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Excitation-contraction coupling in skeletal muscle fibers injected with the $InsP_3$ blocker, heparin

P.C. Pape, M. Konishi, S.M. Baylor and A.P. Somlyo

Department of Physiology and Pennsylvania Muscle Institute, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6085, USA

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Heparin, an inhibitor of inositol trisphosphate ($InsP_3$)-induced Ca^{2+} release in smooth muscle and non-muscle cells, was injected into intact frog skeletal muscle fibres. Ca^{2+} release from the sarcoplasmic reticulum was elicited by the normal action potential mechanism and monitored by both fura-2 fluorescence and an intrinsic birefringence signal. Both optical signals, and hence Ca^{2+} release, were unaffected by high concentrations of heparin. This result argues against a major physiological role of $InsP_3$ as a chemical messenger of excitation-contraction coupling in skeletal muscle.

Excitation-contraction coupling; Inositol trisphosphate; Heparin; Sarcoplasmic reticulum; Ca^{2+}; (Skeletal muscle)

1. INTRODUCTION

The enigma of excitation-contraction (E-C) coupling in skeletal muscle – how depolarization of the transverse tubular membranes causes the release of Ca^{2+} from the sarcoplasmic reticulum (SR) – remains one of the major problems of muscle physiology [1]. One of the two prevalent hypotheses is that depolarization releases a chemical messenger that opens Ca^{2+} channels in the membranes of the SR, while according to the other, an electrical charge movement is directly coupled to a conformational change that opens these channels [2]. The discovery that $InsP_3$ releases Ca^{2+} from the endoplasmic reticulum (ER) in non-muscle cells [3] rapidly led to suggestions that $InsP_3$ may also be the postulated chemical messenger for E-C coupling [4,5].

In smooth muscle, agonists can induce Ca^{2+} release from the SR independently of changes in the surface membrane potential, and it has been suggested that this pharmaco-mechanical Ca^{2+} release is mediated by $InsP_3$ [6,7]. More recently, heparin was shown to inhibit $InsP_3$ binding to cerebral receptors [8] and to block $InsP_3$-induced Ca^{2+} release in liver [9], pancreas [10] and smooth muscle [11] cells. The purpose of the present study was to determine whether heparin also blocks the normal E-C coupling mechanism in skeletal muscle, as might be expected if $InsP_3$ were the physiological messenger.

Correspondence address: S.M. Baylor, Department of Physiology and Pennsylvania Muscle Institute, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6085, USA

2. MATERIALS AND METHODS

Intact single twitch fibres were dissected from leg muscles (semi-tendinosus and iliofibularis) of English frogs (*Rana temporaria*) and mounted in a transparent chamber on an optical bench apparatus for measurement of optical signals related to E-C coupling [12,13]. In order to minimize contributions from movement artifacts in the optical records, the fibres were stretched to long sarcomere spacing (3.8–4.3 μm) and lowered onto pedestal supports. To examine the effect of heparin on Ca^{2+} release from the sarcoplasmic reticulum, single fibres were impaled with a glass micropipette containing a mixture of heparin and the 'free acid' form of the Ca^{2+}-indicator dye fura-2. This mixture was then introduced directly into the myoplasmic space by means of air pressure applied to the back end of the micropipette. The Ringer's solution bathing the fibres contained (in mM): 120 NaCl, 2.5 KCl, 11.8 $CaCl_2$ and 5 Na_2Pipes [sodium

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salt of piperazine-*N,N'*-bis(2-ethanesulphonic acid), pH 7.1], at 16°C. The use of the high-Ca^{2+} Ringer's solution has been found to improve generally fibre viability following microinjection, without significantly altering the amplitude and time course of Ca^{2+} release from the SR when stimulated by a single action potential [13]. Only fibres that gave all-or-none optical responses to action potentials generated by point electrical stimulation were used.

The heparin used in the experiments was of low molecular weight (M_r 4000–6000; product H5640, from Sigma, St. Louis, MO) and was from the same lots as found to have a strong blocking action on $InsP_3$-stimulated Ca^{2+} release from smooth muscle SR [11]. Fura-2 [14] was obtained as the pentapotassium salt of the dye (Molecular Probes, Eugene, OR). Its concentration in the pipette-injection solution was determined by absorbance measurements made in a spectrophotometer. The presence of 4200 μg/ml heparin did not significantly change the absorbance spectrum of Ca^{2+}-free fura-2; nor did this large concentration have any detectable effect on fura-2's dissociation constant for Ca^{2+}, measured by titration in free [Ca^{2+}] solutions buffered with EGTA. Two concentrations of heparin and fura-2 were used in the injection solutions: (i) 833 μg/ml heparin and 3.6 mM fura-2; (ii) 42000 μg/ml heparin and 1.2 mM fura-2. The fura-2 concentrations in the pipette were deliberately kept low in order to avoid the Ca^{2+}-buffering effects of high myoplasmic fura-2 concentrations [13].

Three types of fura-2 signals were measured from the heparin/fura-2 injected fibres, with all measurements carried out by procedures identical to those described [13]: (i) steady-state fura-2 fluorescence from fibres at rest; (ii) changes in fura-2 fluorescence as a result of electrical stimulation; and (iii) changes in fura-2 absorbance as a result of electrical stimulation. For these measurements, a 420 (± 15) nm excitation beam from a tungsten-halogen source was selected by means of a wide-band interference filter (Omega Optical, Brattleborough, VT) positioned between the light source and the muscle fibre. Fluorescence was measured with a 495 nm 'cut-on' filter (OG-495; Schott Optical Glass, Duryea, PA) positioned between the fibre and the photo-detector (a UV-100 silicon diode; E.G. and G., Salem, MA). With these procedures any contribution from fibre intrinsic fluorescence was negligible; thus, the photo-detector output, when corrected for the band-pass overlap of the filters, was proportional to fura-2 fluorescence. Since Ca^{2+}-bound fura-2 has a negligible fluorescence when excited with 420 nm light, the experimental measurements gave direct information about Ca^{2+}-free fura-2 in myoplasm. Although the use of a tungsten-halogen light source, which emits radiation only weakly below 400 nm, did not maximize the signal-to-noise ratio of fura-2's fluorescence, the resting and active dye signals measured by this method were in fact well resolved (cf. figs 1A,2A).

For the fura-2 absorbance measurements, the fibre was trans-illuminated with a spot of light (diameter, 50 μm), the fluorescence cut-on filter was not used, and changes in 420 nm light intensity during activity (ΔI), divided by the steady-state value of transmitted light intensity prior to activity (I), were measured. Changes in fibre absorbance (ΔA) were calculated according to the equation:

$$\Delta A = -(\Delta I/I)/(\log_e 10) \quad (1)$$

In order to obtain the fura-2-related ΔA, it was necessary to correct the raw measurement at 420 nm for changes in fibre intrinsic absorbance, estimated from analogous ΔA measurements made at 480 nm. After correction, the fura-2-related component of ΔA at 420 nm (denoted $\Delta A(420)$) was calibrated in terms of the concentration of Ca^{2+}–fura-2 complex formed during activity (denoted Δ[CaFura-2]) by means of Beer's law:

$$\Delta[\text{CaFura-2}] = \Delta A(420)/(\Delta\epsilon(420)l) \quad (2)$$

In eqn 2, l denotes the path length of the light within the myoplasmic volume and $\Delta\epsilon(420)$ represents the difference in molar extinction coefficients of fura-2 between its Ca^{2+}-bound and Ca^{2+}-free forms [13].

Given the ΔA calibration in eqn 2, the simultaneously measured fura-2 fluorescence signals, both at rest and during activity, could thus be converted to micromolar concentrations of Ca^{2+}-free and Ca^{2+}-bound fura-2, respectively [13]. This calibration procedure was important because the heparin concentrations in myoplasm resulting from the pressure injection were not directly measured. Rather, heparin concentrations were estimated from other measured quantities, namely (i) the ratio of concentrations of fura-2 and heparin in the micropipette injection solution, and (ii) the total myoplasmic concentrations of fura-2 obtained as a function of distance from the injection site. Although the estimated heparin concentrations depend on the value assumed for heparin's diffusion constant in myoplasm (see legend to fig.2B), the peak values estimated for the concentration at the injection site (cf. location 0 in fig.2B) are likely to be a lower limit of the actual concentrations. This follows because heparin's diffusion constant in myoplasm is likely to be smaller than that of fura-2, since its size (M_r 4000–6000) is considerably larger than that of fura-2 (M_r 637). In all cases, the concentrations of heparin and fura-2 given in section 3 have been referred to the myoplasmic water space, under the assumption that myoplasmic water occupies 70% of fibre volume [15].

A second optical signal was also used to assess the effect of injected heparin on E-C coupling, namely, the 'second component' of the intrinsic birefringence signal [12]. In order to measure the birefringence signal, the fibre was positioned between crossed polarisers oriented at ± 45° with respect to the fibre axis and illuminated with 700–750 nm light, a wavelength range beyond the absorbance band of fura-2. The birefringence signal is reported in units of $\Delta I/I$, i.e. the change in light intensity during activity (ΔI) divided by the resting intensity (I). The rising phase, voltage dependence and other properties of this birefringence signal are closely similar to those of the myoplasmic free [Ca^{2+}] transient monitored with Ca^{2+}-indicator dyes [16–19]. This similarity and other evidence [20] indicate that the birefringence signal probably reflects the transient binding of Ca^{2+} to an intrinsic Ca^{2+} receptor. Thus, the birefringence signal and the fura-2 Ca^{2+} signal were expected to give qualitatively similar indications of the effects of heparin on E-C coupling.

3. RESULTS

After injection of any water-soluble substance into myoplasm, including substances of M_r as high as 20000 [21,22], diffusion of the substance

establishes a concentration profile as a function of distance from the injection site. Thus, the concentration dependence of possible effects of injected substances on the E-C coupling process can be estimated in the same fibre at about the same time by measuring the optical signals at different distances from the injection site.

Fig.1 shows the fura-2 fluorescence (panel A) and intrinsic birefringence (panel B) signals elicited by action potential stimulation, for the fibre containing the highest concentration of injected heparin in these experiments. As observed in the absence of heparin [13], the amplitude of the [CaFura-2] response was largest at the measurement site closest to the point of injection (trace labelled + 50 μm), where fura-2 concentration was greatest (fig.2A), while at other locations, the amplitude of the [CaFura-2] response decreased with increasing distance from the injection site. The time course of all fura-2 signals was closely similar (time-to-peak about 20 ms after stimulation, signal half-width about 60 ms). Moreover, signal amplitude at the various locations, assessed as either peak value or rate-of-rise, was approximately linear with fura-2 concentration (fig.2C), as expected for a Ca^{2+}-dye reaction with 1:1 stoichiometry [14] and an underlying free [Ca^{2+}] transient that was similar for all fibre locations. These features, as well as the absolute amplitude of the fura-2 Ca^{2+} signals per unit dye concentration, are identical to those described for fibres injected with fura-2 alone [13], despite the wide range of heparin concentrations involved (see the heparin concentration profile estimated in fig.2B). Thus, the fura-2 measurements indicate that injected heparin had no detectable effect on the myoplasmic free [Ca^{2+}] transient.

Figs 1B and 2D show that the Ca^{2+}-related component of the birefringence signal (early downward deflection in fig.1B) was also very similar in amplitude and time course at the different fibre locations. (Note that variations in the later time

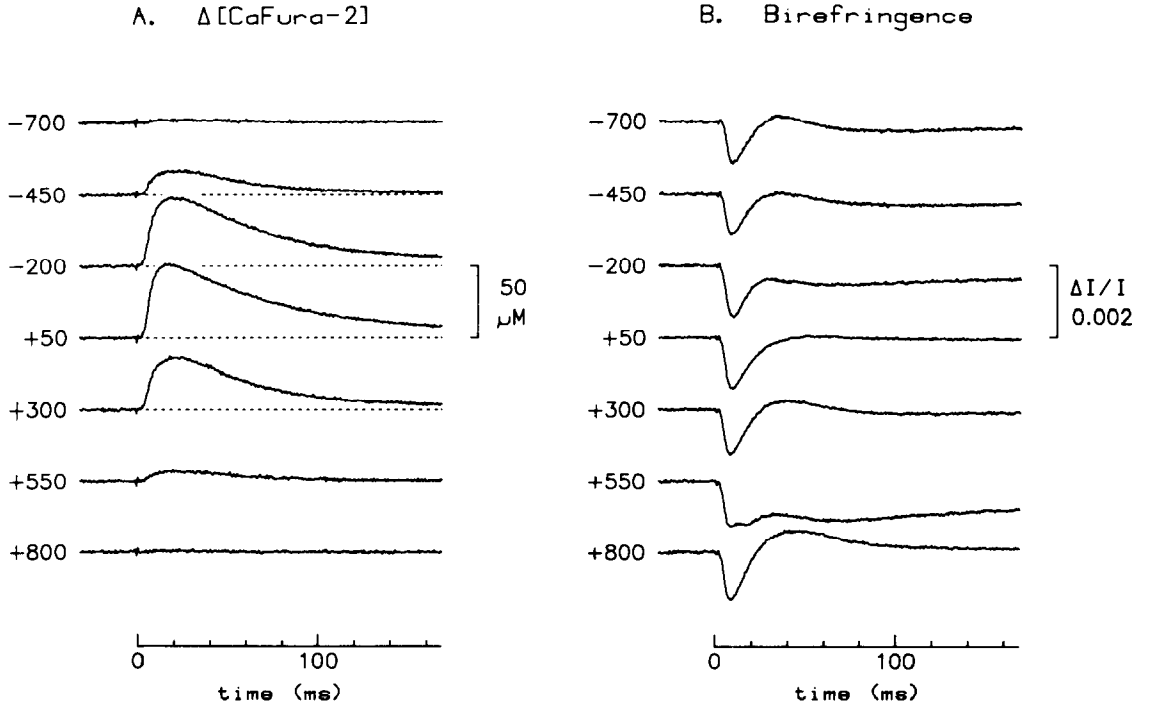

Fig.1. Changes in fura-2 fluorescence (A) and in intrinsic birefringence (B) recorded at different distances (indicated in μm to the left of each trace) from the site of the heparin plus fura-2 injection. Zero time marks the moment of an external shock. The measurements in (A) were made 15–17 min after injection and in (B) 8–10 min after injection. In both parts, the fibre was illuminated with a 90 μm slit of light oriented transversely to the fibre axis. All traces are single sweeps except for those at location + 50 μm, which show the average of 2 such sweeps taken at the beginning and end of each run.

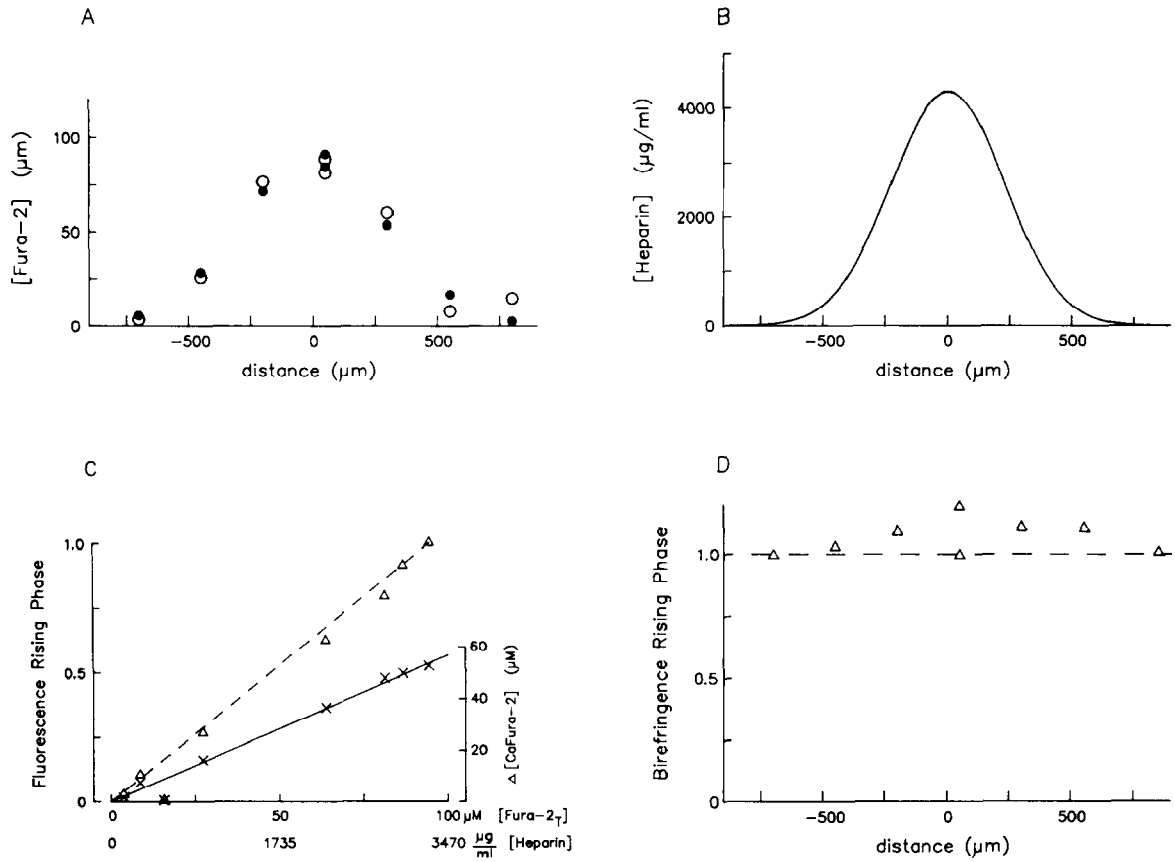

Fig.2. (A) (○) Concentration of Ca^{2+}-free fura-2 prior to stimulation, [Fura-2] (ordinate), as a function of distance from the injection site (abscissa), from the same run as shown in fig.1A. The values of [Fura-2] were estimated from fura-2's resting fluorescence at each measurement location. (●) Best-fit to the fura-2 concentration profile by means of the one-dimensional diffusion equation: $[\text{Fura-2}](x,t) \propto (1/2)(\pi D_{app}t)^{-0.5}\exp(-x^2)/(4D_{app}t)$, where x is distance from the injection site and t is time following injection (cf. [13]). The fitted value of D_{app}, the apparent diffusion constant of fura-2 in myoplasm, was 0.47×10^{-6} cm$^2 \cdot$s^{-1}. (B) Myoplasmic concentration profile of heparin estimated at 9 min after injection, the time of the measurements in fig.1B. The curve was calculated from the one-dimensional diffusion equation using the conservative assumption that the apparent diffusion constant for heparin was the same as estimated for fura-2 in (A). (C) Relationship between the amplitude of the fura-2 signal accompanying activity and the fura-2 concentration. Left-hand ordinate: best-fitted scaling factors (Δ) which relate the amplitude of the rising phase (up to 75% of peak value) of the Ca^{2+}–fura-2 signals in fig.1A to that observed at +50 μm, the location with the largest measured signal. The abscissa plots total fura-2 concentration in myoplasm, [Fura-2$_T$] (assumed to equal 1.06 × [Fura-2]; see [13]), as well as the heparin concentration estimated assuming that the ratio of heparin to [Fura-2$_T$] at each point in the diffusion profile was the same as that in the injection solution. The dashed line corresponds to a linear relationship between the fitted values and the fura-2 concentrations. Right-hand ordinate: peak value (×) of the Ca^{2+}–fura-2 signals in fig.1A vs [Fura-2$_T$]. The solid line shows the best-fitted line to the data. Both rising phase and peak value data were analyzed in order to minimize the effects of indicator saturation associated with the relatively large Δ[CaFura-2] response. (D) Best-fitted scaling factors (Δ) that relate the amplitude of the 'rising' phase (initial downward deflection, to 85% of peak value) of the birefringence signals in fig.1B to the average rising phase of the signals measured at −700 and +800 μm from the site of the heparin/fura-2 injection. The dashed line corresponds to an identical amplitude rising phase at all fibre locations (and heparin/fura-2 concentrations). The scatter about the line is similar to that seen in fibres not containing heparin. The smaller of the two points shown at location +50 μm was obtained at 8 min after injection of heparin.

course of the birefringence signals are attributable to movement artifacts accompanying the development of fibre tension). Since the birefringence signals observed in this experiment were essentially identical to each other and to signals routinely observed in un-injected fibres, the birefringence measurements confirm that the presence of heparin in myoplasm had no significant effect on

the underlying Ca^{2+} transient at any concentration between 0 and 4000 μg/ml.

A similar lack of effect on both optical signals was observed in 5 other fibres containing injected heparin spanning a wide range of concentrations (estimated peak values at the injection site at the time of the optical measurements of 902, 492, 375, 42 and 25 μg/ml).

4. DISCUSSION

Intracellular heparin, at concentrations as high as 4000 μg/ml, was found to be completely ineffective in reducing myoplasmic Ca^{2+} signals resulting from the normal E-C coupling process. The greatest myoplasmic concentration achieved in these experiments is some 300–800-fold larger than the IC_{50} (the concentration for half-inhibition) of $InsP_3$-induced Ca^{2+} release in smooth muscle, 5 μg/ml [11], and in non-muscle cells, 5–15 μg/ml [9,10], as well as of $InsP_3$ binding to receptors in brain, 5 μg/ml [8]. In contrast, in our intact frog skeletal muscle fibres, 5–10-fold smaller quantities of injected ruthenium red, a compound with an IC_{50} for blocking Ca^{2+} release in skeletal muscle [23] that is similar (on a molar basis) to the above heparin values, effectively blocked the Ca^{2+}-related optical signals quantitated by a similar method [24]. Thus, we can report with confidence that heparin has little or no blocking effect on the physiological Ca^{2+} release mechanism in intact skeletal muscle fibres.

The low sensitivity and inconsistent response of skinned skeletal muscle fibres to applied $InsP_3$ [25], the very slow contractions evoked by photolytic release of $InsP_3$ [25], the lack of response of intact (not skinned) frog skeletal muscle fibres to injected $InsP_3$ [26], the low enzyme activity for degradation of $InsP_3$ [27], and the failure reported here of heparin to inhibit Ca^{2+} release from the SR constitute a significant body of evidence against the hypothesis that $InsP_3$ is the chemical messenger of E-C coupling in skeletal muscle. It might be argued that this negative evidence does not conclusively rule out a messenger role. For example, in the intact fibre $InsP_3$-sensitive Ca^{2+} channels might be localized to a compartment not in diffusional contact with injected $InsP_3$ or heparin but to which ruthenium red somehow still has access. Barring demonstration of such a compartment, however, the most straightforward interpretation of this body of experimental results is that $InsP_3$ does not serve as the physiological messenger that triggers Ca^{2+} release in skeletal muscle.

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