Effects of heparin on excitation–contraction coupling in skeletal muscle of toad and rat

G. D. Lamb, G. S. Posterino and D. G. Stephenson

Department of Zoology, La Trobe University, Bundoora, Victoria 3083, Australia

1. Intracellularly applied heparin was found to cause a novel, use-dependent block of excitation–contraction (E–C) coupling in skinned skeletal muscle fibres of the toad. After one to four depolarizations in the presence of 100 μg ml^{-1} heparin, no further depolarization-induced responses could be elicited, even though addition of caffeine or lowering [Mg^{2+}] could still induce massive Ca^{2+} release. This effect could not be reversed by extensive wash-out of the heparin (> 15 min).

2. Heparin (100 μg ml^{-1}) did *not* abolish subsequent depolarization-induced responses if applied while the voltage sensors were in either their resting or inactivated states, that is (a) while a fibre remained fully polarized, (b) when a fibre was already chronically depolarized or (c) after a fibre had been depolarized in the presence of D600 (gallopamil) and then repolarized.

3. When a toad fibre was depolarized in heparin, with the associated Ca^{2+} release blocked by the presence of 10 mM intracellular Mg^{2+}, subsequent E–C coupling was abolished. Heparin did not interrupt E–C coupling when Ca^{2+} release was triggered in the absence of any depolarization, by either caffeine or low [Mg^{2+}]. Thus, the opening of the Ca^{2+} release channels was neither necessary nor sufficient for heparin to abolish E–C coupling.

4. Heparin had direct effects on the contractile apparatus in toad fibres, increasing the Ca^{2+} sensitivity and decreasing the maximum Ca^{2+}-activated force. These effects could only be partly reversed by extensive wash-out of heparin.

5. At 100 μg ml^{-1}, both low molecular weight heparin and pentosanpolysulphate, another highly sulphated polysaccharide, were less effective than heparin in blocking the depolarization-induced response and in changing the properties of the contractile apparatus, and these effects could be substantially reversed by wash-out. Two other polyanions, de-*N*-sulphated heparin (100 μg ml^{-1}), which lacked *N*-sulphate groups, and polyglutamate (500 μg ml^{-1}), had no measurable effect on either E–C coupling or the contractile apparatus.

6. In skinned fibres of the extensor digitorum longus muscle of the rat, 100 μg ml^{-1} heparin had little or no effect on E–C coupling and on the Ca^{2+} sensitivity of the contractile apparatus, but caused a larger reduction of the maximum Ca^{2+}-activated force than in skinned fibres of the toad.

7. These results indicate that heparin blocks E–C coupling in toad muscle if, and only if, it is present when the voltage sensors are activated by depolarization. When the possible heparin binding sites on the voltage sensor/dihydropyridine receptor are considered, these data give further insight into the molecular basis of E–C coupling.

In vertebrate skeletal muscle, an action potential triggers Ca^{2+} release and contraction by activating voltage sensor molecules in the transverse tubular (T)-system, which in turn open the Ca^{2+} release channels in the adjacent sarcoplasmic reticulum (SR) (Lüttgau & Stephenson, 1986; Ashley, Mulligan & Lea, 1991; Rios & Pizarro, 1991). Exactly how the voltage sensors activate the Ca^{2+} release channels is not known. There may be a relatively direct physical connection between the two molecules (Chandler, Rakowski & Schneider, 1976; Brandt, Caswell, Wen & Talvenheimo, 1990) or a diffusable second messenger may act as the link. One such second messenger could be inositol trisphosphate ($Ins P_3$) (Volpe, Salviati, Di Virgilio & Pozzan, 1985; Vergara, Tsien & Delay, 1986; Jaimovich, 1991), although its involvement in excitation–contraction (E–C) coupling has been disputed (Lea, Griffiths, Tregear & Ashley, 1986; Walker, Somlyo, Goldman, Somlyo & Trentham, 1987; Pape, Konishi, Baylor & Somlyo, 1988).

If $InsP_3$ does link the voltage sensors to the release channels, an agent which blocks $InsP_3$ binding should also block normal E–C coupling. Heparin and low molecular weight heparin block $InsP_3$ receptor binding in brain (Worley, Baraban, Supattapone, Wilson & Snyder, 1987) and low molecular weight heparin blocks $InsP_3$-induced Ca^{2+} release in smooth muscle (Kobayashi, Somlyo & Somlyo, 1988), at a half-maximal concentration of 5 μg ml^{-1}. Pape *et al.* (1988) found that injection of up to 4 mg ml^{-1} low molecular weight heparin in skeletal muscle fibres of frog did not affect the Ca^{2+} release induced by action potential stimulation. However, using a scratched fibre preparation, Rojas & Jaimovich (1990) found that the same low molecular weight heparin did not prevent $InsP_3$-induced Ca^{2+} release in frog skeletal muscle, suggesting that this heparin did not block the relevant $InsP_3$ receptor.

Here, using a skinned fibre preparation in which the intracellular environment can be readily altered (Lamb & Stephenson, 1990*a*), we investigated whether either heparin or low molecular weight heparin affected normal E–C coupling in skeletal muscle. In this manner, we could ensure that the heparin concentration within the fibre was raised adequately near the key sites, regardless of the degree of binding to other structures. Surprisingly, we found that heparin caused a unique, *activation-dependent* block of E–C coupling in toad muscle fibres, with little or no effect in rat muscle fibres. The activation dependence of the block in toad muscle is not consistent with the simplest form of the $InsP_3$ second messenger hypothesis but it may be explained by an action of heparin on the voltage sensor.

METHODS

The skinned fibre preparation described previously (Lamb & Stephenson, 1990*a*; 1991*b*) was used. Briefly, cane toads (*Bufo marinus*) were stunned by a blow to the head and then double pithed and the iliofibularis muscles were removed. Long Evans hooded rats (*Rattus norvegicus*) were anaesthetized with diethyl ether and then decapitated and the extensor digitorum longus (EDL) muscles were removed. The excised muscles from either animal were pinned out under paraffin oil and a segment of a single fibre was mechanically skinned and attached to a force transducer (AME875) to measure isometric contractions, which were recorded on a chart recorder (Linear). The resting length (L) and diameter (D) of the fibre were measured in paraffin oil and then the fibre was stretched a further 20% (final sarcomere length 2·6–2·8 μm for toad fibres and 3·1–3·2 μm for rat fibres). The skinned fibre was then placed in a 2 ml Perspex bath filled with potassium hexamethylene-diamine-tetraacetate (HDTA) solution with 1 mM free Mg^{2+} for 2 min before being stimulated by rapidly removing the bath and replacing it with another bath filled with an appropriate solution. All experiments were performed at room temperature (23 ± 2 °C).

Solutions

In the experiments with toad muscle, the potassium HDTA solution contained (mM): K^+, 117; Na^+, 36; $HDTA^{2-}$, 50; total ATP, 8·0; total magnesium, 8·5; creatine phosphate, 10; total EGTA, 0·05; Hepes buffer, 60; NaN_3, 1; with pH 7·10 ± 0·01 and pCa 7·0. The resulting free [Mg^{2+}] was 1 mM and the osmolality was 255 mosmol kg^{-1}. The sodium HDTA solution had the same composition as the potassium solution except that all K^+ (117 mM) was replaced by Na^+. The choline chloride (ChCl) solution contained (mM): choline chloride, 103; Na^+, 42; total magnesium, 8·15; total ATP, 8; creatine phosphate, 10; Hepes buffer, 20; EGTA (total), 0·05; pCa 7·0 and pH 7·10 ± 0·01. Solutions with 0·05 mM free Mg^{2+} had only 2·15 mM total magnesium, but were otherwise similar to the corresponding solutions with 1 mM free Mg^{2+}, in terms of total ATP, creatine phosphate, pH, pCa and osmolality. The potassium HDTA solution with 10 mM free Mg^{2+} had 22·7 mM total magnesium and the corresponding ChCl solution had 19 mM total magnesium, with the K^+ and choline concentrations reduced appropriately to give the standard osmolality. Solutions used with mammalian muscle were similar to the above toad solutions except that the osmolality was raised to about 295 mosmol kg^{-1} by increasing the concentration of Hepes by 30 mM and the concentration of K^+ or Na^+ by about 10 mM, for pH 7·1. (Note: a pH of 7·1 was used here for mammalian solutions, rather than pH 7·4 as in Lamb & Stephenson (1991*b*), to avoid any increase in Ca^{2+} leakage from the SR at the higher pH; the depolarization-induced responses were not noticeably different in the two cases.) The Ca^{2+} loading solution (pCa 5·7, 50 μM EGTA) was made from the standard K^+ solution by adding $CaCl_2$. Solutions in which the HDTA was replaced with 50 mM EGTA or 50 mM CaEGTA, and the total magnesium adjusted to give 1 mM free Mg^{2+} (Stephenson & Williams, 1981), were mixed to give solutions in which the [Ca^{2+}] was heavily buffered to particular levels, in order to determine the maximum Ca^{2+}-activated force and Ca^{2+} sensitivity of a fibre.

For the experiment in which the Ca^{2+} and Na^+ channels in the T-system needed to be blocked, the muscle was bathed in a Ringer-like solution ((mM): NaCl, 110; KCl, 4; $CaCl_2$, 1; $MgCl_2$, 2; Tes buffer (*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid), 10; pH adjusted to 7·1 with NaOH) with 10 mM $CoCl_2$ and 3 μM tetrodotoxin.

Heparins and D600

All heparin compounds and other polyanions were sodium salts and were purchased from Sigma (St Louis, MO, USA). Unless stated otherwise, 'heparin' refers to heparin from bovine lung (product no. H 9133) (MW 5000–25000). As stated in the Results, very similar results were obtained with another high molecular weight heparin from porcine intestinal mucosa (H 3125). Low molecular weight heparin (MW 4000–6000) (H 5640) and de-*N*-sulphated heparin (D 4776) are referred to as such in the text. D600 (gallopamil), a generous gift from Knoll (Tempe, NSW, Australia), was made as a 20 mM stock in ethanol shortly before use.

Labelling convention in figures and data presentation

In all force traces illustrated, the fibre was bathed in the potassium HDTA solution where no bar is shown underneath and in the ChCl solution where an unlabelled bar is present. The ± value given with mean data in the text is the standard error of the mean.

RESULTS

Effect of heparin on depolarization-induced responses in toad fibres

The transverse-tubular (T)-system of a skinned fibre could be depolarized by replacing the potassium HDTA solution

Table 1. Effect of heparin and other polyanions on depolarization-induced responses in skinned muscle fibres of the toad

	No. of depolarizations to reduce response to < 50 %	Percentage recovery after 2–15 min wash-out
Control (no heparin)	19 ± 2 (10)	—
Heparin (100 μg ml^{-1})	2·1 ± 0·2 (26)	1 ± 1 (23)
Heparin (10 μg ml^{-1})	4·2 ± 1·3 (9)	17 ± 11 (8)
Low MW heparin (100 μg ml^{-1})	6·0 ± 1·9 (3)	64 ± 9 (2)
Pentosanpolysulphate (100 μg ml^{-1})	5·3 ± 0·8 (6)	65 ± 9 (6)
De-*N*-sulphated heparin (100 μg ml^{-1})	18 ± 2 (5)	—
Polyglutamate (500 μg ml^{-1})	> 10* (4)	—

Means (± S.E.M.) derived from amplitude of response as a percentage of the initial response in each fibre. Number of fibres shown in brackets.
*Fibres with polyglutamate were only stimulated 10–12 times and showed little reduction in response. Except with polyglutamate and de-*N*-sulphated heparin, all values in column 2 (No. of depolarizations) are significantly smaller than control (Student's *t* test, $P < 0.01$). Recovery upon wash-out was examined after complete or near-complete abolition of responses. The percentage recovery following wash-out of 100 μg ml^{-1} heparin was significantly smaller than all other values in column 3 ($P < 0.01$). Dash indicates 'not applicable'.

bathing the fibre with either the sodium HDTA solution (to lower $[K^+]$) or the ChCl solution (to both lower $[K^+]$ and raise $[Cl^-]$). Such depolarization triggered Ca^{2+} release from the SR, giving a fast, transient force response (e.g. Fig. 1). As described previously (Lamb & Stephenson, 1990*a*), similar responses could be produced by this procedure about 15–25 times in each toad fibre (mean: 19 responses in 10 fibres here), provided that the fibre was repolarized in the potassium HDTA solution for about 30 s in between successive depolarizations.

However, in the presence of 100 μg ml^{-1} heparin, the depolarization-induced response in every toad fibre examined (26 fibres), was completely abolished after between one and four depolarizations (e.g. Fig. 1). The mean number of depolarizations in heparin before the response dropped below 50 % of the initial response was 2·1 ± 0·2 (S.E.M.) (Table 1). Results obtained with heparin from bovine lung (23 fibres) and from porcine intestinal mucosa (3 fibres) were not significantly different from each other, nor were results obtained using either ChCl substitution (22 fibres) or Na^+ substitution (4 fibres), so these results were all pooled (Table 1). In most of the experiments presented here, ChCl substitution was used to ensure the T-system was depolarized as strongly as possible, and care was taken to

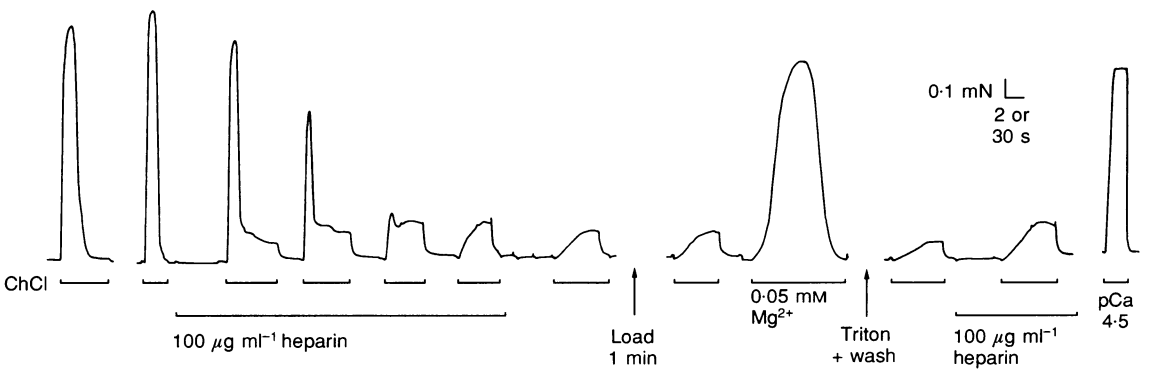

Figure 1. Effect of heparin on E–C coupling and contractile apparatus in a skinned fibre of the toad
Depolarization-induced responses to ChCl substitution were completely abolished after three depolarizations in the presence of 100 μg ml^{-1} heparin, although lowering the $[Mg^{2+}]$ to 0·05 mM still elicited a maximal force response. Heparin exposure also increased the Ca^{2+} sensitivity of the contractile apparatus, resulting in a small increase in the baseline force (i.e. in the potassium HDTA solution) and a substantial force response upon ChCl substitution. ChCl substitution elicited this same force response after all the membranes had been destroyed by treatment in Triton X-100 (3 % v/v for 5 min and wash for 5 min). The maximum force was elicited in a 50 mM CaEGTA solution (pCa 4·5). The load solution was pCa 5·7 (50 μM EGTA). Time scale: 2 s during ChCl substitution and 0·05 mM Mg^{2+} and 30 s elsewhere. Fibre diameter (D) 50 μm, fibre length (L) 2·8 mm.

avoid loading the SR too heavily with Ca^{2+} because this could result in Ca^{2+} release unrelated to depolarization of the T-system (see Lamb & Stephenson, 1990*a*). After several depolarizations in heparin, the only remaining response to ChCl (or sodium HDTA) substitution was due to a direct effect of the ions on the contractile apparatus, as can be seen by the similar response after all the membrane systems had been destroyed by treatment with Triton X-100 (Fig. 1). Except in one case where a small increase was observed, depolarization-induced responses could not be restored by trying to wash out the heparin for up to 15 min (Table 1). Nevertheless, after depolarization-induced responses had been abolished in heparin, addition of 2 mM caffeine or lowering the free $[Mg^{2+}]$ to 0·05 mM (Lamb & Stephenson, 1991*a*), induced Ca^{2+} release and a near-maximal force response in every fibre (e.g. Figs 1 and 5), showing both that the Ca^{2+} in the SR was not depleted and that the Ca^{2+} release channels were still functional.

Heparin interfered with E–C coupling in most toad fibres even at a much lower concentration. In the presence of 10 μg ml^{-1} heparin, depolarization-induced responses were completely blocked after three to seven responses in seven of the nine fibres examined, with there being a much smaller effect in the remaining two fibres. The mean number of depolarizations required to reduce the amplitude of the response to less than 50 % was 4·2 ± 1·3 (Table 1).

Effect of heparin on contractile apparatus in toad fibres

The presence of 100 μg ml^{-1} heparin caused a small increase in the 'baseline' force (i.e. when the fibre was bathed in the standard potassium HDTA solution, pCa 7·0) of about 3–10 % of maximum force in each toad fibre (e.g. Fig. 1). Such an increase in force persisted even after the membranes of the SR and other compartments had been destroyed by treatment in Triton X-100 (Fig. 1). Similarly, when Triton-treated fibres were bathed in ChCl solution (pCa 7·0) (which increases the Ca^{2+} sensitivity of the contractile apparatus relative to that in potassium HDTA solution; Fink, Stephenson & Williams, 1986), they gave little (< 2 %) or no force response before heparin exposure (not shown), and a relatively large force response (10–30 % of maximum; e.g. Fig. 1) after heparin exposure. These observations suggested that the Ca^{2+} sensitivity of the contractile apparatus was greater after exposure to heparin.

Consequently, the effect of heparin on the contractile apparatus was assessed in more detail, over a wide range of $[Ca^{2+}]$, using solutions in which the $[Ca^{2+}]$ was heavily buffered by 50 mM EGTA–CaEGTA (e.g. Fig. 2). In all nine fibres examined with 100 μg ml^{-1} heparin, the maximum Ca^{2+}-activated force was reduced (mean: 86 ± 1 % of force in the absence of heparin) and the Ca^{2+} sensitivity was increased (mean increase in pCa giving half-maximal force: 0·13 ± 0·03 pCa units). With 10 μg ml^{-1} heparin (3 fibres) or 1250 μg ml^{-1} heparin (4 fibres), the mean maximum force was reduced to 83 ± 5 and 55 ± 2 %, respectively, and the Ca^{2+} sensitivity shifted to higher pCa by 0·17 ± 0·06 and 0·30 ± 0·06 pCa units, respectively. Attempted wash-out of the heparin for even 30 min resulted in only a partial reversal of these effects; for example, note that in Fig. 1 the slow force response to ChCl substitution was still present after more than 15 min washing and Triton treatment.

Effect of heparin on E–C coupling in resting and inactivated fibres

In the twenty-six fibres examined, the mean response to the first depolarization in 100 μg ml^{-1} of heparin (present for 1 min) was 89 ± 3 % of the response before addition of heparin (e.g. Fig. 1). Clearly, E–C coupling had not been greatly affected by heparin in that time and the small decrease in the response could be accounted for entirely by the decrease in the maximum Ca^{2+}-activated force described above. After a further 1–1·5 min in heparin and two or three more depolarizations, E–C coupling was completely abolished in every fibre. To verify that this effect was not simply a function of duration of heparin exposure, other fibres were exposed for 4–5 min to the potassium HDTA solution with 100 μg ml^{-1} heparin (with pCa increased to 7·5 to prevent the SR overloading with Ca^{2+}) without being depolarized and then washed in the standard solution. After such heparin exposure, the mean response to the following depolarization was still 83 ± 10 % of the initial control response in the six fibres examined (Table 2). Thus, heparin appeared to have little effect on E–C coupling while the fibre was kept polarized, with its action appearing to depend much more on the number of depolarization-induced responses in heparin than on the duration of the heparin exposure.

This apparent dependence on the number of depolarization-induced responses could result from an underlying dependence on any of a number of associated events, such as activation or inactivation of the voltage sensors, opening of the Ca^{2+} release channels or a rise in myoplasmic $[Ca^{2+}]$.

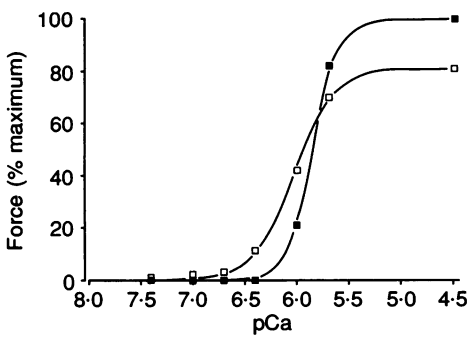

Figure 2. Effect of heparin on Ca^{2+}-activated force in a skinned fibre of the toad

The fibre was bathed in a sequence of solutions heavily buffered with 50 mM EGTA–CaEGTA to indicated $[Ca^{2+}]$ ($pCa = -\log_{10}[Ca^{2+}]$), in the absence (■) and then presence (□) of 100 μg ml^{-1} heparin, and the force expressed as a percentage of the maximum force in the absence of heparin. The maximum Ca^{2+}-activated force was decreased, and the Ca^{2+} sensitivity increased, in the presence of heparin. Data were fitted by Hill curves with coefficients of 4 (■) and 2 (□).

Table 2. Mean peak size (± S.E.M.) of depolarization-induced response for indicated treatment with 100 μg ml^{-1} heparin, as a percentage of response before treatment (see text for details)

A. First depolarization after 1 min in heparin	89 ± 3 % (26)
B. After four depolarizations in heparin	0 ± 0 % (26)
C. After 5 min exposure to heparin without any depolarizations	83 ± 10 % (6)
D. After exposure to heparin while inactivated in Na^+ solution	90 ± 3 % (7)
E. After exposure to heparin while paralysed with D600	82 ± 3 % (3)
F. After two depolarizations in 10 mM Mg^{2+} with heparin	0 ± 0 % (5)
G. After caffeine-induced Ca^{2+} release in heparin	80 ± 13 % (3)
H. After low [Mg^{2+}]-induced Ca^{2+} release in heparin	77 ± 8 % (6)

Number of fibres shown in brackets. The response following either treatment B or F was significantly smaller than that for treatment A (Student's t test, $P < 0.01$), whereas the response following each of the other treatments was not significantly different from A ($P > 0.05$). Note that exposure to 100 μg ml^{-1} heparin directly affected the contractile apparatus, resulting in an irreversible reduction in the maximum Ca^{2+}-activated force to 86 % of control (see text).

A somewhat similar use-dependent abolition of E–C coupling is seen with D600 (gallopamil) in both intact fibres (Eisenberg, McCarthy & Milton, 1983; Berwe, Gottschalk & Lüttgau, 1987) and skinned fibres (Fill & Best, 1989; Lamb & Stephenson, 1990*b*), where depolarization-induced responses are abolished after a single depolarization in the presence of about 10–30 μM D600. If D600 is applied only while a fibre is kept polarized, it does not prevent subsequent responses, and if applied while a fibre is chronically depolarized and the voltage sensors inactivated, D600 prevents subsequent responses. It has been concluded that D600 preferentially binds to the inactivated state of the voltage sensor, keeping it inactivated. In contrast, we found here that heparin did not abolish subsequent E–C coupling if it was applied only after the fibre had already been chronically depolarized. Fibres were depolarized for 1 min in the sodium HDTA solution, which initially induced a response and then made each fibre totally unresponsive to 'depolarization' by ChCl substitution (not shown), and then heparin was applied for 1 min and washed out (e.g. Fig. 3). Following such treatment and repolarization, subsequent depolarization still elicited a large response in all seven fibres examined, the mean response being 90 ± 3 % of initial control response. As might be expected, even though heparin did not abolish E–C

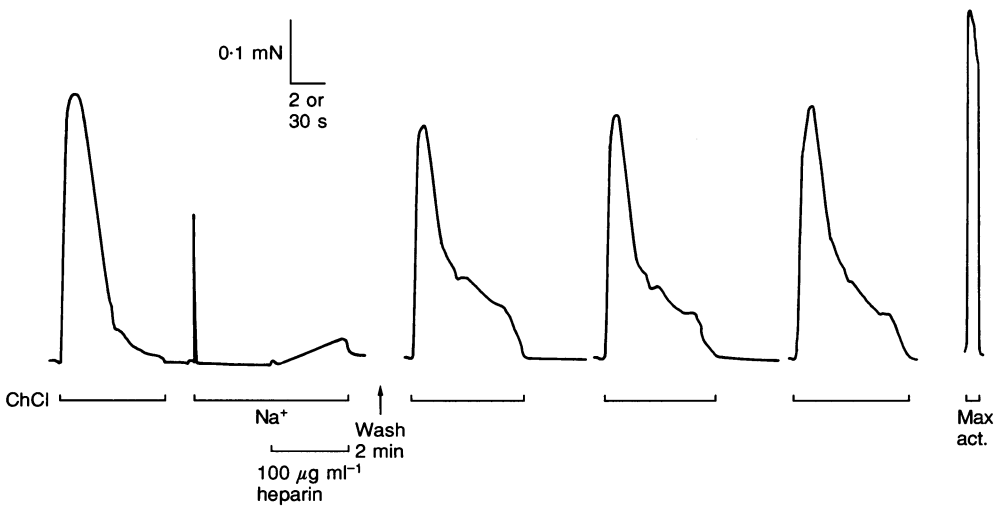

Figure 3. Heparin did not abolish E–C coupling if applied while a fibre was chronically depolarized

Large responses could be induced by depolarization with ChCl after the toad fibre had been exposed to heparin for 1 min while depolarized in the NaHDTA solution. Sixteen such responses were induced in this fibre before the response dropped to less than 50 % of the response in the absence of heparin (not shown). Note that the appearance of a 'pedestal' at the end of each depolarization-induced response indicates that the contractile apparatus was affected by the heparin, even though E–C coupling was not. Maximum activation (Max act.) in pCa 4·5 solution. Time scale: 2 s during ChCl substitution and 30 s elsewhere, including Na^+ substitution. D, 38 μm; L, 2·4 mm.

coupling when applied while the voltage sensors were inactivated, it still had the same direct effect on the contractile apparatus as described above (e.g. Fig. 3). Consequently, even the small reduction in the mean depolarization-induced response observed in this experiment was probably simply due to the reduction in the maximum Ca^{2+}-activated force.

It was also found that repeated depolarizations–repolarizations in the presence of heparin did not abolish E–C coupling, if the voltage sensors were kept in their inactivated state with D600 (e.g. Fig. 4). Each fibre was depolarized once in D600 and then repolarized and depolarized twice in the presence of heparin (100 μg ml^{-1}) with D600 still present. Control experiments showed that after a single depolarization in D600, depolarization could not elicit any response for at least 2 min, indicating that the voltage sensors remained inactivated over that period. In accordance with this, neither depolarization in the presence of heparin gave any fast rise in force in any fibre examined (e.g. Fig. 4). After washing out both the D600 and heparin for 5 min, depolarization could once again elicit a large force response (mean 82 $\pm$ 3 % in 3 fibres). As with the chronic depolarization experiments, heparin still exerted its direct effects on the contractile apparatus, which accounts for the small reduction in the peak of the depolarization-induced response. The protective effect of D600 on E–C coupling indicates that heparin does not simply interfere with the ability of ChCl substitution to depolarize the T-system by some use-dependent action on T-tubular K^+ or Na^+ channels, because D600 is not thought to have a major effect on these channels.

We further tested whether heparin might be chronically depolarizing the T-system by blocking open the voltage-dependent Ca^{2+} channels in the T-system, which are, or are closely related to, the voltage sensors (see Lamb, 1992). After the epimysium had been removed, an iliofibularis muscle was bathed for 30 min in a Ringer-type solution (see Methods) with 10 mM Co^{2+} and 3 μM tetrodotoxin, to respectively block the voltage-dependent Ca^{2+} and Na^+ channels in the T-system. The responses to depolarization by ChCl substitution in skinned fibres from this muscle were no different from control fibres. When exposed to 100 μg ml^{-1} heparin, E–C coupling was completely blocked after two to four depolarizations in all three fibres examined. Thus, it may be concluded that the abolition of E–C coupling seen with heparin is not due to it chronically depolarizing the T-system by blocking open the Ca^{2+} (or Na^+) channels. Consequently, the above experiments together imply that heparin has little or no effect on E–C coupling if applied when the voltage sensors are in their resting or inactivated states.

Heparin abolishes E–C coupling in the absence of Ca^{2+} release

To investigate whether the ability of heparin to abolish E–C coupling depended on the Ca^{2+} release elicited by a depolarization, we examined the effect of heparin when a fibre was depolarized in the presence of 10 mM free Mg^{2+}, which we have shown previously to block depolarization-induced Ca^{2+} release but not activation of the voltage sensors (Lamb & Stephenson, 1991*a*). After equilibration for 1 min in 10 mM Mg^{2+} and 100 μg ml^{-1} heparin, each fibre was depolarized twice (by ChCl substitution) and then returned to the standard potassium HDTA solution with 1 mM Mg^{2+} (e.g. Fig. 5). After such treatment, depolarization could no longer elicit a response in any of the five fibres examined (Table 2), even though addition of 2 mM caffeine induced a maximum

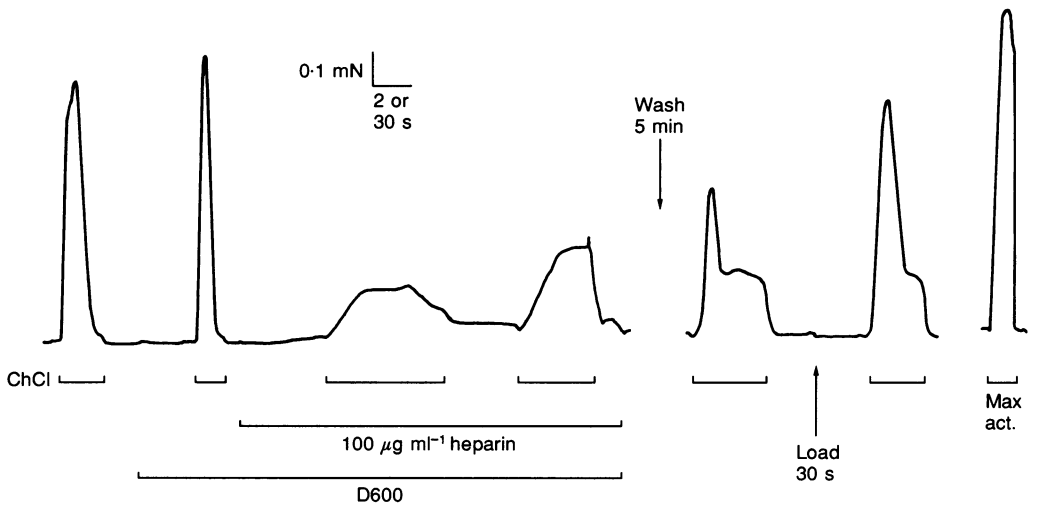

Figure 4. Heparin did not abolish E–C coupling when applied while the voltage sensors were paralysed by D600 (gallopamil)
The toad fibre was depolarized by ChCl substitution once in 20 μM D600, paralysing the voltage sensors, and then depolarized twice more in the presence of heparin. After wash-out of both D600 and heparin, E–C coupling was restored. Time scale: 2 s during ChCl depolarizations and 30 s elsewhere. D, 50 μm; L, 2·1 mm.

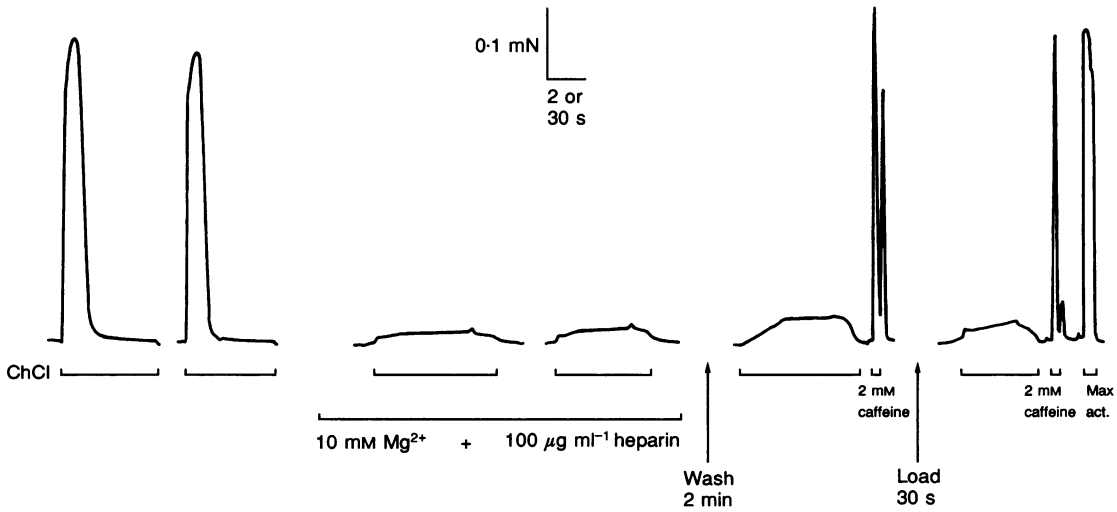

Figure 5. Heparin abolishes E–C coupling in the absence of Ca^{2+} release
The toad fibre was depolarized twice by ChCl substitution in the presence of 100 μg ml^{-1} heparin, but with 10 mM free Mg^{2+} also present to prevent the associated Ca^{2+} release. When the [Mg^{2+}] was returned to 1 mM, depolarization could no longer elicit Ca^{2+} release, even though application of 2 mM caffeine elicited a maximum response. The [Ca^{2+}] in the 10 mM Mg^{2+} solution was lowered to pCa 7·5 (by addition of 100 μM EGTA), to avoid overloading the SR with Ca^{2+} when Ca^{2+} release was abolished. Time scale: 2 s during depolarizations and 30 s elsewhere. D, 45 μm; L, 3·1 mm.

force response in each case. Control experiments showed that E–C coupling was unaffected by depolarizaring a fibre twice in 10 mM Mg^{2+} in the absence of heparin, with the mean response to the first depolarization back in the standard solution being 97 $\pm$ 2 % of the initial response in the three fibres examined. Thus, depolarization in the presence of heparin abolishes E–C coupling in toad fibres, even when the depolarization does not induce any Ca^{2+} release.

Ca^{2+} release in the presence of heparin does not abolish E–C coupling

We then investigated whether heparin affected E–C coupling if Ca^{2+} release was triggered without depolarization and accompanying voltage sensor activation. This was achieved in each of two ways. In the first, after equilibrating the toad fibre in 100 μg ml^{-1} heparin for 1 min, 5 mM caffeine was

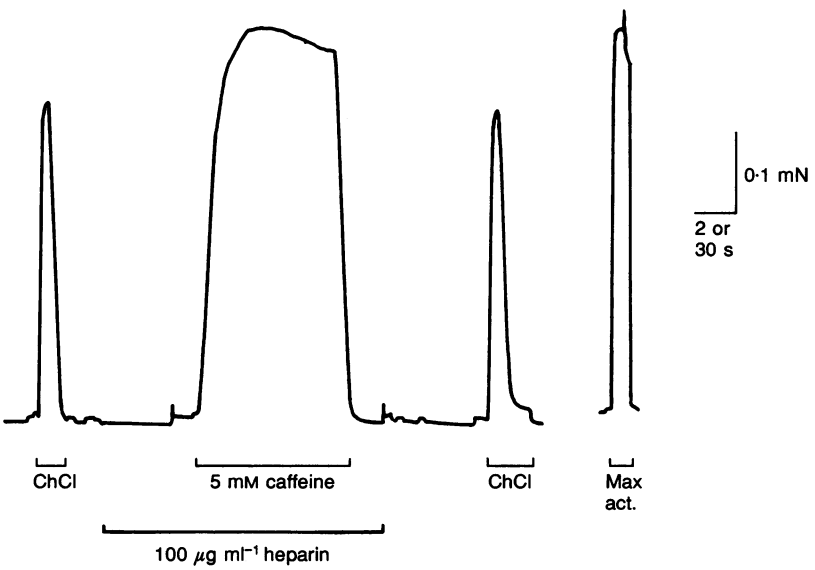

Figure 6. Heparin exposure during caffeine-induced Ca^{2+} release did not abolish subsequent depolarization-induced responses
Toad fibre. Time scale: 2 s during ChCl substitution and caffeine application, 30 s elsewhere. D, 33 μm; L, 1·9 mm.

applied to release sufficient Ca^{2+} to produce near-maximal force for 6–9 s (e.g. Fig. 6). After such treatment, depolarization could still elicit large force responses in each of the three fibres examined; the mean response to the first depolarization was 80 ± 13 % of the initial control response. In the second series of experiments, the free $[Mg^{2+}]$ was rapidly lowered from 1 to 0·05 mM to trigger Ca^{2+} release (Lamb & Stephenson, 1991*a*) in the presence of heparin. This produced a near-maximal force response lasting about 12–15 s in each fibre (not shown), but subsequent depolarization in the standard solutions could still elicit a large force response in each case, with the mean response being 77 ± 8 % of the initial control response in the six fibres examined. After exposure to heparin in both these experiments, fibres commonly showed increases in baseline force and 'pedestals' at the end of each depolarization-induced response (e.g. Fig. 6), indicating that heparin had again directly affected the contractile apparatus. Consequently, most, if not all, of the small decrease in the depolarization-induced response after these treatments was probably due to the associated decrease in maximum Ca^{2+}-activated force in heparin described earlier. Thus, it seems that activating the Ca^{2+} release channels in the presence of heparin, for periods comparable to the duration of two or three depolarization-induced responses (about 6 s), has little if any effect on E–C coupling.

Effects of low molecular weight heparin and other polyanions

The effects of modified heparin compounds and other polyanions were investigated, in order to determine the specificity of the heparin binding site. Low molecular weight heparin is as effective (weight per unit volume) as heparin in blocking $InsP_3$ binding in brain (Worley *et al.* 1987), but is required at 3 times greater weight per unit volume than heparin in order to inhibit dihydropyridine (DHP) binding in mammalian skeletal muscle (Knaus, Scheffauer, Romanin, Schindler & Glossman, 1990). We found that 100 μg ml^{-1} low molecular weight heparin was relatively ineffective at interrupting E–C coupling in toad muscle; about six depolarizations in its presence were required to reduce the depolarization-induced response to less than 50 % of the initial control response in the three fibres examined (Table 1). Even in the one fibre examined with 500 μg ml^{-1} low molecular weight heparin, it took four depolarizations to reduce the response to less than 50 % and the response was not totally abolished after six depolarizations. With both concentrations, the response to depolarization recovered substantially after 2–5 min wash-out (mean recovery as a percentage of decrease: 64 % with 100 μg ml^{-1} and 41 % with 500 μg ml^{-1}); this differed from the complete lack of reversibility observed with heparin. Based on the baseline shift and 'pedestals' seen with the depolarization-induced response, low molecular weight heparin was almost as effective as heparin in shifting the Ca^{2+} sensitivity, although again unlike with heparin, this effect was almost completely reversible on wash-out.

Pentosanpolysulphate was found to be just as effective as heparin at inhibiting DHP binding in a preparation of T-tubules from mammalian skeletal muscle (Knaus *et al.* 1990). In the experiments here, although its actions were generally similar to heparin, 100 μg ml^{-1} pentosanpolysulphate took on average about five depolarizations to reduce the response to less than 50 %, and about eight depolarizations to block it completely (6 fibres). As with the low molecular weight heparin, the response to depolarization recovered substantially upon wash-out (Table 1) and the increase in Ca^{2+} sensitivity could be largely reversed.

In contrast, neither 100 μg ml^{-1} de-*N*-sulphated heparin (in which the *N*-sulphate groups have been removed) nor 500 μg ml^{-1} polyglutamate had any noticeable effect on either E–C coupling (Table 1) or the contractile apparatus (5 and 4 fibres respectively). We note that neither de-*N*-sulphated heparin nor polyaspartate was able to inhibit DHP binding in skeletal muscle (Knaus *et al.* 1990).

Effect of extracellular heparin in toad muscle

As heparin applied intracellularly potently blocked E–C coupling in a unique activation-dependent manner, and as it was possible that the key binding site was located

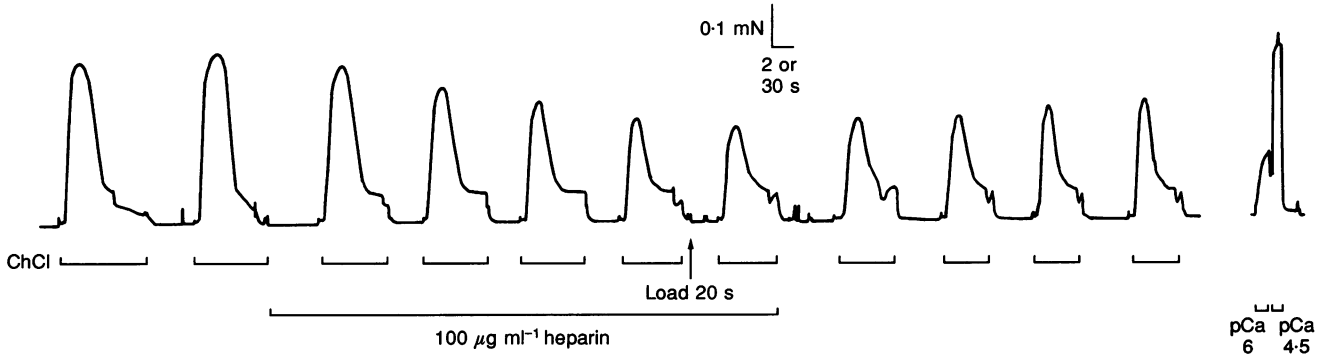

Figure 7. Effect of 100 μg ml^{-1} heparin on depolarization-induced responses in an EDL muscle fibre of the rat

The fibre was depolarized by ChCl substitution. Time scale: 2 s during depolarizations and 30 s elsewhere. D, 43 μm; L, 2·4 mm.

extracellularly in the rest state (see Discussion), we examined the effect of extracellular heparin on E–C coupling. After the epimysium had been removed, an iliofibularis muscle of a toad was bathed for 10 min in Ringer solution with 5000 μg ml^{-1} heparin, before transferring the muscle to paraffin oil and skinning superficial fibres. Depolarization induced a response of at least 90 % of the maximum Ca^{2+}-activated force in all three fibres examined, and in each fibre at least twelve similar responses could be evoked. Thus, extracellular heparin did not appear to interfere with E–C coupling.

Effect of heparin in EDL muscles of rat

In contrast to toad muscle, 100 μg ml^{-1} heparin had relatively little effect on E–C coupling in the EDL muscle of the rat (e.g. Fig. 7). In all six fibres examined (from 3 rats), there was a progressive decline of the depolarization-induced response, with it dropping on the 5th depolarization in heparin to a mean of 56 ± 5 % of the response in the absence of heparin, and then increasing slightly on wash-out. The majority of this decline was accounted for by an accompanying decrease in the maximum Ca^{2+}-activated force, which in three other fibres was observed to decline slowly over a similar period in heparin to 73 ± 3 % of the control level. (Note that the effect of heparin on both E–C coupling and the contractile apparatus could not be examined in the same fibres, because its effects on the contractile apparatus were only partly reversible.) At 500 μg ml^{-1} heparin, the maximum Ca^{2+}-activated force was reduced even further (to 44 ± 7 % of control in 3 fibres). In contrast to toad muscle, there was no significant shift in the $[Ca^{2+}]$ producing half-maximal force at either heparin concentration (shift to higher pCa of $0{\cdot}01 \pm 0{\cdot}04$ pCa units, $n = 3$, for 100 μg ml^{-1} and $0{\cdot}01 \pm 0{\cdot}05$, $n = 3$, for 500 μg ml^{-1}). The reduction of maximum force with 500 μg ml^{-1} heparin was even larger in the presence of the ChCl and sodium solutions, making it very difficult to quantify any effect on E–C coupling. Nevertheless, it was clear that E–C coupling in rat muscle was not completely abolished by even four or five depolarizations in 500 μg ml^{-1} heparin and that responses of up to 50 % of the initial response could be obtained on wash-out of heparin.

DISCUSSION

We have shown that heparin causes a novel, use-dependent block of E–C coupling in toad skeletal muscle (Fig. 1). Heparin does not block E–C coupling if the muscle fibre is (a) kept polarized, (b) already chronically depolarized (Fig. 3) or (c) paralysed by a preceding depolarization in D600 (Fig. 4). Thus, it appears that heparin only exerts its inhibitory effect when the voltage sensors are activated, and not while they are in either their resting or inactive states. Furthermore, opening of the SR Ca^{2+} release channels and Ca^{2+} efflux, which normally accompany activation of the voltage sensors, are neither necessary (Fig. 5) nor sufficient (Fig. 6) to permit this effect of heparin. Moreover, direct activation of the release channels (by caffeine or low $[Mg^{2+}]$) is not affected after heparin has abolished normal E–C coupling (Figs 1 and 5). This last finding is consistent with the observation that Ca^{2+}-induced Ca^{2+} release in SR vesicles is not affected by even 2·5 mg ml^{-1} heparin (Valdivia, Vaughan, Potter & Coronado, 1992).

Pape *et al.* (1988) found no effect of low molecular weight heparin on Ca^{2+} release stimulated by single action potentials in intact fibres of the frog. Our results are not in disagreement with this earlier study, as (a) we found that low molecular weight heparin was much less effective than heparin at abolishing E–C coupling and (b) we showed that, even in heparin, it required between one and three depolarizations to abolish E–C coupling, indicating that the voltage sensors had to be activated for the order of a second or more for heparin to bind and exert its effect. Thus, we would predict that heparin would have little, if any, effect when a fibre was depolarized for several milliseconds only, as occurs with single action potential stimulation. Finally, our results indicate that there are a large number of tight binding sites for heparin on the contractile apparatus, which could mean that the concentration of free heparin produced by heparin injection in intact fibres of the frog may have been very much lower than that assumed by Pape *et al.* (1988).

Rojas & Jaimovich (1990) reported that low molecular weight heparin actually induced a small amount of Ca^{2+} release in scratched fibres. In our experiments, neither heparin nor low molecular weight heparin caused any obvious Ca^{2+} release. Even with the increased Ca^{2+} sensitivity of the contractile apparatus we could see no evidence of Ca^{2+} release upon addition of heparin (e.g. Figs 1, 4 and 6) and there was no apparent depletion of Ca^{2+} in the SR after 5 min exposure to heparin in a solution (pCa 7·5, 150 μM EGTA) in which Ca^{2+} uptake was very low (see Results). Thus, any such Ca^{2+} release must have been very small. It is possible that a direct effect of the highly charged heparin molecule on aequorin contributed to the increase in the aequorin light signal observed by Rojas & Jaimovich (1990).

Heparin and InsP_3

The role of InsP_3 as a second messenger has been questioned on a number of grounds (see Introduction). Donaldson, Goldberg, Walseth & Huetteman (1988) found that InsP_3 injection in skinned mammalian fibres caused greater Ca^{2+} release when the fibres were depolarized for a prolonged period, and they suggested that depolarization could sensitize the SR to InsP_3. However, Donaldson (1989) subsequently concluded that it was *inactivation* of the voltage sensors, rather than T-system depolarization, which caused this sensitization, which meant that this mechanism could not be used to support a role for InsP_3 in normal E–C coupling. Recently, Hannon, Lee, Yandong & Blinks (1992) reported that injection of even very high concentrations of InsP_3 did not cause Ca^{2+} release in intact fibres in either the resting or inactivated state, but did so if the fibres were depolarized to close to contraction threshold. They concluded that the lack of effect of InsP_3 in resting fibres was strong evidence against the proposed second messenger role of InsP_3, and

instead suggested that InsP_3 can trigger Ca^{2+} release under some circumstances because it activates a low-level depolarizing current, perhaps by opening the voltage-dependent Ca^{2+} channels in the T-system (Vilven & Coronado, 1988).

Although we found that heparin did affect normal E–C coupling in toad muscle, this should not be taken as evidence that InsP_3 acts as a simple second messenger in vertebrate muscle. Firstly, the activation dependence of heparin's effect on E–C coupling is strikingly different from the simple competitive antagonism of heparin on the binding and action of InsP_3 (Worley *et al.* 1987; Ghosh, Eis, Mullaney, Ebert & Gill, 1988; Kobayashi *et al.* 1988). Secondly, low molecular weight heparin, which blocks InsP_3 binding in brain and InsP_3-induced Ca^{2+} release in smooth muscle with half-maximal effects at 5 μg ml^{-1} (Worley *et al.* 1987; Kobayashi *et al.* 1988), was relatively ineffective at abolishing E–C coupling in toad muscle, even at 100 μg ml^{-1}. Finally, heparin had little if any effect in abolishing E–C coupling in rat muscle (Fig. 7).

Thus, unless (a) the InsP_3 binding site is pharmacologically very different from that in other tissues (as suggested by Rojas & Jaimovich, 1990) *and* (b) this site is only revealed upon activation of the voltage sensors, InsP_3 does not act as a second messenger in E–C coupling. Instead, the abolition of E–C coupling in toad muscle observed here may result from heparin binding to a site unrelated to the putative InsP_3 receptor/release channel in the SR.

Heparin and the voltage sensor

The unique activation dependence of heparin on E–C coupling of course would be consistent with heparin binding to the activated voltage sensor. Furthermore, the data of Vilven & Coronado (1988), showing that InsP_3 opens the voltage-dependent Ca^{2+} channels in the T-system, also suggests that heparin may bind to the voltage sensors, because the dihydropyridine (DHP) receptor complex in the T-system may actually be both voltage sensor and voltage-dependent Ca^{2+} channel (see Lamb, 1992) and heparin binds to other InsP_3 binding sites.

Consequently, it is worth briefly examining where on the voltage sensor heparin might bind. We assume here that the general structure of the voltage sensor in toad muscle is similar to the voltage sensor/DHP receptor described in mammalian muscle (Tanabe *et al.* 1987; Tanabe, Beam, Powell & Numa, 1988) and that the difference in the action of heparin in the two species (cf. Figs 1 and 7) is due to a difference in the accessibility of the key site or the duration of its exposure during depolarization, or to a minor difference in the structure of the binding site. Heparin is a polyanion and so we expect that it binds to a region of positively charged amino acids which is revealed upon activation. One such site could be the S4 segment in any of the four homologous 'repeats' of the DHP receptor, which actually acts as the voltage-sensing element (Tanabe *et al.* 1987). However, this seems unlikely, as upon activation such sites apparently move towards the extracellular, not intracellular, space (Guy & Conti, 1990). A second possible site is on the 'extracellular' loop between the S5 and S6 transmembrane segments of the first repeat, which appears to be the site at which heparin interferes with DHP binding to the mammalian skeletal muscle DHP receptor (Knaus *et al.* 1990; Knaus, Moshammer, Friedrich, Kang, Haugland & Glossmann, 1992). Interestingly, upon activation, part of this loop (the so-called SS1–SS2 region) appears to move into the membrane region to form part of the ion conduction pathway (Guy & Conti, 1990; Heinemann, Terlau, Stühmer, Imoto & Numa, 1992), suggesting that the heparin binding site might be extracellular in the rest state and intracellular during activation. However, this too seems unlikely, as the region of positive amino acids forming the putative heparin binding site appears to be quite distant from the region proposed to move intracellularly. Furthermore, extracellularly applied heparin did not seem to affect E–C coupling in intact fibres (see Results), even though it might have been expected to interfere with the DHP receptor activation.

Finally, a likely site for heparin's action is the intracellular loop between the second and third repeats of the DHP receptor. Part of this loop is very positively charged (Tanabe *et al.* 1987), and strongly binds heparin (Knaus *et al.* 1990; residues 679–693, which includes the principal cAMP-dependent phosphorylative site) and the loop itself has been shown to be essential for normal coupling with the Ca^{2+} release channels (Tanabe, Beam, Adams, Niidome & Numa, 1990). Thus, activation of the voltage sensor/DHP receptor may cause a conformational change which allows heparin to bind to this loop and prevent it activating the Ca^{2+} release channel. In any case, it is interesting that the activation dependence of heparin's effect could imply that a positive region of the voltage sensor is revealed intracellularly during the coupling with the Ca^{2+} release channel; this is intriguingly like the simplistic model proposed in Fig. 2 of Lamb & Stephenson (1992) to indicate how activation of the voltage sensor could lead to opening of the release channels by lowering their affininity for Mg^{2+}.

REFERENCES

Ashley, C. C., Mulligan, I. P. & Lea, T. J. (1991). Ca^{2+} and activation mechanisms in skeletal muscle. *Quarterly Review of Biophysics* **24**, 1–73.

Berwe, D., Gottschalk, G. & Lüttgau, H. Ch. (1987). Effects of the calcium antagonist gallopamil (D600) upon excitation–contraction coupling in toe muscle fibres of the frog. *Journal of Physiology* **385**, 693–707.

Brandt, N. R., Caswell, A. H., Wen, S.-R. & Talvenheimo, J. A. (1990). Molecular interactions of the junctional foot protein and di-hydropyridine receptor in skeletal muscle triads. *Journal of Membrane Biology* **113**, 237–251.

Chandler, W. K., Rakowski, R. F. & Schneider, M. F. (1976). Effects of glycerol treatment and maintained depolarization on charge movement in skeletal muscle. *Journal of Physiology* **254**, 285–316.

Donaldson, S. K. (1989). Inactivated state rather than transverse tubule depolarization increases peeled skeletal muscle fiber sensitivity to inositol trisphosphate. *Biophysical Journal* **55**, 236a.

DONALDSON, S. K., GOLDBERG, N. D., WALSETH, T. F. & HUETTEMAN, D. A. (1988). Voltage dependence of inositol 1,4,5-trisphosphate-induced Ca^{2+} release in peeled skeletal muscle fibers. *Proceedings of the National Academy of Sciences of the USA* **85**, 5749–5753.

EISENBERG, R. S., MCCARTHY, R. T. & MILTON, R. L. (1983). Paralysis of frog skeletal muscle fibres by the calcium antagonist D-600. *Journal of Physiology* **341**, 495–505.

FILL, M. D. & BEST, P. M. (1989). Block of contracture in skinned frog skeletal muscle fibers by calcium antagonists. *Journal of General Physiology* **93**, 429–449.

FINK, R. H. A., STEPHENSON, D. G. & WILLIAMS, D. A. (1986). Potassium and ionic strength effects on isometric force of skinned twitch muscle fibres of the rat and toad. *Journal of Physiology* **370**, 317–337.

GHOSH, T. K., EIS, P. S., MULLANEY, J. M., EBERT, C. L. & GILL, D. L. (1988). Competitive, reversible, and potent antagonism of inositol 1,4,5-trisphosphate-activated calcium release by heparin. *Journal of Biological Chemistry* **263**, 11075–11079.

GUY, H. R. & CONTI, F. (1990). Pursuing the structure and function of voltage-gated channels. *Trends in Neurosciences* **13**, 201–206.

HANNON, J. D., LEE, N. K.-M., YANDONG, C. & BLINKS, J. R. (1992). Inositol trisphosphate ($InsP_3$) causes contraction in skeletal muscle only under artificial conditions: evidence that Ca^{2+} release can result from depolarization of T-tubules. *Journal of Muscle Research and Cell Motility* **13**, 447–456.

HEINEMANN, S. H., TERLAU, H., STÜHMER, W., IMOTO, K. & NUMA, S. (1992). Calcium channel characterisitics conferred on the sodium channel by single mutations. *Nature* **256**, 441–443.

JAIMOVICH, E. (1991). Chemical transmission at the triad: $InsP_3$? *Journal of Muscle Research and Cell Motility* **12**, 316–320.

KNAUS, H.-G., MOSHAMMER, T., FRIEDRICH, K., KANG, H. C., HAUGLAND, R. P. & GLOSSMANN, H. (1992). In vivo labeling of L-type Ca^{2+} channels by fluorescent dihydropyridines: Evidence for a functional, extracellular heparin-binding site. *Proceedings of the National Academy of Sciences of the USA* **89**, 3586–3590.

KNAUS, H.-G., SCHEFFAUER, F., ROMANIN, C., SCHINDLER, H.-G. & GLOSSMANN, H. (1990). Heparin binds with high affinity to voltage-dependent L-type Ca^{2+} channels. *Journal of Biological Chemistry* **265**, 11156–11166.

KOBAYASHI, S., SOMLYO, A. V. & SOMLYO, A. P. (1988). Heparin inhibits the inositol 1,4,5-trisphosphate-dependent, but not the independent calcium release induced by guanine nucleotide in vascular smooth muscle. *Biochemical and Biophysical Research Communications* **153**, 625–631.

LAMB, G. D. (1992). DHP receptors and excitation–contraction coupling. *Journal of Muscle Research and Cell Motility* **13**, 394–405.

LAMB, G. D. & STEPHENSON, D. G. (1990*a*). Calcium release in skinned muscle fibres of the toad by transverse tubular depolarization or by direct stimulation. *Journal of Physiology* **423**, 495–517.

LAMB, G. D. & STEPHENSON, D. G. (1990*b*). Control of calcium release and the effect of ryanodine in skinned muscle fibres of the toad. *Journal of Physiology* **423**, 519–542.

LAMB, G. D. & STEPHENSON, D. G. (1991*a*). Effect of magnesium ions on the control of calcium release in skeletal muscle fibres of the toad. *Journal of Physiology* **434**, 507–528.

LAMB, G. D. & STEPHENSON, D. G. (1991*b*). Excitation–contraction coupling in skeletal muscle fibres of rat and toad in the presence of GTPγS. *Journal of Physiology* **444**, 65–84.

LAMB, G. D. & STEPHENSON, D. G. (1992). Importance of Mg^{2+} in excitation–contraction coupling in skeletal muscle. *News in Physiological Science* **7**, 270–274.

LEA, T. J. GRIFFITHS, D. J., TREGEAR, R. T. & ASHLEY, C. C. (1986). 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. *FEBS Letters* **207**, 153-161.

LÜTTGAU, H. CH. & STEPHENSON, D. G. (1986). Ion movements in skeletal muscle in relation to the activation of contraction. In *Physiology of Membrane Disorders*, ed. ANDREOLI, T. E., HOFFMAN, J. F., FANESTIL, D. D. & SCHULZ, S. G., chap. 28, pp. 449–468. Plenum Press, NY, USA.

PAPE, P. C., KONISHI, M., BAYLOR, S. M. & SOMLYO, A. P. (1988) Excitation–contraction coupling in skeletal muscle fibers injected with the $InsP_3$ blocker, heparin. *FEBS Letters* **235**, 57–62.

RIOS, E. & PIZARRO, G. (1991). Voltage sensor of excitation–contraction coupling in skeletal muscle. *Physiological Reviews* **71**, 849–908.

ROJAS, C. & JAIMOVICH, E. (1990). Calcium release modulated by inositol trisphosphate in ruptured fibers from frog skeletal muscle. *Pflügers Archiv* **416**, 296–304.

STEPHENSON, D. G. & WILLIAMS, D. A. (1981). Calcium-activated force responses in fast- and slow-twitch skinned muscle fibres of the rat at different temperatures. *Journal of Physiology* **317**, 281–302.

TANABE, T., BEAM, K. G., ADAMS, B. A., NIIDOME, T. & NUMA, S. (1990). Regions of the skeletal muscle dihydropyridine receptor critical for excitation–contraction coupling. *Nature* **346**, 567–569.

TANABE, T., BEAM, K. G., POWELL, J. A. & NUMA, S. (1988). Restoration of excitation–contraction coupling and slow calcium current in dysgenic muscle by dihydropyridine receptor complementary DNA. *Nature* **336**, 134–139.

TANABE, T., TAKESHIMA, H., MIKAMI, A., FLOCKERZI, V., TAKAHASHI, H., KANGAWA, K., KOJIMA, M., MATSUO, H., HIROSE, T. & NUMA, S. (1987). Primary structure of the receptor for calcium channel blockers from skeletal muscle. *Nature* **328**, 313–318.

VALDIVIA, C., VAUGHAN, D., POTTER, B. V. & CORONADO, R. (1992). Fast release of $^{45}Ca^{2+}$ induced by inositol 1,4,5-tris-phosphate and Ca^{2+} in the sarcoplasmic reticulum of rabbit skeletal muscle: evidence for two types of Ca^{2+} release channels. *Biophysical Journal* **61**, 1184–1193.

VERGARA, J., TSIEN, R. Y. & DELAY, M. (1986). Inositol 1,4,5-trisphosphate: A possible chemical link in excitation–contraction coupling in muscle. *Proceedings of the National Academy of Sciences of the USA* **82**, 6352–6356.

VILVEN, J. & CORONADO, R. (1988). Opening of dihydropyridine calcium channels in skeletal muscle membranes by inositol trisphosphate. *Nature* **336**, 587–589.

VOLPE, P., SALVIATI, G., DI VIRGILIO, F. & POZZAN, T. (1985). Inositol (1,4,5) trisphosphate induces calcium release from sarcoplasmic reticulum of skeletal muscle. *Nature* **316**, 347–349.

WALKER, J. W., SOMLYO, A. V., GOLDMAN, Y. E., SOMLYO, A. P. & TRENTHAM, D. R. (1987). Kinetics of smooth and skeletal muscle activation by laser pulse photolysis of caged inositol 1,4,5-trisphosphate. *Nature* **327**, 249–252.

WORLEY, P. F., BARABAN, J. M., SUPATTAPONE, S., WILSON, V. S. & SNYDER, S. H. (1987). Characterization of inositol trisphosphate receptor binding in brain. *Journal of Biological Chemistry* **262**, 12132–12136.

Acknowledgements

We are grateful to Mrs R. Cafarella for excellent technical assistance. This work was supported by the NH and MRC of Australia.

Received 28 April 1993; accepted 2 July 1993.