambs. J Comp Pathol 1990;102:443–55.

[12] Frost DV, Lish PM. Selenium in biology. Rev Annu Rev Pharmacol 1975;15:259–84.

[13] Ishikawa T, Yamamoto F, Ohashi T, Shimada Y, Kagisaki K, Kumada Y, et al. The effects of Ebselen upon post-ischemic functional recovery in rat heart. Nippon Kyobu Geka Gakkai Zasshi 1995;43:458–65.

[14] Franconi F, Manghi N, Giotti A, Martini F, Dini G. Effect of selenium on the contractile force of isolated and perfused Guinea-pig heart. Acta Pharmacol Toxicol (Copenh) 1980;46:98–104.

[15] Aviado DM, Drimal J, Watanabe T, Lish PM. Cardiac effects of sodium selenite. Cardiology 1975;60:113–20.

[16] Heinrich MA, Canon DM. Some effects of sodium selenite on the cardiovascular system. Toxicol Appl Pharmacol 1960;2:33–45.

[17] Liu S, Fu W, Lin-Shuau S. Effects of sodium selenite on neuromuscular junction of the mouse phrenic nerve-diaphragm preparation. Neuropharmacology 1989;28:733–9.

[18] Lin-Shiau S, Liu S, Fu W. Neuromuscular actions of sodium selenite on chick biventer cervicis nerve–muscle preparation. Neuropharmacology 1990;29:493–501.

[19] Ganther HE. Selenotyrosine and related phenylalanine derivatives. Bioorg Med Chem 2001;9:1459–66.

[20] MacLennan DH. Purification and properties of an adenosine triphosphatase from sarcoplasmic reticulum. J Biol Chem 1970;245:4508–18.

[21] Lai FS, Erickson HP, Rousseau E, Liu QY, Meissner G. Purification and reconstitution of the Ca^{2+} release channel from skeletal muscle. Nature 1988;331:315–9.

[22] Pessah IN, Stambuk RA, Casida JE. Ca^{2+}-activated ryanodine binding: mechanisms of sensitivity and intensity modulation by Mg^{2+}, caffeine, and adenine nucleotides. Mol Pharmacol 1987;31:232–8.

[23] Bers D, Patton C, Nuccitelli R. Methods Cell Biol 1994;40:3–29.

[24] Abramson JJ, Cronin JR, Salama G. Oxidation induced by phthalocyanine dyes causes rapid calcium release from sarcoplasmic reticulum vesicles. Arch Biochem Biophys 1988;263:245–55.

[25] Liu G, Abramson JJ, Zable AC, Pessah IN. Direct evidence for the existence and functional role of hyperreactive sulfhydryls on the ryanodine receptor–triadin complex selectively labeled by coumarin maleimide 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin. Mol Pharmacol 1994;45:189–200.

[26] Favero TG, Webb J, Papiez M, Fisher E, Trippichio RJ, Broide M, et al. Hypochlorous acid modifies calcium release channel function from skeletal muscle sarcoplasmic reticulum. J Appl Physiol 2003;94:1387–94.

[27] Wang HT, Yang XL, Zhang ZH, Lu JL, Xu HB. Reactive oxygen species from mitochondria mediate SW480 cells apoptosis induced by Na_2SeO_3. Biol Trace Elem Res 2002;85:241–54.

[28] Zhong W, Oberley TD. Redox-mediated effects of selenium on apoptosis and cell cycle in the LNCaP human prostate cancer cell line. Cancer Res 2001;61:7071–8.

[29] Eu JP, Xu L, Stamler JS, Meissner G. The skeletal muscle calcium release channel: coupled O_2 sensor and NO signaling functions. Cell 2000;102:499–509.

[30] Combs GF, Combs, SB. The role of selenium in nutrition. New York: Academic Press; 1986. p. 179–204.