An examination of the ability of inositol 1,4,5-trisphosphate to induce calcium release and tension development in skinned skeletal muscle fibres of frog and crustacea

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We have examined the ability of inositol 1,4,5-trisphosphate ($InsP_3$) to cause contractions of mechanically skinned muscle fibres of frog and barnacle. $InsP_3$ (10–500 μM) did not cause any tension development in 25 frog skinned fibres and 26 barnacle myofibrillar bundles, although contractions could be readily evoked by caffeine and by replacement of an impermeant anion by Cl^-, treatments known to release calcium from the sarcoplasmic reticulum (SR). Four barnacle bundles did give responses to $InsP_3$. $InsP_3$ did not modify responses to caffeine or calcium-induced calcium release. Free Mg^{2+} was lowered to 40 μM and 15 mM D-2,3-diphosphoglycerate was added in order to inhibit the possible breakdown of $InsP_3$ by inositol trisphosphatase. Neither measure revealed a response to $InsP_3$. Arsenazo III absorbance measurements failed to detect any binding of Mg^{2+} (0–0.5 mM) by 0.35 mM $InsP_3$ in our solutions. Inhibitors of SR calcium uptake (cadmium, quercetin, furosemide), omission of EGTA from the solution and varying the temperature from 4° to 22°C also failed to reveal a response of frog skinned fibres to $InsP_3$. The nucleotide GTP, which has been reported to enhance $InsP_3$-induced calcium release from rat liver microsomes, had no effect at 50 μM on the response of frog fibres to $InsP_3$. It is concluded that under conditions in which other calcium release mechanisms operate well, $InsP_3$ is relatively ineffective at releasing calcium from the SR in amounts sufficient to induce contraction. Although we have been unable to find evidence to support the proposed role of $InsP_3$ as an essential link in excitation-contraction coupling of skeletal muscle, we cannot entirely reject its role if essential cofactors are lost in the skinned preparations.

(Skinned muscle fiber) *Sarcoplasmic reticulum* *Inositol 1,4,5-trisphosphate* *Ca^{2+}* *Muscle contraction*

1. INTRODUCTION

In many cells external stimuli can cause the hydrolysis of the inositol lipid, phosphatidylinositol 4,5-bisphosphate, which is located in the cell membrane, releasing $InsP_3$ into the cytoplasm [1]. There is now evidence from a variety of cells that $InsP_3$ can then mobilise calcium from a cytoplasmic store, probably the endoplasmic reticulum [1]. In this way $InsP_3$ appears to act as a messenger between an extracellular stimulus such as acetylcholine and the resulting rise in intracellular free calcium. $InsP_3$ has also been reported to release calcium from SR vesicles of cardiac [2] and skeletal muscle [3] and from the SR of skinned, vascular smooth muscle [4–6] and also to induce tension development in skinned skeletal muscle fibres [3,7] and skinned smooth muscle preparations [5]. These findings have led to the proposal that $InsP_3$ may be the specific chemical transmitter at the triadic junction responsible for linking the depolarisation of the transverse tubule to the release of calcium from the SR during muscle activation [3,7]. However, some workers have been unable to confirm that $InsP_3$ can release calcium from the SR of either cardiac [26] or

Abbreviations: $InsP_3$, inositol 1,4,5-trisphosphate; DPG, D-2,3-diphosphoglycerate; SR, sarcoplasmic reticulum

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skeletal muscle [23,24]. We have compared the effects of $InsP_3$ with those of other SR Ca^{2+}-releasing agents using mechanically skinned muscle fibres of barnacle, crab and frog and have examined conditions under which $InsP_3$ action might be accentuated. We find little evidence to suggest that $InsP_3$-induced calcium release could be large enough to justify its proposed sole involvement in excitation-contraction coupling.

2. MATERIALS AND METHODS

Calcium release from the SR was examined by recording isometric tension of skinned muscle fibres of the barnacle *Balanus nubilus*, the spider crab *Maia* and the frog *Rana temporaria* [8]. Single muscle fibres of the barnacle were immersed in paraffin oil and longitudinally divided by forceps. Bundles of myofibrils, 200 μm in diameter, were removed and clamped between two pairs of forceps, one of which was attached to the anode peg of an RCA 5734 force transducer, the output of which was fed into a bridge circuit and a Pantos pen recorder. Muscle fibres of the frog sartorius muscle were mechanically skinned with needles under a solution containing 20 mM EGTA and then glued at each end using cyanoacrylate adhesive to steel pins attached to the forceps of the transducer apparatus. Bathing solutions (100 μl–1.5 ml) were contained in a series of cuvettes or perspex blocks which were held in a spring-mounted sample chamber, through which was circulated coolant to maintain thermostatically the solution temperature at 4 or 15°C.

Aequorin light output was measured through one side of the solution cuvette by an EMI 9635B photomultiplier tube, via a shutter and short perspex light guide. The photocathode was maintained at −700 V by a stabilised voltage supply (Oakfield Instruments, Eynsham, Oxford) and the output from the anode was passed through a current-to-voltage converter (1 mV/nA, 20 ms time constant) into the pen recorder.

The standard bathing solution (referred to as 'low relaxing') contained (mM): 90–150 K^+, 90–150 Cl^-, 30 Na^+, 1.0 Mg^{2+}, 5.0 ATP, 10 creatine phosphate (CP), 60 Tes, 0.1 EGTA, 20 U/ml creatine phosphokinase, pH 7.1. Contaminating calcium was found by atomic absorption spectrometry to be about 30 μM; this was estimated to give a p*Ca* of about 7.0 and this was confirmed by calcium electrode measurements. Barnacle solutions in some experiments contained in addition 900 mM sucrose. Free calcium and magnesium concentrations were varied as described in the text; the compositions of the solutions were calculated by a computer programme [9], using published affinity constants [30] with added temperature corrections. The affinity constants employed (M^{-1}) for pH 7.1 and 15°C were as follows: CaEGTA = 4.7×10^6, MgEGTA = 53, CaATP = 4.8×10^3, MgATP = 7.5×10^3, Mg,CaCP = 20, Mg,CaTes = 1.

To examine the importance of the main anion, KCl in the standard solution was replaced by equimolar K propionate or K methylsulphate.

$InsP_3$ was kindly supplied in three separate batches by Dr R.F. Irvine. Three samples were also obtained from Sigma: their $InsP_3$ is predominantly the 1,4,5-isomer with some 2,4,5-isomer present. A stock solution of 10 mM $InsP_3$ was prepared in 20 mM KTes, pH 7.1. Aequorin was obtained from J.R. Blinks, Mayo Clinic, Rochester, USA.

Arsenazo III absorbance measurements of free Mg^{2+} concentrations in solutions containing $InsP_3$ and ATP were made on a Perkin-Elmer Lambda 5 UV/VIS spectrophotometer. Differential spectra were obtained by using identical solutions in both reference and sample beams, with the exception that $MgCl_2$ was added to the sample solution.

3. RESULTS

$InsP_3$ was tested on barnacle and frog skinned muscle fibres in concentrations up to 500 μM. In 4 out of 30 barnacle preparations tension responses

Fig.1. The effects of $InsP_3$ (170–500 μM) on the isometric tension of four separate barnacle myofibrillar bundles (diameters 200 μm). (a,b) In low relaxing Cl^- solution at 1.0 mM free Mg^{2+}. (c) The effects of the inhibitors quercetin and DPG. The initial contraction was found to be due to quercetin. (d) In low relaxing propionate solution at reduced free Mg^{2+} (0.3 mM) and MgATP (0.2 mM). The initial contraction was always found on exposing bundles to low Mg^{2+}. The first control consisted of moving the bundle to the solution to which $InsP_3$ is later added. The second control is the addition of KTes (pH 7) (the $InsP_3$ stock solution was prepared in KTes). p*Ca* = 5.2 (a), 6.4 (b,c), 6.7 (d). Temperature, 15°C.

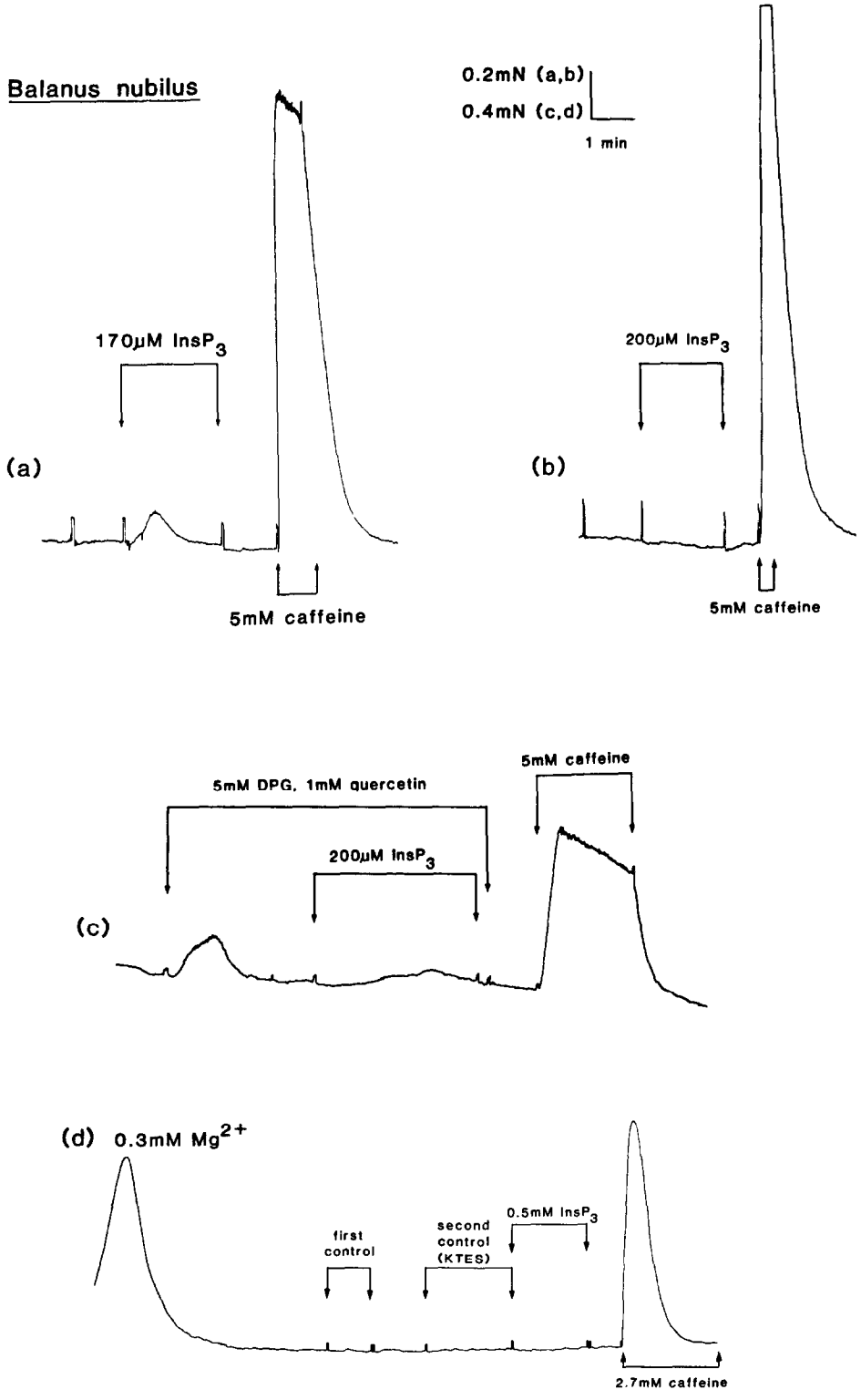

Balanus nubilus
0.2mN (a,b)
0.4mN (c,d)
1 min
170μM InsP3
(a)
5mM caffeine
200μM InsP3
(b)
5mM caffeine
5mM caffeine
5mM DPG, 1mM quercetin
200μM InsP3
(c)
(d) 0.3mM Mg2+
first control
second control (KTES)
0.5mM InsP3
2.7mM caffeine

Table 1

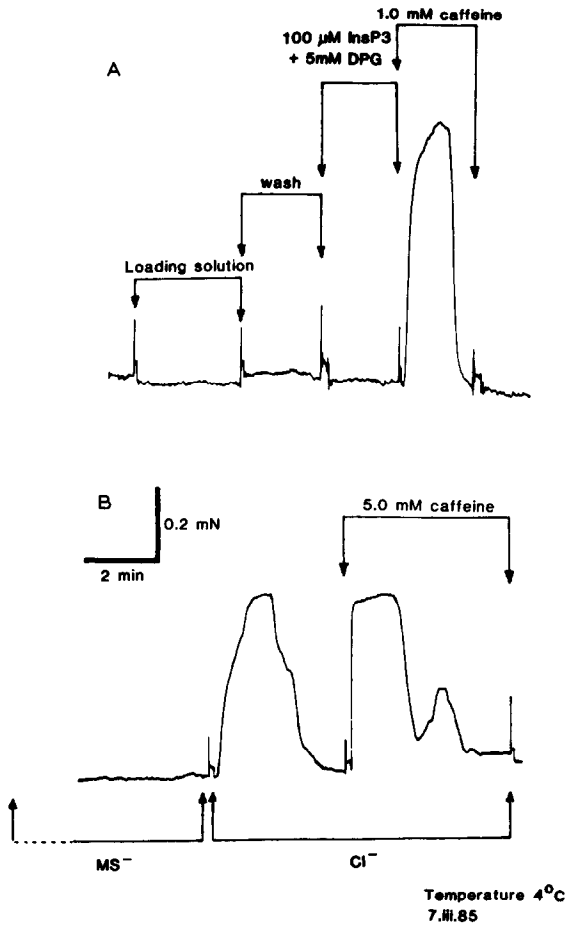
Effects of $InsP_3$ on *Balanus nubilus* myofibrillar bundles

Main anion in low relaxing solution	$[Mg^{2+}]$ (mM)	[MgATP] (mM)	Number of bundles giving contraction	Number of bundles tested
Cl^-	1.0	4.4	2[a]	10
Cl^-, methylsulphate, propionate	0.5	2.2	2[b]	12
Cl^-, propionate	0.04–0.3	0.2–0.9	0	8

[a] See fig.1 (a,c)

[b] These two responses, which were 40 and 96% of the response to 5 mM caffeine in amplitude, could not be repeated with other bundles in a replacement $InsP_3$-containing solution. For this reason it is suspected that they arose from calcium contamination in the first solution used

$InsP_3$ concentrations = 170–500 μM. Temperature, 14°C



were detected, although two of these were attributed to calcium contamination in the low relaxing solution which contained 0.1 mM EGTA (fig.1a,c; table 1). In the remaining 26 barnacle preparations (e.g. fig.1b) and 25 frog fibres (fig.2) there was no response to $InsP_3$. Releasable calcium was present in the SR of these fibres since 5 mM caffeine gave contractions (figs 1,2). Contractions could also be evoked from frog skinned fibres under the same conditions by replacing a relaxing solution containing methylsulphate as the predominant anion with one containing Cl^- (fig.2b); this is the phenomenon of 'depolarisation-induced' calcium release [10]. In relation to this, $InsP_3$ was tested on barnacle preparations in low relaxing solutions containing Cl^-, propionate or methylsulphate as the main anion (table 1).

Fig.2. Frog skinned muscle fibre exposed to 100 μM $InsP_3$ gives no response (A), whereas 'depolarisation-induced' and caffeine-induced calcium release can be demonstrated (B). (A) Fibre was first exposed to loading solution (p*Ca* 5.2) for 3 min, before a wash in 'Ca^{2+}-free' solution (p*Ca* 7). $InsP_3$ in the presence of DPG gave no response at p*Ca* 7, whereas caffeine did. Diameter 60 μm (7.iii.85). (B) A skinned fibre, showing no response to 100 μM $InsP_3$ plus 10 mM DPG, does contract on replacement of 130 mM methylsulphate (MS^-) by 130 mM Cl^- and also to caffeine. p*Mg* 3.0, p*Ca* 7.0. Diameter 60 μm (7.iii.85). Temperature, 4°C.

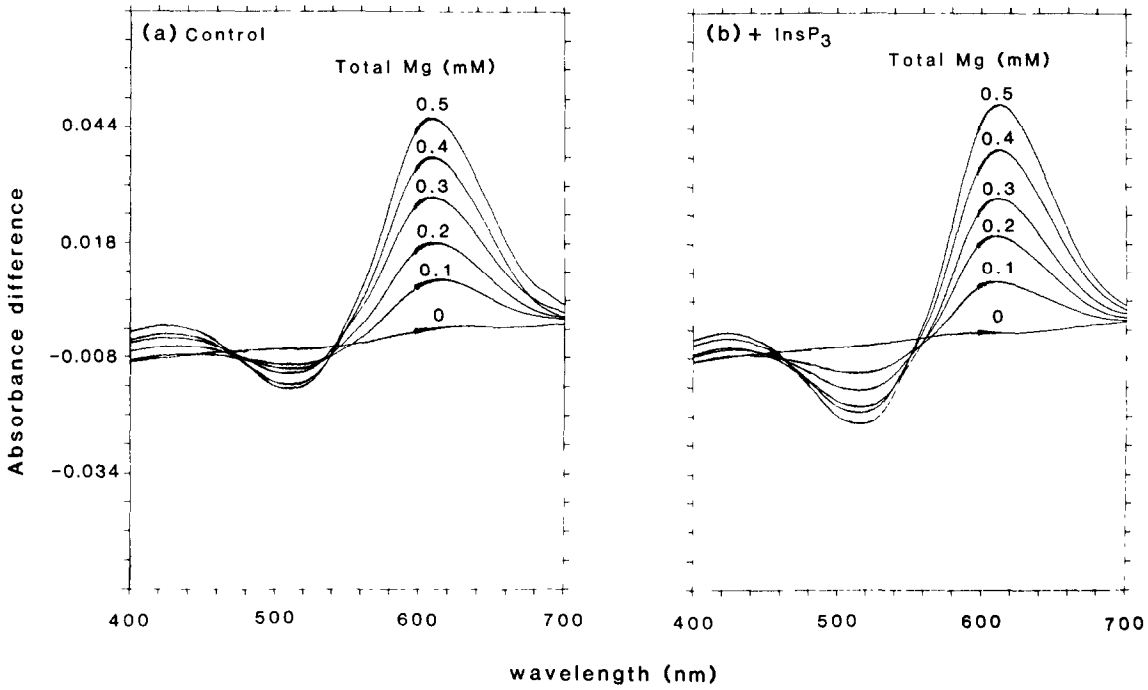
An experimental protocol with two controls was used whenever possible for the above experiments; this is shown in fig.1d. The bundle or skinned fibre was first moved to a solution to which the $InsP_3$/20 mM KTes would be added and secondly to another solution to which was added the appropriate volume of 20 mM KTes alone. Some barnacle preparations contracted in one or other of these control solutions; these preparations and the solutions were discarded.

A possible reason for the absence of an $InsP_3$-induced response under the conditions employed was that released calcium was being taken up immediately by the SR, thus preventing any force development. If this were the case, inhibition of the SR calcium pump might reveal a response to $InsP_3$. Consequently, $InsP_3$ was tested in the presence of three different calcium pump inhibitors: 1 mM quercetin [11,12], 1 mM furosemide [13] and 0.1 mM cadmium chloride [7,14,15]. However, these compounds were not successful in revealing an $InsP_3$-dependent response in frog fibres, despite the report of $InsP_3$-induced contractions in the presence of 0.1 mM $CdCl_2$ [7]. A slow response to $InsP_3$ was detected in a barnacle preparation in the presence of quercetin when absent without it, but it was smaller than the contraction due to the initial exposure to quercetin (fig.1c). Quercetin, in addition to inhibiting the SR calcium uptake pump, also has a caffeine-like action in releasing calcium from the SR [16]. Experiments with cadmium were carried out in the absence of EGTA from the bathing solutions, to avoid displacement of calcium from CaEGTA by cadmium and consequent tension development. The possibility that quercetin and furosemide interfered with the mechanism of an $InsP_3$-induced calcium release cannot be discounted.

A second explanation that we considered for the ineffectiveness of $InsP_3$ in our experiments was that the compound was being hydrolysed by enzyme(s) in the skinned muscle fibre, before it had diffused to the SR. The enzyme inositol trisphosphatase has been isolated from human erythrocyte ghosts and has been shown to remove phosphate from the 5-position of $InsP_3$ to give inositol 1,4-bisphosphate, a compound which possesses no calcium-releasing activity [1,17]. Both low free magnesium concentrations and DPG inhibit the action of this enzyme and both have been reported to enhance $InsP_3$-induced contractions from frog skinned fibres [7]. The K_i for DPG is 3.5 10^{-4} M and the K_m for the enzyme is 2×10^{-5} M [17], so that in the presence of 100 μM $InsP_3$, assuming simple Michaelis-Menten kinetics for competitive inhibition, it is estimated that 15 mM DPG should reduce the rate of breakdown of $InsP_3$ by 90%. We therefore included 15 mM DPG in the bathing solutions to inhibit the action of the enzyme if it is present in skinned muscle fibres. Nevertheless, no $InsP_3$ response was obtained in either barnacle or frog preparations when the inhibitor was present (e.g. fig.2). Similarly, the free Mg^{2+} concentration was lowered from 1 mM to 0.5, 0.3 mM and 40 μM without revealing any contractions on addition of $InsP_3$ (fig.1d, table 1). Barnacle myofibrillar bundles gave a phasic contraction on being placed in low Mg^{2+} solutions (fig.1d); loading of the SR after this in solutions of p*Ca* 6.4 for 2 min did not produce a positive result with $InsP_3$.

A low free Mg^{2+} concentration might have been expected to potentiate calcium release in a second way, since it has been reported to enhance both Ca^{2+}-induced and caffeine-induced calcium release mechanisms. Mg^{2+} appears to compete with the calcium binding site on the SR and additionally block the calcium channel [18]. Indeed, it occurred to us that the reported $InsP_3$-induced contractions in low free Mg^{2+} concentrations [7] might be due to binding of Mg^{2+} in solution by $InsP_3$ and a reduction in the free Mg^{2+}. Since $InsP_3$-metal binding constants could not be found in the literature, we used arsenazo III absorbance at 624 nm to measure the free Mg^{2+} levels in modified low relaxing solutions (0.2 mM EGTA, no ATP or creatine phosphate). The presence of 350 μM $InsP_3$ in solutions containing 0–500 μM $MgCl_2$ did not reduce the estimated free Mg^{2+} (fig.3); there was a small increase in fact but this is attributed to experimental error. Measurements which were made in a similar way in the presence of 350 μM ATP were consistent with a binding constant for MgATP of 5400 M^{-1}, agreeing approximately with published constants. It is concluded that the binding of Mg^{2+} by $InsP_3$ in our solutions was negligible compared with that by ATP.

The preceding experiments with frog skinned



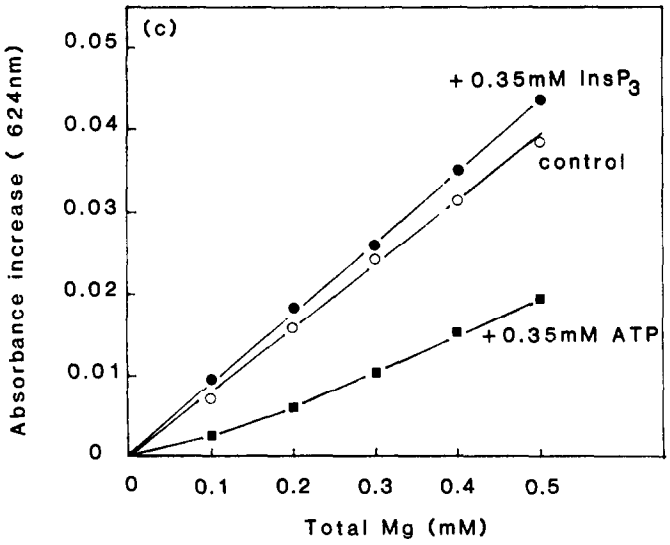


Fig.3. Does $InsP_3$ bind Mg^{2+}? (a,b) Differential absorption spectra of Arsenazo III in the presence (b) and absence (a) of 0.35 mM $InsP_3$ for different concentrations of total Mg^{2+} (added as $MgCl_2$ in 20 mM Tes, pH 7.1). Solutions contained (mM): 150 KCl, 60 Tes, 0.2 EGTA, 0.02 Arsenazo III, pH 7.1, p*Ca* 8; temperature, 20°C. In the control solution the free Mg^{2+} concentration is 99% of the total Mg (calculated using the metal binding programme). (c) Absorbance increase at 624 nm wavelength as a function of total Mg added: control (○), with 0.35 mM $InsP_3$ (●) and 0.35 mM ATP (■). The free Mg^{2+} in the ATP solution was measured by treating the control curve as a calibration for free Mg^{2+}. A binding constant for MgATP of 5400 M^{-1} was obtained from a Scatchard plot of Mg_{bound}/Mg_{free} against Mg_{bound}. There was no evidence of binding of Mg^{2+} by $InsP_3$.

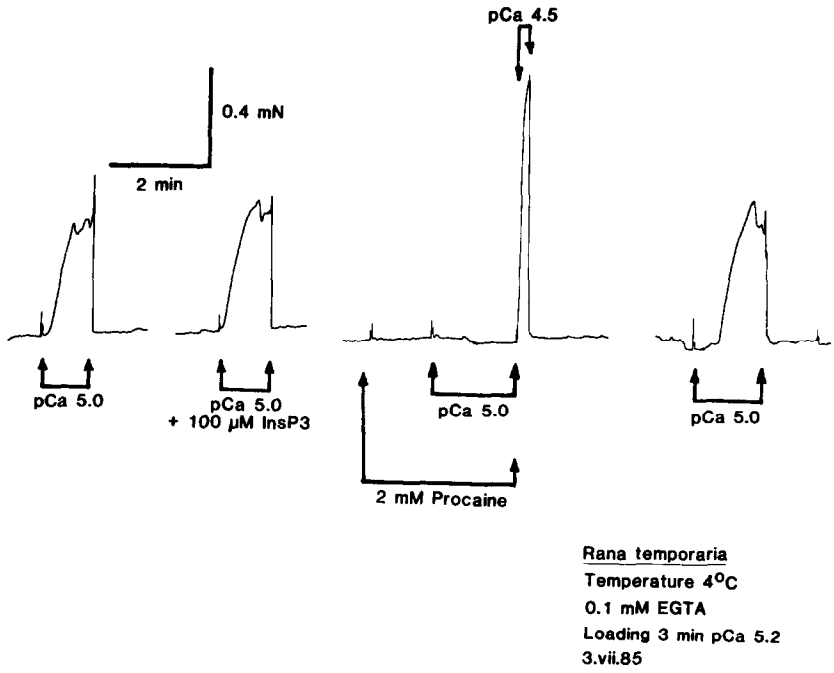


Fig.4. $InsP_3$ action on Ca^{2+}-induced Ca^{2+} release from frog skinned muscle fibre. 2 mM procaine, which has no effect on the force-p*Ca* curve, suppresses the tension response to p*Ca* 5.0, suggesting that this response is due to SR Ca^{2+} release. Subsequent activation at p*Ca* 4.5 in the presence of 25 mM EGTA gives a measure of maximum isometric tension. Fibre was exposed to a p*Ca* 5.2 loading solution for 3 min before each response. (3.vii.85). Fibre diameter: 100 μm.

muscle fibres were carried out at a p*Ca* of 7.0. An increase in solution free Ca^{2+} to 10^{-5} M in the presence of 0.1 mM EGTA is sufficient to cause a contraction (fig.4) which is most likely due to calcium-induced calcium release [18]. The responses to p*Ca* 5.0 were inhibited by the SR calcium release inhibitor procaine at a concentration (2 mM) which does not affect the p*Ca*-tension curve [20], demonstrating that they were the result of calcium release from the SR. An addition of 100 μM $InsP_3$ to the p*Ca* 5.0 solution did not affect the amplitude or the rise time of the resulting contraction. The contractions were submaximal in amplitude as demonstrated by the subsequent application of a p*Ca* 4.5 solution (fig.4). Oscillating contractions in response to p*Ca* 5.0 were sometimes obtained; neither the falling phase of the contraction, nor the frequency of the oscillation was affected by $InsP_3$ (100 μM).

Caffeine (2 mM) is an effective releaser of SR calcium at a p*Ca* of 7; the addition of 100 μM $InsP_3$ did not affect the amplitude or the time course of the phasic contractions due to caffeine in either frog or barnacle. Removal of EGTA from the bathing solution to reduce the buffering of any released calcium and variation of temperature from 4 to 22°C failed to reveal any response to $InsP_3$. The guanidine nucleotide GTP has been implicated in the cellular reactions in which $InsP_3$ is released from the cell membranes and then mobilises stored calcium [21,22,25]. At a concentration of 50 μM, it has been reported to enhance the $InsP_3$-induced release of calcium from liver microsomes [25]. However with frog skinned fibres, we did not find that 50 μM GTP revealed any response to $InsP_3$.

The force-p*Ca* relationship of frog skinned muscle fibres was investigated in solutions containing 25 mM total EGTA at 1 mM free Mg^{2+}; $InsP_3$ (250 μM) was without effect on this curve.

A more sensitive method of detecting calcium release from the SR of myofibrillar bundles was also employed, namely the measurement of the light output from bundles containing the calcium-

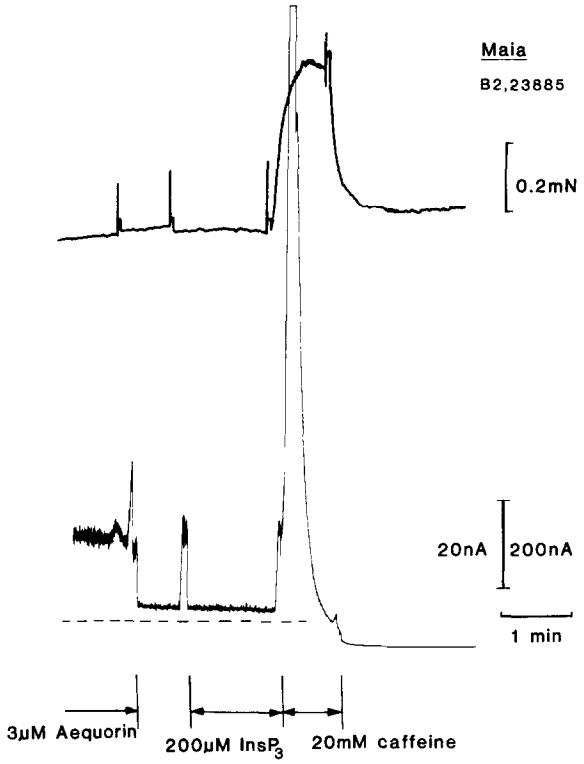


Fig.5. Isometric tension (top trace) and aequorin light (bottom trace) from a *Maia* myofibrillar bundle (diameter 200 μm) exposed to $InsP_3$ and caffeine. The bundle was first 'loaded' with aequorin by immersion for 7 min in a relaxing solution containing 3 μM aequorin; the beginning of the traces shows the end of this loading period. Caffeine caused the light signal to go off scale on the pen recorder, so the recording sensitivity was reduced by a factor of 10 during the response. The dashed line represents zero light at the initial recording sensitivity. The peak light during the response to caffeine is estimated to be 60-times the size of the solution changing artefact seen at the start of the exposure to $InsP_3$, i.e. 1.2 μA. Bathing solutions as for barnacle: 900 mM sucrose, p*Ca* = 7.0, p*Mg* = 3.0, pH = 7.1. Temperature, 14°C. Experiment B2, 23885.

sensitive photoprotein, aequorin (e.g. [27]). A *Maia* bundle was first immersed in relaxing solution containing aequorin (3 μM) for 7 min; this should have allowed time for the concentration of the aequorin in a bundle of 200 μm diameter to reach 95% of that in the bathing solution [28]. The bundle was then transferred to aequorin-free solutions. Fig.5 shows that 200 μM $InsP_3$ failed to increase the light level from an aequorin-'loaded' bundle, whereas the subsequent transfer to a solution containing caffeine caused an immediate increase in both light and isometric tension. A similar result was obtained from a second myofibrillar bundle. The increase in light caused by caffeine demonstrates that there was a significant aequorin concentration in the bundle when $InsP_3$ was applied. This is also confirmed by studies on the rate of aequorin loss from barnacle myofibrillar bundles, which have shown that at the time of transfer to $InsP_3$ the aequorin concentration in a bundle of 200 μm diameter would have been 35% of the initial loading concentration [29].

4. DISCUSSION

We have been unable to obtain the significant $InsP_3$-induced contractions that have been reported by two other groups using frog [7] and rabbit [3] skinned muscle fibres; for example Volpe et al. [3] found contractions which had a rate of rise as high as 50% of that due to 10 mM caffeine. Our conditions were similar to those used by Vergara et al. [7]; in particular, they reduced the free Mg^{2+} concentration to the low values of 0.5–0.04 mM and used $InsP_3$ concentrations of up to 500 μM in the presence of 0.1 mM EGTA. The method of skinning of the muscle fibres does not seem to be important since $InsP_3$-induced contractions were reported for both mechanically [7] and chemically skinned muscle fibres [3]; we did not test $InsP_3$ on chemically skinned fibres, however. The different sources of the $InsP_3$ could be a possible cause of the differences in its effect, although Volpe et al. [3] report that $InsP_3$ derived from ox brain gave comparable results to those obtained with $InsP_3$ samples which they prepared from human erythrocyte ghosts. It is not clear what the free Ca^{2+} concentrations were in the $InsP_3$-containing solutions of Volpe et al. [3]; the absence of EGTA from their solutions may have meant that the free Ca^{2+} level, being only buffered by ATP, was close to the threshold for calcium-induced calcium release. Propionate [3] and aspartate [7], anions with relatively low membrane permeabilities, were used in the bathing solutions by the other two groups. We found that barnacle myofibrillar bundles were still unresponsive to $InsP_3$ when the main anion of the bathing solution was propionate or methylsulphate.

We have considered two other reasons for our

inability to detect $InsP_3$-induced contractions of skinned fibres. First, the immediate uptake of released calcium back into the SR may have prevented the development of any tension response. However, the negative results obtained with inhibitors of calcium uptake such as quercetin and cadmium do not support this. Second, the presence in the skinned fibres of enzymes capable of hydrolysing $InsP_3$ may have prevented the compound from reaching a sufficiently high concentration in the vicinity of the SR for it to activate calcium release. This explanation is not considered likely in view of the failure of two inhibitors of the enzyme inositol trisphosphatase, DPG and low Mg^{2+}, to reveal a positive response to $InsP_3$. The possibility that muscle fibres possess enzymes other than inositol trisphosphatase which are capable of inactivating $InsP_3$ cannot be discounted at present.

Another reason for the lack of effect of $InsP_3$ on barnacle preparations compared with the reported effects on frog and rabbit skinned fibres could be that the physiological calcium release mechanism of invertebrate SR differs fundamentally from that of vertebrate SR.

Although we have obtained little evidence from barnacle or frog muscle to support the proposed role of $InsP_3$ as an essential link in excitation-contraction coupling [3,7], we are unable to reject this theory totally since we cannot exclude the possibility that there exists a powerful $InsP_3$ inactivation system in muscle which is insensitive to DPG and low Mg^{2+}, or that other essential cofactors are required for the proposed action of $InsP_3$ and are lost in the skinned preparation.

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