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CAFFEINE AND MICROMOLAR Ca^{2+} CONCENTRATIONS CAN RELEASE Ca^{2+} FROM RYANODINE-SENSITIVE STORES IN CRAB AND LOBSTER STRIATED MUSCLE FIBRES

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Summary

Ca^{2+} release mechanisms were studied in striated muscle from the walking legs of crabs using isometric tension recordings from isolated myofibrillar bundles. Caffeine-induced phasic contractions had properties consistent with Ca^{2+} release from a sarcoplasmic store, which could be optimally loaded in the presence of ATP at pCa 6.4–6.1. Ryanodine (10 μmol l^{-1}) abolished the caffeine-induced contractions and in solutions with low Ca^{2+} buffering (0.1 mmol l^{-1} EGTA) itself caused phasic contractions, indicative of Ca^{2+} release. Ca^{2+}-induced Ca^{2+} release (CICR) was observed in a pCa 5.8 solution (buffered by 1 mmol l^{-1} EGTA) as a phasic contraction of variable nature, inhibited by ryanodine (10 μmol l^{-1}), procaine (10 mmol l^{-1}) or benzocaine (5 mmol l^{-1}). Ca^{2+} release was measured as a function of releasing pCa by using the force–time integral of the caffeine-induced contraction as an estimate of the Ca^{2+} remaining in the store. After the Ca^{2+} store had been loaded for 2 min at pCa 6.6, CICR was measured in the presence of 1 mmol l^{-1} Mg^{2+}, 1 mmol l^{-1} EGTA and 5 mmol l^{-1} ATP. The threshold pCa for CICR was 6.0–6.4 under these conditions and more than 90 % of stored Ca^{2+} was released in 1 min by pCa values in the range 3.5–5.3. Benzocaine totally inhibited the release and promoted extra Ca^{2+} loading. Preliminary experiments showed a similar caffeine-releasable store in lobster abdominal muscle, which was slightly less sensitive to free [Ca^{2+}]. It is concluded that in crustacean muscle caffeine and micromolar [Ca^{2+}] can release Ca^{2+} from a ryanodine-sensitive store, which in many respects is similar to the sarcoplasmic reticulum of vertebrate skeletal and cardiac muscle.

Key words: crab, lobster, muscle, *Carcinus maenas*, *Homarus vulgaris*, caffeine, Ca^{2+}-induced Ca^{2+} release, ryanodine.

Introduction

There is increasing evidence that excitation–contraction coupling in crustacean striated muscle is dependent on the operation of Ca^{2+}-induced Ca^{2+} release (CICR) from the sarcoplasmic reticulum (SR) (for reviews, see Ashley *et al.* 1993; Palade and Györke, 1993). Extracellular Ca^{2+} is essential for electrically induced contractions of crustacean striated muscle. In experiments with voltage-clamped muscle fibres from crab walking legs, Mounier and Goblet (1987) found that the amplitudes of both the contraction and the inward Ca^{2+} current (I_{Ca}) fell to zero when extracellular Ca^{2+} was replaced by a Ca^{2+}-free EGTA saline. In addition, the amplitude of the contractile response (phasic component) varied in a bell-shaped manner with membrane potential, closely following the relationship between I_{Ca} and membrane potential. Ca^{2+} release from the SR was found to be essential for the development of the contraction since procaine, which inhibits Ca^{2+} release from the SR, abolished the contraction despite the continued generation of an inward Ca^{2+} current (Mounier and Goblet, 1987). These authors also showed that CICR can occur in crab muscle since skinned fibres with an intact SR developed tension when bathed in solutions containing low concentrations of Ca^{2+} (micromolar) insufficient to activate the contractile proteins directly (Goblet and Mounier, 1986).

This combined evidence from the crab muscle is consistent with a mechanism of excitation–contraction coupling in which the generation of I_{Ca} across the sarcolemma leads to an increase in sarcoplasmic [Ca^{2+}] and this releases Ca^{2+} from SR stores through CICR. In this respect, crustacean striated muscle may have more in common with vertebrate cardiac muscle than with vertebrate skeletal muscle, where the primary Ca^{2+}-releasing mechanism is thought to be a mechanical coupling between a voltage sensor in the transverse (T-) tubule and the ryanodine receptor (Ca^{2+}-release) channel in the membrane of the SR (Rios *et al.* 1991).

The SR of crustacean striated muscle has many similarities with that of vertebrate skeletal and cardiac muscle. A major protein in crustacean SR has a molecular mass of about 100 kDa, which is typical of vertebrate SR Ca^{2+}-ATPases, and

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SR vesicles can be prepared from crustacean muscle which show an ATP-dependent Ca^{2+} uptake in the presence of oxalate (Ashley *et al.* 1993; Ushio and Watabe, 1993). The Ca^{2+}-release channel of the junctional SR membrane from vertebrate skeletal and cardiac muscle has been identified as a high molecular mass protein with a high-affinity binding site for the alkaloid ryanodine (for reviews, see Lai and Meissner, 1989; Fleischer and Inui, 1989; Meissner, 1994). The protein occurs as a tetramer, identified as the foot structure in the triadic gap between the T-tubule and the terminal cisternae of the SR (Lai and Meissner, 1989; Fleischer and Inui, 1989). Similar foot structures have been found in barnacle and crayfish muscle (Hoyle *et al.* 1973; Loesser *et al.* 1992). Ryanodine receptor proteins have been purified from crayfish and lobster muscle and these show Ca^{2+} channel activity when incorporated into planar lipid bilayers (Formelova *et al.* 1990; Arispe *et al.* 1992; Seok *et al.* 1992). The lobster ryanodine receptor has been characterised in detail (Seok *et al.* 1992). It was localised using immunofluorescent methods to the junctions of the A and I bands, where the diads and triads occur in crustacean muscle. The lobster Ca^{2+} channel required millimolar [Ca^{2+}] for optimal activation (i.e. opening), in marked contrast to the skeletal and cardiac muscle channels which can be activated optimally by micromolar [Ca^{2+}] (Seok *et al.* 1992; Meissner, 1994). The lobster channel appeared to lack the high-affinity regulatory Ca^{2+} binding site found in skeletal and cardiac receptors. It also appeared to lack the high sensitivity to caffeine shown by the skeletal and cardiac receptors, since the rate of ^{45}Ca efflux from isolated SR vesicles from lobster muscle was only modestly increased by 10 mmol l^{-1} caffeine.

In vertebrate skeletal muscle, electron probe analysis has demonstrated that caffeine releases Ca^{2+} from the terminal cisternae of the SR (Yoshioka and Somlyo, 1984). Although such a direct demonstration has not been reported for crustacean muscle, there is indirect evidence which shows that caffeine releases Ca^{2+} from a sarcoplasmic store in barnacle muscles and that this store is probably the SR (Ashley *et al.* 1993).

This paper presents results from an isolated myofibrillar bundle preparation demonstrating that crab and lobster striated muscle fibres also possess caffeine-releasable Ca^{2+} stores and that these are sensitive to both ryanodine and micromolar [Ca^{2+}].

Materials and methods

Preparations

The extensor muscle from the meropodite of walking legs of the shore crab *Carcinus maenas* (L.) was used in this study. Fibres from this muscle respond to electrical stimulation with a small graded depolarisation and phasic and tonic contraction components (Mounier and Vassort, 1975; Mounier and Goblet, 1987). They are similar to the 'intermediate' fibre type (Atwood *et al.* 1965), being neither exclusively 'fast' (phasic) nor 'slow' (tonic). Like other crustacean muscle fibres, the meropodite extensor fibres have a voltage-dependent inward Ca^{2+} current but no tetrodotoxin-sensitive currents (Mounier and Vassort, 1975). Some preliminary experiments were also performed on unidentified fibres from the abdominal flexor muscles of the lobster *Homarus vulgaris* (Milne-Edwards).

In experiments at the Plymouth Marine Laboratory, animals were kept in circulating sea water. In experiments in Oxford, animals were obtained from the Plymouth Marine Laboratory and the Millport Marine Laboratory, Scotland, and were kept in a tank through which filtered, artificial sea water at 8 °C was circulated. Single muscle fibres were isolated and split longitudinally under paraffin oil to produce myofibrillar bundles 100 μm in diameter and 5 mm long. These were mounted for isometric tension recording with one end held between the tips of a fixed pair of watchmaker's forceps and the other glued with cyanoacrylate to a vertical carbon fibre rod attached to the filament of a tension transducer (silicone AE801, SensoNor, Norway). The output of the transducer was fed *via* a bridge circuit into a Rostol pen recorder and stored either in a Nicolet Explorer digital oscilloscope (206) or on tape (a JVC video cassette recorder with a modified Sony digital audio processor, PCM).

Bathing solutions for the 'skinned fibres' were contained in a series of 1.5 ml cuvettes (or sometimes 0.15 ml Perspex containers) held in a spring-mounted sample chamber, through which thermostatically controlled coolant was circulated to maintain solution temperature at 12 °C.

Solutions

All bathing solutions contained (in mmol l^{-1}): K^+, 156–166; Na^+, 30.0; total magnesium, 5.0; free Mg^{2+}, 1.0; propionate, 130; Cl^-, 10.0; ATP, 5.0; creatine phosphate, 10.0; Tes, 60; pH 7.0 at 12 °C. The concentration of EGTA was 0.1, 0.2 or 1.0 mmol l^{-1} in Ca^{2+}-releasing solutions and 5.0 mmol l^{-1} in Ca^{2+}-loading and Ca^{2+}-activating solutions. Creatine phosphokinase (20 units ml^{-1}) was added in some experiments (1 unit will transfer 1.0 μmole of phosphate from phosphocreatine to ADP per minute at pH 7.4 and 30 °C). The ionic strength of all bathing solutions was between 0.20 and 0.24 mol l^{-1}. Solutions of known pCa values were made with added volumetric $CaCl_2$, according to a computer program which calculates free metal ion concentrations for solutions containing a mixture of metal ions and ligands (Perrin and Sayce, 1967; modified by P. J. Griffiths, unpublished; see Lea, 1986, for details).

Potassium propionate was obtained from K & K Laboratories, Cleveland, Ohio; Tes (Ultrol) from Calbiochem; ryanodine from Penick Laboratories, USA (a gift from Dr T. Powell); and procaine and benzocaine from Sigma. Benzocaine was prepared as a 1.0 mol l^{-1} stock solution in dimethyl sulphoxide (DMSO), so that in a 5 mmol l^{-1} benzocaine aqueous solution the DMSO concentration was 0.5 %. This concentration of DMSO does not affect either the caffeine-induced contraction in skinned fibres from frog muscle (Du *et al.* 1994) or Ca^{2+} release from caffeine-releasable stores in myofibrillar bundles from barnacle muscle (Lea, 1986).

Use of the caffeine-induced contraction to estimate the amount of stored Ca^{2+}

The amount of Ca^{2+} in the Ca^{2+} stores of the crustacean myofibrils was estimated from the force–time integral of the response to caffeine following Endo (1977). The amplitude of contraction is a less reliable indicator because it can reach its maximum (i.e. P_{max}) before the loading capacity of the store has been reached. The tension–time integral was obtained either graphically from the printed trace or by tranferring the oscilloscope trace to the disc of a personal computer and using an integrating program.

Protocol for Ca^{2+} loading experiments

The Ca^{2+} store was first emptied by applying 20 mmol l^{-1} caffeine in the presence of 5 mmol l^{-1} EGTA. Run-down of the caffeine-induced contractions following repeated stimulation (see Fig. 3) made it necessary to obtain control caffeine-induced contractions (1 min loading at pCa 6.1, 5 mmol l^{-1} EGTA) before and after each test loading (1 min loading at the test pCa) (see Fig. 2A). The integral of the test contraction was then expressed as a fraction of the mean of the two controls.

Protocol for the Ca^{2+} release experiments using Endo's method

Endo (1977) characterised the CICR process in frog muscle using caffeine to assess the Ca^{2+} content of the SR and thus to estimate how much Ca^{2+} had been released by a test pCa solution. The releasing solutions often lacked ATP, so that re-uptake of released Ca^{2+} was prevented. With the crab preparation, treatment for 5 min with a rigor solution (ATP-free and Mg^{2+}-free) caused a substantial loss of caffeine-releasable Ca^{2+} even at a pCa value of approximately 9 (1 mmol l^{-1} EGTA with no added Ca^{2+}). The caffeine-induced contraction was reduced by approximately 70 % by this treatment. The addition of 0.9 mmol l^{-1} free Mg^{2+} to the ATP-free solution did not prevent this effect. For this reason, ATP was included in the releasing solution for the crab experiments.

A control caffeine-induced contraction was obtained before and after each test release by loading for 2 min at pCa 6.6 and then exposing the bundle to a solution containing 1 mmol l^{-1} EGTA and no added Ca^{2+} for 3 min, before recording the caffeine-induced contraction. The pCa of this control solution was estimated to be 9 using a value for the contaminant Ca^{2+}, estimated by atomic absorption spectrometry, of 10 μmol l^{-1}. The releasing solution (test pCa at 1 mmol l^{-1} EGTA) was applied for 1 min and was then washed off before assaying the Ca^{2+} remaining in the store with caffeine. The resulting force–time integral of the contraction was expressed as a fraction of the mean value of the two controls. When the effect of benzocaine was tested, the same protocol was used, except that after Ca^{2+} loading the bundle was transferred to the pCa 9 solution containing benzocaine (5 mmol l^{-1}) for 30 s, then the releasing solution containing benzocaine for 1 min, then a wash pCa 9 solution for 90 s before testing the response to caffeine.

Results

A caffeine-releasable Ca^{2+} store in crab myofibrillar bundles

When a myofibrillar bundle from the crab extensor muscle was exposed to 20 mmol l^{-1} caffeine, a phasic increase in isometric tension was recorded (Fig. 1A). The following three results indicate that caffeine causes a release of Ca^{2+} from sarcoplasmic stores. First, in the continual presence of caffeine, the tension returned to resting values 30–70 s after the bundle was immersed in the caffeine solution (Fig. 1A). After the myofibrillar bundle had been bathed in caffeine-free solutions of low free $[Ca^{2+}]$ (pCa 9), a second exposure to caffeine could not induce a contraction until the bundle had been exposed to an ATP-containing solution of a sufficiently high free $[Ca^{2+}]$, i.e. pCa 7 or lower (Figs 1, 2). The amplitude and the force–time integral of the resulting contraction increased with

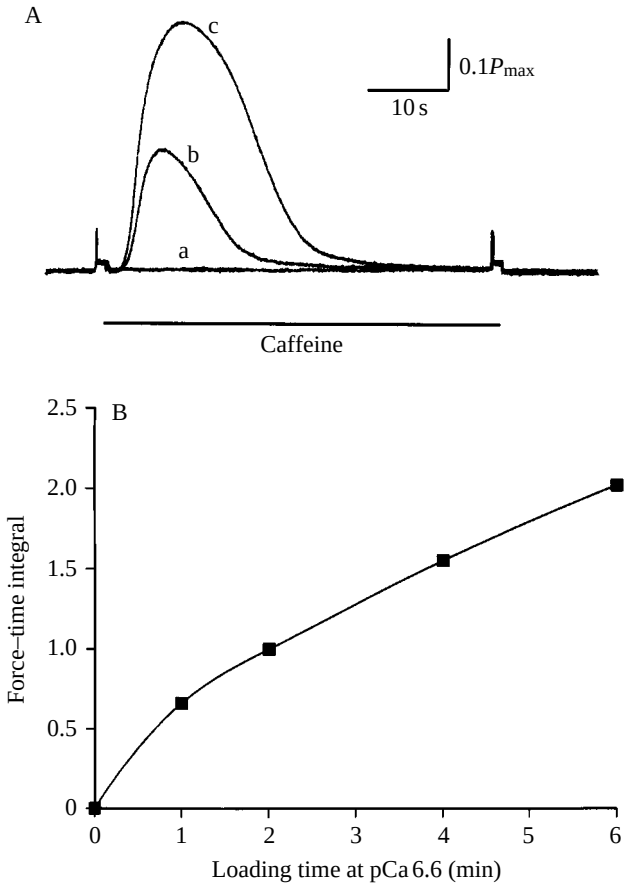

Fig. 1. (A) Caffeine-induced contractions from a 100 μm diameter myofibrillar bundle taken from a crab meropodite extensor muscle. The caffeine concentration was 20 mmol l^{-1}. Superimposed responses are shown for three loading times at pCa 6.0: (a) 0, (b) 1.0 and (c) 2.0 min. Maximum Ca^{2+}-activated tension (P_{max}) was 0.75 mN. (B) The dependence of Ca^{2+} loading on the loading time for a single myofibrillar bundle. The force–time integral of the caffeine-induced contraction is plotted against the loading time in a pCa 6.6 solution containing 5 mmol l^{-1} EGTA. The response for 2 min is taken as 1.0; the responses at the other times are expressed as a fraction of the mean of two control values with 2 min loading which were obtained before and after each response.

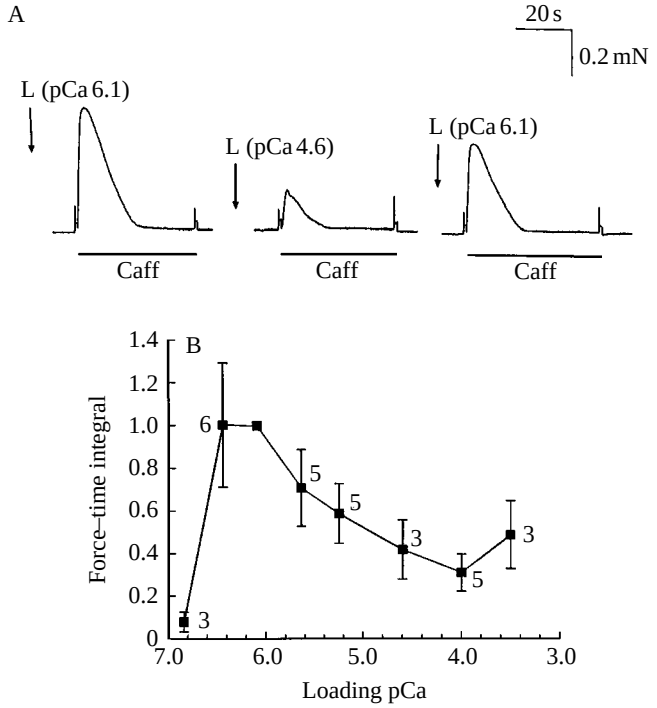

Fig. 2. The dependence of the caffeine-induced contraction on the pCa of the loading solution. (A) Successive responses to 20 mmol l^{-1} caffeine (Caff). Here Ca^{2+} loading at pCa 4.6 (for 1 min) is compared with control responses (1 min loading at pCa 6.1), which were measured before and after the test response. Loading periods are not shown on the tension traces but are represented by the arrows labelled L. (B) The force–time integral of the caffeine-induced contraction is plotted against the pCa of the loading solution and is expressed as a fraction of the average of two control responses at pCa 6.1. Each point is the mean value from 3–6 bundles (numbers next to each point); bars show ± S.E.M. Loading time 1 min.

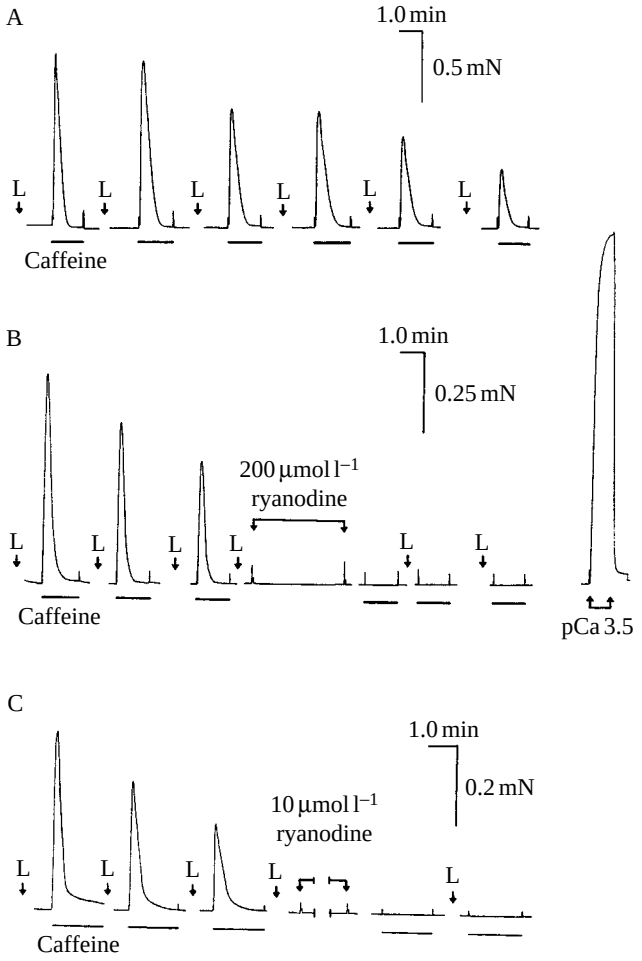

Fig. 3. Ryanodine inhibits the caffeine-induced contraction of crab myofibrillar bundles. (A) Control experiment: successive caffeine-induced contractions were obtained by reloading Ca^{2+} stores for 1 min at pCa 6.0 (arrow labelled L) after each challenge with caffeine (bars) (20 mmol l^{-1}). Run-down of the response is evident. (B) Ryanodine application at 200 µmol l^{-1} for 4 min in the relaxing solution containing 1 mmol l^{-1} EGTA irreversibly abolished the caffeine-induced contractions, although the bundle was still responsive to activating solutions of pCa 3.5. (C) Ryanodine at a concentration of 10 µmol l^{-1} similarly abolished the caffeine-induced contractions.

the duration of exposure to the Ca^{2+} loading solution (from 0 to 6 min) at pCa 6.0 (e.g. Fig. 1A) and at pCa 6.6 (e.g. Fig. 1B) (N=3 bundles), a result consistent with a requirement for an ATP-dependent Ca^{2+} uptake into a caffeine-releasable Ca^{2+} store.

Second, the caffeine-induced contraction was abolished by exposing the myofibrillar bundles to the detergent Triton X-100 (at 0.5 % w/v for 20 min), a treatment known to destroy myofibrillar membranes including the SR. The direct activation of the myofibrillar contractile proteins by added Ca^{2+} was unaffected by the treatment with the detergent.

Third, for a given amount of Ca^{2+} loading, the amplitude of the caffeine-induced contraction was inversely proportional to the EGTA concentration in the solution bathing the myofibrillar bundle, over the range 0.1–5 mmol l^{-1} (results not shown). This is consistent with competition between EGTA and the myofilaments for the released Ca^{2+}. For the experiments reported in this paper, an EGTA concentration of 1 mmol l^{-1} was chosen so that the amplitude of the caffeine-induced contraction was below the maximum tension of the fibre (P_{max}).

The relationship between the free [Ca^{2+}] of the loading solution and the rate of loading of the Ca^{2+} store in crab muscle was examined in detail. An optimum loading pCa value of between 6.4 and 6.1 was found for the caffeine-induced contraction (Fig. 2B). Loading at higher free Ca^{2+} values (i.e. pCa values of less than 6.1) produced smaller responses to caffeine (e.g. Fig. 2A).

Ryanodine inhibits responses to caffeine

A series of caffeine-induced contractions could be obtained in a single myofibrillar bundle preparation by reloading the Ca^{2+} stores after each exposure to caffeine (Fig. 3A). Run-down of the response always occurred. The addition of a protease inhibitor, leupeptin (100 µmol l^{-1}), did not prevent this run-down (results not shown). The effects of ryanodine

were tested on the caffeine-induced responses of crab myofibrillar bundles. Exposure to ryanodine for 4 min caused a total and irreversible loss of the response to caffeine, even though the subsequent loading and caffeine solutions contained no ryanodine (Fig. 3B,C). This was found with two preparations at 10 μmol l^{-1} ryanodine and three preparations at 200 μmol l^{-1} ryanodine. These effects of ryanodine are not considered to be due to inhibition of Ca^{2+} activation of tension at the site of the myofilaments, since ryanodine was not present in the caffeine solution and 100 μmol l^{-1} ryanodine does not affect the pCa–tension curve of other skinned muscle fibres (Su, 1988). Certainly, the myofibrillar bundles still responded to pCa 3.5 solutions after exposure to 200 μmol l^{-1} ryanodine (Fig. 3B).

Ryanodine also releases Ca^{2+} from sarcoplasmic stores

In the preceding experiments in the presence of 1 mmol l^{-1} EGTA, the addition of 200 μmol l^{-1} ryanodine produced no increase in resting tension over 4 min, even though the Ca^{2+} stores were first reloaded for 1 min at pCa 6.0 (Fig. 3B). However, when the loading time was increased to 5 min, 200 μmol l^{-1} ryanodine produced a slow phasic contracture after a 40 s delay (results not shown). This dependence on Ca^{2+} loading time suggests that ryanodine was releasing Ca^{2+} from a sarcoplasmic store. From the force–time integral of the contracture, it was estimated that ryanodine released the equivalent of approximately one-third of the Ca^{2+} present in the caffeine-sensitive store.

When the EGTA concentration of the relaxing solution was reduced to 0.1 mmol l^{-1}, ryanodine at concentrations of 2 μmol l^{-1} and higher produced contractions in myofibrillar bundles (N=6) having a Ca^{2+}-loaded store (Fig. 4). The contractions in response to the lower concentrations of ryanodine were oscillatory in nature over a period of 50 min. Before the onset of the ryanodine-induced contractions there was a delay, the duration of which decreased with increasing concentrations of ryanodine (Fig. 4). At 200 μmol l^{-1} ryanodine, this delay was 40 s (data not shown). In the presence of 1 mmol l^{-1} EGTA, by contrast, 200 μmol l^{-1} ryanodine produced no contraction over a 4 min period (Fig. 3B). This provides further evidence that ryanodine is releasing Ca^{2+} from a sarcoplasmic store, since the absence of a contractile response can be accounted for by the higher EGTA concentration causing the chelation of the released Ca^{2+}, thus preventing it from binding to the tension-generating troponin sites on the myofilaments.

The duration of the delay (nearly 7 min) in the onset of contraction after exposure to 10 μmol l^{-1} ryanodine was too long to be explained solely by the time taken for ryanodine to diffuse into the bundles. Assuming that the diffusion coefficient for ryanodine in myofibrils is similar to that of a larger molecule, Fura 2 (Timmerman and Ashley, 1986), and that it is not significantly affected by binding to sarcoplasmic sites, it can be calculated that the mean ryanodine concentration in a 100 μm diameter bundle will reach 95 % of the bulk solution concentration in 30 s. Similar delays in the onset of contraction have been observed in the effects of ryanodine on barnacle myofibrillar bundles (Ashley *et al.* 1993) and toad skinned fibres, in which the slow binding of ryanodine to the SR receptors was thought to be responsible (Lamb and Stephenson, 1990).

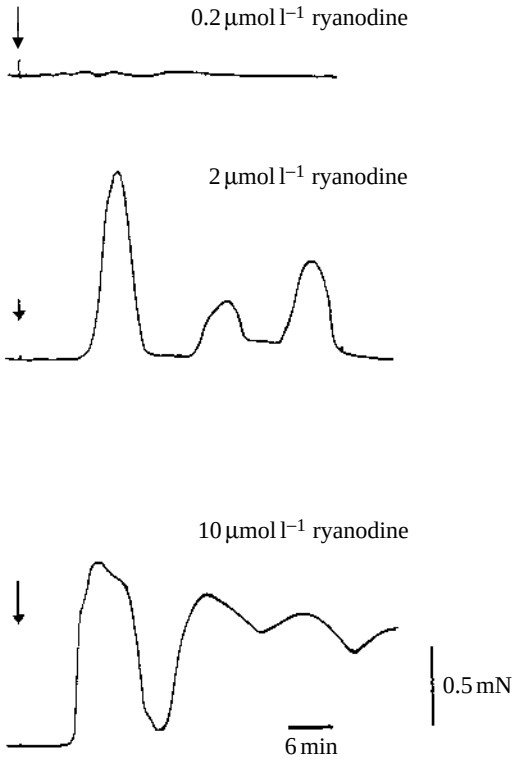

Fig. 4. Ryanodine-induced contractions in three separate crab myofibrillar bundles in relaxing solutions containing 0.1 mmol l^{-1} EGTA. Tension responses to three concentrations of ryanodine (0.2, 2 and 10 μmol l^{-1}) are shown. Ca^{2+} loading time prior to transfer to a solution containing ryanodine was 1 min at pCa 6.4 after initial emptying of the Ca^{2+} store with caffeine.

CICR force responses in crab myofibrillar bundles

Fabiato (1983, 1985) measured CICR in skinned cardiac cells by raising the free Ca^{2+} concentration in solutions containing physiological levels of Ca^{2+} buffering (typically 100 μmol l^{-1} EGTA) and quantifying the resulting tension or free Ca^{2+} transients. The tension method was adopted for looking at CICR in crab myofibrillar bundles, but the concentration of EGTA was raised to 1 mmol l^{-1} EGTA.

In myofibrillar bundles in which the Ca^{2+} stores had been loaded for 2 min at pCa 6.0 (with 5 mmol l^{-1} EGTA), transfer to a pCa 5.8 solution containing 1 mmol l^{-1} EGTA (following a wash in a solution also containing 1 mmol l^{-1} EGTA) produced a phasic contracture (Fig. 5). This was variable in form between preparations, sometimes showing more than one peak, and usually with a slow onset (mean time to peak 54.4±14.4 s, S.E.M., N=7). Treating the preparation with ryanodine (10 μmol l^{-1}) for 5 min abolished this contracture (and also the caffeine response), leaving a small tonic response

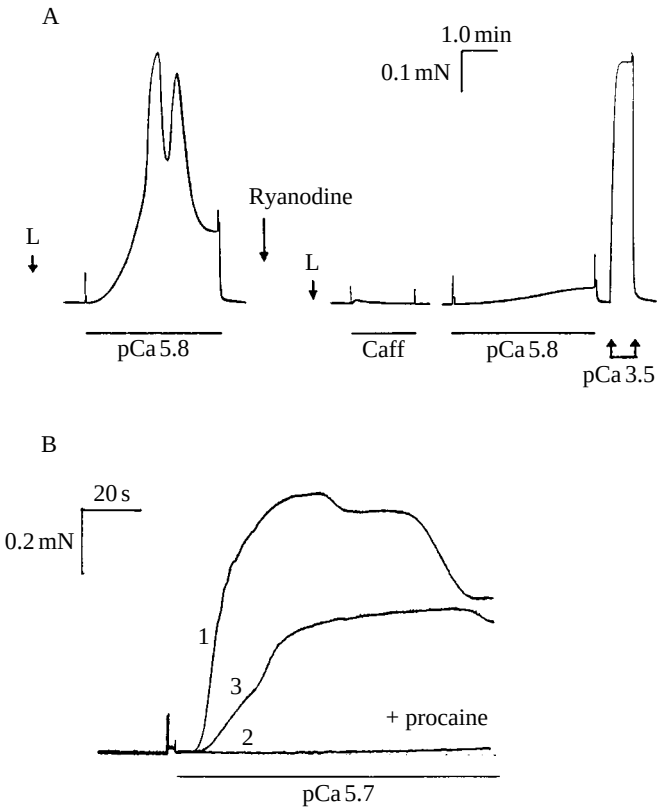

Fig. 5. Ca^{2+}-induced Ca^{2+} release (CICR) from crab myofibrillar bundles. (A) After loading for 2 min at pCa 6.0 (arrow labelled L), a pCa 5.8 solution (with 1 mmol l^{-1} EGTA) produced a biphasic contraction. Ryanodine, applied for 5 min at 10 μmol l^{-1}, inhibited the contraction induced by caffeine (Caff, 20 mmol l^{-1}) and virtually abolished the response to pCa 5.8, in spite of further Ca^{2+} loading after exposure of the bundle to ryanodine. The bundle still contracted when exposed to a pCa 3.5 activating solution. (B) The force response of a crab myofibrillar bundle to pCa 5.7 (1 mmol l^{-1} EGTA) (trace 1) was inhibited by 10 mmol l^{-1} procaine after a 1 min preincubation (trace 2). The Ca^{2+} store was loaded for 2 min at pCa 6.0 prior to the start of each trace. After each exposure to pCa 5.7, the store was emptied using caffeine (not shown). Procaine was included in a preincubation solution and the pCa 5.7 solution only. The numbers on the traces refer to the order in which they were obtained. Trace 3 was obtained in procaine-free solutions and indicates that the inhibition was reversible, the reduction in response amplitude compared with trace 1 probably being due to run-down.

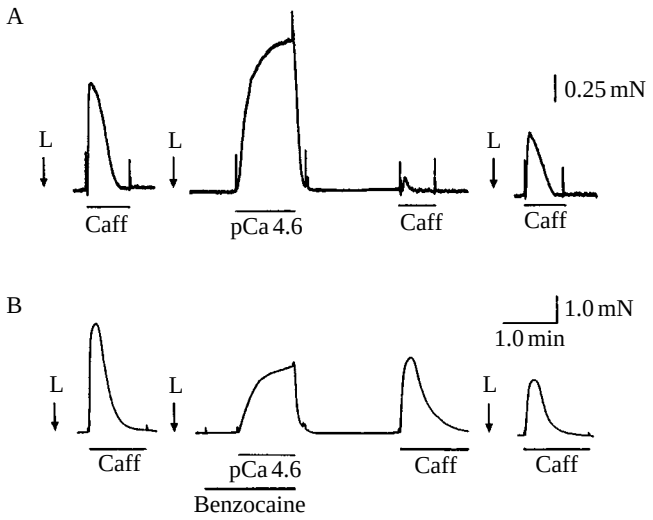

Fig. 6. (A) Ca^{2+}-induced Ca^{2+} release (CICR) in crab myofibrillar bundles and (B) its inhibition by benzocaine (5.0 mmol l^{-1}). Transfer of the bundle to a pCa 4.6 releasing solution for 1 min reduced the size of the subsequent caffeine-induced contraction (Caff) in A but not in B. Two controls (releasing pCa 9) are shown before and after the exposure to the pCa 4.6 releasing solution. Ca^{2+} loading (arrow labelled L) was for 2 min at pCa 6.6.

(Fig. 5A). Larger responses (not shown) were obtained in pCa 5.8 solutions containing only 0.2 mmol l^{-1} EGTA, especially if the Ca^{2+} store in the bundle was allowed to keep its endogenous level of Ca^{2+} loading, rather than having this emptied and then refilled at pCa 6.0 for 2 min. However, it is possible that the endogenous Ca^{2+} load in the bundles may be artificially high as a result of uptake of Ca^{2+} from broken extracellular clefts during the preparation of the myofibrillar bundle.

The phasic contraction in response to pCa 5.7–5.8 was also strongly inhibited by the local anaesthetics procaine (Fig. 5B) and benzocaine. Local anaesthetics are well-known inhibitors of CICR in vertebrate skinned fibres (Thorens and Endo, 1975). They also inhibit caffeine-induced Ca^{2+} release in barnacle myofibrillar bundles without affecting Ca^{2+}-activated tension significantly (Lea, 1984). The contractile response of crab myofibrillar bundles to pCa 5.7 was virtually abolished within 1 min by the addition of 10 mmol l^{-1} procaine (N=2) or 5 mmol l^{-1} benzocaine (N=3; not shown) and this effect was reversible (Fig. 5B). This effect of procaine was confirmed as an inhibition of Ca^{2+} release by using the subsequent caffeine-induced response as a measure of the Ca^{2+} content of the caffeine-releasable store (see below). In the experiment shown in Fig. 5B, the Ca^{2+} content after exposure to the pCa 5.7 solution with procaine was 3.8 times that after exposure to the pCa 5.7 solution in the absence of procaine (not shown).

CICR from crab myofibrillar bundles: Endo's method

The results of typical experiments using Endo's method are shown in Fig. 6 (see Materials and methods for protocol). The releasing solutions contained 5 mmol l^{-1} total ATP, 1 mmol l^{-1} EGTA and 1.0 mmol l^{-1} free Mg^{2+}; Ca^{2+} loading was for 2 min at pCa 6.6. In Fig. 6A, the crab myofibrillar bundle was exposed to the pCa 4.6 releasing solution for 1 min; this reduced the force–time integral of the subsequent caffeine-induced contraction to 4.2 % of the mean of the two controls (in which the releasing solution was pCa 9). In the experiment shown in Fig. 6B (from a separate bundle), both the pCa 4.6 releasing solution and a preincubation solution contained 5 mmol l^{-1} benzocaine; the subsequent caffeine-induced contraction was 119 % of the mean control value. The pCa 4.6 solution produced tension in the presence of benzocaine presumably as a result of direct Ca^{2+}-activation of the

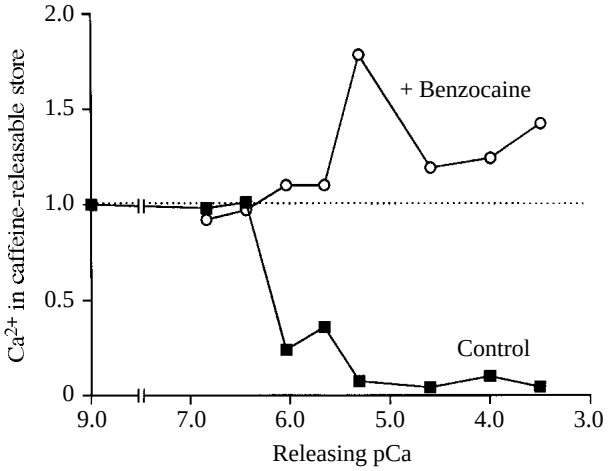

Fig. 7. The dependence of Ca^{2+}-induced Ca^{2+} release (CICR) in crab myofibrillar bundles on releasing pCa and the effect of benzocaine (5.0mmol l^{-1}). The points are single measurements and were obtained from experiments similar to those depicted in Fig. 6 using 16 myofibrillar bundles. Ca^{2+} loading was for 2min at pCa6.6; each bundle was then transferred for 1min to a releasing solution (1mmol l^{-1} EGTA) of given pCa. The force–time integral of the subsequent caffeine-induced contraction is expressed as a fraction of the mean of two control values (releasing pCa9.0) and is taken as a measure of the Ca^{2+} content of the caffeine-releasable store.

myofilaments. The results from the two experiments taken together can be interpreted as follows: pCa 4.6 released 95.8 % of the caffeine-releasable Ca^{2+} in 1 min, and benzocaine inhibited this release and promoted a further 19 % Ca^{2+} loading. This makes the assumption that the force–time integral is linearly proportional to the Ca^{2+} content of the store.

The dependence of Ca^{2+} release on pCa (Fig. 7) was examined in eight experiments of the type shown in Fig. 6A, with each experiment testing a different pCa value. The relative force–time integral of the caffeine-induced contraction following a test release is plotted against the pCa of the releasing solution. The threshold for Ca^{2+} release was between pCa6.0 and 6.4. More than 90% of the loaded Ca^{2+} was released in 1min by pCa values of 5.3–3.5, whereas less was released at pCa6.0 and 5.6, indicating the graded nature of the response. The effect of benzocaine (5mmol l^{-1}) was examined in eight experiments similar to that shown in Fig. 6B, with each experiment testing a different pCa value. CICR was inhibited totally at all pCa values of 6.0 and below, with additional Ca^{2+} loading being promoted at these pCa values. Procaine has a similar effect on the CICR curve in frog skinned fibres (Thorens and Endo, 1975).

Separate Ca^{2+} loading experiments showed that 5 mmol l^{-1} benzocaine actually causes a small inhibition (15 %) of Ca^{2+} uptake into the caffeine-sensitive store at pCa 6.4 (data not shown); no correction was made for this. Corrections to the curves in Fig. 7 to allow for the non-linearity of the Ca^{2+} loading curve (Fig. 1B) do not affect the conclusions regarding CICR. The releasing solutions contained only

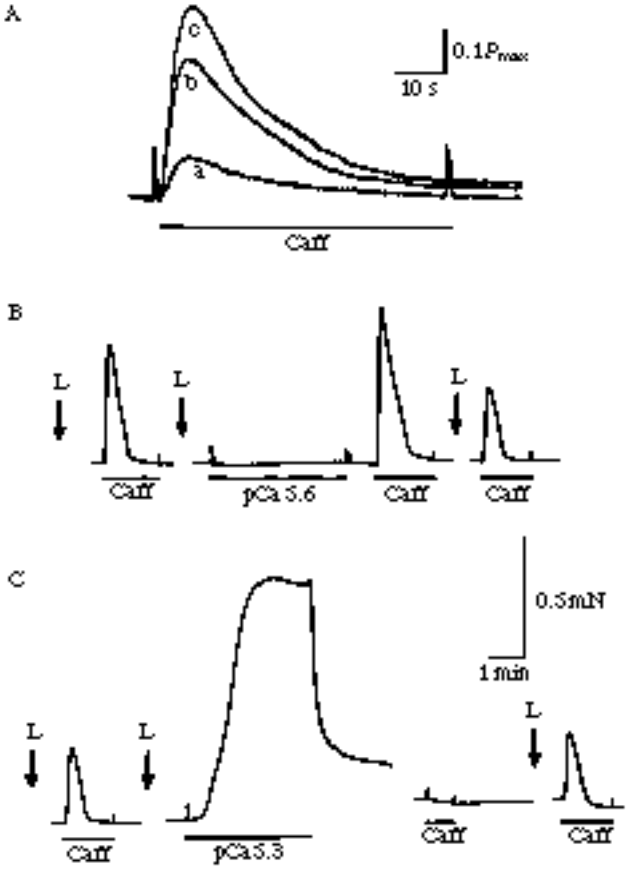

Fig. 8. Ca^{2+} release from a caffeine-releasable store in myofibrillar bundles from the abdominal flexor muscles of the lobster. (A) Caffeine-induced contractions at three Ca^{2+} loading times: (a) 1.0, (b) 2.0 and (c) 3.0min at pCa5.8. The maximum Ca^{2+}-activated tension (P_{max}) was 0.66mN. (B) Exposure to a pCa5.6 solution (1mmol l^{-1} EGTA) caused an increase in the response to 20mmol l^{-1} caffeine (Caff) after a Ca^{2+} loading period of 2min at pCa5.9 (indicated as L). (C) Exposure of the same bundle as in B to a pCa5.3 solution (1mmol l^{-1} EGTA) for 3min caused a contraction and inhibition of the subsequent response to caffeine. Ca^{2+} loading was for 2min at pCa5.9 (L).

1 mmol l^{-1} total EGTA, compared with the 10 mmol l^{-1} EGTA in the work of Endo (1977). This concentration may have been too low to prevent some regenerative Ca^{2+} release at the lower free [Ca^{2+}] values, i.e. extra Ca^{2+} release triggered by the initial release.

Ca^{2+} stores in lobster abdominal muscle

Myofibrillar bundles isolated from lobster abdominal flexor musculature contracted phasically when exposed to 20mmol l^{-1} caffeine in the presence of 1mmol l^{-1} EGTA (Fig. 8A). The size of the response was dependent on the Ca^{2+} loading time. A loading time of 1.5–3min at pCa5.8 was necessary to give responses of amplitude equal to 30–50 %P_{max} (N=14 bundles). As with the crab preparation, a series of responses to caffeine could be obtained from a single lobster bundle by reloading the Ca^{2+} store after each exposure to

caffeine. Exposure to ryanodine (200 μmol l^{-1}) for 4min irreversibly abolished the response to caffeine (N=2). Of 17 fibres randomly chosen from the flexor muscle, 14 gave caffeine-induced contractions but three gave no response after Ca^{2+} loading at pCa5.8. This may reflect the fact that the flexor muscle contains both fast and slow fibre types (Jahromi and Atwood, 1969).

The threshold [Ca^{2+}] for CICR in the lobster preparation appeared to be higher than in crab. In a single experiment, a pCa5.6 solution failed to release loaded Ca^{2+} and actually caused further loading (Fig. 8B). However, application of a pCa5.3 solution for 3min released all the loaded Ca^{2+} (Fig. 8C).

Discussion

CICR and excitation–contraction coupling in crab muscle

The results from isolated myofibrillar bundles which are presented in this paper demonstrate that striated muscle fibres of crab and lobster possess ryanodine-sensitive sarcoplasmic stores of Ca^{2+} that can be released by both caffeine and micromolar [Ca^{2+}] (i.e. CICR). In crab fibres, Ca^{2+} release by micromolar [Ca^{2+}] was demonstrated by three different methods. First, the Ca^{2+} loading experiments showed that as the free [Ca^{2+}] of the loading solution was increased above 1 μmol l^{-1} (i.e. pCa less than 6.0) the degree of loading was reduced. This can be explained by a triggering of CICR causing the release of some of the loaded Ca^{2+}. Such an interpretation has been given to similar results from skinned trabeculae of rat heart (Zhu and Nosek, 1991) and frog skinned fibres (Horiuti, 1986; Endo and Iino, 1988). Second, the application of micromolar [Ca^{2+}] to crab myofibrillar bundles resulted in phasic contractions which could be inhibited by the Ca^{2+}-release inhibitors ryanodine, procaine and benzocaine. Third, micromolar [Ca^{2+}] reduced the SR Ca^{2+} content of the caffeine-releasable store, assessed by the size of the subsequent caffeine-induced contraction.

These three independent demonstrations of CICR add support to a suggested mechanism of excitation–contraction coupling in crab muscle in which an inward Ca^{2+} current across the sarcolemma leads to a rise in sarcoplasmic [Ca^{2+}] which, in turn, releases Ca^{2+} from the SR by triggering the CICR mechanism (Mounier and Goblet, 1987). There is increasing evidence that in other crustacean striated muscles, for example those of the crayfish and the barnacle, excitation–contraction coupling works in the same way (Györke and Palade, 1992; Palade and Györke, 1993; Ashley *et al.* 1993).

Could the CICR responses be spontaneous Ca^{2+} release?

The possibility that the CICR responses of the crab myofibrillar bundles were a type of spontaneous Ca^{2+} release, as described for rabbit skeletal muscle (Volpe *et al.* 1983) and cardiac myocytes (Fabiato, 1983), was considered. Such a release occurs from the light fraction of rabbit SR vesicles (presumably, therefore, in the absence of the ryanodine receptor which is located mainly in the heavy fraction) and is triggered by submicromolar [Ca^{2+}] after a characteristic lag phase, during which Ca^{2+} uptake occurs until a critical level of loading is reached. Some of the responses in crab and lobster myofibrillar bundles exposed to pCa 5.7–5.8 solutions showed such a lag phase, but this may simply reflect the time required for the free [Ca^{2+}] directly at the site of the Ca^{2+} store to reach the value in the bathing solution. In barnacle myofibrillar bundles, a similar lag occurs and this can be abolished by using the rapid photorelease of Ca^{2+} from caged Ca^{2+} to circumvent diffusional delays (Lea and Ashley, 1990). A more conclusive point which argues against spontaneous release in the crab experiments is that in the rabbit light SR vesicles this type of release can only be inhibited by procaine (10 mmol l^{-1}) after a long preincubation (hours), whereas the contractions induced by pCa 5.8 in the crab myofibrillar bundles were virtually abolished by 10 mmol l^{-1} procaine within 1 min.

Threshold for CICR in crab myofibrillar bundles

The threshold pCa for CICR in the crab Ca^{2+} store was found to be 6.0–6.4 using the caffeine method of Endo (1977). Lower threshold concentrations of free [Ca^{2+}] were reported by Goblet and Mounier (1986) who used a whole skinned-fibre preparation from the crab extensor muscle and found that, in the absence of EGTA in the bathing solution, a free [Ca^{2+}] of 10^{-7} mol l^{-1} or less (pCa7.0 or higher) could elicit some release of stored Ca^{2+}. In the absence of Ca^{2+} buffering, there may have been some regenerative Ca^{2+} release, i.e. a triggering of further CICR by the released Ca^{2+}. This would effectively amplify a small Ca^{2+} release, at say pCa7.0, which could not be detected by the Endo method in which the bathing solutions contained 1 mmol l^{-1} EGTA.

The results from the crab preparation are in broad agreement with those from the barnacle, in which the threshold pCa for CICR, measured either as a rise in myofibrillar free [Ca^{2+}] (Lea and Ashley, 1989) or as a rise in tension (Lea and Ashley, 1990), was estimated to be about 6.7.

Ryanodine releases Ca^{2+} in crab myofibrillar bundles

The observations that the caffeine-induced contraction was inhibited by ryanodine and that ryanodine itself produced phasic contractions suggest that ryanodine can release Ca^{2+} from the caffeine-releasable store, although the presence of a separate ryanodine-releasable Ca^{2+} store cannot be excluded. The ability of ryanodine to release stored Ca^{2+} is supported by the report that 10 μmol l^{-1} ryanodine can induce an open subconductance state in the lobster ryanodine receptor in planar lipid bilayers (Seok *et al.* 1992).

One difference in the action of ryanodine on crab fibres compared with its effect on toad fibres was its ability to inhibit the caffeine-induced contractions in the presence of 1 mmol l^{-1} EGTA (estimated pCa9), whereas in the toad fibres ryanodine was ineffective at this pCa (Lamb and Stephenson, 1990). This appears to indicate that ryanodine can bind to the crab ryanodine receptor when the Ca^{2+} channel is in the closed state, unlike in the skeletal ryanodine receptor.

Release of Ca^{2+} from the myofibrillar store compared with activation of the isolated ryanodine receptor

Ryanodine receptor proteins which act as Ca^{2+}-release channels have been isolated from crustacean striated muscles (Formelova *et al.* 1990; Arispe *et al.* 1992; Seok *et al.* 1992). When the ryanodine receptor from lobster abdominal muscle was incorporated into planar lipid bilayers, the sensitivities of the Ca^{2+} channel to caffeine and micromolar [Ca^{2+}] were found to be much lower than those of skeletal and cardiac muscle ryanodine receptors (Seok *et al.* 1992). The lobster channel appeared to lack the caffeine-sensitive, high-affinity regulatory Ca^{2+}-binding site present on the skeletal and cardiac receptors. This absence was not evident in the preliminary results from lobster myofibrillar bundles described in this paper. Caffeine (20mmol l^{-1}) readily released Ca^{2+} from a ryanodine-sensitive store in myofibrillar bundles taken from the abdominal musculature, confirming previous reports of its action on lobster abdominal fibres (Jahromi and Atwood, 1969), minced fibres from leg muscle (Rojas *et al.* 1987) and skinned fibres from the specialised remotor muscle (Tang *et al.* 1990). In addition, 5μmol l^{-1} free Ca^{2+} solutions (pCa5.3) released all the caffeine-releasable Ca^{2+} in lobster myofibrillar bundles within 3min. In these respects, therefore, the caffeine-releasable Ca^{2+} stores in the lobster skinned fibre preparation appeared to behave in a similar way to those in the crab preparation and also to those of skeletal and cardiac skinned muscle preparations (Endo, 1977; Fabiato, 1983).

Without a more detailed study of Ca^{2+} uptake and release in lobster myofibrillar preparations, one can only speculate on the apparent differences between the isolated ryanodine receptor and the caffeine-releasable Ca^{2+} store of myofibrillar bundles. It is possible that there are two ryanodine receptor isoforms with different properties in lobster muscle; one isoform could have dominated the myofibrillar responses, while the other was the protein characterised by Seok *et al.* (1992). Two distinct isoforms of the ryanodine receptor have been isolated from frog skeletal muscle (Lai *et al.* 1992). Another possible explanation is that a high-affinity Ca^{2+}-activating site on the lobster ryanodine receptor was impaired or lost during the isolation; in their paper, Seok *et al.* (1992) state that there was probably partial inactivation of the receptor during purification.

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