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SULPHYDRYL REAGENTS TRIGGER Ca^{2+} RELEASE FROM THE SARCOPLASMIC RETICULUM OF SKINNED RABBIT PSOAS FIBRES

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SUMMARY

1. By analogy with studies on sarcoplasmic reticulum (SR) vesicles, Ca^{2+} release induced by heavy metals and mercaptans (e.g. cysteine) was investigated in rabbit skinned psoas fibres through measurements of isometric tension.

2. Heavy metals (at 2–5 μM) elicited phasic contractions by triggering Ca^{2+} release from the SR and had the following order of potency: $Hg^{2+} > Cu^{2+} > Cd^{2+} > Ag^{+} > Ni^{2+}$. Higher concentrations produced tonic contractions due to maintained high Ca^{2+} permeability of SR membranes.

3. Contractions induced by heavy metals required a functional and Ca^{2+}-loaded SR, were dependent on $[Ca^{2+}]_{free}$, blocked by Ruthenium Red (RR), inhibited by free Mg^{2+} and reduced glutathione (GSH) but not by oxidized glutathione (GSSG). Such contractions were not elicited through direct interaction(s) of heavy metals with the myofilaments.

4. In the presence of catalytic concentrations of Hg^{2+} or Cu^{2+} (2–5 μM), additions of cysteine (25–100 μM) elicited rapid twitches, producing 70% of maximal force with a time to half-peak of 2 s. Contractions induced by cysteine plus a catalyst required a functional SR network, were dependent on free $[Mg^{2+}]$ and were blocked by RR or GSH but not by GSSG.

5. In the presence of Hg^{2+} (2–5 μM), low concentrations of cysteine (10 μM) elicited tonic contractures, but subsequent or higher additions of cysteine (50–100 μM) caused further SR Ca^{2+} release and tension, followed by rapid and full relaxation.

6. High cysteine (200–250 μM, without Cu^{2+} or Hg^{2+}) blocked contractions elicited by Cl^{-} induced depolarization of sealed T-tubules. High cysteine probably acted as a sulphydryl reducing agent which promoted rapid relaxation of the fibres through the closure of Ca^{2+}-release channels and ATP-dependent re-uptake of Ca^{2+} by the SR.

7. In some batches of skinned fibres ($\sim$ 10%), cysteine (5–50 μM) alone (without Hg^{2+} or Cu^{2+} catalyst) produced rapid twitches. This implied that the catalyst(s) necessary to promote the sulphydryl oxidation reaction with exogenously added cysteine may be present in intact fibres but is usually lost by the skinning procedure.

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8. The data demonstrate that skeletal fibres contain a highly reactive and accessible sulphydryl site on an SR protein which can be reversibly oxidized and reduced to respectively, open and close SR Ca^{2+}-release channels. A model of sulphydryl-gated excitation–contraction coupling is proposed where the voltage sensor on the T-tubule membrane directly oxidizes sulphydryl sites on SR Ca^{2+}-release channels.

INTRODUCTION

Force generation in muscle is regulated by the cytosolic levels of Ca^{2+}. In skeletal muscle, the sarcoplasmic reticulum (SR) is the intracellular compartment from which Ca^{2+} release and re-uptake produces muscle twitches. A central question in muscle physiology is the mechanism coupling the firing of an action potential at the plasma membrane to the release of Ca^{2+} from the SR network. Current theories propose that the dihydropyridine receptor acts as the voltage sensor at the transverse tubules (Rios & Brum, 1987). The L-type, voltage-gated Ca^{2+} channels at the T-tubules may activate SR Ca^{2+} release across a 12 nm gap through the influx of Ca^{2+} (Ford & Podolsky, 1972), the synthesis and diffusion of the chemical messenger inositol 1,4,5-trisphosphate across the gap (Vergara, Tsien & Delay, 1985), or by direct physical connections via 'feet' spanning the gap (Ferguson, Schwartz & Franzini-Armstrong, 1984).

The SR Ca^{2+}-release channel was identified by several laboratories as a high molecular weight protein (MW = 565000) through its high affinity binding to ryanodine, a plant alkaloid known to interfere with the release process (Pessah, Waterhouse & Casida, 1985; Pessah, Stambuk & Casida, 1987). The ryanodine receptor complex was purified from detergent-solubilized junctional or heavy SR proteins using radiolabelled ryanodine and linear sucrose gradients (Lai, Erickson, Rousseau, Liu & Meissner, 1988). Incorporation of the ryanodine receptor in planar bilayers revealed a Ca^{2+}-permeable pore with a large unitary conductance (Lai *et al.* 1988; Smith, Imagawa, Ma, Fill, Campbell & Coronado, 1988). Its channel properties were similar to those observed for Ca^{2+} release from skinned fibres (Volpe & Stephenson, 1986), heavy SR vesicles (Meissner, Darling & Eveleth, 1986), or 'native' SR Ca^{2+} channels, incorporated by fusing SR vesicles with planar bilayers (Smith, Coronado & Meissner, 1986). These criteria suggested that the 565 kDa or 'feet' proteins are the physiological SR release channels which communicate by an unknown mechanism with dihydropyridine receptors, or voltage sensors on the T-tubules.

In addition to the traditional activators of SR Ca^{2+} release, we have demonstrated that sulphydryl reagents trigger release from SR vesicles. Heavy metals like Hg^{2+}, Ag^{+}, Cu^{2+}, Cd^{2+}, and Zn^{2+} were shown to induce Ca^{2+} release from SR vesicles by binding to an accessible free sulphydryl group (SH) on a protein (Abramson, Trimm, Weder & Salama, 1983). The concentrations of heavy metals used to elicit rapid and complete release of Ca^{2+} were too low to affect the Ca^{2+}, Mg^{2+}-ATPase and their potency was similar to their relative binding affinity to SH groups. More detailed experiments revealed that at low concentrations, Ag^{+} interacted directly with the Ca^{2+} release mechanism of cardiac (Prabhu & Salama, 1990) and skeletal (Salama & Abramson, 1984) SR and not with Ca^{2+}, Mg^{2+}-ATPase. Thus, heavy metals (2–20 μM)

most likely bind to a 'critical' free SH group (but not a disulphide bond) on SR protein(s) resulting in the opening of the physiological Ca^{2+}-release channel (Salama & Abramson, 1984). Since heavy metals are clearly not physiological triggers of SR Ca^{2+} release, other sulphydryl reagents were examined to test the notion that free SH group(s) on SR proteins could be modified to reversibly open and close Ca^{2+}-release channels.

Mercaptans (i.e. the amino acids cysteine, cysteamine and homocysteine) had no effect on either Ca^{2+} uptake or release by SR vesicles. However, in the presence of Cu^{2+} ions (0·5–5 μM), the same mercaptans elicited Ca^{2+} release (Trimm, Salama & Abramson, 1986). These results were consistent with the notion that Ca^{2+}-release channels, in the closed state, contain SH group(s) which would not be expected to react with exogenously added mercaptans unless a catalyst (Cu^{2+} ions) was available to promote the oxidation of the fixed SH sites on the proteins with the exogenously added SH sites on cysteine molecules (Cavallini, DeMarco, Dupre & Rotiolio, 1969). The formation of mixed disulphide bonds resulted in the opening of the channel and SR Ca^{2+} release. Consistent with this interpretation, the subsequent addition of millimolar concentrations of sulphydryl reducing agents such as dithiothreitol (DTT) or reduced glutathione (GSH) reduced the newly formed disulphide bonds. This resulted in the regeneration of free SH groups on SR proteins, closure of the channel and active re-uptake of Ca^{2+} by SR vesicles (Trimm *et al.* 1986).

An alternative chemical reaction to oxidize free SH sites is through sulphydryl–disulphide interchange reactions which do not require the presence of a heavy metal catalyst. In this case, the SH group on a protein attacks the disulphide bond on an exogenously added reagent which results in the formation of a new disulphide bond between the protein and the added reagent. Sulphydryl–disulphide interchange reactions typically exhibit slow reaction times and require high substrate concentrations, except for a class of compounds called 'reactive disulphides'.

Reactive disulphides, compounds with a pyridyl ring adjacent to a disulphide are known to be absolutely specific to free sulphydryls which they oxidize via sulphydryl–disulphide exchange reactions at high reaction rates and low substrate concentrations (10–50 μM). In line with their well-established chemistry, the reactive disulphides, 2,2′- and 4,4′-dithiodipyridines, oxidized SH sites on SR Ca^{2+} release channels, resulting in Ca^{2+} release in both isolated vesicles and skinned rabbit psoas fibres. Their effect was completely reversed by reducing the newly formed disulphide bonds using DTT or GSH (Zaidi, Lagenaur, Abramson, Pessah & Salama, 1989*a*).

By analogy with our work on SR vesicles, Ag^{+} and Hg^{2+} ions induced Ca^{2+} release from the SR network of skinned frog fibres and appeared to act by binding to a sulphydryl site on Ca^{2+}-induced Ca^{2+} release channels (Aoki, Oba & Hotta, 1985; Oba, Iwama & Aoki, 1989). In intact frog fibres, the same heavy metals elicited contractions by an unknown action on T-tubule membrane proteins (Oba & Hotta, 1985).

In this report, we studied the effect of heavy metal ions and cysteine (in the presence or absence of catalytic concentrations of Cu^{2+} or Hg^{2+}) on Ca^{2+} release from the SR network of chemically and mechanically skinned mammalian skeletal muscle fibres. The data show that the heavy metals probably bind to and cysteine (plus a

catalyst) oxidizes 'critical' and accessible SH groups on SR protein(s) resulting in an increase in SR Ca^{2+} permeability and thus, muscle contractions.

METHODS

Preparation of SR vesicles

New Zealand White rabbits were killed with an intravenous injection of sodium pentobarbitone (Nembutal, 50 mg kg^{-1}) then psoas and hindleg white skeletal muscles were removed to prepare skinned fibres and sarcoplasmic reticulum (SR) vesicles, respectively. SR vesicles enriched with heavy or terminal cisternae SR were prepared from white skeletal muscle of rabbit hindleg as previously described (Salama & Abramson, 1984). After the last centrifugation step, the isolated SR (8–12 mg protein ml^{-1}) was suspended in a medium consisting of 0·9 M-sucrose and 10 mM-histidine (pH 6·8) and stored in liquid nitrogen until used. Protein concentrations were determined by the method of Lowry, Rosebrough, Farr & Randall (1953).

Measurement of Ca^{2+} transport by SR vesicles

Calcium uptake and efflux from SR vesicles was measured spectrophotometrically using Arsenazo III (Ars III) or Antipyrylazo III (Ap III) as an indicator of ionized extravesicular Ca^{2+} concentration in the suspending medium. The differential absorption changes of Ars III or Ap III were kinetically measured with a time-sharing, dual-wavelength spectrophotometer (SDB-3A, University of Pennsylvania Biomedical Instrumentation Group, Philadelphia, PA, USA), at 675–685 nm and 720–790 nm, respectively (Salama & Scarpa, 1983). In a typical experiment, SR vesicles (0·3–1 mg ml^{-1}) were suspended in a 2 ml cuvette containing 100 μM-Ca^{2+} indicator dye, 100 mM-KCl, 0–10 mM-$MgCl_2$, and 20 mM-HEPES, at pH 6·8 and 23 °C. The cuvette was placed in the spectrophotometer and aliquots of Ca^{2+} were manually but rapidly (1–2 s) added to calibrate the absorption changes of the metallochromic indicator. Ca^{2+} transport was initiated by the addition of 0·2 mM-MgATP and monitored for several minutes; that is, until the ATP-dependent Ca^{2+} uptake process was completed and slow passive efflux of Ca^{2+} began. The time course of Ca^{2+} efflux from Ca^{2+}-loaded SR vesicles was quantitatively measured following additions of a heavy metal, or sequential additions of Cu^{2+} then cysteine, or cysteine then Cu^{2+}. In other experiments, cysteine was added to the cuvette at the onset of the experiment, then catalytic concentrations of Cu^{2+} were added to Ca^{2+}-loaded vesicles to oxidize cysteine and accessible SH groups on the SR proteins. Controls were carried out to show that Hg^{2+} (1–50 μM), Ag^{+} (1–50 μM), Cd^{2+} (1–200 μM), Ni^{2+} (2–500 μM) and Cu^{2+} (0·5–20 μM) did not interfere with the differential absorption changes of Ars III and Ap III or quantitative measurements of $[Ca^{2+}]_{free}$. The rate of Ca^{2+} release (in nm (mg protein)$^{-1}$ s^{-1}) was calculated from the amount of Ca^{2+} released during the initial 5 s after the addition of a sulphydryl reagent.

Fibre preparation

Chemically skinned fibres were prepared from psoas muscles of New Zealand White rabbits, as previously described by Eastwood, Wood, Bock & Sorenson (1979). Small bundles of several hundred psoas fibres were removed from the muscle, tied to Teflon sticks at 110–120% of resting length and 'skinned' in a solution of the following composition (in mM): 170 potassium gluconate, 2·5 magnesium gluconate, 2·5 Na_2ATP, 5 EGTA and 5 imidazole at pH 7·0. After 24 h at 4 °C the bundles were transferred to a solution of the same ionic composition but made up in 50% glycerol for storage at −20 °C for up to 6 weeks. Solutions containing 2, 5, 7 or 10 mM-EGTA appeared to effectively skin muscle fibres after several days, as evidenced by phasic Ca^{2+}-induced Ca^{2+}-release contractions elicited by nanomolar quantities of ionized Ca^{2+}.

Some control experiments were carried out in split fibres to verify that exposure of the fibres to EGTA and glycerol did not affect the results. These single fibres were dissected from freshly excised bundles of psoas fibres following several wash cycles in relaxing solution (see below), then split by microdissection and mounted on a tension transducer within 20 min. Alternatively, freshly dissected fibres were mounted on Teflon sticks and skinned for 20 min in a saponin solution containing (in mM): 170 potassium gluconate, 2·5 magnesium gluconate, 2·5 Na_2ATP and 5 imidazole, plus saponin, 50 μg ml^{-1}, at pH 7·0 and 23 °C. For control experiments requiring the absence of an SR network, fibres were skinned with Triton X-100 (1% v/v) for 2–4 h to solubilize

the SR membrane while maintaining a functional contractile apparatus. Fibres prepared in this manner were used to determine the direct effects of sulphydryl reagents on the contractile apparatus and to demonstrate that these reagents acted primarily at the level of the SR network.

Procedures and bathing solutions

Following skinning, bundles of two to four fibres were mounted between two stainless-steel rods and held with an acetone-based glue or a pair of microclamps. One of the rods or clamps was fixed and the other was attached to the headstage of a force transducer (Cambridge, Model 400A) which was mounted on a micromanipulator to adjust the length of the fibres. Fibres were stretched to 120% resting length and suspended in a relaxing solution (2 ml) containing (in mM): 170 potassium gluconate, 10 3-(*N*-morpholino)propane sulphonic acid (MOPS), 2·5 Na_2ATP, 1 $MgSO_4$. Experiments were carried out at pH 6·75 to avoid spontaneous twitches but qualitatively similar results were also obtained at pH 7·0. Bathing solutions were maintained at 23 °C and were vigorously stirred with a magnetic bar throughout the experiments. All solutions were made up in double-distilled, de-ionized water and then passed through a Chelex 100 (Bio-Rad, Rockville Center, NY, USA) ion-exchange column to remove Ca^{2+} and possible heavy metal contaminants such as Cu^{2+}, Fe^{2+}, Fe^{3+} and Pb^{2+}. To prepare the column (1 × 31 cm), the resin was washed three times in 10 mM-EGTA, at pH 7–7·4, three times in 0·1 N-HCl, then twice with 1 N-KOH and poured into the column. De-ionized water was passed through the column until the pH of the effluent was equal to the pH of the water on top of the column. Background levels of Ca^{2+} contamination in all solutions were determined by atomic absorption using chelexed water as the standard solution for a zero calibration. The chelexed water contained less than 10^{-8} M-free Ca^{2+} according to aequorin measurements (Borle & Snowdowne, 1986). Free Mg^{2+} and Ca^{2+} levels in the relaxing solution were determined to be based on a modified version of the calculator program of Fabiato & Fabiato (1979). Calculations of free Ca^{2+} were confirmed with a Ca^{2+}-selective electrode (Cal 1, WPI, New Haven, CT, USA) and in some experiments, free Ca^{2+} was continuously measured using ion-selective electrodes during contractions elicited by sulphydryl reagents, Ca^{2+}-induced Ca^{2+} release, or caffeine. The effect of ionized Mg^{2+} on the rate and extent of Ca^{2+} release was examined by setting the levels of free Mg^{2+} at 40 μM, 100 μM, 400 μM or 1·5 mM by respectively adding 0·5 mM, 1 mM, 2·5 mM or 5 mM-$MgSO_4$ to the medium. Calculations of free metal concentrations were based on stability constants from Sillen & Martell (1964). At the start of each run, fibres were loaded with Ca^{2+} by successive additions of calcium gluconate to lower pCa ($-\log[Ca^{2+}]$) from 6·8 to 6·5–6·3. At such pCa levels, Ca^{2+} loading occurred in a few minutes, and the SR clearly accumulated sufficient Ca^{2+} to produce large phasic contractions upon additions of caffeine (1–5 mM), or 100–300 nM-Ca^{2+}_{free} to elicit Ca^{2+}-induced Ca^{2+} release.

Under our experimental conditions, aliquots of heavy metal stock solution (1–20 μl) altered free Ca^{2+} by a maximum of 5 nM when added to the fibres' bathing medium. In many experiments, pH was continuously monitored with a small, fast-response electrode (Ingold Electrodes Inc. 6028-01, Andover, MA, USA) to insure that aliquots of stock solutions added to the bath did not alter pH.

Determination of free heavy metal concentrations

Background concentrations of heavy metals in our experimental fibres and solutions were estimated to be less than 10^{-8} M. The estimate was based (1) on the possible contaminating levels of metal in salts purchased from Sigma, (2) the removal of heavy metals by passing the solutions through a Chelex-100 cation-exchange column (1 × 31 cm) and (3) the removal of heavy metals intrinsic to the fibres during the skinning procedure where muscles are incubated in 5 mM-EGTA for more than 10 days before use.

The most significant source of heavy metal contamination came from the 170 mM-potassium gluconate (< 5 p.p.m.) compared to 2·5 mM-$MgSO_4$ (40 p.p.m.), 2·5 mM-Na_2ATP (< 20 p.p.m.), 10 mM-MOPS (< 5 p.p.m.) and 250 μM-calcium gluconate (< 20 p.p.m.). Total heavy impurities in potassium gluconate (< 5 p.p.m.) probably consisted of Pb^{2+}, some Zn^{2+} and Hg^{2+}. The maximum concentration of heavy metal contamination would be 3·4 μM if all the impurities consisted exclusively of Ni^{2+}, the metal with the lowest molecular weight, and the minimum would be 0·8 μM if the impurities consisted exclusively of Pb^{2+}, the highest molecular weight metal that is likely to contaminate the solutions. The ion-exchange column can reduce the heavy metal concentrations by at least a factor of 100 such that of the total (bound and free) concentration of heavy metal contaminants was conservatively estimated at less than 10^{-8} M.

Table 1 lists the stability constants of heavy metal–ATP, gluconate, penicillamine (a mercaptan with a mid-range affinity for heavy metals), and EGTA complexes. The last two columns list the total heavy metal concentration (at concentrations used in this study) and estimates of free heavy metal in the bathing medium. Free heavy metal concentrations were calculated from the stability constants of the heavy metals with ATP (2·5 mM), gluconate (170 mM), taking into account the

TABLE 1. Stability constants of heavy metal (HM) complexes and heavy metal concentrations in the bathing medium

	log stability constants							
			Penicillamine		EGTA			
HM	ATP	Glu	K_1	K_2	K_1	K_2	$[HM]_{total}$* (μM)	$[HM]^h_{free}$ (M)
Ag^+	2·88[a]	0·23[b]	12·42	—	7·06	—	10	$3·5 \times 10^{-6}$
							50	$1·75 \times 10^{-5}$
Cd^{2+}	4·60[c]	2·09[d]	11·4	7·1	16·50	—	20	$4·4 \times 10^{-8}$
							100	$22·0 \times 10^{-8}$
Cu^{2+}	6·13	18·3[e]	16·5	5·2	17·57	—	5	$1·5 \times 10^{-9}$
							20	6×10^{-9}
							50	$1·5 \times 10^{-8}$
Hg^{2+}	17·5[f]	8·43[g]	17·5	6·0	21·64	—	20	1×10^{-12}
Ni^{2+}	5·02	1·82	11·4	10·9	13·50	—	50	4×10^{-7}
							200	$1·6 \times 10^{-6}$
Zn^{2+}	4·85	1·70	10·0	8·9	12·60	—	200	$2·3 \times 10^{-6}$
Ca^{2+}	3·85	1·21	—	—	10·70	5·3	1	$8·0 \times 10^{-8}$
							250	$2·5 \times 10^{-5}$

[a] log apparent dissociation constant (K_{app}) at pH 6·8 using an Orion Ag^+-selective electrode.
[b] log K_{app} at pH 7·0.
[c] log K_{app} at pH 7·1, from Stephenson & Thieleczek (1986).
[d] log β_2 $[Cd^{2+} + 2\ Glu^- = Cd\ (Glu)_2]$.
[e] log K $[Cu^{2+} + Glu^- + 2·5\ OH^- = CuGlu(OH)^{1·5}_{2·5}]$.
[f] Estimate based on: log K $[Hg^{2+} + OH^- + P_2O_7^{4-} = Hg(OH)P_2O_7^{3-}]$.
[g] Estimate based on: log β_2 $[Hg^{2+} + 2(acetate^-) = Hg(acetate)_2]$.
[h] Calculated for: pH 6·8, $[ATP]_{total}$ = 2·5 mM, $[Glu]_{total}$ = 170 mM, $[Mg^{2+}]_{total}$ = 1 mM.

Note: stability constants for Cu–Glu and Hg–Glu do not represent simple one-to-one heavy metal Glu complexes; a stability constant for Hg–Glu was not available and a value for Hg–$(acetate)_2$ was offered as an approximation. Unless stated, all stability constants (K_1, K_2 and β_2) were as defined and obtained from Sillen & Martell (1964). For a metal (M) and a ligand (L), $K_n = \frac{[ML_n]}{[ML_{n-1}][L]}$, where K_n is a stepwise constant for binding a metal to a ligand and n defines the stoichiometry of the metal–ligand complex. However, when the K_n values are not known, $\beta_n = \frac{[ML_n]}{[M][L]^n}$ values are used as cummulative constants for binding of the metal to the ligand.

* The data throughout this paper are expressed in terms of total heavy metal concentrations added to the medium, except for Figs 1 and 5. The last two columns of this table serve to determine free heavy metal from total heavy metal in the range of total heavy metal used for this study.

values of $[Mg^{2+}]_{total}$ (1 mM), pCa (6·5), and pH (6·75). The calculations of free heavy metal concentration do not take into account the binding of heavy metals to sulphydryl groups on muscle proteins.

Table 1 indicates that the high binding affinity of heavy metals to EGTA (column 6) would make EGTA an unsuitable buffer for free Ca^{2+} in the presence of heavy metals. The stability constants of heavy metal EGTA complexes are greater than the Ca–EGTA complex (except for Ag^+ ions)

such that in solutions buffered with Ca–EGTA, heavy metals would preferentially bind to EGTA and decrease pCa. The problem would be particularly severe in studies of Ca^{2+}-induced Ca^{2+} release in skinned fibres where solutions are typically buffered with low [EGTA] (50–200 μM) since higher [EGTA] would chelate Ca^{2+} released from the SR and block contractions. On the other hand, the weak Ca^{2+} buffer, gluconate (Glu) was highly suitable to study Ca^{2+}-induced Ca^{2+} release because additions of heavy metal did not alter pCa through the formation of heavy metal–Glu complexes due to the large excess of $[Glu]_{free}$. This was experimentally verified by measuring pCa with a Ca^{2+}-selective electrode (Cal 1, WPI) and systematically adding heavy metals to a 170 mM-gluconate solution with an initial pCa of 5·5, 6·0 or 6·5. The concentrations of heavy metals used in this study did not affect the Ca^{2+} electrode nor did they alter the electrode's response as a function of free Ca^{2+}.

Stability constants of heavy metal–EGTA complexes are greater than the heavy metal–penicillamine complexes (Table 1) which implies that heavy metal binding to EGTA competes with heavy metal–protein binding. The stability constants of heavy metal–penicillamine were chosen as representative values for heavy metal–protein complexes because penicillamine binding to heavy metals is in the middle of the range for heavy metal binding to SH sites on proteins (A. Martell, personal communication).

Even though these stability constants were measured under different ionic strengths and temperatures, correction factors were used to obtain accurate calculated values of free heavy metals (Sillen & Martell, 1964). For Hg–ATP and Hg–Glu, stability constants were not available but were substituted by values for Hg–pyrophosphate and Hg–acetate, respectively. In the latter case, calculated values for free $[Hg^{2+}]$ were probably correct to within an order of magnitude. Table 1 indicates that the lowest concentrations of free heavy metals used to elicit SR Ca^{2+} release by Ag^{+}, Cd^{2+}, Cu^{2+}, Hg^{2+} and Ni^{2+} were respectively 3·5 μM, 44 nM, 1·5 nM, 0·5 pM and 40 μM. The estimates of free heavy metal in the bathing medium are not likely to apply in the cytosol of skinned fibres because of the high binding affinity of heavy metals to sulphydryl groups. The stability constants of heavy metals to SH sites can be as low as 10^{10}–10^{43} for Hg–cysteine (Stricks & Kolthoff, 1953). Thus, nearly all the added heavy metals diffusing into the cytosol were probably bound to SH sites on muscle proteins.

Analysis of tension recordings

The magnitudes of phasic or tonic contractions were expressed as a percentage of maximum tension generated by the fibres. The maximum tension was determined by adding caffeine (2 mM) to release enough Ca^{2+} from the SR to saturate the myofilaments and by directly adding Ca^{2+} (250 μM) to saturate the myofilaments. The two methods yielded equivalent levels of tension (within 10%). Caffeine-induced contractions confirmed that the SR network was functional, Ca^{2+} loaded and could release sufficient Ca^{2+} to saturate the myofibrils.

Upon the addition of a low concentration of a heavy metal, there was a delay followed by a rapid phasic contraction. With higher heavy metal concentrations, the initial delay was reduced and the rate of rise of contractions was increased. To quantify the relative potency of different SH reagents and different concentrations of the same reagent, the inverse time-to-peak (s^{-1}) was plotted as a function of free heavy metal concentration. The time-to-peak (s) was the interval measured from the time a reagent was added to the time of maximum force. This interval was dependent on (i) mixing time in the chamber ($\sim$ 0·5 s), (ii) diffusion of the reagent into the fibres, (iii) reaction rate of the reagent with the SR Ca^{2+} release channel, (iv) rate of SR Ca^{2+} release, and (v) activation of the myofilaments by Ca^{2+}. Assuming that mixing time in the chamber and activation of the myofilaments at a given $[Ca^{2+}]_{free}$ remained constant for these SH reagents, then differences in times-to-peak depended on the diffusion, reaction rate, and rates of Ca^{2+} release induced by the SH reagent. Note that contractions elicited by different heavy metal concentrations often had similar maximum rates of rise but different times-to-peak because the intervals from the addition of heavy metal to the onset of contraction were reduced at higher heavy metal concentrations. Thus, the inverse time-to-peak (s^{-1}) compared the relative potency of SH reagents at triggering SR Ca^{2+}-release.

Tests and precautions

On the absence of an ATP-regenerating system. Studies of SR Ca^{2+} release from skinned fibres are traditionally carried out in the presence of ATP-regenerating systems to maintain [ATP] within the myofibrils, during activation of the fibres by Ca^{2+} and high rates of ATP hydrolysis. As a result,

force measurements reflect changes in pCa within the fibres and are not dependent on local depletion of ATP in diffusionally restricted spaces. However, ATP regenerating systems were not compatible with our experimental protocols because enzymes like creatine and pyruvate kinase are inhibited by micromolar concentrations of heavy metal, including Cd^{2+}, Zn^{2+}, Cu^{2+}, Ag^{+}, Ni^{2+}, and

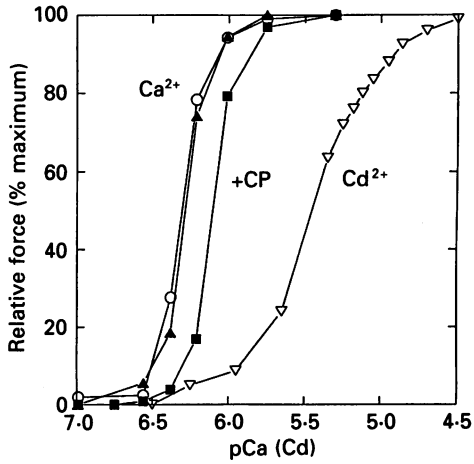

Fig. 1. Force *vs.* pCd and pCa in various media. Fibres were chemically skinned in EGTA and exposed to Triton X-100 for 2–4 h to solubilize the SR membrane. Bundles (2–4 fibres) were mounted in the chamber and bathed in: 50 mM-MOPS, 2·5 mM-Na_2ATP, 3·5 mM-$MgCl_2$, pH 6·8, plus various [Ca^{2+}] to adjust pCa and (i) 170 mM-KGlu (▲), (ii) 170 mM-KCl, 2 mM-EGTA, (○), or (iii) 105 mM-KCl, 15 mM-creatine phosphate (CP), 2 mM-EGTA (■). Force *vs.* pCa curves are identical in solutions containing KCl plus EGTA or KGlu. The presence of CP caused at most a 0·3 pCa right-shift of the curve. Each point represents the mean from three experiments, from two separate batches of skinned fibres and is expressed relative to the maximum tension generated at pCa 3·0. The standard error from the mean (S.E.M.) was within 10% of the mean. To measure force *vs.* pCd, bundles (3–4 fibres) were suspended in 170 mM-KGlu, 10 mM-MOPS, 2·5 mM-Na_2ATP, 3·5 mM-$MgSO_4$, pCa of 6·80 at pH 6·8 then various heavy metal concentrations were added to measure force as a function of heavy metal concentrations. Of the metals tested (Cd^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+}), only Cd^{2+} activated the myofilaments. Relative force was plotted as a function of pCd (▽). Each point represented the mean of four to six experiments from five separate batches of skinned fibres and was expressed relative to the maximum tension generated at pCa 3·0; S.E.M. was within 10%; pCd = $-\log[Cd^{2+}]_{free}$ was calculated from the stability constants in Table 1.

Hg^{2+} (Kayne, 1973). However, the omission of ATP-regenerating systems is of little consequence to force measurements when fibre diameters are in the range of 100 μM and the diffusion of ATP to the centre of the fibres is rapid compared to rates of ATP hydrolysis, as is the case for fibres bathed in millimolar ATP (Ferenczi, Goldman & Simmons, 1984) and high ionic-strength solutions to reduce ATPase activity (Yanagida, Kuranaga & Inoue, 1982). Previous studies on the interaction of Cd^{2+}, Ag^{+} or Hg^{2+} with skinned fibres also omitted ATP-regenerating systems (Stephenson & Thieleczek, 1986, Oba *et al.* 1989). Force *vs.* pCa curves measured from fibres bathed in KCl plus EGTA buffer (Fig. 1, ○) were indistinguishable from curves generated in potassium gluconate and no additional Ca^{2+} buffer (Fig. 1, ▲) ($n = 3$, each). The presence of 15 mM-creatine phosphate (CP) shifted the pCa for 50% of maximum force from 6·4 to 6·1 (Fig. 1, ■). In three out of six experiments, the force *vs.* pCa curves obtained with CP (in the presence of endogenous creatine kinase, CK) (Fig. 1, ■) were similar to measurements obtained with or without CP (15 mM) plus exogenously added CK (50–75 U ml^{-1}) which indicated that the ATP trans-phosphorylating system had little effect on force measurements. In other controls, the presence of

an ATP-regenerating system (15 mM-CP plus 50 U ml^{-1} CK did not change either the time course or the amplitude of forces generated during Ca^{2+}-induced Ca^{2+} release ($n = 6$) or caffeine ($n = 4$) contractions, or contractures induced by a pCa of 2 ($n = 6$) (not shown).

Possible fibre rundown. Precautions were taken to insure that fibre degradation and rundown did not contribute to changes in twitch tension attributed to sulphydryl reagents. For each experiment, fibres were first incubated at pCa = 6·80 ± 0·3, then aliquots of Ca^{2+} were added to lower pCa to 6·60 ± 0·03 and load the SR network. Further additions of Ca^{2+} were made to elicit a Ca^{2+}-induced Ca^{2+}-release twitch followed by a caffeine-induced contraction. The fibres were re-set by washing with pCa 6·80, then aliquots of Ca^{2+} were added to reload the SR with Ca^{2+} before testing a sulphydryl reagent. After recording a sulphydryl-induced twitch, a second caffeine contraction was recorded and compared to the previous caffeine contraction. Experiments were considered valid when the peak forces of the two caffeine contractions were similar, within 10%. For Hg^{2+} and Ag^{+}, the second caffeine contraction was smaller than the first because these heavy metals inhibited the actomyosin ATPase. Consequently, contractions elicited by Hg^{2+} or Ag^{+} were compared to the initial caffeine contractions measured in the absence of heavy metals.

Strength of contractions as a function of heavy metal concentration. Separate experiments were performed for each heavy metal concentration because once fibres were exposed to a heavy metal, no practical measurement was available to verify that heavy metals were successfully washed out before testing a different concentration on the same fibres. In principle, heavy metals could be effectively washed out by bathing the preparation with high [EGTA]. However, washing the fibres with EGTA did not ensure complete removal of heavy metals because the stability constants of heavy metals to the various endogenous sulphydryl groups were not known and can vary widely. For instance, Hg–cysteine complexes (Stricks & Kolthoff, 1953) can vary from 10^{+9} to 10^{+43} (in units of M^{-1}).

Gluconate as a Ca^{2+} buffer. Because EGTA (50–200 μM) introduced technical problems in studies with heavy metals, we used 170 mM-potassium gluconate solutions to regulate $[Ca^{2+}]_{free}$. The approach required that Ca^{2+} contamination in water and salts be reduced to pCa > 7 by passing the solution through a Chelex-100 column, then adding known concentrations of calcium gluconate (CaGlu) to adjust $[Ca^{2+}]_{free}$. Calculations of $[Ca^{2+}]_{free}$ from $[Ca^{2+}]_{total}$ resulted in a linear relationship between $[Ca^{2+}]_{free}$ and $[Ca^{2+}]_{total}$ for additions of 1–500 μM-CaGlu. An addition of 1 μM $[Ca^{2+}]_{total}$ produced an increase of 80 nM $[Ca^{2+}]_{free}$ (i.e. a ratio of bound/free Ca^{2+} of 11·5), as verified with Ca^{2+}-selective electrodes placed in the bathing medium. Heavy metals (in the range of concentrations used in this study) did not alter pCa as expected from the stability constants (Table 1) and as confirmed with Ca^{2+}-selective electrodes. The concentrations of heavy metals used in this study did not affect the Ca^{2+} electrodes with respect to their responses to Ca^{2+} nor their Ca^{2+} calibration curves.

Gluconate as a weak Ca^{2+} buffer maintained a constant pCa in the bathing medium, without inhibiting Ca^{2+}-induced Ca^{2+} release and caffeine contractions by buffering Ca^{2+} in the interior of the fibres. These observations were supported by estimates of the maximum $[Ca^{2+}]$ that can be sequestered and released by the SR network. For instance, the maximum Ca^{2+} concentration in the lumen of the SR vesicles is 36 mM since the maximum uptake of Ca^{2+} (in the absence of Ca^{2+} chelating agents) is 180 nmol (mg SR protein)$^{-1}$ and the luminal volume is 5 μl (mg SR)$^{-1}$ (Salama & Scarpa, 1983). The SR lumen is about 5% of fibre volume (Peachey, 1965). Hence, the $[Ca^{2+}]$ in the interior of the fibres can increase by a maximum of 1·8 mM $[Ca^{2+}]_{total}$ or 144 μM $[Ca^{2+}]_{free}$ in gluconate buffer. The volume of a fibre 100 μm in diameter × 1 cm in length equals 0·785 μl and for three fibres bathed in 2 ml, the maximum change of Ca^{2+} in the bath would be 2·1 μM-total or 168 nM-free Ca^{2+}. Another positive feature of the gluconate Ca^{2+} buffer is that the binding and unbinding of Ca^{2+} to gluconate is expected to occur on a microsecond to a submillisecond time scale (A. Martell, personal communication).

Interaction of heavy metals with actomyosin ATPase. Of the heavy metals tested, only Ag^{+} and Hg^{2+} ions interacted directly with SH groups associated with actomyosin ATPase and thus caused a reduction of maximum tension generated by Ca^{2+}_{max} (the Ca^{2+} concentration needed to elicit the maximum force that can be generated by the fibres). To measure actomyosin ATPase activity in the presence of heavy metals, fibres were homogenized (Polytron; Brinkmann, Westbury, NY, USA) for 15 s in ice-cold buffer, the homogenized proteins were diluted (0·1 mg ml^{-1}) in 170 mM-potassium gluconate, 10 mM-MOPS (pH 6·8), 2·5 mM-Na_2ATP, 3·5 mM-magnesium gluconate (pCa 5·0) at 23 °C, for 10 min, ± various heavy metal concentrations. The myofibrils were removed

by Millipore filtration (0·45 μM) and aliquots of the filtrates were tested for inorganic phosphate content as described by Martin & Doty (1949). Ag^+ and Hg^{2+} inhibited ATPase activity at concentrations as low as 10 μM. It was not possible to separate the action of Ag^+ ions on SR Ca^{2+} release (which required a minimum of 20 μM-Ag^+), from its toxicity to myosin ATPase. On the other hand, Hg^{2+} acted at the level of the SR and elicited rapid contraction at 2·5–5 μM; that is, at concentrations below its toxic effects. Moreover, toxic levels of Hg^{2+} (25–50 μM) still elicited a single large contraction equal to maximum force before inhibiting atomyosin ATPase activity and subsequent contractions elicited by Hg^{2+}, Ca^{2+}-induced Ca^{2+} release, caffeine or Ca^{2+}_{max}.

Interaction of heavy metals with troponin C. Heavy metals (i.e. Ba^{2+}, Ni^{2+} and Cd^{2+}) have been shown to directly interact with myofilaments to elicit force generation in skinned frog fibres (Stephenson & Thieleczek, 1986). The effect of these metals on skinned psoas fibres lacking an SR network was examined by pre-treatment with Triton X-100 (1·0% v/v) for 2–4 h and by measuring isometric force as a function of heavy metal concentration, at a constant pCa of 6·80. Additions of up to 100 μM-Hg^{2+} and 1 mM-Cu^{2+} or Ni^{2+} did not activate mammalian myofibrils (less than 10% of maximum force). Of all the heavy metals tested, only Cd^{2+} ions activated the contractile apparatus of rabbit psoas fibres by a direct interaction with myofilaments. Figure 1 ($\bigtriangledown$) shows a plot of isometric force *vs.* pCd ($-\log[Cd^{2+}]_{free}$); pCd required to elicit 50% of maximum force was 5·5 compared to 6·8 for calcium. $[Cd^{2+}]_{free}$ values were calculated from the log of stability constants for Cd–Glu of 2·09 (Sillen & Martell, 1964) and 4·6 for Cd–ATP (Stephenson & Thieleczek, 1986). Under these conditions, an addition of 50 μM-$CdCl_2$ corresponded to a pCd of 6·3 which produced less than 10% of maximum tension (Fig. 1 $\bigtriangledown$).

Osmotic effects. Small decreases in osmolarity (about 2–3%) of the bathing solutions were found to elicit phasic contractions by triggering SR Ca^{2+} release. For instance, additions of 60 μl of water (a 3% decrease in osmolarity) had no effect on EGTA-skinned fibres, when the SR was depleted of Ca^{2+}. However, when the SR was loaded with Ca^{2+}, an addition of water (60 μl) elicited twitches of about 50% of the maximal force that could be generated by the fibres. Such volumes of H_2O contained less than 5 nM $[Ca^{2+}]_{free}$, insufficient to elicit Ca^{2+}-induced Ca^{2+} release interactions. Contractions induced by osmotic changes were highly reproducible ($n = 12$) but were reliably avoided by keeping all volumetric additions to the bathing medium at 10 μl or less and by preparing all stock solutions of sulphydryl reagents in iso-osmotic medium.

Materials

All reagents were of analytical grade. Ruthenium Red (RR) was obtained from Sigma Chemical Co. (St Louis, MO, USA) and final concentrations were adjusted to take into account the 45% purity of the commercial product. Arsenazo III, anhydrous caffeine, $ZnSO_4.7H_2O$, $NiCl_2.6H_2O$, $CdCl_2$, $CuSO_4$, $AgNO_3$, $HgCl_2$, L-cysteine and oxidized and reduced forms of glutathione were obtained from Sigma. Oxidized glutathione and cysteine stock solutions were freshly prepared, kept ice-cold and used the same day. Antipyrylazo III (Lot-36695-A) was obtained from ICN Pharmaceuticals (Plainview, NY, USA).

RESULTS

Heavy metals induce SR Ca^{2+} release in skinned fibres

The addition of a heavy metal was found to cause Ca^{2+} release from the SR network of chemically skinned skeletal fibres and to generate transient contractions. Figure 2*A* describes the experimental protocol. The SR network was loaded with Ca^{2+} by gradually lowering pCa from 6·8 to 6·63 through additions of CaGlu (1–2·5 μM). After a Ca^{2+} loading phase, a twitch was elicited by Ca^{2+}-induced Ca^{2+} release followed by a caffeine-induced (2 mM) contraction to determine the force generated by a maximum release of Ca^{2+} from the SR. The fibres were washed, reloaded with Ca^{2+}, a Ca^{2+}-induced Ca^{2+}-release twitch was recorded, then an addition of 10 μM-$CuCl_2$ elicited a rapid twitch of similar kinetics and magnitude to the Ca^{2+}-induced Ca^{2+}-release twitch. At the end of each run, caffeine, then saturating levels of free Ca^{2+} (Ca^{2+}_{max} = 250 μM-total Ca^{2+}) were added to evaluate

the amount of releasable Ca^{2+} from the SR network compared to the maximum force that could be generated by the fibres. The magnitudes of caffeine contractions at the beginning and end of the run, and the maximum force generated by Ca^{2+}_{max} did not vary by more than 10%. Reproducible levels of force induced by caffeine and Ca^{2+} indicated a lack of fibre rundown and was used as a criterion to include the data in our analysis. A major concern is whether additions of Cu^{2+} elicited contractions by dissociating calcium bound to gluconate, ATP or residual fragments of sarcolemma on the fibres, thereby increasing $[Ca^{2+}]_{free}$ in the medium, and inducing Ca^{2+}-induced Ca^{2+}-release contractions. In Fig. 2*B*, force measurements were repeated (bottom trace) while continuously recording free $[Ca^{2+}]$ in the bathing medium with an ion-selective electrode (top trace). Additions of 2·5 μM-Ca^{2+} lowered pCa from 6·63 to 6·36 and caffeine elicited a contraction through SR Ca^{2+} release. After washing and reloading the fibres, 5 μM-Cu^{2+} elicited a twitch by triggering SR Ca^{2+} release, but did not release bound Ca^{2+} to alter free Ca^{2+} in the surrounding medium, as indicated by Ca^{2+}-electrode recordings. Additions of up to 200 μM-Cu^{2+} did not increase $[Ca^{2+}]_{free}$ by releasing bound Ca^{2+} and did not alter the pH of the bathing medium (not shown). Low concentrations of Cu^{2+} produced phasic twitches, whereas, higher concentrations elicited phasic contractions followed by tonic contractions (contractures) and an inability of the fibres to fully relax (Fig. 2*C*). As anticipated from SR vesicle studies, Ruthenium Red (RR) blocked Cu^{2+}-induced contractions (Fig. 2*D*). Ca^{2+} also blocked caffeine contractions (95% reduction of force, $n = 5$) by inhibiting caffeine-induced SR Ca^{2+} release and did not act at the level of the myofibrils since the force generated by direct additions of Ca^{2+} was not altered by Cu^{2+} (Fig. 2*C*). Of all the heavy metals tested, only Cu^{2+} inhibited caffeine-induced contractions. The suppression of caffeine contractions by Cu^{2+} ions was dependent on $[Cu^{2+}]$ and time. For instance, caffeine elicited normal contractions if added within 5–10 s after an addition of Cu^{2+} (250 μM) (i.e. 93·3 ± 5·4% of control caffeine contractions; $n = 3$). On the other hand, caffeine added 3 min after the addition of Cu^{2+} (250 μM) did not cause SR Ca^{2+} release and caffeine contractions were blocked (i.e. 4·8 ± 0·9% of controls, $n = 3$). Lower concentrations of Cu^{2+} (50 and 100 μM) could block caffeine contractions if fibres were incubated with Cu^{2+} for 6–10 min. Cu^{2+} blocked caffeine-induced contractions but did not deplete Ca^{2+} in the SR network since subsequent SR Ca^{2+} release could be elicited through either Ca^{2+}-induced Ca^{2+} release, or heavy metal-induced contractions (not shown).

Figure 3 shows the effects of low and high concentrations of Cd^{2+} and Ni^{2+} on skinned fibres. After loading, depleting and re-setting the SR of skinned fibres, addition of 5 μM-Cd^{2+} had no effect but a second addition of 10 μM-Cd^{2+} elicited a rapid twitch (Fig. 3*A*). Still higher concentrations of Cd^{2+} (50 μM) elicited rapid contractions followed by a tonic contracture (Fig. 3*B*) or slower spontaneous contractions (not shown). These levels of free Cd^{2+} were too low to directly activate the myofilaments (see Fig. 1) and caused contractions only if the SR network was functional and loaded with Ca^{2+}. Nickel ions were considerably less effective at triggering contractions and a minimum of 100 μM-Ni^{2+} was necessary to elicit contractions (Fig. 3*C*) and higher concentrations (250 μM-Ni^{2+}) were often followed by spontaneous twitches (Fig. 3*D*).

Mercury and silver ions triggered SR Ca^{2+} release and induced twitches at

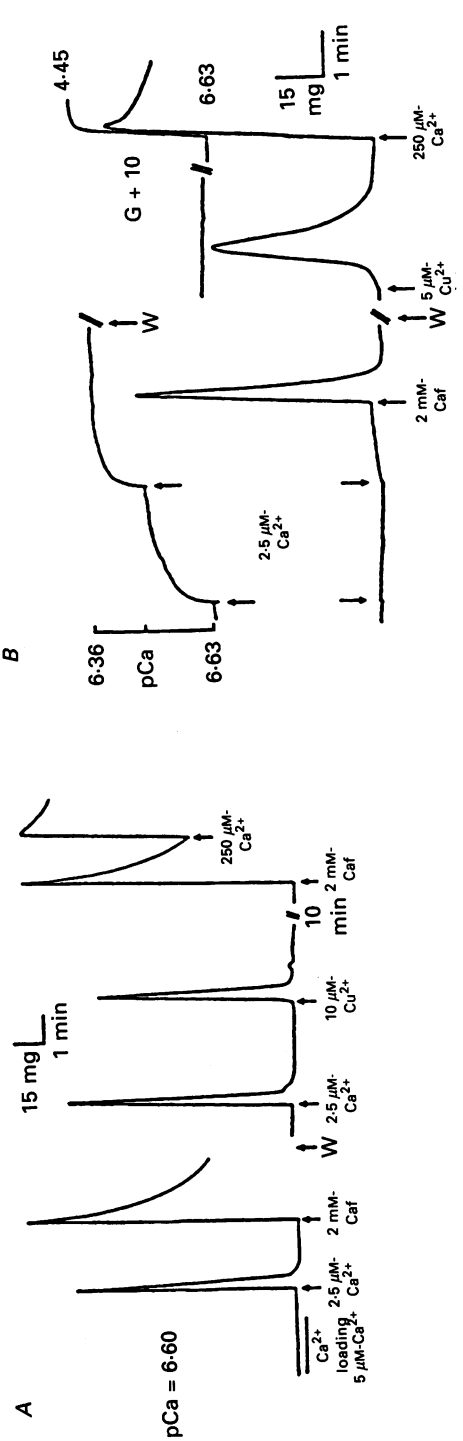

Fig. 2. For legend see facing page.

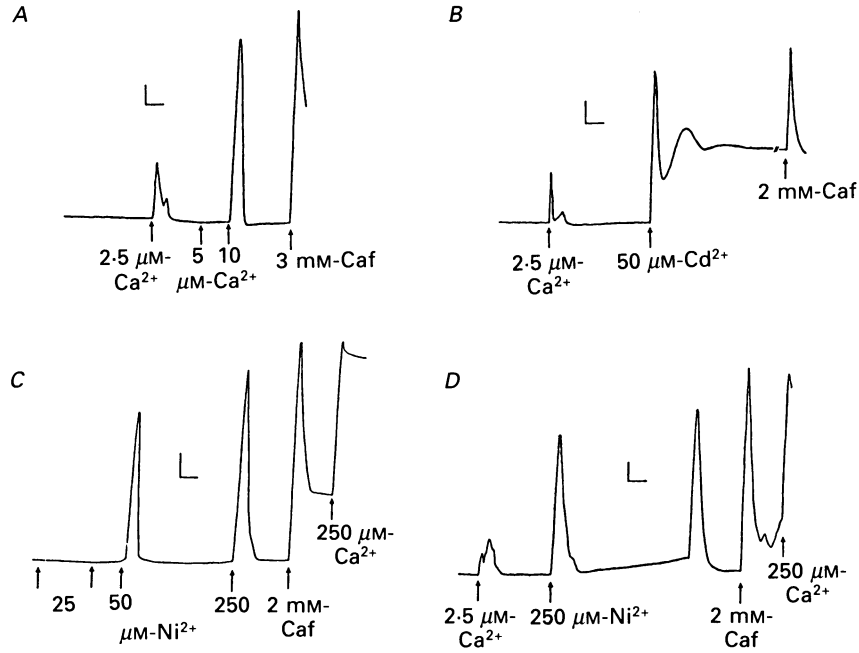

Fig. 3. Contractions induced by Cd^{2+} and Ni^{2+}. As for Fig. 2, fibres were Ca^{2+} loaded, control caffeine contractions were measured, then the fibres were washed and re-set at pCa 6·60 before adding: 5 then 10 μM-Cd^{2+} (total addition of 15 μM; *A*); 50 μM-Cd^{2+} (*B*); 25, 25, 50 then 250 μM-Ni^{2+} (*C*); or 250 μM-Ni^{2+} (*D*). Low Cd^{2+} (15 μM) elicited rapid phasic contractions (*A*) whereas high Cd^{2+} (50 μM) elicited rapid force generation followed by a partial relaxation and maintenance of a contracture (*B*). Low Ni^{2+} (50 μM) elicited twitches (*C*) and high Ni^{2+} (250 μM-free) elicited contractions followed by spontaneous twitches (*D*). Vertical and horizontal bars represented 10 mg and 1 min, respectively.

Fig. 2. Cu^{2+}-induced contractions. Skinned fibres were mounted on the tension transducer, stretched to 120% of their resting length, and bathed in 2 ml of continuously stirred potassium gluconate solution at pCa 6·8, pH 6·75 and pMg 4·0. *A*, skinned fibres were Ca^{2+} loaded at pCa 6·60, then tested for Ca^{2+}-induced Ca^{2+} release and caffeine-induced contractions by adding 200 nM-free Ca^{2+} and 2 mM-caffeine (Caf), respectively. The fibres were washed, re-loaded with Ca^{2+} at pCa 6·60, re-tested for a Ca^{2+}-induced Ca^{2+}-release contraction by the addition of 200 nM-free Ca^{2+}. Cu^{2+} (10 μM) induced a rapid twitch, then a caffeine contraction and the maximum force were measured by adding 2 mM-caffeine and Ca^{2+}_{max}. *B*, force generated by the fibres (bottom trace) and $[Ca^{2+}]_{free}$ were simultaneously measured during two additions of 2·5 μM-Ca^{2+} and a control caffeine contraction. Direct additions of Ca^{2+} to the bath lowered pCa while caffeine elicited a twitch but did not alter pCa in the bulk solution. After washing, reloading the SR and setting pCa at 6·63, Cu^{2+} (5 μM) elicited a twitch and like caffeine contractions, did not alter free Ca^{2+} in the surrounding medium. The gain for the ion-selective electrode (G) was reduced by a factor of 10 and pCa was lowered to 4·45 to measure the maximum force of the fibres. *C*, after a caffeine contraction, fibres were washed and reloaded; 250 μM-Cu^{2+} resulted in a rapid rise in force followed by a partial relaxation. Cu^{2+} blocked SR Ca^{2+} release by a 2nd caffeine addition but did not inhibit force generated by a direct addition of Ca^{2+}. *D*, after loading the SR, measuring a caffeine contraction, washing and re-loading the SR, a Ca^{2+}-induced Ca^{2+}-release contraction was elicited by adding 2·5 μM-Ca^{2+}. An addition of 2·5 μM-Ruthenium Red (RR) blocked subsequent Cu^{2+}-induced contractions.

minimum concentrations of 2·5 and 25 μM, respectively. Hg^{2+}, at 2·5 or 5 μM, elicited weak twitches and did not suppress subsequent contractions elicited by caffeine or Ca^{2+}_{max} (not shown). Higher [Hg^{2+}] (10 and 25 μM) produced stronger twitches but suppressed subsequent measurements of force induced by caffeine or Ca^{2+}_{max} because Hg^{2+} inhibited actomyosin ATPase activity (Fig. 4*A* and *B*). Ag^{+} suppressed myosin

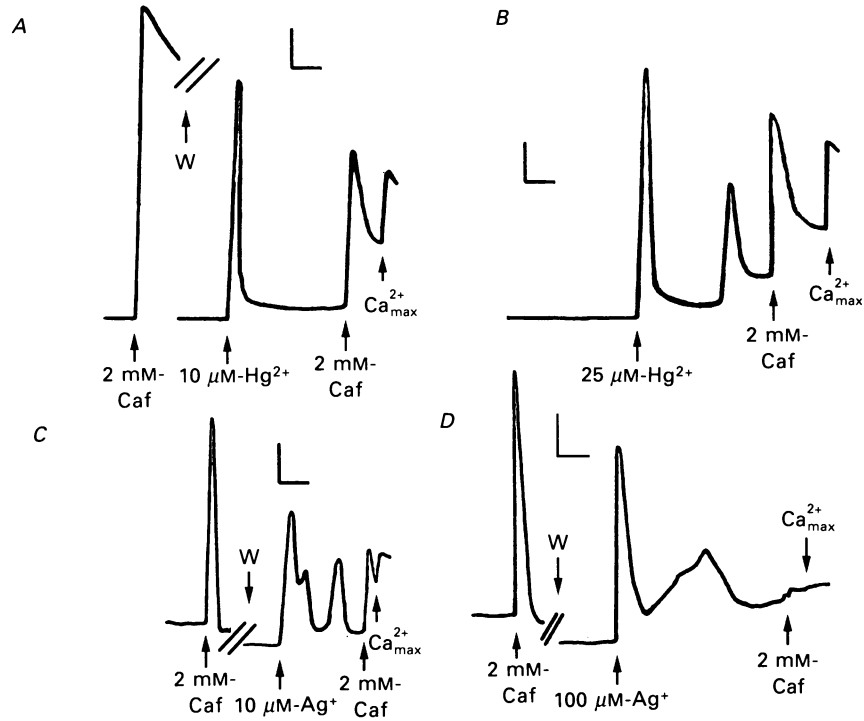

Fig. 4. Hg^{2+}- and Ag^{+}-induced contractions. Fibres were treated as described for Fig. 2, washed, reloaded with Ca^{2+}, set at pCa 6·60, then different concentrations of Hg^{2+} were added to elicit SR Ca^{2+} release and contractions: 10 μM (*A*) and 25 μM (*B*). After adding Hg^{2+}, caffeine (2 mM) then 250 μM-Ca^{2+} were added to evaluate the levels of Ca^{2+} stored in the SR and the maximum force of the fibres, respectively. *A* and *B* were normalized to the first caffeine-induced contraction shown in *A*. In *C*, 25 μM-Ag^{+}, and in *D*, 100 μM-Ag^{+} were added which elicited twitches followed by spontaneous contractions. In the presence of Ag^{+}, subsequent additions of caffeine then Ca^{2+}_{max} resulted in considerably weaker forces compared to control caffeine contractions. Vertical and horizontal bars represent 15 mg and 1 min, respectively.

ATPase activity and force, at lower concentrations than Hg^{+}. In Fig. 4*C* and *D*, control caffeine contractions were measured, the fibres were washed and the SR network was reloaded with Ca^{2+} before adding 25 or 100 μM-Ag^{+}. At the end of each run, additions of Ca^{2+}_{max} indicated that the presence of Ag^{+} reduced the maximum force generated by the myofilaments. Ag^{+} and Hg^{2+} suppressed force induced by both caffeine or Ca^{2+}_{max} by suppressing myosin ATPase activity rather than caffeine-induced Ca^{2+} release ($n = 25$). The stimulation of SR Ca^{2+} release by Hg^{2+} was more rapid than the suppression force because the addition of Hg^{2+} (and to a lesser extent Ag^{+}) always elicited a full-size contraction before suppressing subsequent contractions. The other heavy metals did not alter the force that could be generated by

the fibres at various $[Ca^{2+}]_{free}$. Zn^{2+}, Fe^{3+} or Fe^{2+} did not induce SR Ca^{2+} release at concentrations as high as 250 μM. Measurements of actomyosin ATPase activity in the presence of these heavy metals were in agreement with the force measurements since only Ag^{+} and Hg^{2+} inhibited the ATPase activity of myofibrils, as described in Results (tests and precautions).

Several experiments suggested that these heavy metals interact with an SH site on an SR protein to cause Ca^{2+} release. First, tension responses induced by heavy metals required an intact and Ca^{2+}-loaded SR network since treatment of fibres with either (i) Triton X-100 to solubilize SR membranes, (ii) quercetin (100 μM) to block SR Ca^{2+} pumps, or (iii) A23187 (10 μg) for 20–30 min to collapse Ca^{2+} gradients across the SR, all abolished heavy metal-induced contractions (not shown). Secondly, contractions induced by these heavy metals were blocked by Ruthenium Red (2–20 μM), a most potent blocker of SR Ca^{2+} release induced by Ca^{2+} or T-tubule depolarization (Volpe & Stephenson, 1986). Thirdly, reduced glutathione (GSH, 1–3 mM) blocked heavy metal-induced contractions whereas oxidized glutathione (GSSG) had no effect (not shown). GSH interfered with the action of heavy metals on SR Ca^{2+} release probably by providing a pool of SH sites to bind the metals. Fourthly, heavy metals did not interact directly (like Ca^{2+} ions) with tropinin-C to activate the contractile apparatus. This was demonstrated by treating skinned psoas fibres with Triton X-100 (1% v/v) and measuring isometric tension as a function of heavy metal concentrations. Hg^{2+} (100 μM), Cu^{2+} (1 mM), or Ni^{2+} (1 mM) ions did not activate mammalian myofibrils (less than 10% of maximum force). Only cadmium directly activated the mammalian contractile apparatus but the pCd ($-\log[Cd^{2+}]_{free}$) required to induce 50% of maximum force was 5·5 compared to a pCa of 6·8 needed to generate the same levels of force (Fig. 1). Moreover, 10 μM-Cd^{2+} (i.e. 50 nM-Cd^{2+}_{free}) elicited large phasic contractions ($\sim$ 100% of maximum force), yet 250 nM-free Cd^{2+} (pCd = 6·3) produced less than 10% of maximum tension by direct interaction with myofibrils.

Heavy metals inducing muscle contractions had the following order of potency: $Hg^{2+} > Cu^{2+} > Cd^{2+} > Ag^{2+} > Ni^{2+}$. The results are summarized in Fig. 5 where the inverse time-to-peak tension (Fig. 5*B*) and the percentage maximum force generated by the fibres (Fig. 5*A*) were plotted against the negative logarithm of the free heavy metal (M) in the bath. The concentrations of free Hg^{2+}, Cu^{2+}, Ag^{2+} and Ni^{2+}, which produced 50% of maximum force, were respectively, 0·28 pM, 1·14 nM, 41·5 nM, 850 nM and 10·4 μM. Cu^{2+} produced the highest rate of force development with a time to half-peak of 1 s. With Ag^{2+} and Hg^{2+}, the inverse time-to-peak tension and the percentage maximum force were calculated with respect to initial caffeine contractions measured in the absence of these ions. The force of contractions increased then dropped off with increasing concentrations of Hg^{2+} and Ag^{+} (Fig. 5*A* and *B*) because the higher concentrations of these ions suppressed force development. As a result, the potency of Ag^{+} and Hg^{2+} compared to the other heavy metals was underestimated when determined through the force generated by the fibres rather than through their potency at triggering Ca^{2+} release from isolated SR vesicles (Abramson *et al.* 1983).

Contractions elicited by heavy metals were modified by agents that modulate Ca^{2+}-induced Ca^{2+}-release contractions. For instance, Cu^{2+}-induced contractions

were dependent on background concentrations of ionized Ca^{2+} (Fig. 6*A*). The percentage maximum force and the inverse time-to-peak of Cu^{2+} contractions (2·5 μM) increased with increasing $[Ca^{2+}]_{free}$, from pCa of 6·75 to 6·2. The effects of still higher $[Ca^{2+}]$ on Cu^{2+}-induced contractions could not be tested because of

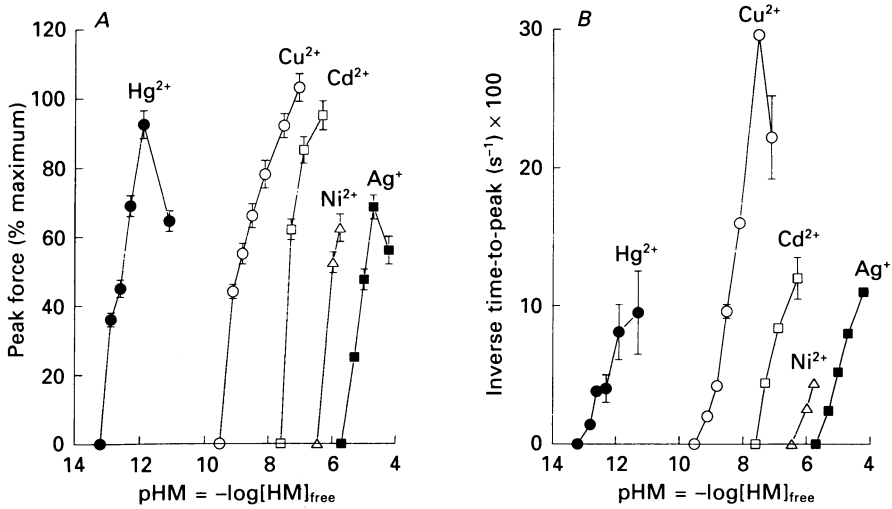

Fig. 5. Development of isometric force as a function of heavy metal concentration. Skinned fibres were Ca^{2+} loaded at pCa 6·50, pH 6·75, and pMg 4·00. A control caffeine-induced contraction was measured, the fibres washed, reloaded with Ca^{2+} for 2 min at pCa 6·50 before making a single addition of a heavy metal. Peak force induced by the addition of a heavy metal was expressed as percentage maximum force induced by caffeine (2 mM) at the beginning of the run, in *A*, and rate of rise of tension expressed as the inverse time-to-peak (in s^{-1}) of phasic contractions were plotted against the negative logarithm of the free metal concentration (M) (pHM = −log[free heavy metal]) in *B*. Up to 0·5 mM-total Zn^{2+} did not evoke muscle contractions. Each datum point was the mean of three to five separate experiments and S.E.M. was within 10%, unless shown.

spontaneous Ca^{2+}-induced Ca^{2+}-release contractions. RR (2–15 μM) blocked (Fig. 2*D*) and Mg^{2+} (0·1–4 mM) inhibited contractions induced by all the heavy metals (not shown).

The possibility that the skinning procedure influenced contractions induced by heavy metals was examined by repeating some key experiments with mechanically split and saponin-skinned fibres, without exposure to EGTA. Regardless of the skinning procedure, the addition of these heavy metals resulted in quantitatively similar tension responses.

Cysteine-induced contractions

In SR vesicles, Ca^{2+} release was induced by the addition of a mercaptan (i.e. cysteine) but only in the presence of a catalyst (i.e. 1–5 μM-Cu^{2+}) which was required to promote the oxidation of the added mercaptan with an endogenous SH group on an SR protein to form a mixed disulphide bond (Trimm *et al.* 1986). By analogy with isolated SR vesicles, the effect of cysteine was examined on skinned skeletal muscle fibres in the presence or absence of a heavy metal catalyst. As shown in Fig. 7*A*,

cysteine (10 μM) alone elicited rapid phasic contractions with kinetics and amplitudes similar to Ca^{2+}-induced Ca^{2+}-release contractions. In 10% of the fibres, contractions occurred immediately upon the addition of cysteine (Fig. 7*A* and *B*) and in 15% of the fibres, contractions occurred with delays of 10–20 s after cysteine additions. The

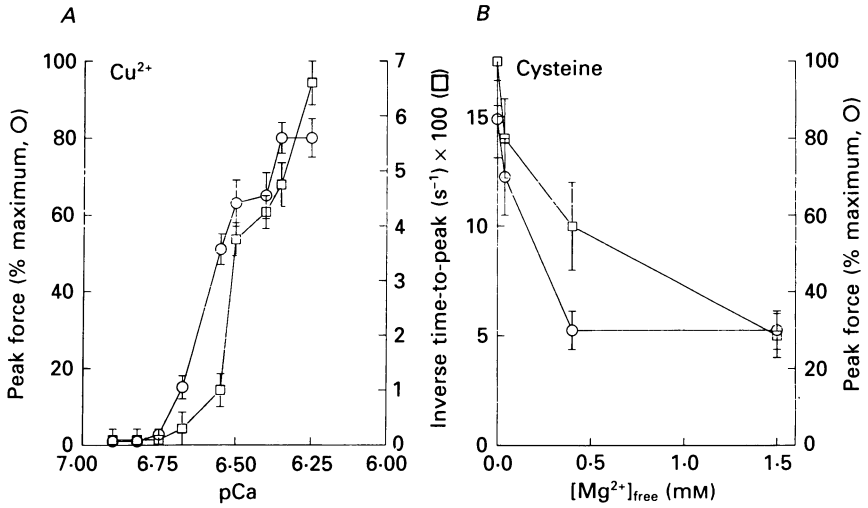

Fig. 6. *A*, effect of background free Ca^{2+} on Cu^{2+}-induced contractions. After recording a caffeine contraction, fibres were washed, loaded with Ca^{2+} for 2 min at pCa 6·25, then washed in solution at pCa 6·82, pH 6·75 and pMg 4·00. Free Ca^{2+} was continuously monitored with an ion-selective electrode and Ca^{2+} was added in aliquots of 1–2·5 μM (i.e. 80–200 nM-free Ca^{2+}) to lower pCa to the desired value before adding Cu^{2+} (2·5 μM). For the datum point taken at pCa 6·9, EGTA was added to raise pCa and total Cu^{2+} was raised to maintain a constant concentration of free Cu^{2+}. Contractions elicited by Cu^{2+} at different pCa values were compared with respect to inverse time-to-peak (□, in s^{-1}) and percentage maximum force (○) with respect to maximum force induced by 250 μM-Ca^{2+} (pCa 4·45). Each set of points represents the mean from three experiments; S.E.M. was within 10%. *B*, effect of $[Mg^{2+}]_{free}$ on cysteine contractions. MgGlu was added to adjust the free concentration of Mg^{2+} in the bathing medium. Total $[Mg^{2+}]$ of 0·5, 1, 2·5 and 5 mM were added to obtain respectively 40, 100, 400 and 1500 μM free $[Mg^{2+}]$. After recording control caffeine contractions, fibres were reloaded with Ca^{2+} (pCa 6·50), pH 6·75 at different values of pMg. After an addition of Hg^{2+} (5 μM), fibres were allowed to stabilize back to baseline tension before adding cysteine (25 μM) to elicit Hg^{2+}-catalysed cysteine contractions. The percentage maximum force (○) and the inverse time-to-peak (in s^{-1}) (□) were plotted as a functions of free $[Mg^{2+}]$. Each point corresponds to the mean ± S.E.M. of four to five measurements from different preparations.

ability of cysteine to elicit SR Ca^{2+} release and muscle contractions in the absence of exogenously added catalyst was surprising and implied that an endogenous catalyst may be present in some skinned fibres. Cysteine-induced contraction seemed to be independent of skinning procedure since the probability of eliciting such contractions were similar for mechanically split fibres (not shown).

Approximately 75% of the fibres did not respond to cysteine additions alone. However, cysteine (5–25 μM) did elicit contractions, if preceded by an addition of 5 μM-Hg^{2+} (Fig. 7*C*) or 1 μM-Cu^{2+} (Fig. 7*D*). Ruthenium Red (25 μM) blocked Ca^{2+} release induced by cysteine plus Hg^{2+} or Cu^{2+} (not shown). Similarly, GSH (5 mM)

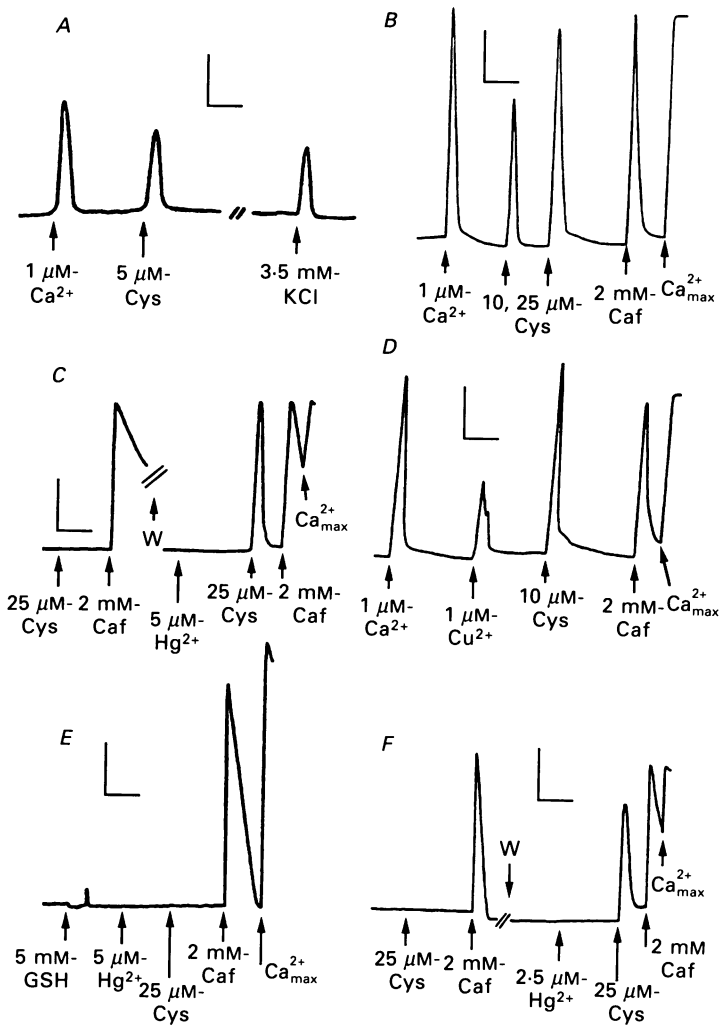

Fig. 7. Cysteine-induced contractions. *A*, a chemically skinned fibre was loaded with Ca^{2+}, and an addition of Ca^{2+} (1 μM) elicited a Ca^{2+}-induced Ca^{2+}-release twitch. At pCa 6·5, an addition of cysteine (Cys) (5 μM) elicited a twitch of comparable magnitude and kinetics to contractions induced by Ca^{2+}-induced Ca^{2+} release, or a Cl^--induced depolarization elicited by an addition of 3·5 mM-KCl. *B*, *mechanically* split fibres were Ca^{2+} loaded and a Ca^{2+}-induced Ca^{2+}-release contraction was recorded. At pCa 6·6, additions of 10 then 25 μM-cysteine elicited rapid twitches. Subsequent additions of caffeine and Ca^{2+}_{max} demonstrated the relative potency of cysteine contractions. Contractions were induced by cysteine plus a heavy metal catalyst, Hg^{2+} or Cu^{2+}. *C*, chemically skinned fibres which did not respond to direct additions of cysteine were washed, re-set and exposed to catalytic concentrations of Hg^{2+} (5 μM). This addition of Hg^{2+} did not elicit a contraction but cysteine (25 μM) evoked an immediate twitch with a maximum force equal to the maximum response of the fibres evoked by caffeine or Ca^{2+}_{max}. The oxidized form of cysteine, cystine did not elicit release. *D*, as for *C*, except that Cu^{2+} (1 μM) was used to catalyse a contraction evoked by cysteine (10 μM). *E*, as for *C*, except that prior exposure of fibres to GSH (5 mM) blocked Hg^{2+} plus cysteine contractions but did not alter force elicited by caffeine and Ca^{2+}_{max}. *F*, as for *E*, but with freshly split fibres which did not

blocked contractions induced by cysteine plus Hg^{2+} (Fig. 7*E*) or Cu^{2+} (not shown). Hg^{2+} and Cu^{2+} catalysed cysteine-induced contractions whereas Ag^{+}, Cd^{2+}, Zn^{2+} and Ni^{2+} did not. Contractions could be induced by sequential additions of Hg^{2+} (or Cu^{2+}) then cysteine or by reversing the order of additions, first cysteine then Hg^{2+} (or Cu^{2+}). Figure 7*F* shows an example of mechanically split fibres which did not respond to additions of cysteine, alone, but after a wash period, sequential additions of Hg^{2+} then cysteine elicited a phasic contraction ($n = 3$).

Contractions induced by sequential additions of Hg^{2+} and cysteine were measured as a function of Hg^{2+} and cysteine concentrations. At constant cysteine (25 μM), the strongest and fastest contractions were elicited by prior additions of 5 μM-Hg^{2+} and decreased at higher or lower [Hg^{2+}] (Fig. 8*A*). This decrease of peak force at higher Hg^{2+} concentrations was probably due to the direct suppression of myosin ATPase by Hg^{2+}, as shown in Fig. 4 (*A* and *B*). With constant [Hg^{2+}] (5 μM_{total}) in the bath, the rate and strength of contractions increased with increasing [cysteine] from 10–250 μM (Fig. 8*B*).

The effect of Mg^{2+} on contractions induced by SH oxidation was measured using 5 μM-Hg^{2+} and 25 μM-cysteine. The rate and magnitude of contractions elicited by SH oxidation decreased with increasing free [Mg^{2+}] from 40 μM to 1·5 mM-free Mg^{2+} (Fig. 6*B*). These results were quantitatively similar to the effect of Mg^{2+} on Ca^{2+} release from SR vesicles triggered by sulphydryl oxidation using cysteine plus Cu^{2+} (Trimm *et al.* 1986).

Cysteine-induced contraction exhibited different characteristics at low and high cysteine concentrations. In the presence of Hg^{2+} (5 μM), low [cysteine] produced a tonic contraction (Fig. 9*A* and *B*) whereas higher [cysteine] ($\geqslant$ 25 μM) elicited rapid phasic contractions (Fig. 7*C* and *F*). In fibres exposed to Hg^{2+} (5 μM), contractions induced by cysteine were followed by faster rates of relaxation as the cysteine concentration was raised. With 5 μM-Hg^{2+}, 10 μM-cysteine resulted in the generation of force followed by a partial relaxation and a tonic contraction which could be maintained as long as the fibre was viable (Fig. 9*A* and *B*; $n = 8$). The sustained tonic tension indicated that Ca^{2+} levels in the interior of the fibres remained elevated and were not re-accumulated by the SR network since additions of EGTA relaxed the fibres (not shown; $n = 3$). A second addition of 100 μM-cysteine resulted in an initial increase in tension followed by a rapid re-uptake of Ca^{2+} by the SR and a rapid relaxation back to baseline tension (Fig. 9*A* and *B*). Alternatively, when a single addition of 250 μM-cysteine was made, developed force was followed by a rapid relaxation which indicated rapid rates of Ca^{2+} release and re-uptake by the SR network (Fig. 9*C*; $n = 6$). Approximately 25% of the fibres exhibited spontaneous contractions which were abolished by adding mercaptans to the bathing solution (i.e. cysteine $\geqslant$ 250 μM; dithiothreitol (DTT) or GSH $\geqslant$ 2 mM; $n = 3$, for each mercaptan). In Fig. 9*B*, an addition of Hg^{2+} (5 μM), followed by two additions of cysteine at 10 then 100 μM elicited respectively, tonic and phasic contractions, but the fibres did not fully relax. However, a further addition of 250 μM-cysteine resulted in full

respond to cysteine alone. After a caffeine-induced contraction, washing and reloading the SR, Hg^{2+} (2·5 μM) plus cysteine elicited a twitch about 75% of the initial caffeine contraction. Vertical and horizontal bars represent 15 mg and 1 min, respectively.

relaxation of the fibres and blocked contractions elicited by Cl^--induced depolarization (Fig. 9*B*). Note that high [cysteine] ($\geqslant 250\ \mu M$) relaxed the fibres but did not deplete Ca^{2+} from the SR and did not act at the level of the myofibrils since caffeine-induced contractions were normal (Fig. 9*B*). After washing cysteine from the

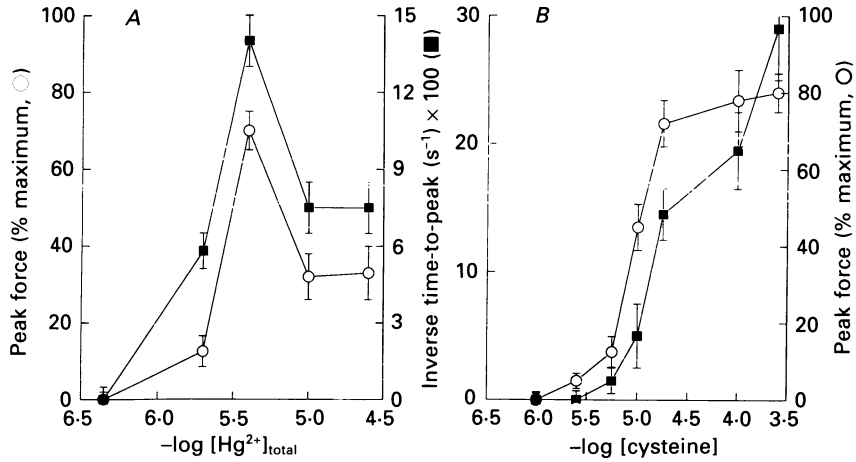

Fig. 8. Dose–response relationships of cysteine plus Hg^{2+} on force development in skinned psoas fibres. After a caffeine-induced contraction, fibres were Ca^{2+} loaded in KGlu buffer with pCa 6·50, pH 6·75 and pMg 4·00 for 2 min before inducing a contraction with Hg^{2+} plus cysteine. Twitches due to an initial addition of Hg^{2+} were allowed to stabilize to a stable baseline before adding cysteine. *A*, the percentage maximum force (○) and the inverse time-to-peak (s^{-1}; ■) were plotted against the negative logarithm of the total Hg^{2+} concentration (M), $-\log[Hg^{2+}]_{total}$, keeping a constant concentration of cysteine (25 μM) to evoke contractions. With $Hg^{2+} > 5\ \mu M$, the maximum force decreased because the higher $[Hg^{2+}]$ inhibited myosin ATPase activity. *B*, the same parameters were plotted as a function of $-\log$[cysteine], keeping a constant concentration of Hg^{2+} (5 μM). Each point consisted of the mean ± S.E.M. of three to six experiments obtained from different skinned fibre preparations. The forces were normalized with respect to the first caffeine contraction of each experiment.

fibres, Cl^--induced depolarization could again elicit phasic contractions (Fig. 9*B*; $n = 4$). Figure 9*C* tested the same concepts and showed that a Hg^{2+}-cysteine contraction could be followed by a full relaxation of the fibres by making a single addition of 250 μM-cysteine. Again, the presence of cysteine blocked subsequent Cl^--induced contractions and slowed down the time course of Ca^{2+}-induced Ca^{2+}-release contractions. Thus, in the presence of Hg^{2+}, cysteine at 5–10 μM elicited SR Ca^{2+} release and contractions, whereas 25–100 μM elicited contractions followed by rapid relaxation. As the cysteine concentration was increased from 10–250 μM, the rates of force generation and subsequent relaxation were both increased.

Actions of heavy metals and cysteine

To better understand the different effects of low *versus* high concentrations of heavy metals or cysteine on skinned fibres, the action of these reagents was re-examined in SR vesicles. In fibre experiments, the increased rates of relaxation with increasing [cysteine] could be due to stimulation of SR Ca^{2+} uptake, or to more abrupt closure of SR Ca^{2+}-release channels or both. Studies on SR vesicles had

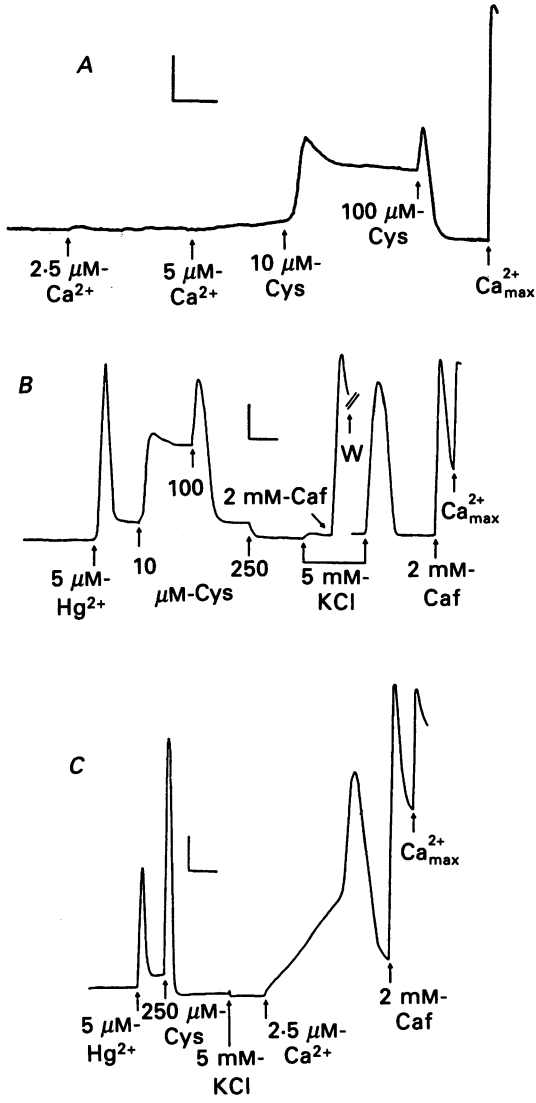

Fig. 9. High [cysteine] promotes relaxation and blocks SR Ca^{2+} release induced by Cl^- diffusion potentials. After control caffeine contractions, skinned fibres were loaded at pCa 6·50, pH 6·75 and pMg 4·00 for 2 min. *A*, pCa was set at 6·70, 5 μM-Hg^{2+} was added, followed by 10 μM-cysteine which evoked tension development through SR Ca^{2+} release and a partial relaxation. However, tension remained elevated, indicating increased levels of free Ca^{2+} in the myofilament spaces. A second addition of 100 μM-cysteine caused a further increase in tension followed by re-uptake of Ca^{2+} by the SR and full relaxation of the fibres ($n = 4$). An addition of Ca^{2+} (pCa 4·45) was made to measure the maximum force of the fibres. *B*, fibres were set as in *A* but at pCa 6·60; as a result, 5 μM-Hg^{2+} elicited a twitch, 10 μM-cysteine produced a contracture and 100 μM-cysteine evoked further Ca^{2+} release followed by a rapid and nearly complete relaxation. A third addition of 250 μM-cysteine promoted further relaxation of the fibres and blocked contractions induced by a Cl^- diffusion potential through an addition of 5 mM-KCl. Caffeine elicited a contraction indicating that the SR network was still Ca^{2+} loaded and functional. The fibres were washed, reloaded and Cl^--induced contractions were successfully elicited with 5 mM-KCl

indicated that Cu^{2+} plus cysteine triggered SR Ca^{2+} release and had no direct effect on ATP-dependent Ca^{2+} uptake (Trimm *et al.* 1986). The dual effects of cysteine, that is the opening and closure of SR Ca^{2+}-release channels at low and high [cysteine] was investigated in isolated SR vesicles. Heavy SR vesicles were pre-incubated (30 min) in various concentrations of cysteine; 0 (control), 20, 50, 100 and 1000 μM, then loaded with Ca^{2+} by adding 50 μM-Ca^{2+} followed by 0·2 mM-MgATP to initiate ATP-dependent Ca^{2+} uptake (Fig. 10*A*). The time course and extent of active Ca^{2+} uptake was not affected by low or high concentrations of cysteine in the medium. However, when sulphydryl oxidation of SR proteins was initiated by adding Cu^{2+} (10 μM), Ca^{2+} release from the vesicles was triggered in the presence of low cysteine concentrations (< 20 μM), inhibited by increasing cysteine (> 20 μM) and blocked at 1 mM-cysteine (Fig. 10*A*). The same result was obtained when the vesicles were first incubated with Cu^{2+} (10 μM), loaded with Ca^{2+} then release was elicited by adding various concentrations of cysteine.

The percentage Ca^{2+} efflux (Fig. 11, top) compared to the total releasable pool of Ca^{2+} in the presence of the Ca^{2+} ionophore, A23187 and the rate of Ca^{2+} release (Fig. 11, bottom) were plotted as a function of cysteine concentration (0–100 μM) in the presence of 0·5, 1 or 2·5 μM-Cu^{2+} in the medium. A family of curves are thus generated to describe Cu^{2+}-catalysed cysteine-induced Ca^{2+} release (Fig. 11). The data show that increasing [Cu^{2+}] increased the rate and extent of SR Ca^{2+} release, at low cysteine concentrations (0–20 μM). Maximum rates of and extent of release were obtained at 2·5 μM-Cu^{2+} and 30 μM-cysteine. Higher Cu^{2+} concentrations (5–10 μM) did not substantially increase extent or rates of release (not shown). However, a most important observation was the decrease in the rates of SR Ca^{2+} release at cysteine concentrations above 30 μM (regardless of the Cu^{2+} concentration in the medium). At 1 mM-cysteine, there was no release of Ca^{2+} and the passive leak of Ca^{2+} from the lumen of the vesicles was reduced compared to control measurements in the absence of cysteine (Fig. 10*A*). These results accounted for the data on skinned muscle fibres and indicated that high [cysteine] did not stimulate Ca^{2+} uptake but promoted the closure of a Ca^{2+}-release pathway perhaps by reducing the 'critical' SH sites on the release channel or by acting at a second SH group, on or near the Ca^{2+}-release channel.

The characteristics of contractions induced by heavy metals were also dependent on the heavy metal concentrations. Contractions induced by low heavy metal concentrations were phasic and exhibited rapid relaxation phases (e.g. Figs 2*A* and *B*, 3*A* and 4*A*). The kinetics of these phasic contractions indicated that Ca^{2+} was released and re-accumulated by the SR since the rapid relaxation could not be due to the slow diffusion of Ca^{2+} from the interior of the fibres to the surrounding bath. On the other hand, high heavy metal concentrations elicited rapid force generation followed by slower partial relaxations, tonic contractions (contractures) or

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($n = 4$). *C*, as in *B* but in this case, a single addition of 250 μM-cysteine was made after the addition of Hg^{2+}. High cysteine resulted in full relaxation of the fibres and a blockade of Cl^--induced contraction ($n = 6$). A subsequent addition of Ca^{2+} elicited a Ca^{2+}-induced Ca^{2+}-release contraction with a remarkably slow rise time ($n = 4$). Vertical and horizontal bars represented 15 mg and 1 min, respectively. Note, Hg^{2+} was kept at 5 μM to avoid inhibition of actomyosin ATPase and elicited phasic contractions at low pCa (6·6) in *B* and *C* but not high pCa (6·70) in *A*.

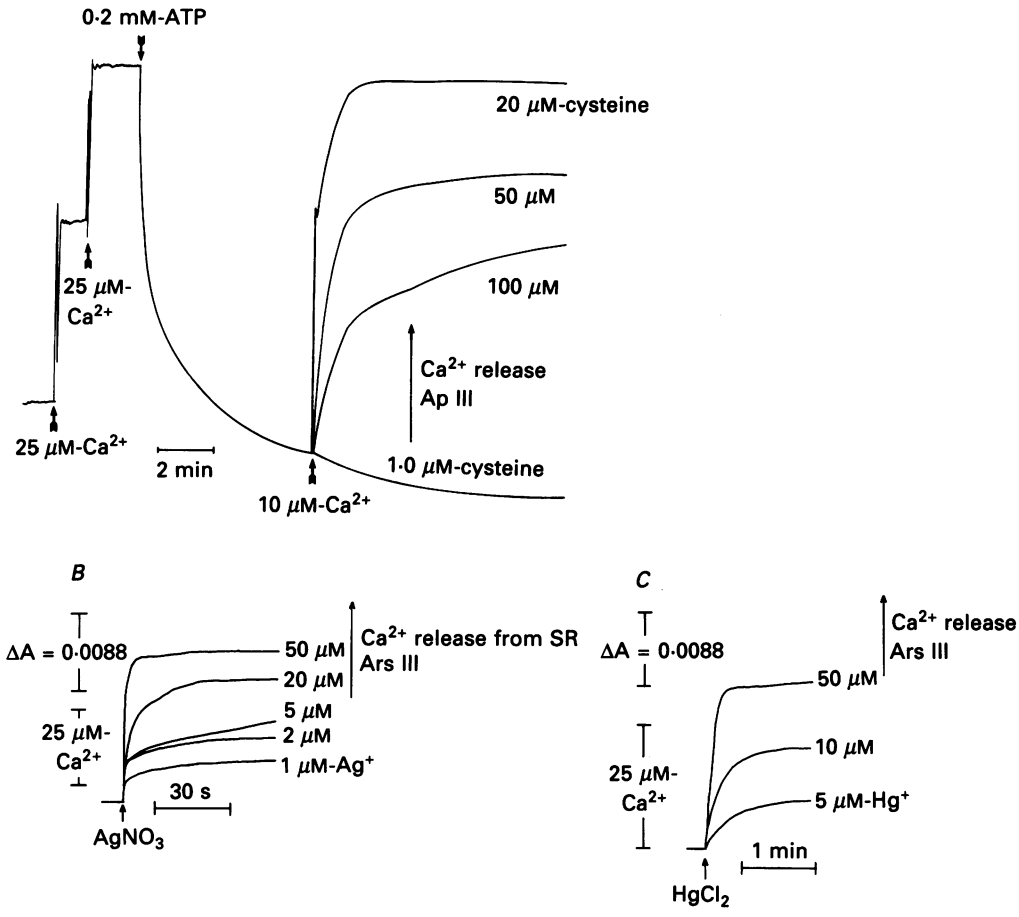

Fig. 10. Action of low and high concentrations of SH reagents on SR vesicles. *A*, Ca^{2+} release from isolated heavy SR vesicles induced by cysteine plus Cu^{2+}. SR vesicles (0·6 mg ml^{-1}) were incubated in a 2 ml cuvette containing 100 μM-Ap III (absorption measured at 720–790 nm), 100 mM-KCl, 1 mM-$MgCl_2$, 20 mM-HEPES, pH 6·8 at 23 °C in various concentrations of L-cysteine. Two additions of 25 μM-Ca^{2+} served to calibrate the dye signal and the subsequent addition of 0·2 mM-MgATP initiated active accumulation of Ca^{2+} by the SR vesicles. The addition of 10 μM Cu^{2+} did not induce Ca^{2+} release (control trace) unless L-cysteine was added. Cu^{2+} catalysed the formation of disulphide bonds between cysteine and an SR protein such that in the presence of cysteine (5–30 μM), Cu^{2+} triggered SR Ca^{2+} release. However, increasing concentrations of L-cysteine (50, 100 or 1000 μM) suppressed Ca^{2+} efflux. *B* and *C*, Ca^{2+} release from SR vesicles induced by various [Ag^+] and [Hg^{2+}]. SR vesicles (0·5 mg ml^{-1}) were incubated in a 2 ml cuvette containing 100 μM-Ars III (absorption measured at 675–685 nm), 100 mM-KCl, 1 mM-$MgCl_2$, 20 mM-HEPES, at pH 6·8, 23 °C. After two additions of 25 μM- Ca^{2+}, 0·2 mM-MgATP was added to initiate active Ca^{2+} uptake by the SR vesicles. Once uptake was completed and the differential absorption changes (ΔA) of Ars III reached a stable baseline, Ag^+ (*B*) or Hg^{2+} (*C*) was added to trigger Ca^{2+} release. Increasing concentrations of the metals elicited graded levels of Ca^{2+} release from the vesicles.

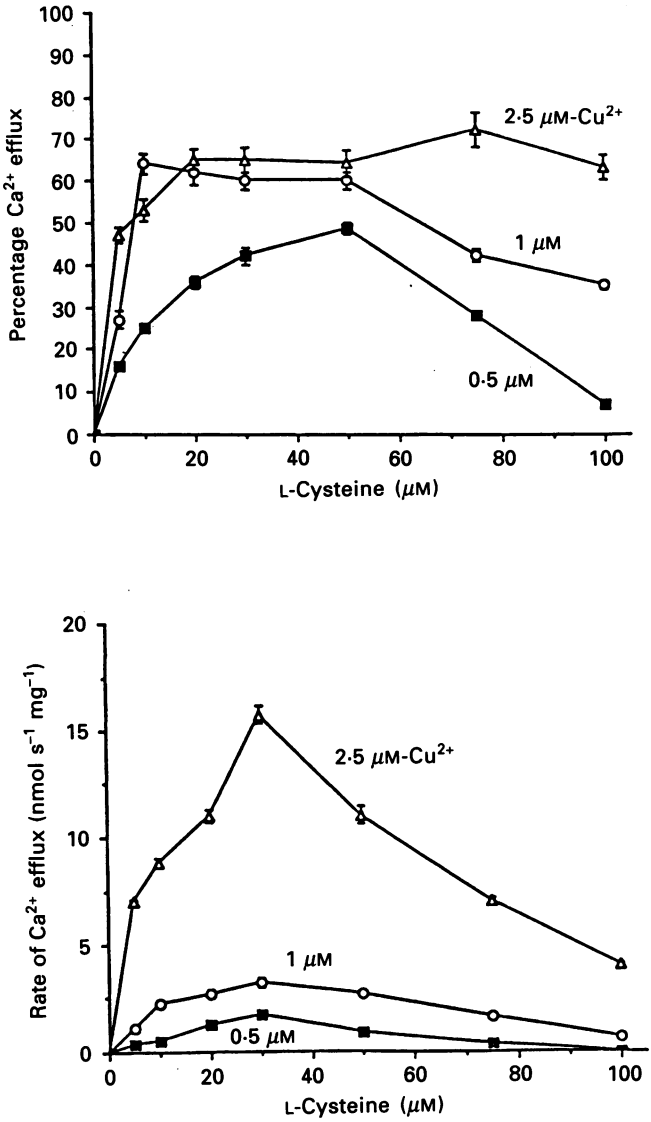

Fig. 11. Rate and extent of Ca^{2+} efflux from actively loaded SR vesicles induced by various concentrations of L-cysteine and Cu^{2+}. SR vesicles were incubated in the same medium as for Fig. 10*A* but with various concentrations of Cu^{2+} instead of cysteine. MgATP (0·2 mM) was added to initiate active Ca^{2+} uptake, followed by various concentrations of cysteine to induce Ca^{2+} release. The rate of Ca^{2+} release was determined by measuring the amount of Ca^{2+} released during the first 5 s after the addition of cysteine. The percentage Ca^{2+} efflux induced by Cu^{2+} plus cysteine was calculated from the ratio of Ca^{2+} released by Cu^{2+} plus cysteine to the total pool of releasable Ca^{2+} determined by adding the Ca^{2+} ionophore A23187.

spontaneous contractions (Figs 2*C*, 3*B* and *D* and 4*B–D*). In the latter case, heavy metals elicited release but the re-accumulation of Ca^{2+} was reduced due to an inhibition of Ca^{2+} pumps and/or greater stimulation of release.

In SR vesicles, heavy metals at low and high concentrations elicited Ca^{2+} release but no subsequent re-uptake of the released Ca^{2+}. Nevertheless, we re-examined the rates and extent of Ca^{2+} release from SR vesicles as a function of heavy metal concentration. As shown in Fig. 10, Ca^{2+} was released in a stepwise manner as higher $[Ag^+]$ (*B*) and $[Hg^{2+}]$ (*C*) were added, which indicated that the percentage of Ca^{2+}-permeable vesicles increased proportionately with heavy metal concentration. An explanation for the rapid relaxations of skinned fibres is that low heavy metal concentrations caused a small portion of the SR to undergo an irreversible increase in Ca^{2+} permeability and the remaining SR network could actively take-up the released Ca^{2+} to bring about relaxation. With higher heavy metal concentration, a greater portion of the SR released its Ca^{2+} such that the remaining fraction of SR lacked the storage capacity to take up the released Ca^{2+} and impart a state of relaxation. As a result, high heavy metal concentration standardly produced tonic contractures (e.g. Figs 2*C*, 3*B* and 4*D*).

DISCUSSION

The main results of this study are that the binding of heavy metals or the oxidation via cysteine ($\pm$a catalyst) of SH groups on SR proteins initiates a rapid release of Ca^{2+} and thus elicits contractions in skinned muscle fibres. By analogy with studies on isolated SR vesicles, both types of sulphydryl reagents induced Ca^{2+} release in skinned fibres by interacting with SH groups on the apparent physiological Ca^{2+}-release channel protein. The data are interpreted in terms of a highly reactive SH site which is associated with SR Ca^{2+} release at the triadic junction and is readily accessible to sulphydryl reagents from the cytosolic milieu.

Effect of heavy metals on SR vesicle and skinned fibres

In SR vesicles isolated from rabbit white skeletal muscle, heavy metals triggered Ca^{2+} release with a relative potency similar to their binding sequence to mercaptans like *S*-methylcysteine and penicillamine (Abramson *et al.* 1983). Several lines of evidence indicated that heavy metals acted at what appears to be the physiological or Ca^{2+}-induced Ca^{2+}-release site of release (Abramson *et al.* 1983; Salama & Abramson, 1984). (1) Ag^+ ions (at 30 nmol (mg SR protein)$^{-1}$) elicited the fastest rates of release observed at high $[Mg^{2+}]$ and low [ATP]. (2) Ruthenium Red, procaine and tetracaine, all of which block SR Ca^{2+} release, also blocked release induced by Ag^+. (3). Release was 6 times greater in heavy *versus* light SR vesicles. (4) Low concentrations of heavy metals used to elicit release resulted in a stimulation of the Ca^{2+}, Mg^{2+}-ATPase activity as expected when SR vesicles increase their Ca^{2+} permeability. (5). Ag^+ ion and reactive disulphides at 2–5 μM dissociated [^{3}H]ryanodine from its receptor site (Pessah *et al.* 1987; Zaidi *et al.* 1989*a*).

The present data on skinned fibres also suggests that heavy metals bind to SH sites on an SR protein to cause Ca^{2+} release. Heavy metals elicited contractions only if the SR network was kept intact and was Ca^{2+} loaded. Fibres pre-treated with Triton X-100, A23187, or quercetin (not shown) did not have functional, Ca^{2+}-loaded SR and thus did not respond to heavy metals but did contract upon direct additions of high Ca^{2+} concentrations. Contractions induced by heavy metals were blocked by RR and

GSH, and were inhibited by ionized Mg^{2+}. The effect of heavy metals was dependent on $[Ca^{2+}]_{free}$ and this dependence was examined in detail for Cu^{2+} ions. Cu^{2+} contractions were stimulated by increasing concentrations of ionized Ca^{2+} for pCa values in the range of 6·8–6·2. These data plus the results obtained on isolated heavy SR vesicles indicate a common site of action with Ca^{2+}-induced Ca^{2+} release. The relative potency of heavy metals at eliciting contractions in skinned fibres was different from that observed in SR vesicles. However, the differences were attributed to the high affinity of Ag^{+} and Hg^{2+} to other SH groups, namely sulphydryls on myosin ATPase. Qualitatively similar results were obtained on frog skinned fibres where Ag^{+} and Hg^{2+} produced tension transients followed by an inhibition of contractions at the level of the myofilaments (Aoki *et al.* 1985).

Heavy metals elicit Ca^{2+} release by binding to a 'critical' sulphydryl site on SR proteins

In both SR vesicles and skinned fibre experiments, heavy metals cause Ca^{2+} release by binding to sulphydryl sites and not by promoting the oxidation of adjacent free sulphydryls to form new disulphide bonds. This interpretation is based on the following observations.

(a) Heavy metals exhibit high binding affinities to free sulphydryls as demonstrated from measurements of stability constants. For any heavy metal, its stability constants vary widely depending on the sulphydryl-containing compound; for instance log $K_1 = 16·6$ for Hg^{-2}-mercaptoethyliminodiacetic acid and log $K_1 = 43·57$ for Hg-cysteine (see Table 1 for definition of K_1). Because of these high stability constants, all the exogenously added heavy metals were probably bound to SH-containing proteins of the skinned muscle fibres.

(b) Of the heavy metals tested, only Cu^{2+} and Hg^{2+} (albeit, more weakly) ions have been shown to act as catalysts which can promote the oxidation of free sulphydryls. Ag^{+}, Cd^{2+} and Ni^{2+} did not promote the oxidation of free sulphydryls, yet were effective at causing SR Ca^{2+} release in both SR vesicles and skinned fibres. This implied that the binding of Ag^{+}, Cd^{2+} and Ni^{2+} to SH sites had to account for SR Ca^{2+} release.

(c) Heavy metals induced Ca^{2+} release from heavy SR vesicles that are essentially devoid of transverse tubules such that their site of action is most likely on SR proteins (Abramson *et al.* 1983; Salama & Abramson, 1984). In skinned fibres, heavy metals also appear to act by binding to SR Ca^{2+}-release channel proteins since agents that activate or inhibit Ca^{2+}-induced Ca^{2+} release have similar effects on Ca^{2+} release induced by sulphydryl reagents (Zaidi *et al.* 1989*a*; Zaidi, Lagenaur, Hilkert, Xiong, Abramson & Salama, 1989*b*). Another less likely but intriguing possibility is that heavy metals bind to SH sites on T-tubules which indirectly causes SR Ca^{2+} release.

Mechanism and site of action of sulphydryl reagents

Reagent disulphide compounds were recently used to show that the oxidation then reduction of a critical sulphydryl group on an SR protein reversibly opened and closed a Ca^{2+}-release channel (Zaidi *et al.* 1989*a*). Sulphydryl chemistry was then used to identify and purify a 106 kDa protein containing this critical SH site. Immunological analysis using polyclonal and monoclonal antibodies indicated that

the 106 kDa protein was distinct from Ca^{2+}, Mg^{2+}-ATPase and 565 kDa junctional feet protein (Zaidi *et al.* 1989*a*). Incorporation of 106 kDa proteins in planar bilayers revealed the presence of SR Ca^{2+}-release channels with characteristics similar to those described for junctional feet proteins (Zaidi *et al.* 1989*b*; Hilkert, Zaidi, Nigam, Shome, Lagenaur & Salama, 1992). Moreover, sulphydryl oxidizing (i.e. 10–20 μM-2,2′-dithiodidipyridine) and reducing (i.e. 20–50 μM-cysteine, 1–4 mM-DTT) agents respectively, increased and decreased the open-time probability of 106 kDa Ca^{2+}-release channels in planar bilayers (Hilkert *et al.* 1991). Similar results were obtained from native SR Ca^{2+}-release channels incorporated in planar bilayers through the fusion of SR vesicles with black lipid membranes (R. J. Hilkert, N. F. Zaidi, C. Teremana, C. F. Lagenaur, M. Nigam & G. Salama, unpublished results). The reactive disulphide compound 2,2′-dithiodidipyridine is known to interact with and oxidize free sulphydryl groups with absolute specificity. Moreover, like heavy metals and cysteine (plus a catalyst), 2,2′-dithiodidipyridine (5–20 μM) triggered SR Ca^{2+} release in skinned rabbit psoas fibres and thus elicited rapid twitches (Zaidi *et al.* 1989*b*). The evidence suggests that heavy metals and cysteine (plus a catalyst) interact with the critical sulphydryl site(s) on the 106 kDa Ca^{2+}-release channel protein of sarcoplasmic reticulum.

Cysteine-induced contractions

Heavy metals are clearly not a physiological trigger for Ca^{2+} release but can be used to probe the gating mechanism of an SR Ca^{2+}-channel protein. On the other hand, Ca^{2+} release induced by the amino acid cysteine raises the possibility that the opening and closing of the Ca^{2+}-release channel may be modulated by sulphydryl oxidation and reduction under physiological or pathophysiological conditions. In SR vesicles, cysteine, from 2 to 20 μM, triggered an increasingly faster and greater release of Ca^{2+} but only in the presence of 1–5 μM-Cu^{2+} in the reaction medium (Figs 10 and 11). The most likely interaction between cysteine and the SR is the sulphydryl oxidation of SH groups on SR proteins with the SH moiety of cysteine to form a mixed disulphide (Trimm *et al.* 1986). The sulphydryl oxidation reaction requires the presence of a catalyst like Cu^{2+} ions (Cavallini *et al.* 1969) and the order in which the mercaptan (cysteine) and the catalyst (Cu^{2+}) are added to the medium can be reversed with no changes in the rate and extent of Ca^{2+} efflux. Pre-mixed stock solutions of Cu^{2+} plus cysteine could not be used to trigger Ca^{2+} release since Cu^{2+} promotes the auto-oxidation of cysteine to cystine and cystine had no effect on Ca^{2+} transport by the SR. The site of action of cysteine was related to physiological Ca^{2+} release because it was blocked by Ruthenium Red, was predominantly observed in heavy SR vesicles, was stimulated by adenine nucleotides, inhibited by free Mg^{2+}, and modulated by free Ca^{2+} (Trimm *et al.* 1986). The oxidation of sulphydryl groups on SR proteins and Ca^{2+} release could be reversed by adding SH reducing agents like dithiothreitol. This resulted in the closure of the Ca^{2+}-release pathway and in ATP-dependent Ca^{2+} re-uptake by SR vesicles (Trimm *et al.* 1986).

In skinned rabbit psoas fibres, both Cu^{2+} and Hg^{2+} catalysed cysteine-induced Ca^{2+} release and thereby elicited phasic contractions. While sulphydryl oxidation catalysed by Cu^{2+} ions has been well documented (Cavallini *et al.* 1969), there is little information regarding Hg^{2+}-catalysed sulphydryl oxidation reactions. Still, Hg^{2+}

was found to act weakly as a catalyst for cysteine-induced Ca^{2+} release in isolated SR vesicles (data not shown) as well as skinned fibre preparations. The sequential additions of the metal catalyst and the mercaptan produced contractions in all skinned fibres that have an intact and Ca^{2+}-loaded SR network. These phasic contractions were inhibited by free Mg^{2+} and blocked by Ruthenium Red or reduced glutathione. As in isolated SR vesicles, cysteine induced Ca^{2+} release and evoked contractions by oxidizing an SH site probably on the Ca^{2+}-release channel protein.

In a small fraction of fibres (10%), phasic contractions could be repeatedly elicited, with no delays, by additions of cysteine without the addition of exogenous catalysts to promote sulphydryl oxidation reactions. These fibres were treated under identical conditions and with solutions that should not retain endogenous heavy metals after long exposures and washes with 5 mM-EGTA. Yet, such fibres seemed to contain endogenous catalysts which promoted sulphydryl interactions between added cysteine and the SR. These observations imply that intact skeletal muscle may contain cytosolic enzymes to control the redox state of SH groups and the Ca^{2+} permeability of the SR.

Possible role of sulphydryl oxidation–reduction in excitation–contraction (E–C) coupling

The occurrence of cysteine-induced contractions (at 10–20 μM, $\pm Cu^{2+}$) suggests that a sulphydryl oxidation reaction between SR proteins and adjacent SH-containing proteins can cause the opening of SR Ca^{2+}-release channels. Conversely, the blockade of Cl^--induced contractions by cysteine (at 200 μM) indicate that an SH-reducing agent can block a physiological trigger for Ca^{2+} release. These observations provide the basis for a theory of excitation–contraction coupling that involves a sulphydryl oxidation–reduction to gate SR Ca^{2+} release. A possible scheme is shown in Fig. 12. Voltage sensors on T-tubule membranes (i.e. the dihydropyridine receptor, DHP-R) communicate directly with Ca^{2+}-release channels on the SR across a 12 nm gap separating T-tubule and SR membranes (Fig. 12*A*). At resting membrane potential (−90 mV) across the plasma membrane, DHP-R and SR Ca^{2+}-release channels (which may include the 106 kDa Ca^{2+}-release channel protein) possess free SRs, respectively labelled as *a* and *b*. The membrane depolarization at T-tubules may alter the redox potential of SH sites on a cytosolic moiety of DHP-Rs (SH site labelled *a*) and thus promote the rapid formation of disulphide bonds between DHP-Rs and SR Ca^{2+}-release channels (Fig. 12*B*).The oxidation reaction between the voltage sensor and Ca^{2+}-release channels opens the channel resulting in Ca^{2+} release and muscle contraction. The subsequent reduction of the disulphide bond formed between these proteins results in the closure of SR Ca^{2+}-release channels (Fig. 12*C*). The reduction of disulphide bonds between DHP-Rs and SR Ca^{2+} release channels may occur through a sulphydryl–disulphide interchange reaction. The third SH site (*c*) involved in the closure of the Ca^{2+}-release channel may be located on either DHP-Rs Ca^{2+}-release channels or an 'unknown' protein found in triadic junctions. Part *C* of the model proposes that there is a second SH site on SR proteins (*c*) (with a lower reactivity than SH *b*) which can be oxidized to promote the closure of Ca^{2+}-release channels. Evidence for two SH sites (*b* and *c*) comes from the activation of SR Ca^{2+}-release channels at low heavy metal concentrations and an inhibition by high heavy metal concentrations (Moutin, Abramson, Salama &

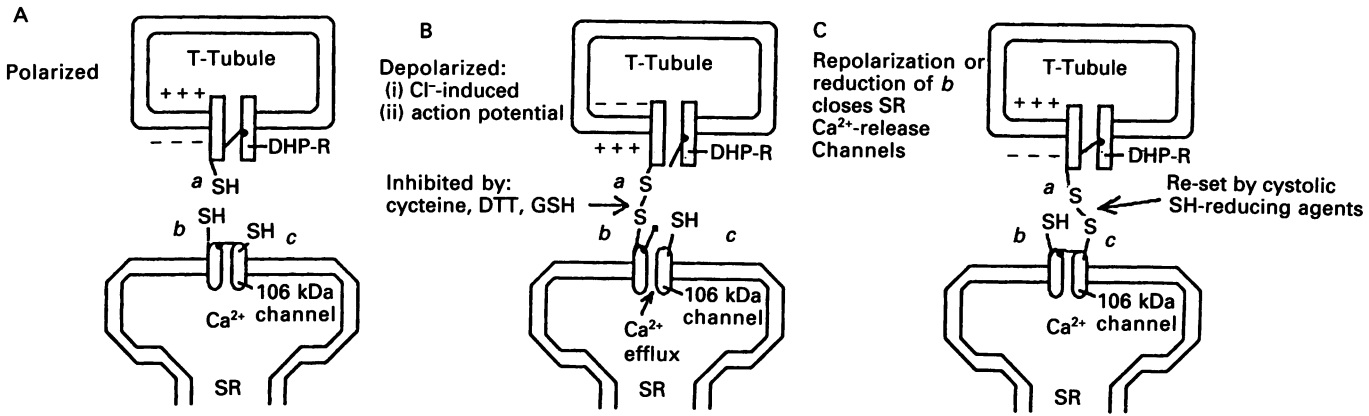

Fig. 12. Model of excitation–contraction coupling gated by sulphydryl oxidation–reduction. *A*, at resting membrane potential (−90 mV intracellular potential), SH sites on dihydropyridine receptors (DHP-R) (*a*) and on 106 kDa SR Ca^{2+}-release channels (*b*) are in the reduced form and both channels are in the closed state. *B*, depolarization of T-tubule membranes by a Cl^- diffusion potential or an action potential shifts the pK_a of SH sites on DHP-Rs which promotes the oxidation of SH sites *a* and *b* resulting in the formation of a disulphide bond between DHP-Rs and SR Ca^{2+}-release channels. The oxidation of SH site *b* promotes the opening of SR Ca^{2+}-release channels. The binding of heavy metals to SH site *b* or the oxidation of SH site *b* by cysteine plus Cu^{2+} also opens SR Ca^{2+}-release channels. Conversely, SH reducing agents like cysteine, DTT or GSH block the formation of disulphide bonds between DHP-Rs and SR Ca^{2+}-release channels. *C*, reduction of sulphydryl site *b* occurs after the repolarization phase of the action potential to promote the closure of SR Ca^{2+}-release channels. The reduction of the disulphide bond between sulphydryls *a* and *b* may be directly caused by the repolarization of T-tubules or may require an intermediate SH site (*c*) to undergo a sulphydryl–disulphide interchange reaction. The exchange reaction results in the formation of an intermediate disulphide bond between *a* and *c* which is subsequently reduced by cytosolic reagents to re-set the system back to *A*.

Dupont, 1989). The reduction of the disulphide bond (shown in Fig. 12*C*) re-sets the system back to its resting state. The latter step may be kinetically slower and modulated by the sulphydryl redox state of the cytosol (i.e. the ratio of reduced to oxidized glutathione: GSH/GSSG). A key issue is whether a change in membrane potential can drive a sulphydryl redox reaction at rates compatible with E–C coupling. In other biological systems, altered SH redox states of membrane-bound proteins driven by changes in membrane potential have been demonstrated experimentally and modelled for theoretical analysis (see Robillard & Konings, 1982).

A number of experiments support the general features of the model. The opening of Ca^{2+}-release channels in SR vesicles was promoted by (i) the binding of heavy metals to SH site *b* (Salama & Abramson, 1984); the oxidation of SH site *b* by (ii) cysteine in the presence of a catalyst (Trimm *et al.* 1986), or by (iii) reactive disulphide compounds (Zaidi *et al.* 1989*a*, *b*). Contractions induced by Cl^- diffusion potentials were reproducibly inhibited by sulphydryl reducing agents. More specifically, they were blocked by 200–250 μM-cysteine (Fig. 9) and inhibited by 5 mM-DTT or -GSH (not shown). It is important to note that Cl^--induced contractions are generally thought to be invoked by the same process as excitation–contraction coupling in intact fibres. Such contractions elicited by an abrupt addition of Cl^- (i.e. membrane-permeable anions) to fibres bathed in a potassium gluconate solution (i.e. containing impermeable anions) are primarily attributed to the depolarization of sealed T-tubules which in turn triggers SR Ca^{2+} release (Stephenson, 1985).

Brunder, Dettbarn & Palade (1988) concluded that sulphydryl reactions were not involved in E–C coupling because DTT or GSH introduced into cut frog fibres inhibited Ag^+-induced contractions but did not block contractions elicited by voltage-clamp steps. They based their interpretation on the questionable premise that 1 mM-GSH or -DTT should block or inhibit SR Ca^{2+} release even though physiological levels of GSH in skeletal fibres are already in the millimolar range and do not block contractions. Their experiments with GSH did not assess the SH redox state of the fibres because the latter is not determined by the [GSH] but by the ratio of GSH/GSSG. A more meaningful test of SH-gated Ca^{2+} release should examine more potent SH reducing agents (i.e. cysteine or 2,2′-dithiodipyridines) because GSH and DTT require relatively high substrate concentrations and long reaction times to reduce disulphide bonds. When a higher concentration of DTT (10 mM) was tested, there was a significant (2- to 3-fold) prolongation of the minimum stimulus duration, indicating substantial inhibition of contractions elicited by voltage-clamp steps (Brunder *et al.* 1988). The effects seen at 10 mM-DTT were highly consistent with the participation of SH oxidation in physiological Ca^{2+} release. Yet, Brunder *et al.* (1988) considered the effects of 10 mM-DTT, to be unrelated to SH protection of the release channel, despite their evidence supporting the involvement of sulphydryl chemistry in the process of E–C coupling.

The proposed model makes speculative but testable predictions: (a) the voltage sensors thought to be DHP-Rs activate Sr Ca^{2+}-release channels through a sulphydryl oxidation reaction which results in a transient cross-linking of these two Ca^{2+}-channel proteins and (b) the sulphydryl oxidation–reduction state in the cytosol is essential for normal excitation–contraction coupling.

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