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Cardiovascular Research 32 (1996) 351–361

Cardiovascular Research

Oxidative effects of selenite on rat ventricular contractility and Ca movements

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Received 12 September 1995; accepted 15 February 1996

Abstract

Objective: The study aimed at characterizing the effects of selenite, known for its reactivity with thiols, on cardiac contractility and excitation–contraction coupling. **Methods:** The inotropic effects of selenite were studied on rat papillary muscles. Freshly isolated rat ventricular myocytes were used to determine the selenite-induced alterations in thiol contents, free Ca^{2+} levels (in fura-2 loaded cells), Ca^{2+} currents and contractile properties of skinned cells. **Results:** Selenite, at concentrations ≥ 0.1 mM, affected muscle contractions by inducing a transient positive inotropic effect (up to $120 \pm 3\%$ of control in 1 mM selenite) followed by a gradual decline of developed tension together with an increase in resting tension (respectively to 37 ± 3 and $166 \pm 5\%$ of their control values after 20 min exposure). These changes, irreversible on washout, could be reversed by the disulfide reducing agent dithiothreitol (DTT, 1 mM). Lowering temperature from 35° to 22°C or preincubating the muscles with the disulfonic stilbene SITS (0.2 mM) completely prevented the selenite-induced transient positive inotropy and rise in resting tension. In isolated myocytes, 10 min exposure to 1 mM selenite induced a $40 \pm 9\%$ decrease of total sulfhydryl content. At this concentration, selenite rapidly caused a rise of basal $[Ca^{2+}]_i$ together with a diminution of the Ca^{2+} spike amplitude (respectively to 165 ± 15 and $45 \pm 9\%$ of their control values after 5 min exposure). In addition, selenite significantly enhanced at each Ca^{2+} concentration the force generated by skinned myocytes. Ca^{2+} currents, measured at 22°C, decreased by $28 \pm 8\%$ in the presence of 1 mM selenite.These effects were reversed by DTT. **Conclusions:** The results demonstrate that selenite, through alterations of cellular thiol redox status, induced a dual action on muscle contraction that can be imputed to a combined action on Ca^{2+} channels, Ca^{2+} transporters and contractile proteins. Extracellular negative effects of selenite are due to a partial reduction of Ca^{2+} current magnitude. Intracellular effects are mediated both by a diminution of Ca^{2+} handing by intracellular organelles and by a sensitization of the contractile to Ca^{2+} ions. The results further indicate that selenite uptake into the cardiac cells occurs mainly through the temperature-sensitive anion exchanger.

Keywords: Selenite; Contractile proteins; Contractile function; Calcium channel, L-type; Calcium, intracellular concentration; Disulfonic stilbene; Rat, ventricle

1. Introduction

Selenium, a component of glutathione peroxidase [1], is known today to be an essential trace element in the mammalian diet, and sodium selenite (Na_2SeO_3) is commonly used as a dietary supplement for the treatment of selenium deficiency [2]. On the other hand, selenium toxicity in livestock that consumed selenium accumulator plants can be traced back to Marco Polo [3]. Experimental chronic selenium toxicity in animals affect the major organs including the liver, spleen, heart, kidneys and pancreas; more particularly sodium selenite has been shown to cause cellular dysfunction in a number of tissues including ery-

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Time for primary review 28 days.

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PII S0008-6363(96)00071-5

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throcytes [4], hepatocytes [5], lens [6,7], and skeletal muscle [8]. Moreover, due to their cytotoxicity, selenium compounds, namely selenoglutathione, are effective carcinostatic agents. The exact mechanisms underlying these cytotoxic effects remain to be understood but appear to involve, at least partially, alteration of intracellular Ca^{2+} homeostasis following oxidative damage. It is known that selenite catalyzes oxidation of glutathione (GSH) [9] and can react intracellularly with other sulfhydryl compounds [10–13].

The effects of selenite on heart function have yet to be characterized although a positive inotropic effect has been observed in dog heart [14]. In fact, its putative oxidative actions could lead to potentially important effects inasmuch as alteration of protein thiols in heart is known to affect substantially excitation–contraction coupling. For instance, oxidation of cardiac protein thiols by hypochlorous acid (HOCl) has been shown to depress contractility and alter Ca^{2+} homeostasis [15,16]—effects that were not reversible on washout of the oxidant but that could be restored by the disulfide-reducing agent, dithiothreitol (DTT). These studies are in line with observations demonstrating that oxidants can inactivate various cellular ion regulatory proteins, including the sarcolemmal Ca^{2+} pump [17], the Na^{+}–Ca^{2+} exchanger [18], and Ca^{2+}-ATPase of the sarcoplasmic reticulum (SR) [19], via the oxidation of protein thiols.

Considering the importance of selenite as a supplement in selenium deficiency and its possible specific effects as an oxidant of protein thiols, the present study aimed at characterizing its direct actions on rat heart. The results demonstrated that selenite, at concentrations $\geq 10^{-4}$ M, altered contractility, intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), Ca^{2+} current and calcium sensitivity of myofilaments through mechanisms mainly attributable to alterations of protein thiol redox status. Furthermore, it could be proposed that the intracellular transport of this three-oxygen containing anion occurs via the Cl/HCO_3 exchanger.

2. Methods

2.1. Recording of contractions from papillary muscles

Unless otherwise specified, experiments involving contraction recordings on muscle were performed in a well-oxygenated (95% O_2 and 5% CO_2) modified Krebs solution that contained (in mmol $\cdot l^{-1}$): NaCl 120, $CaCl_2$ 2.5, KCl 5, $MgCl_2$ 1.2, NaH_2PO_4 2, Na_2SO_4 1.2, $NaHCO_3$ 25, glucose 10, pH 7.4. Adult Wistar rats (250–300 g) were heparinized and anesthetised with sodium pentobarbitol (30 mg/kg). The hearts were quickly removed and placed into a low-Ca^{2+}-containing (0.63 mM $CaCl_2$) Krebs solution maintained at 4°C and papillary muscles from the left ventricule were then dissected.

Measurements of isometric tension were carried out on samples that were initially equilibrated for 40–50 min in a warmed (35°C) Krebs solution. Papillary muscles were attached at one end to a fixed arm and at the other to a force transducer (Grass Model FT-03). Electrical stimulation (2 ms pulses, twice the threshold) was achieved by two Ag/AgCl electrodes fixed at one end of the preparation. Unless otherwise stated, the stimulation frequency was 0.1 Hz. The muscles were stretched gradually until a maximal tension was developed on stimulation. Forces were monitored with a digital storage oscilloscope (Tektronix, Model 7704/P7001) and simultaneously stored on-line into the hard disk of a microcomputer (386SX-25) at a sampling rate of 1 kHz.

2.2. Isolation of ventricular myocytes

Myocytes from the hearts of Wistar rats (200–300 g) were isolated according to a method described elsewhere [20]. Briefly, the heart was first perfused at 37°C with a HEPES-buffered solution containing (in mmol $\cdot l^{-1}$): NaCl 123, KCl 5.4, $NaHCO_3$ 5, NaH_2PO_4 2, $MgCl_2$ 1.6, glucose 10, taurine 20, HEPES 20 and bubbled with 100% O_2. The pH was adjusted to 7.1 with NaOH. After 5 min, fresh buffer supplemented with 1–1.5 mg/ml collagenase was recirculated (8–10 ml/min) for 20–30 min. At the end of the collagenase perfusion, the ventricles were cut off and stirred to disperse the myocytes. The cells were then suspended at 37°C in the HEPES-buffered solution that also contained 1 mM $CaCl_2$ and 0.5% bovine serum albumin (pH adjusted to 7.4 with NaOH). Five to 7 000 000 cells were generally obtained with a 70 to 80% yield of well-elongated cells. Dissociations with lower cell amount and yield were discarded. The myocytes were kept under this condition until use for experiments the same day.

2.3. Determination of sulfhydryl groups in isolated myocytes

The myocytes were first washed with a HEPES-buffered solution containing (in mmol $\cdot l^{-1}$): NaCl 123, KCl 5.4, $MgCl_2$ 1.7, $CaCl_2$ 1.8, glucose 10, HEPES 10, pH 7.4. The cells were then divided into two groups: the control group that represented cells kept in the HEPES-buffered solution, and the selenite-treated group that consisted of cells incubated in this same solution supplemented with 1 mM Na_2SeO_3 (Sigma). After 10 min incubation at 37°C, the cells from both groups were washed with the HEPES-buffered solution and kept frozen until use.

Total and acid-soluble sulfhydryl (SH) groups were estimated with Ellman's reagent by a modification of the methods described by Sedlak and Lindsay and Eley et al. [15,21] . The cells were thawed and lysed in 0.2 M Tris/HCl buffer, pH 8.1, containing 2% sodium dodecylsulfate. For the determination of total SH groups, 0.05 ml aliquots of cell lysates were mixed with 0.8 ml distilled

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water and 0.1 ml of 2 mM 5,5′-dithiobis-(2-nitrobenzoic acid). The tubes were let stand for 20 min for color development. Absorbances of the supernatants obtained from $3000 \times g$ centrifugation were read at 412 nm (Shimatzu UV-120-02 spectrophotometer). After correction of the absorbances with sample and reagent blanks, concentrations of SH groups in each sample were calculated employing an extinction coefficient of 1.31 $mM^{-1} \cdot mm^{-1}$. To determine the acid-soluble SH groups, 0.7 ml aliquots of cell lysates were mixed with 0.35 ml of 20% trichloroacetic acid (TCA). After 10 min, the tubes were centrifuged at $13000 \times g$ for 10 min. The precipitates were washed with 0.2 ml of 20% TCA in a similar manner and the supernatants were combined and brought to pH 8 with NaOH. The SH content of the supernatants was measured as described above for total SH measurements.

2.4. Measurement of intracellular Ca^{2+} in single cells

Isolated myocytes were loaded with the membrane-permeant fluorophore fura-2-acetoxymethyl ester (fura-2 AM; Molecular Probes Inc.). Loading conditions consisted of a 1 h incubation at 37°C in a HEPES-buffered solution (in $mmol \cdot l^{-1}$: NaCl 120, KCl 5.4, Na_2PO_4 1.2, $MgCl_2$ 1.7, $CaCl_2$ 1.8, glucose 10, HEPES 10, pH 7.4) containing 2 μM fura-2 AM and 0.00125% pluronic acid F-127 (Molecular Probes Inc.). The cells were then washed and kept for another hour in fresh HEPES-buffered solution prior to use. The myocytes were then transferred in a small superfusion chamber mounted on the stage of an inverted fluorescence microscope (Diaphot, Nikon). After adherence to the glass bottom of the chamber, cells were superfused at a flow rate of $\simeq 5$ ml/min with the HEPES-buffered solution. Unless otherwise specified, bath temperature was maintained at 37°C. The myocytes were stimulated at 0.2 Hz from small platinum electrodes placed in the superfusion chamber.

Fura-2 fluorescence, which was monitored as described previously [16], was determined at excitation wavelengths of 350 and 380 nm with emission light centred at 505 nm. Wavelengths were alternated at 100 Hz using a computer-controlled dual beam spectrofluorometer (SPEX industries). The concentration of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) was calculated as previously described [16] from the standard method that utilized the ratio of fluorescence at the two excitation wavelengths [22], and as determined from extracellular calibration solutions containing (in mM): KCl 145, HEPES 5, EGTA 5, fura-2 (pentapotassium salt) 0.002, pH 7.2, with pCa values ranging between 3 and 9. Since background and cell autofluorescence were usually about 10 times less than that measured from fura-2 loaded myocytes, they were considered negligible and no correction was made in the calculation of $[Ca^{2+}]_i$. In any case, the main conclusions from the present study depend entirely on detection of changes in intracellular free Ca^{2+} levels and should not be affected by limitations related to possible systematic errors in calculating $[Ca^{2+}]_i$. Furthermore, sodium selenite (1 mM) and dithiothreitol (1 mM) caused no detectable changes in the ratio 350/380 when measured from the calibration solutions containing 2 μM fura-2 and either 0.01 or 10 μM free Ca^{2+}. Similarly, exposure of unloaded myocytes to 1 mM sodium selenite, with or without dithiothreitol, did not cause any detectable change in cell autofluorescence.

2.5. Recording of Ca^{2+} currents from whole-cell patch clamp

To record Ca^{2+} currents (I_{Ca}), the ventricular myocytes were placed in a tissue culture dish and superfused by gravity with solutions containing (in $mmol \cdot l^{-1}$): CsCl 20, NaCl 117, $CaCl_2$ 1.8, $MgCl_2$ 1.7, glucose 10 and HEPES 10, pH 7.4. The superfusing solutions also contained 50 μM tetrodotoxin (TTX) in order to block the sodium current. The internal solution in the patch electrode (resistance of 0.8–1.2 MΩ) contained (in $mmol \cdot l^{-1}$): CsCl 120, $MgCl_2$ 6.8, Na_2ATP 5, Na_2-creatine phosphate 5, Na_2GTP 0.4, EGTA 5, $CaCl_2$ 0.06 and HEPES 20, pH adjusted to 7.2 with CsOH. Extracellular solutions could be changed by positioning the cell at the extremity of one of 6 capillaries. Experiments were carried out at 22° and 35°C.

Whole-cell recording and analysis of I_{Ca} were performed as described previously [23]. Currents recordings were acquired by a microcomputer at a sampling rate of 10 kHz. I_{Ca} amplitude was measured on-line as the difference between peak inward current and the current at the end of 200 ms voltage pulses. These values were normalized with respect to cell capacitance that was measured from the time constant of the capacitive current elicited from 2 mV pulses [23].

2.6. Mechanical measurement on the skinned single ventricular cells

Tension measurements were performed as previously reported [20]. In brief, after isolation, myocytes were decanted and immediately incubated by shaking for 6 min at 20°C in a relaxing solution that contained 0.3% vol/vol Triton X-100 to allow for fast skinning. Skinned cells were then rinsed twice with the same solution without Triton X-100 and maintained at 4°C for up to 8 h. The adequacy of the skinning protocol has been checked previously by measuring myosin ATPase activity on a batch of skinned cells using a fluorimetric-coupled enzymatic assay. The relaxing and activating solutions were calculated according to Fabiato [24], with the following exceptions: 30 mM imizadole and acetic acid were used as a buffer to adjust solutions to pH 7.1, and acetate was used instead of chloride anions. Also note that dithiothreitol was omitted during the time of experiment except when indicated.

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Both ends of skinned cardiomyocytes were glued to a thin glass with optical glue (Norland Products Inc., North Brunswick, NY) which were polymerised in UV light for 3 min. The system for recording tension consisted of a piezo-resistant strain gauge (model AE 801, SensoNor a.s., Horten, Norway) with a thin 3-cm-long glass rod. It was connected to an amplifier to yield a sensitivity of 60 mV/μN with a noise level below 0.3 μN. The first amplification stage was achieved by means of a low-noise transducer amplifier (model 1B32, Analog Devices, Boston). Compliance of the strain gauge was 1 μm/10 μN. The tension–pCa curves were fitted according to the Hill equation: T (relative tension) = $[Ca]^{n_H}/K + [Ca]^{n_H}$ that allows one to calculate the Hill coefficient n_H, and pCa for half-maximal activation, $pCa_{50} = (-\log K)/n_H$.

2.7. Statistical analysis

All data are reported as mean ± s.e.m. Statistical comparisons were performed from Student's t-test. When more than two groups were tested simultaneously, significance from ANOVA was first established before paired comparisons. Tests were considered statistically significant when $P < 0.05$.

3. Results

3.1. Effects of selenite on the contractile performance of papillary muscles

In papillary muscles maintained under normal conditions, both resting and developed forces remained within 2.5% of their initial values during the 30 min period of recording ($n = 5$). The presence of selenite in the bath medium (maintained at 35°C) caused a dose- and time-dependent alteration of the muscle contractility as illustrated in Fig. 1. Thus, selenite at concentrations of 10^{-4} M and higher had a biphasic effect on the developed force; selenite elicited an initial increase within 5 min (to 115 ± 2% of control with 10^{-4} M selenite) that was followed by a progressive decline below the control level (85 ± 3% of control after 20 min exposure). Increasing selenite concentration from 10^{-4} to 10^{-3} M and 3.3×10^{-3} M enhanced the negative inotropy (down to respectively 37 ± 3 and 10 ± 4% of control after 20 min) as well as the transient positive inotropic phase (up to 120 ± 3 and 146 ± 5% of control). The resting force was also affected by selenite exposure as it gradually increased with time (112 ± 2, 166 ± 5 and 190 ± 6% of control after 20 min exposure to 10^{-4}, 10^{-3} and 3.3×10^{-3} M selenite, respectively). It should further be mentioned that washout of selenite after the 20 min exposure did not cause restoration of contractile performance which rather continued to decline. This effect is apparent in the typical recording depicted in the upper tracing of Fig. 1. In fact, after 20 min exposure to 1 mM selenite, all the studied muscles became unresponsive to electrical stimulation and did not show any sign of recovery even 40 min after washout of selenite. One should note, however, that this sustained tension associated with the contracted state never exceeded the developed force recorded during the positive inotropic phase.

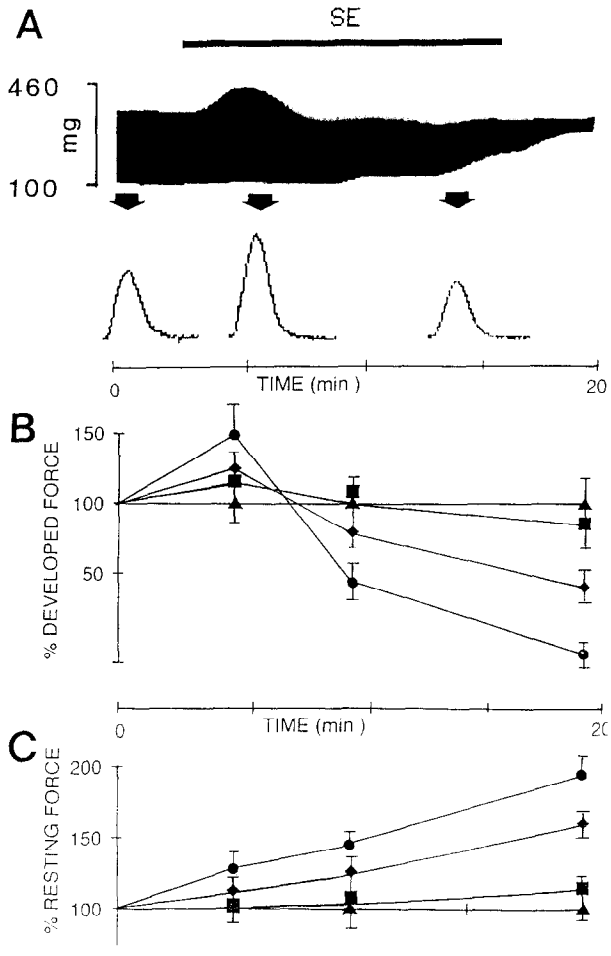


Fig. 1. Effect of selenite on contraction of papillary muscles. (A) The upper tracing represents a typical recording from a muscle exposed to 10^{-3} M selenite (SE) during the time indicated by the horizontal bar as well as in insets below individual contractions on an expanded time scale. The graphs give the average values of developed (B) and resting (C) forces measured at selenite concentrations of 10^{-5} (▲; $n = 8$), 10^{-4} (■; $n = 10$), 10^{-3} M (◆; $n = 10$) and 3.3×10^{-3} M (•; $n = 5$). Stimulation frequency was 0.1 Hz. n = number of muscles. Data are given as mean ± s.e.m. Temperature = 35°C.

The experiments described in Fig. 1 were performed at a stimulation frequency of 0.1 Hz, but similar results were obtained with papillary muscles stimulated at either 0.016 or 1 Hz. This therefore indicates that, in papillary muscles, the inotropic effects of selenite are independent of stimulation frequency.

3.2. Effect of low temperature and SITS on selenite-induced changes of contractile properties

As illustrated in Fig. 2 (upper panel), selenite-induced changes of both developed and resting tensions were greatly

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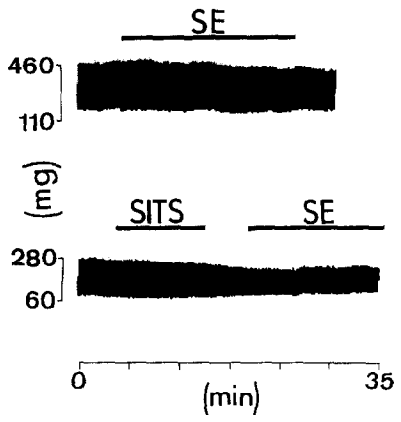


Fig. 2. Effect of low temperature and SITS on selenite-induced changes of contraction from papillary muscles. *Upper panel:* After equilibration in a superfusion chamber kept at room temperature (22°C), the muscle was exposed to 10^{-3} M selenite (SE). Low temperature incubation resulted in a complete absence of selenite effect on resting tension while the developed force slightly decreased, from 295 mg before selenite addition to 270 mg at the end of the 20 min exposure. *Lower panel:* The muscle was exposed to 10^{-3} M selenite at 35°C after 10 min application of 0.2×10^{-3} M SITS and 5 min washout that caused a small decrease of developed tension. Then, selenite did not induce a transient positive followed by a negative inotropy nor did it affect resting tension. Stimulation frequency was 0.1 Hz for both recordings.

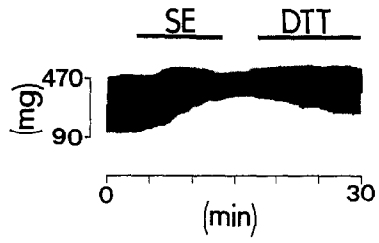


Fig. 3. Effect of dithiothreitol (DTT) on selenite-induced changes in contraction. The papillary muscle was successively exposed to 10^{-3} M selenite and 10^{-3} M DTT, as indicated by the horizontal bars. Contrary to selenite washout, addition of DTT induced recovery of developed and resting tensions. Stimulation frequency = 0.1 Hz. Temperature = 35°C.

attenuated when the muscles were maintained at room temperature (22°C). Thus, no change in resting tension could be detected in all 4 papillary muscles exposed for at least 20 min to 10^{-3} M selenite, while the developed force slowly declined but stayed within 90% of control value after that time. To test whether this preventive effect of low temperature was due to inhibition of selenite uptake through temperature-sensitive transport mechanisms, contractions were also measured at 35°C following exposure to the anion transport inhibitor, SITS (4-acetamido-4'-isothiocyanotostilbene-2,2'-disulfonic acid). An example of such an experiment is also given in Fig. 2 (lower panel). Addition of 2×10^{-4} M SITS alone resulted in a small but reproducible decrease of developed force (to $87 \pm 6\%$ of control after 10 min exposure, $n = 6$). This change of force persisted even 20 min after washout of SITS (data not shown), indicative of a relatively irreversible process. Under those conditions, the contractile effects of selenite were also attenuated. Thus, no selenite-induced transient positive inotropy could be detected in SITS-treated muscles. Similarly, diastolic tension did not significantly change during 20 min incubation in 10^{-3} M selenite although the active force gradually declined during that period (Table 1).

3.3. Effect of dithiothreitol on selenite-induced contractile alterations and measurement of intracellular sulfhydryls

As mentioned above, the inotropic effects of selenite were not reversible on washout. On the other hand, a marked restoration of contractile function could be observed by subsequent exposure to the disulfide-reducing agent, DTT. This is demonstrated in Fig. 3 which shows that the addition of 10^{-3} M DTT into the medium some 5 min following selenite washout induced recovery in both developed and resting forces towards control levels. On average, developed tension increased from $54 \pm 5\%$ ($n = 5$; after 10 min selenite exposure plus 5 min washout) to $90 \pm 6\%$ following 15 min exposure to DDT (a $79 \pm 4\%$ recovery). Similarly, resting tension decreased from $158 \pm 5\%$ to $113 \pm 4\%$ during this period (i.e., a $78 \pm 5\%$ recovery).

The observation that DTT caused a restoration of selenite-induced inotropic effects indicates that these effects occur through oxidation of protein thiols. To directly test this hypothesis, total and acid-soluble SH concentrations were measured from control and 10^{-3} M selenite-treated myocytes. The results, summarized in Table 2, demonstrate that both total and soluble SH levels were signifi-

Table 1
Effects of selenite on contractile activity of rat papillary muscles: prevention by SITS, recovery by DTT

	Se (20 min)		Se + SITS	Se (10 min)	DTT (15 min)
	10^{-4} M	10^{-3} M	(10^{-3} M + 2×10^{-4} M)	(10^{-3} M)	(10^{-3} M)
Resting force	$112 \pm 2\%$*	$166 \pm 5\%$*	$110 \pm 5\%$	$158 \pm 5\%$*	$113 \pm 4\%$
Active force	$85 \pm 3\%$*	$37 \pm 3\%$*	$90 \pm 8\%$	$54 \pm 5\%$*	$90 \pm 6\%$

Values are mean ± s.e.m. from 5–6 preparations. They are expressed relative to the values obtained on preparations under control conditions. After 20 min, in the presence of SITS (2×10^{-4} M), active force was reduced to $87 \pm 6\%$ of control value. DTT (10^{-3} M) was added after the preparations had been exposed for 10 min to selenite (Se) and washed for 5 min.
* Significantly different from the respective control value ($P < 0.05$).

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Table 2
Effect of selenite on sulfhydryl content of isolated myocytes

	Control	Selenite-treated
Total SH (μmol/mg wet weight)	5.2±0.9	3.1±0.3*
Free SH (μmol/mg wet weight)	1.9±0.4	0.9±0.2*

Values are mean ± s.e.m. (n = 5 hearts). Myocyte suspension was divided into two groups that were then incubated for 10 min at 37°C in the absence (Control) or the presence (Selenite-treated) of 10^{-3} M selenite. For each group, total and free sulfhydryl (SH) contents were determined as described in 'Methods'.
* Significantly different from the respective control value ($P < 0.05$).

cantly ($P < 0.05$) decreased to, respectively, 60 and 47% of their control values following 10 min exposure to selenite.

3.4. Steady-state $[Ca^{2+}]_i$ and Ca^{2+} transients

Exposure of fura-2 loaded myocytes (stimulated at 0.2 Hz) to 10^{-4} M selenite did not cause any detectable change in fluorescence signals during at least 5 min ($n = 4$). On the other hand, and as shown in Fig. 4, the addition of 10^{-3} M selenite rapidly induced a substantial rise in intracellular Ca^{2+} level over time. This effect on basal $[Ca^{2+}]_i$ was observed in all cells studied, with the average value significantly increasing ($P < 0.05$) from 203 ± 31 to 335 ± 49 μM before and 5 min after the addition of selenite, respectively ($n = 10$). Conversely, the amplitude of the Ca^{2+} transients progressively declined after the addition of selenite. This can be readily seen in the lower panel of Fig. 4, which represents the time derivative of the Ca^{2+} transients. On average, the amplitude of these transients significantly decreased ($P < 0.05$) from 253 ± 50 to 114 ± 20 μM before and 5 min after the addition of selenite ($n = 10$). Further note from the figure that this decline occurred without transitory increase in the spike amplitude. In some cells (3 out of 10), peak Ca transient did increase transiently (e.g., see Fig. 5), but this increase occurred only in the presence of a concurrent rise in basal $[Ca]_i$ such that the amplitude of the Ca transient was unchanged even in these cells.

Panel B of Fig. 4 demonstrates that, in a manner similar to muscle contractions, the effects of selenite on intracellular Ca^{2+} were almost completely abolished by superfusion at room temperature. As shown, addition of 10^{-3} M selenite at 22°C failed to induce any detectable change in basal $[Ca^{2+}]_i$ while the amplitude of the Ca^{2+} transients remained within 90% of the control values after 5 min exposure to selenite. This prevention of selenite effects by

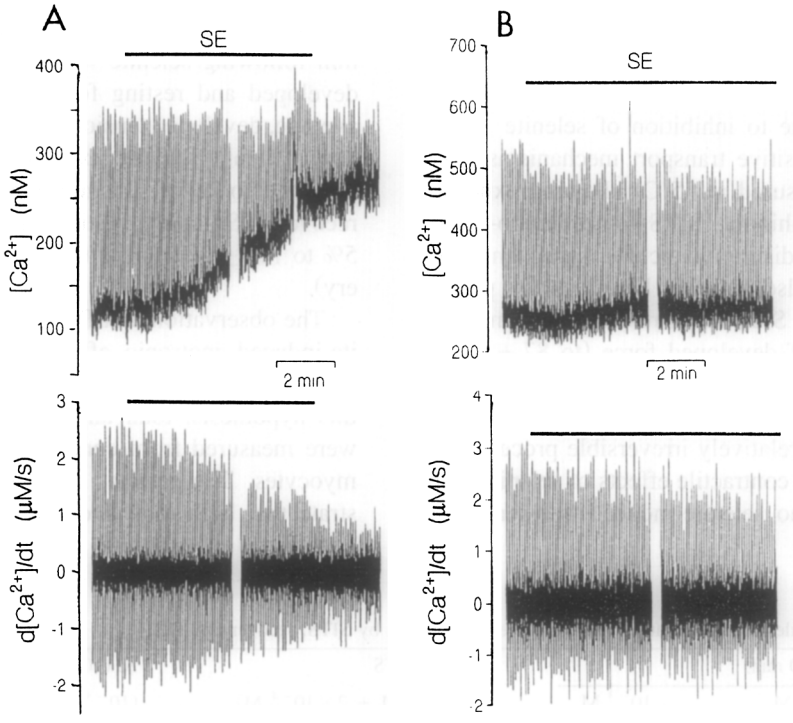


Fig. 4. Examples of selenite effects on Ca^{2+} transients in isolated ventricular myocytes incubated at different temperatures. Fura-2 loaded myocytes, superfused in solutions maintained at 37°C (Panel A) and 22°C (Panel B), were exposed to 10^{-3} M selenite (SE) during the time periods indicated by the horizontal bars. Upper tracings represent intracellular free Ca^{2+} concentrations calculated from fluorescence ratios while the lower tracings are the corresponding time derivatives. At 37°C, selenite caused a rapid increase of resting $[Ca^{2+}]_i$ together with a decline in Ca^{2+} transient amplitude and rate of rise. On the other hand, superfusion at 22°C completely prevented the selenite-induced increase in resting $[Ca^{2+}]_i$ while the Ca^{2+} transient amplitude and rate of rise only slowly declined with time, decreasing to 90% of control value after 5 min exposure to selenite. The blank spaces in the middle of the tracings correspond to a 20 s period used to reset computer data acquisition. Stimulation frequency = 0.2 Hz.

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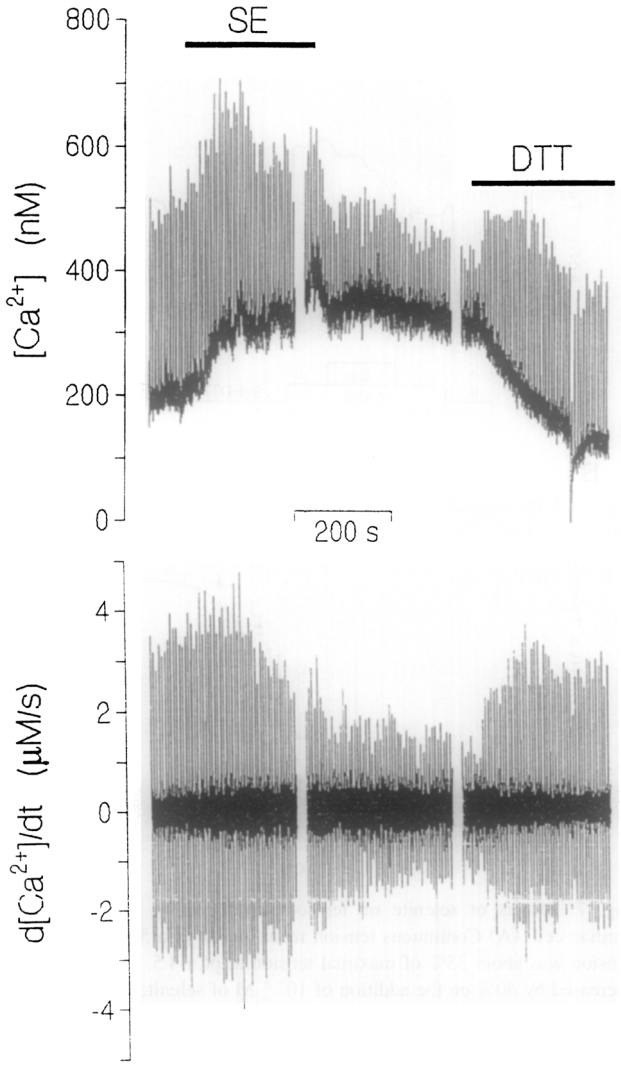


Fig. 5. Example of the effect of dithiothreitol on selenite-induced changes in Ca^{2+} transients. The fura-2 loaded ventricular myocyte was first exposed to 10^{-3} M selenite (SE) for 250 s, as indicated by the horizontal bar. After 5 min washout of selenite, the superfusion solution was switched to solution containing 10^{-3} M dithiothreitol (DTT). The blank spaces in the tracings correspond to 20 s periods used to reset computer data acquisition. Stimulation frequency = 0.2 Hz; temperature = 37°C.

low temperature was observed in all 5 cells studied under these conditions.

Washout of selenite after 5 min of exposure failed to attenuate the oxyanion effect observed at 37°C. Besides, complete reversal could be obtained by subsequent superfusion of the myocytes with DTT. As shown in Fig. 5, the addition of DTT some 5 min after washout of selenite promptly induced a return of both basal $[Ca^{2+}]_i$ and Ca^{2+} transients towards their control values. As one can see from the derivative trace, the restoration of Ca^{2+} transients appeared to occur faster than that of resting $[Ca^{2+}]_i$. For the 5 cells studied under those conditions, basal $[Ca^{2+}]_i$ decreased from 407 ± 62 μM following washout of selenite to 243 ± 27 μM after 5 min exposure to DTT. Similarly, the amplitude of the Ca^{2+} transients increased from 112 ± 35 to 306 ± 50 μM during that period. These values measured after exposure to DTT were not significantly different from the respective control values measured prior to selenite addition (269 ± 40 and 287 ± 98 μM for basal $[Ca^{2+}]_i$ and Ca^{2+} transients amplitude, respectively), thereby indicating that full restoration of intracellular Ca^{2+} level by DTT was achieved within 5 min. It should finally be mentioned that DTT alone added prior to selenite exposure did not cause detectable changes in resting $[Ca^{2+}]_i$ and Ca^{2+} transients (data not shown).

Overall, these data show that the effects of selenite on intracellular Ca^{2+}, together with the preventive effects of low temperature and DTT, parallel well those observed on contraction, with one major difference: the transient positive inotropic effect of selenite could not be correlated to a transient increase in Ca^{2+} spike amplitude.

3.5. Effect of selenite on Ca^{2+} current

In order to test the possibility that selenite-induced modification of intracellular Ca^{2+} occurred through alteration of membrane Ca^{2+} conductances, the selenite effect on the slow inward Ca^{2+} current (I_{Ca}) was directly determined in single cells. As shown in Fig. 6 (top panel), 10^{-5} M selenite had only a small inhibitory effect on peak I_{Ca} ($12 \pm 7\%$ decrease, $n = 20$ cells). This inhibition was enhanced when selenite was applied at a concentration of 10^{-3} M. On average, peak I_{Ca} amplitude significantly decreased by $28 \pm 8\%$ of control ($P < 0.05$; $n = 18$ cells) following 5 min exposure to 10^{-3} M selenite. This calculation took into consideration the small rundown of I_{Ca} by extrapolating from the slope of the declining peak values measured at the time of selenite application. These effects were not reversible on washout of selenite. Similar decreases in peak I_{Ca} amplitude were observed at 37°C. After 3 min exposure the inhibitory effect was 11 ± 2 and $39 \pm 6\%$ ($n = 7$) in the presence of 10^{-5} and 10^{-3} M selenite, respectively.

The bottom panel of Fig. 6 illustrates the effect of 10^{-3} M selenite on the current–voltage relationship of peak I_{Ca}. As shown, the overall effect of selenite can be seen as a scaling-down of the current amplitude at the voltage range of −60 to +60 mV. In other words, the relative selenite-induced reduction of I_{Ca} was independent of membrane potential. Furthermore, selenite did not affect significantly the voltage-dependent inactivation properties of I_{Ca}, although a leftward shift by a few mV was generally observed. Similar effects were observed in 5 cells. The effects of selenite were partially reversed by DTT (not shown).

3.6. Effect of selenite on myofibrillar Ca sensitivity at single cell level

The effects of selenite are shown in Fig. 7A on the continuous record of tension developed by a chemically

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skinned single cell submitted to EGTA-buffered solutions at near half-maximal and maximal Ca activation. At pCa 5.875, the addition of 10^{-3} M selenite induced a marked increase in developed tension that reached its maximum after 35 s. This effect was not reversible on removal of the anion. However, the disulfide-reducing agent, dithiothreitol (DTT), added for 10 min at 0.3×10^{-3} M allowed for full recovery of force elicited by this Ca concentration. Incidentally, selenite had no effect on the resting tension (at pCa 9) which also was not affected by the presence of DTT. The last part of the record further demonstrates that the addition of selenite to the high Ca-containing solution (pCa 4.5) clearly further increased the maximal activated tension. Such an observation confirms that the effects of

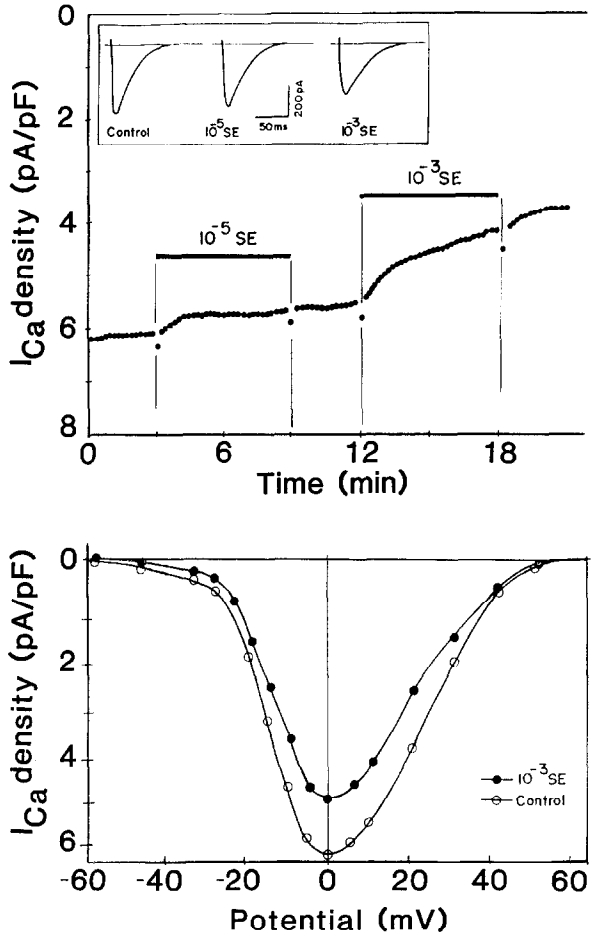


Fig. 6. Effect of selenite on Ca^{2+} current. *Top panel:* Time course of peak I_{Ca} during exposures to 10^{-5} and 10^{-3} M selenite, as indicated by the horizontal bars. I_{Ca} was elicited with 200 ms depolarization pulses to 0 mV every 4 s. Holding potential was −70 and 50 μM TTX was continuously present to inhibit the fast Na^+ current. Values are normalized with respect to membrane capacitance. *Inset:* Actual recordings of I_{Ca}. The "control" tracing corresponds to the current measured 3 min after initiating the recording (i.e., just before the addition of 10^{-5} M selenite), while the other two tracings were taken 6 min after the addition of 10^{-5} and 10^{-3} M selenite, respectively. *Bottom panel:* Current–voltage relationships of peak I_{Ca} before (○) and 6 min after (•) the addition of 10^{-3} M selenite. The relative selenite-induced decrease in I_{Ca} remained constant throughout the voltage range. Temperature = 22°C.

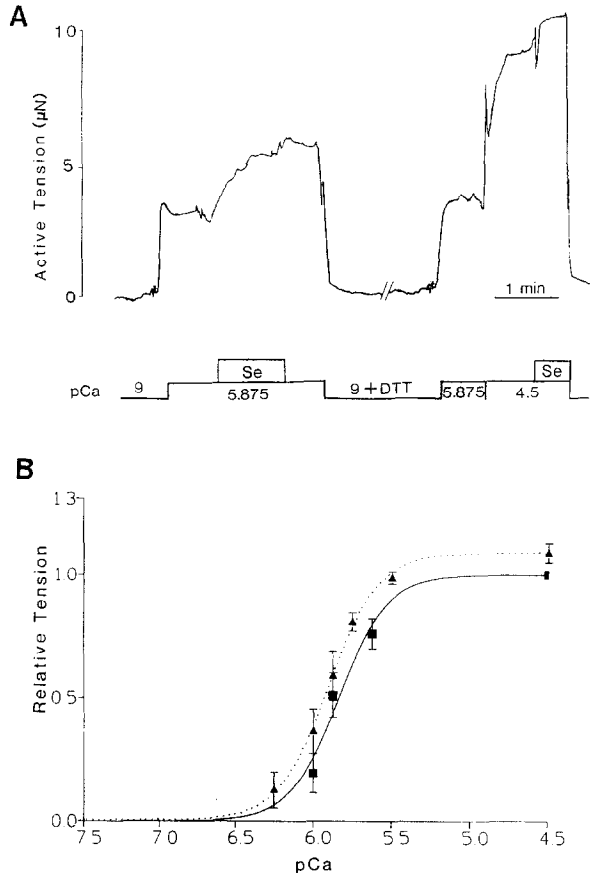


Fig. 7. Effects of selenite on tension developed by a single skinned cardiac cell. (A) Continuous tension recording at pCa 5.875; the elicited tension was about 35% of maximal tension at pCa 4.5. This tension was increased by 40% on the addition of 10^{-3} M of selenite, an effect which did not wash out on selenite removal. However, after 10 min in the relaxing solution at pCa 9 containing 0.3×10^{-3} M DTT, the selenite effect was reversed. The last part of the tracing shows that a second application of selenite induced a significant increase in the maximal tension elicited at pCa 4.5. (B) Tension–pCa relationships summarizing the effects of applying 10^{-3} M selenite on 7 skinned cells. The curves established before and after exposure to selenite were fitted according to the Hill equation with $pCa_{50} = 5.82$ and 5.90, and with $n_H = 2.84$ and 2.78, respectively. Temperature = 22°C.

selenite are reversed by DTT and are reproducible. Fig. 7B shows averaged tension/pCa relations. The presence of selenite significantly increased the tension developed at intermediate and maximal Ca concentrations. Maximal tension was increased by $8 \pm 2\%$ and the relations were best fitted with $pCa_{50} = 5.82 \pm 0.01$ and 5.90 ± 0.01 ($n = 7$, $P < 0.01$) and $n_H = 2.84 \pm 0.10$ and 2.78 ± 0.04, respectively, in control and 10^{-3} M selenite solutions.

4. Discussion

4.1. Selenite-induced alterations of cardiac contractility result from changes of both intracellular Ca^{2+} levels and myofilament contractile properties

By virtue of its regulatory effect through glutathione peroxide, selenite is protective at low concentration. How-

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ever, selenite has biphasic effects on tissue, higher concentrations having long been known to be toxic [3]. Selenium plasma concentration in the order of 1 μM can vary several 10-fold in the rat according to the diet [25]. A daily amount of 1 mg/kg body weight may be sufficient to produce chronic intoxication in man with the lethal toxicity limit for humans being 10–30 mg/kg body weight [26]. The present study demonstrates that selenite, applied acutely in the millimolar range, affects ventricular cardiac contractility in a pattern that comprised two phases: an initial transient increase in developed force followed by a progressive decline of this force that occurred concomitantly with an increase in resting tension. Our measurements of $[Ca^{2+}]_i$ and contractility in isolated ventricular myocytes suggest that these events are the expression of two distinct mechanisms: perturbation of Ca^{2+} homeostasis and alteration of contractile apparatus. In fura-2 loaded myocytes, selenite induced a progressive rise of basal $[Ca^{2+}]_i$ together with a decline of the amplitude of the Ca^{2+} spikes without any detectable transient enhancement of these spikes (Fig. 4). Thus, these changes in $[Ca^{2+}]_i$ can account for the late effect of selenite on contractile performance of muscle strips. The fact that these changes were faster in single cells than in muscle strips can be readily explained by differences between single cells and multicellular preparations in diffusion distances for selenite. On the other hand, this could hardly account for the observed selenite-induced transient inotropy in muscle strips, where the developed force reached levels actually higher than during the contracted state elicited after 20 min superfusion with selenite (Fig. 1). One could argue that the apparent absence of a selenite-induced transient increase in Ca^{2+} spike amplitude could have been due to the fact that selenite actions occurred more rapidly in single cells than in papillary muscles, thereby masking an early transient stimulation. There are, however, two major observations that do not support this possible explanation. First, a 3.3-fold increase in selenite concentration (which should compensate for the rapid diffusion in single cells) still caused a further transient increase in the developed force from papillary muscles. Second, exposure of single cells to 10^{-4} M selenite did not cause any detectable effects on Ca^{2+} spikes although inducing a significant positive inotropy in papillary muscles. Thus, this positive effect must have occurred through mechanisms independent of perturbations of cellular free Ca^{2+}. This additional effect of selenite was directly demonstrated with skinned myocytes where Ca^{2+}-induced contraction was significantly augmented in the presence of selenite (Fig. 7). Although our experiments were performed at 22°C, one can reasonably assume that a sensitizing effect would occur at 37°C.Taken together, the results from isolated myocytes therefore indicate that the transient positive inotropy is at least in part due to a direct effect of selenite on the myofilaments while the subsequent decline in developed force and rise in resting tension are the consequence of diminished Ca^{2+} transients and elevation of basal $[Ca^{2+}]_i$.

The selenite-induced dual change in force development is qualitatively similar to that reported with some exogenous oxidants [27–29], although both positive and negative inotropic effects have been attributed to changes in intracellular $[Ca^{2+}]_i$ in these studies. The most direct evidence comes from the recent report by Goldhaber and Liu [29] who indicated that exposure of isolated ventricular myocytes to 10^{-3} M hydrogen peroxyde (H_2O_2) caused a short-lived positive inotropy that occurred together with a small increase of the Ca^{2+} transients and diastolic $[Ca^{2+}]_i$. On the other hand, the secondary H_2O_2-induced decline in Ca transients appeared to occur much faster than that of active tension (the former, but not the latter, being significantly smaller than control after some 8 min exposure to H_2O_2). Although not discussed, the contribution of altered myofilament properties could in fact explain such observations.

4.2. *Selenite effects occur through alteration of protein thiol redox status*

The results further indicate that the effects of selenite occur mainly through oxidative alteration of protein thiols. The most direct evidence comes from the observation that selenite-induced changes in contractile activity and $[Ca^{2+}]_i$ were irreversible on washout but could be restored by the disulfide-reducing agent, DTT. In support of these data, both reduced glutathione and protein thiol levels significantly decreased in cells exposed to selenite (Table 2). These observations are in line with the known effects of selenite as a catalyst of glutathione protein oxidation [9,11,12]. In fact, our observed effects of selenite are qualitatively similar to those reported for oxidants that mainly affect protein thiol redox status. For instance, HOCl has been shown to cause contracture of rat papillary muscles [15], an effect that could be reversed by DTT and that was interpreted as an alteration of Ca^{2+} transport mechanisms [16]. It is now well recognized that several transport processes such as Na–Ca exchange [18] and Ca^{2+}-ATPase [19] are inhibited following oxidative stress, thereby leading to intracellular Ca^{2+} overload. Our direct measurements of $[Ca^{2+}]_i$ in fura-2 loaded myocytes can be interpreted in the same fashion whereby selenite, through inhibition of active Ca^{2+} transport mechanisms at the sarcolemma and the sarcoplasmic reticulum should lead to increased basal levels of cytosolic Ca^{2+} and depletion of Ca^{2+} from the SR stores. The complete reversibility by DTT on Ca^{2+} transients and basal $[Ca^{2+}]_i$ (Fig. 5) further points to the specificity of the action of selenite on protein thiols.

The effect of selenite on myofilaments can also be explained by an induced formation of disulfide bonds on the contractile proteins. The selenite-induced increase in

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Ca^{2+} sensitivity of myofilaments occurred at both intermediate and optimal Ca^{2+} concentrations. This would indicate that disulfide bond formation slows down cross-bridge turnover so that they are attached more of the time during each crossbridge cycle. Ca^{2+} sensitization was readily reversed by DTT and was reproduced on re-applying selenite. Selenite-induced Ca^{2+} sensitization was at first surprising in view of the report that superoxide anions depress peak force without altering Ca^{2+} sensitivity of chemically skinned cardiac muscle of rat [30]. Nevertheless, the two oxidizing agents are different and it should be kept in mind that two classes of SH groups have been demonstrated in cardiac myosin, the alkylation of one or the other class induces activation or inhibition of Ca^{2+}-dependent myosin ATPase [31]. It should finally be mentioned that the effects of selenite were considered irreversible because of the absence of recovery of contractile function even 40 min after washout. In fact, the deleterious effects of selenite on both contractions and $[Ca^{2+}]_i$ continue to develop after washout. It is interesting to note that this effect following selenite washout is similar to that reported for HOCl [15,16]. Considering that HOCl is membrane-permeant and should consequently be rapidly washed out, the persistence in the development of the deleterious effects may therefore be attributed to the slowness of the cellular processes triggered after oxidation.

4.3. Cellular uptake of selenite is achieved through the SITS-sensitive anion exchanger

Reducing the temperature to 22°C had a strong inhibitory effect on selenite-induced contractile responses and $[Ca^{2+}]_i$ variations. Two mechanisms could account for this protective effect: the temperature dependency of protein thiol oxidation and/or that of selenite uptake by the cardiac myocytes. The former possibility is likely to play a minor role considering the low energy of activation required for thiol oxidation by selenite [9], which in fact can occur readily even at 4°C [12]. The very observation that selenite increased tension in skinned fibers maintained at room temperature, but not in preparations with intact membranes, supports this concept. On the other hand, lowering the temperature from 35° to 22°C may cause a substantial inhibition of selenite transport mechanisms and hence of its intracellular oxidative effects in intact cells. The fact that the anion exchange inhibitor, SITS, prevented both selenite-induced positive inotropy and the development of contracture points to the Cl/HCO_3 exchange as the main carrier, a transport mechanism well known for its strong temperature dependency and its low specificity for various anions [32]. In support of this conclusion, selenite (in the form SeO_3^-) has been shown to be carried by the Cl/HCO_3 exchanger in human erythrocytes with an apparent affinity similar to that of HCO_3^- and maximal rate of transport only some 6 times less than for HCO_3^- [33]. It should finally be mentioned that SITS caused a small but irreversible decrease in the developed force in papillary muscles (Fig. 2), as also observed in other cardiac preparations [34]. The mechanisms underlying this small inhibitory effect remain to be elucidated, but may be related to the intracellular acidification induced by these disulfonic stilbene compounds [35]. Such an acidification is unlikely to interfere with the effects of selenite, however, inasmuch as catalysis of protein oxidation by selenite does not appear to be pH-sensitive [12].

4.4. Extracellular inhibitory effect of selenite on the Ca^{2+} current

Overall, the results involving low temperature and SITS suggest that selenite acts mainly on intracellular targets following transmembrane movement through SITS-sensitive anion exchangers. Conversely, the observation that selenite induced an inhibition of the Ca^{2+} current at room temperature (Fig. 6) can be attributed to an extracellular effect. This notion is in fact directly supported by the results showing that both SITS application and lowering temperature unveiled a small selenite-induced negative inotropy (Fig. 2), an effect also observed with the Ca^{2+} transients recorded from cells incubated at room temperature (Fig. 4). These results may therefore be interpreted as an extracellular blockade of Ca^{2+} channels that would lead to negative inotropy.

An external effect of selenite has been demonstrated on rat liver glucocorticoid receptor [36], whereby hormone binding could be inhibited by selenite and reversed by DTT. This indicated that the hormone binding site comprises critical sulfhydryl groups involved in the receptor–agonist interaction. Whether the observed selenite-induced inhibition of Ca^{2+} conductance also occurs through modification of thiol groups remains to be determined. Ca currents have been shown to be relatively insensitive to oxidants [16,37,38]; other reports showed a significant reduction in the presence of hydrogen peroxide at high concentration when oxygen radicals were generated by dihydroxyfumarate [29,39] and after exposure to 2,2′-dithiopyridine (DTDP), a specific lipophilic oxidizer of sulfhydryl groups [40]. In the latter studies and ours, the relative selenite-induced inhibition of peak Ca-current is voltage-independent. Selenite effects were only partially reversed by DTT at 1 mM, and a direct effect of selenite anions on the Ca channel without formation of disulfide bonds cannot be completely excluded.

The present results demonstrate that selenite can alter substantially the contractile function of rat myocardium, mainly due to a direct effect on contractile proteins that results in a transient positive inotropy followed by a gradual Ca^{2+} overload which leads to an eventual state of contracture. These effects are mostly related to specific alteration of protein thiol redox status. In that regard, selenite may prove to be an important tool to study the function of cardiac regulatory proteins whose modulation involves sulfhydryl interactions.

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Acknowledgements

C. Vannier was supported by a grant from Glaxo-France. Thanks are due to Dr. M. Pucéat for helpful discussion and to H. Chevassus for his help with skinned cell tension measurements.

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