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Negative inotropic effect of heparin on tension development in rat skinned skeletal muscle fibres

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Abstract

Heparin inhibits inositol trisphosphate receptors, particularly in smooth muscle, but its effect on skeletal muscle is controversial. Our study showed that heparin induced a decrease in the amplitude of 10 mM caffeine-induced contracture in slow and fast saponin-skinned fibres. Moreover, measurements on Triton X-100-skinned fibres in soleus muscle showed that heparin alone decreased maximal Ca^{2+}-activated tension and Ca^{2+} sensitivity of contractile proteins, whereas no significant effect was observed in extensor digitorum longus muscle. However, in the presence of caffeine, heparin decreased maximal Ca^{2+}-activated tension in both muscles. It would appear that the heparin-induced decrease in the amplitude of caffeine contracture in rat skeletal muscle was not related to a direct inhibition of Ca^{2+} release from sarcoplasmic reticulum but to a desensitising effect of heparin and caffeine on myofilaments.

Keywords: Heparin; Caffeine contracture; Sarcoplasmic reticulum; Skinned fiber; Skeletal muscle, mammalian

1. Introduction

Calcium ions (Ca^{2+}) play a major role in cellular function, and any alteration in intracellular Ca^{2+} concentration modifies cellular responses (Ebashi and Endo, 1968). In skeletal muscle, depolarisation of the transverse tubule membrane triggers Ca^{2+} release from sarcoplasmic reticulum, resulting in contraction activation by Ca^{2+} binding to contractile proteins (Costantin, 1975). The mechanisms involved in the events linking an electrical stimulus to a mechanical response, i.e. in excitation-contraction coupling, have been studied extensively (Ebashi, 1976; Caputo, 1978; Rios et al., 1992). Different models have been proposed depending on the nature of the triggering signal sensed by sarcoplasmic reticulum Ca^{2+} release channels (Schneider and Chandler, 1973; Mathias et al., 1980; Vergara et al., 1985). In an electrical model, depolarisation of sarcolemma results in the opening of a high-conductance pathway between the transverse tubule and the sarcoplasmic reticulum (Mathias et al., 1980). However, this hypothesis has not received experimental support. In a mechanical model, depolarisation is sensed by a voltage-dependent molecule in transverse tubule membrane which undergoes a conformational change propagated to the 'foot structure' of the ryanodine receptor in the sarcoplasmic reticulum membrane, thereby opening sarcoplasmic reticulum Ca^{2+} channels (Schneider and Chandler, 1973). In a chemical model, depolarisation releases an intracellular messenger, inositol trisphosphate ($InsP_3$), which activates sarcoplasmic reticulum Ca^{2+} release by binding to the $InsP_3$ receptor-Ca^{2+} channels in the sarcoplasmic reticulum membrane (Vergara et al., 1985). Indeed, in skeletal muscle, $InsP_3$-induced Ca^{2+} release is apparently dependent on transverse tubule membrane polarisation (Donaldson et al., 1988; Hannon et al., 1992).

The chemical model was described for smooth muscle (Somlyo et al., 1985) and for skeletal muscle (Walker et al., 1987), but its application to skeletal fibres has proved controversial. Previous studies showed (i) that sarcoplasmic reticulum Ca^{2+} release induced when $InsP_3$ was applied to mechanically skinned fibres from skeletal muscle was not due to opening of ryanodine receptors (Rojas and Jaimovich, 1990), (ii) that microinjection of $InsP_3$ into intact skeletal fibres caused local contraction (Lopez and Parra, 1991) and (iii) that two types of Ca^{2+} channels exist on sarcoplasmic reticulum vesicles, one Ca^{2+}-sensitive and the other $InsP_3$-sensitive (Valdivia et al., 1992). However,

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conflicting results were obtained by Mikos and Snow (1987) and Kobayashi et al. (1989) on the same muscle preparations.

Heparin, a sulphated polysaccharide, can block $InsP_3$-induced Ca^{2+} release in smooth muscle (Kobayashi et al., 1988) and liver (Hill et al., 1987) as well as inhibit $InsP_3$ binding to cerebral receptors (Worley et al., 1987). Thus, heparin could be a helpful tool for the recognition of $InsP_3$ receptors in different tissues. However, the effect of heparin in skeletal muscle is controversial. Ritov et al. (1985) found that it induced Ca^{2+} release from sarcoplasmic reticulum in rabbit skeletal muscle, whereas in frog skeletal muscle it was ineffective on sarcoplasmic reticulum Ca^{2+} release resulting from the normal excitation-contraction coupling process (Pape et al., 1988) and failed to block $InsP_3$-induced Ca^{2+} release (Rojas and Jaimovich, 1990). However, it was previously shown that intracellular injection of heparin caused a use-dependent block of excitation-contraction coupling in skinned skeletal fibres in the toad (Lamb et al., 1994). In fact, these conflicting results are of little use in analysing the role of heparin relative to $InsP_3$ effects in mammalian skeletal muscle.

As caffeine is known to release Ca^{2+} from ryanodine-sensitive pools in striated muscle (Endo, 1975), we used the caffeine-induced contracture to test whether the effect of heparin on sarcoplasmic reticulum Ca^{2+} release was due to ryanodine receptors. These investigations were performed on two types of mammalian skeletal muscle in order to compare the effect of heparin on sarcoplasmic reticulum Ca^{2+} release and the Ca^{2+} sensitivity of contractile proteins in fast- and slow-twitch muscles. The results indicate that the reduced amplitude of caffeine contractures induced by heparin could result from its decreasing effect on maximal Ca^{2+}-activated tension and the Ca^{2+} sensitivity of the contractile apparatus.

2. Materials and methods

All procedures in this study were in accordance with the stipulations of the Helsinki Declarations for the care and use of laboratory animals. Adult male Wistar rats weighing 475 ± 75 g ($n = 11$) were heavily anaesthetized by an ether vapour flow. After respiratory arrest, the extensor digitorum longus (edl) and soleus muscles were excised and placed in oxygenated HEPES-buffered physiological solution in a dissecting dish at room temperature.

2.1. Chemically skinned fibre preparation

Small bundles 0.15–0.20 mm in diameter and 2–3 mm in length were dissected from freshly isolated soleus and edl muscles. Chemical skinning was carried out immediately after dissection.

For Triton-skinned fibres, preparations were incubated for 1 h in a relaxing solution (pCa 9, see composition below) containing 1% Triton X-100 (v/v) to solubilise membranes and then transferred to relaxing solution without detergent. Following skinning, some fibres were stored at −20°C in relaxing solution containing 50% glycerol (v/v). Saponin skinning was performed by incubating the bundles for 30 min in relaxing solution containing 50 μg/ml of saponin. This treatment preserved the ability of sarcoplasmic reticulum to accumulate and release Ca^{2+} (Fano et al., 1989).

After the skinning procedure, fibres were transferred to a chamber and mounted between two stainless-steel tubes (Huchet and Léoty, 1993). One end of the muscle was snared in a loop of fine hair pulled into a tube glued to a fixed rod that was part of the transducer (Kaman KD 2300, 0.5 SU, Colorado Springs, CO, USA). The other end of the preparation was similarly snared to a tube glued to a rod which formed the arm of the transducer. The diameter and length of skinned muscle fibres were measured under a binocular microscope. The preparation was adjusted to slack length and then stretched step by step until the tension developed at pCa 4.5 became maximal. Maximal tension was generally reached when the resting length was increased by 20%. All experiments were performed at 22°C.

2.2. Experimental protocol

Tension-pCa relationships ($pCa = -\log[Ca^{2+}]$) were obtained by exposing Triton-skinned fibres sequentially to solutions of decreasing pCa. At the beginning of each experiment, a full set of solutions containing different concentrations of Ca^{2+} was prepared, and each Ca^{2+} concentration was then divided into appropriate aliquots, one serving as the control and the other containing heparin. After steady-state tension was reached in the control, the fibre was immersed in the same Ca^{2+} concentration containing heparin and then returned to the free Ca^{2+} concentration to test reversibility. An identical protocol was performed for fast- and slow-twitch fibres. Isometric tension was continuously recorded on chart paper (Linear Bioblock 1200, Reno, NV, USA), and baseline tension was established at the steady state measured in relaxing solution. Data for relative tensions above 10% and below 90% were fitted using a modified Hill equation (Huchet and Léoty, 1993):

$$\text{Relative tension} = T/T_{max} = [Ca^{2+}]^{n_H}/(K + [Ca^{2+}])^{n_H}$$

The Hill coefficient, n_H, and the pCa for half-maximal activation, $pCa_{50} = (-\log 10\ K/n_H)$, were calculated for each experiment by using linear regression analysis. The Hill coefficient for each type of fibre was calculated as the slope of the fitted straight lines. Resting tension was that in pCa 9, and maximal tension was obtained in pCa 4.5. Tension is expressed in mN/mm^2.

2.3. Ca^{2+} uptake and release in sarcoplasmic reticulum

For experiments on saponin-skinned fibres, the preparation was immersed sequentially in 5 different solutions, first to load the sarcoplasmic reticulum with Ca^{2+} and then to release it with caffeine. Application of 10 mM caffeine generates a transient contracture (Su, 1987, 1988).

The ionic composition of solutions was the same as that of the relaxing solution, except for the concentration of magnesium, EGTA and Ca^{2+}, which varied as described below.

Solution 1 (pCa 9) was a high-EGTA (10 mM), high-Mg^{2+} (1 mM) and high-caffeine (25 mM) solution used to deplete the sarcoplasmic reticulum of calcium. Solution 2 was a caffeine-free wash solution similar to solution 1. Solution 3 (pCa 7) was a high-EGTA (10 mM) and high-Mg^{2+} (1 mM) solution used to load the sarcoplasmic reticulum with Ca^{2+}. Solution 4 (pCa 7.5) was a low-EGTA (0.1 mM) and low-Mg^{2+} (0.1 mM) solution used to prime Ca^{2+} release. Solution 5 was similar to solution 4 but contained 10 mM caffeine to release Ca^{2+} from the sarcoplasmic reticulum.

The incubation time in each solution was 2 min, except for that of solution 5, which was based on contracture duration. Application of caffeine induced a transient contracture whose amplitude ($mN \cdot mm^{-2}$), time to peak (s) and relaxation constant (s) were measured. At the beginning of the experiments, 2 or 3 challenges of 10 mM caffeine contracture were performed. To elicit consistent contracture and to limit the sensitising effect of caffeine on contractile proteins, 10 mM caffeine was used instead of 25 mM (Wendt and Stephenson, 1983). In the absence of any further Ca^{2+} loading step, subsequent application of caffeine did not elicit a contractile response. In previous reports, various concentrations of heparin (100–10000 $\mu g/ml$) were used in striated muscles (Pape et al., 1988; Lamb et al., 1994; Lopez and Terzic, 1996). In the present study, two concentrations of heparin were tested in this range: 400 $\mu g/ml$ was the lowest concentration producing a significant effect on caffeine contractures, and 1200 $\mu g/ml$ was the highest concentration allowing partially reversible effects. The experimental protocol consisted of a control cycle in the absence of heparin, followed by a study of the effect of heparin (400 and 1200 $\mu g/ml$) added to solution 4, 2 min before the caffeine contracture was elicited. The reversibility of effects due to heparin was tested in a subsequent control cycle. The amplitude of the 10 mM caffeine contracture was measured under each of these conditions.

2.4. Solutions

The control physiological solution contained (in mM): NaCl 140, KCl 6, $CaCl_2$ 3, $MgCl_2$ 2, glucose 5 and HEPES 5. The pH was adjusted to 7.4 with Tris base.

2.5. Skinned-fibre solutions

The calcium concentration of relaxing (pCa 9, solution A) and activating (pCa 4.5, solution B) solutions was calculated using the computer program of Godt and Nosek (1986).

All solutions were calculated to contain 10 mM EGTA, 30 mM imidazole, 30.6 mM Na^+, 1 mM Mg^{2+}, 3.16 mM MgATP, 12 mM phosphocreatine and 0.3 mM dithiothreitol. The pH was adjusted to 7.1 with HCl or KOH. An ionic strength of 160 mM was achieved by adding KCl. In saponin-skinned fibre experiments, solutions also contained phosphocreatine kinase (17.5 IU/ml) and sodium azide (1 mM). For Triton- and saponin-skinned fibre experiments, solutions with intermediate Ca^{2+} concentrations were obtained by mixing solutions A and B in appropriate proportions. EGTA, phosphocreatine and heparin (low-molecular weight 6000 g/mol, H2149) were obtained from Sigma (St. Louis, MO, USA). Heparin was prepared as a stock solution (16.6 mM) in deionised and distilled water and added to solution 4 before the caffeine contracture was elicited to obtain a final concentration of 400 and 1200 $\mu g/ml$.

2.6. Statistical analysis

All values are expressed as means $\pm$ standard error of the mean ($\pm$S.E.M.). Student's unpaired *t*-test was used to compare the different parameters between groups. Statistical significance was reached when $P \leq 0.05$.

3. Results

3.1. Effects of heparin on sarcoplasmic reticulum Ca^{2+} release in saponin-skinned skeletal muscle fibres

Experiments were performed by bathing fibres previously loaded for 2 min with Ca^{2+} (pCa 7.0) in a solution (pCa 7.5) containing 400 or 1200 $\mu g/ml$ of heparin for 2 min. In edl and soleus muscles, caffeine (10 mM) induced a similar transient contracture, with a time to peak contracture of 9.6 ± 0.5 s ($n = 9$) and 12.0 ± 1.1 s ($n = 8$), respectively, and a relaxation constant of 13.5 ± 1.8 s ($n = 9$) and 21.0 ± 5.1 s ($n = 8$), respectively. The increase in tension was greater in edl (130.6 ± 12.1 mN/mm^2, $n = 10$) than in soleus muscles (109.1 ± 21.5 mN/mm^2, $n = 10$). In slow and fast fibres, the maximal tension induced by 10 mM caffeine represented $65.4 \pm 5.4\%$ ($n = 4$) and $82.4 \pm 3.1\%$ ($n = 5$) of maximal Ca^{2+}-activated tension, respectively. Furthermore, from pCa-tension relationships (Fig. 3) it was seen that in soleus muscle, 65% of the maximal Ca^{2+}-activated tension corresponded approximately to pCa 6.0 while in edl muscle, 80% of the maximal Ca^{2+}-activated tension corresponded approximately to pCa 4.5.

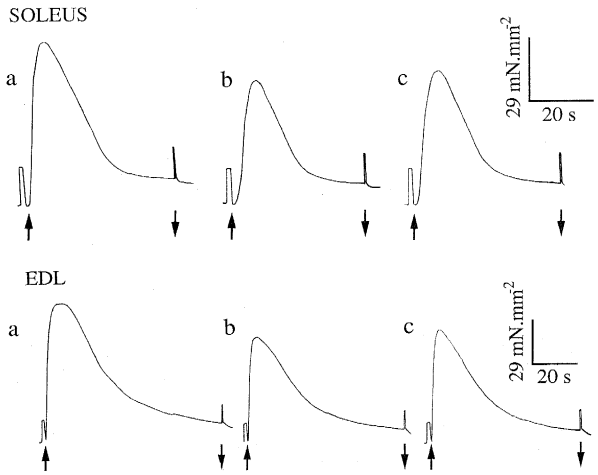

Fig. 1. Effects of 1200 μg/ml of heparin on sarcoplasmic reticulum Ca^{2+} release induced by 10 mM of caffeine in slow and fast saponin-skinned fibres when heparin was applied for 2 min before the caffeine contracture was elicited. Isometric tension was recorded from soleus and edl skeletal muscles. (a) Control response to 10 mM of caffeine, (b) 10 mM caffeine contracture after application of heparin and (c) 10 mM caffeine contracture after the return to control conditions. Arrows indicate the beginning and the end of caffeine application.

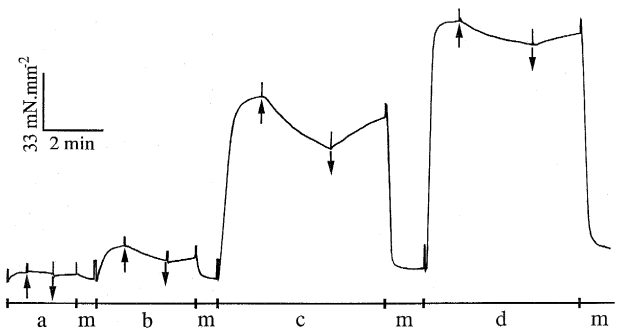

Fig. 2. Effect of 400 μg/ml of heparin on Ca^{2+}-activated tension and maximal Ca^{2+}-activated tension (pCa 4.5) of soleus slow-twitch fibres. Triton-skinned fibre was bathed in submaximal Ca^{2+} concentrations: a, pCa 7.0, b, pCa 6.5 or c, pCa 6.0 and maximal Ca^{2+} concentration d, pCa 4.5 (T_{max} = maximal tension) (indicated by the bar). After steady state was reached under control conditions, the fibre was immersed in the same Ca^{2+} concentration containing heparin before being returned to the free Ca^{2+} concentration to test reversibility. The application and the withdrawal of heparin are indicated by arrows. The preparation was then exposed to the relaxing solution, m = pCa 9.0.

In slow- and fast-twitch muscles (Fig. 1), heparin (1200 μg/ml) applied for 2 min before the application of caffeine (10 mM) induced a decrease in contracture amplitude. However, the time to peak tension and the relaxation constant were not significantly affected in either type of muscle (Table 1). Heparin (1200 μg/ml) induced a similar reduction in the amplitude of the caffeine contracture in fast- ($17.6 \pm 3.2\%$, $P < 0.05$) and slow-twitch fibres ($18.9 \pm 3.2\%$, $P < 0.05$) (Table 1). The effects of heparin (1200 μg/ml) were not fully reversible. After the return to control conditions for 2 min, caffeine contractures remained reduced in fast- and slow-twitch fibres (10.1 ± 3.0 and $7.3 \pm 4.4\%$, respectively). With a concentration of 400 μg/ml of heparin, the decrease in the amplitude of the 10 mM caffeine contracture was less pronounced, corresponding in edl and soleus skinned fibres to $10.7 \pm 3.6\%$ ($n = 5$, $P < 0.01$) and $11.7 \pm 3.6\%$ ($n = 4$, $P < 0.05$), respectively. As noted above, the alterations due to heparin (400 μg/ml) were not fully reversible in either type of muscle (Table 1).

This decrease in the amplitude of the caffeine contracture may have been related to reduced sarcoplasmic reticulum Ca^{2+} release due to the inhibitory effects of heparin on $InsP_3$ receptors. This would lend support to the notion that these receptors exist in mammalian skeletal muscle. This assumption implies that caffeine acts directly on $InsP_3$ receptors by releasing Ca^{2+}. Another possibility is that heparin affects the properties of the contractile apparatus. Accordingly, the effects of heparin on the apparent Ca^{2+} sensitivity of contractile proteins were tested in both types of skeletal muscles, using Triton-skinned fibres. Moreover, as heparin was tested on the caffeine contracture, we also investigated its effect on maximal Ca^{2+}-activated tension in the presence of caffeine.

Table 1
Effects of heparin (400 and 1200 μg/ml) on the caffeine contracture in saponin-skinned fibres from soleus and edl muscles

	Control	Heparin 400 μg/ml	Washout	Control	Heparin 1200 μg/ml	Washout
Soleus						
Amplitude (mN/mm^2)	112.1 ± 32.0	96.8 ± 29.7 [a]	83.9 ± 19.4	105.4 ± 32.7	86.8 ± 20.6 [a]	99.4 ± 22.7
Relaxation constant (s)	21.6 ± 7.3	17.6 ± 7.5	14.3 ± 4.7	19.3 ± 6.6	16.3 ± 4.4	21.0 ± 7.8
Time to peak (s)	12.3 ± 1.5	13.6 ± 2.1	14.3 ± 2.3	11.2 ± 1.3	11.2 ± 1.4	10.7 ± 1.2
n	4	4	4	4	4	4
edl						
Amplitude (mN/mm^2)	132.9 ± 16.0	112.1 ± 17.1 [b]	109.4 ± 15.6 [b]	127.0 ± 21.3	103.6 ± 16.6 [a]	109.8 ± 16.1 [a]
Relaxation constant (s)	10.4 ± 1.5	12.7 ± 2.4	13.9 ± 3.2	22.0 ± 1.9	20.9 ± 1.7	21.9 ± 2.4
Time to peak (s)	9.5 ± 0.7	9.2 ± 1.0	8.5 ± 0.8	9.9 ± 0.7	10.2 ± 1.2	9.0 ± 1.1
n	5	5	5	4	4	4

Letters indicate significant differences from the value under control conditions at [a] $P \leq 0.05$ and [b] $P < 0.01$ as determined by Student's paired *t*-test; *n* represents the number of fibres.

Table 2
Effects of 400 and 1200 μg/ml of heparin on maximal Ca^{2+}-activated tension (pCa 4.5) in Triton-skinned fibres from soleus and edl muscles

	T_{max} (mN/mm^2)					
	Control	Heparin 400 μg/ml	Washout	Control	Heparin 1200 μg/ml	Washout
Soleus	184.2 ± 15.2	164.6 ± 13.0 [c]	168.5 ± 13.9 [b]	150.0 ± 14.0	129.6 ± 12.3 [c]	136.6 ± 13.6 [c]
n	6	6	6	13	13	13
edl	161.2 ± 23.2	158.9 ± 23.4	159.3 ± 23.3	166.9 ± 18.0	162.7 ± 18.2 [a]	164.0 ± 18.6
n	6	6	6	7	7	7

Letters indicate significant differences from the value under control conditions at [a] $P \leq 0.05$, [b] $P < 0.01$, and [c] $P < 0.001$. Values are expressed as means ± S.E.M.; *n* represents the number of fibres.

3.2. Effects of heparin on Ca^{2+}-activated tension in Triton-skinned skeletal muscle fibres

The maximal Ca^{2+}-activated tension and apparent Ca^{2+} sensitivity of contractile proteins were estimated in the absence or presence of 400 or 1200 μg/ml of heparin. As illustrated in Fig. 2, heparin (400 μg/ml) in soleus skinned fibres (but not in those of edl) induced a decrease in the force developed by the contractile proteins at each Ca^{2+} concentration tested.

3.2.1. Maximal Ca^{2+}-activated tension

In soleus muscle, the tension generated by the contractile apparatus when maximally activated by Ca^{2+} (pCa 4.5) was decreased by 10.5 ± 0.8% and 13.6 ± 1.9% ($P < 0.001$) in the presence of 400 and 1200 μg/ml of heparin, respectively (Table 2). This effect was not fully reversible. In slow-twitch muscle, maximal tension returned to control levels after 2 min and was decreased after application of 400 and 1200 μg/ml of heparin by 8.5 ± 1.7% ($n = 6$, $P < 0.01$) and 9.4 ± 1.5% ($n = 13$, $P < 0.001$), respectively. In edl skinned fibres, 400 μg/ml of heparin produced a non-significant decrease of maximal Ca^{2+}-activated tension (1.6 ± 0.7%), while a small but significant decrease in maximal Ca^{2+}-activated tension was found in the presence of 1200 μg/ml of heparin (2.6 ± 0.7%) ($P < 0.05$) (Table 2).

The present results show that in soleus and in edl muscles, the effect of heparin on the maximal Ca^{2+}-activated tension was almost reversible. Indeed, after return to control conditions, the maximal Ca^{2+}-activated tension was close to the control value (Table 2).

3.2.2. Ca^{2+} sensitivity

In slow-twitch fibres, heparin induced a shift to the right of the relative tension-pCa curves, i.e. apparent Ca^{2+} sensitivity decreased in a dose-dependent manner (Fig. 3). For example, at pCa 6.0, Ca^{2+}-activated tension decreased by 22.9 ± 1.4% ($n = 6$) and 33.1 ± 3.4% ($n = 13$) in the presence of 400 and 1200 μg/ml of heparin, respectively. The reduction in pCa_{50} below control values (ΔpCa_{50}) under the influence of heparin was −0.088 ± 0.001 and −0.213 ± 0.002 ($P < 0.05$) for 400 and 1200 μg/ml, respectively (Table 3). Thus, the amount of Ca^{2+} required to develop 50% of the maximal Ca^{2+}-activated tension ($[Ca^{2+}_{50}]$) increased in the presence of heparin. However, in this type of fibre, the Hill coefficient, n_H, was not significantly affected by heparin (Table 3).

Conversely, in fast-twitch fibres, no significant difference in apparent Ca^{2+} sensitivity was noted between tension-pCa curves recorded in the presence of either 400 or 1200 μg/ml of heparin (Fig. 3).

These results suggest that heparin decreases Ca^{2+} sensitivity and the maximal Ca^{2+}-activated tension of contractile proteins in slow-twitch skeletal fibres, whereas the properties of the contractile apparatus in fast-twitch skeletal fibres are not affected by heparin.

3.3. Effects of heparin on maximal Ca^{2+}-activated tension in the presence of caffeine

It was previously shown that the properties of contractile proteins could be affected differently by caffeine in the presence or absence of another pharmacological tool (Bonnet and Léoty, 1996). Thus, we studied the effects of

Table 3
Effect of heparin on variations of pCa_{50} and the Hill coefficient in slow- and fast-twitch fibres from soleus and edl muscles

	Soleus			edl		
	Control	Heparin		Control	Heparin	
		400 μg/ml	1200 μg/ml		400 μg/ml	1200 μg/ml
pCa_{50}	6.23 ± 0.04	6.09 ± 0.02 [a]	6.04 ± 0.04 [a]	6.20 ± 0.01	6.18 ± 0.01	6.19 ± 0.02
n_H	1.93 ± 0.12	2.41 ± 0.09	1.63 ± 0.14	4.44 ± 0.28	3.75 ± 0.20	4.03 ± 0.30
n	19	6	13	13	6	7

The means (± S.E.M.) of the pCa_{50} were obtained by fitting the curves to the Hill equation. The means (± S.E.M.) of the Hill coefficient corresponding to the affinity sites of troponin C were obtained from the Hill plot curves; *n* represents the number of fibres.

heparin on maximal Ca^{2+}-activated tension in Triton-skinned fibres in the presence of caffeine. To estimate the effect of heparin on caffeine contractures, we first tested the effect of caffeine (10 mM) on the Ca^{2+} sensitivity of contractile proteins. In slow- and fast-twitch fibres, 10 mM of caffeine decreased maximal Ca^{2+}-activated tension by 3.4 ± 1.4 and 4.6 ± 1.4%, respectively (Fig. 4). After application of 1200 μg/ml of heparin, maximal Ca^{2+}-activated tension in the presence of 10 mM caffeine decreased by 18.9 ± 1.6% ($n = 3$, $P < 0.05$) and 14.9 ± 3.4% ($n = 2$, $P < 0.05$), respectively in soleus and edl fibres (Fig. 4).

These results show that in both types of muscle caffeine potentiated the effect of heparin on the maximal Ca^{2+}-activated tension of the contractile apparatus and that the effect was more pronounced in edl than in soleus muscle. In fast-twitch fibres, heparin (1200 μg/ml) had little effect on maximal Ca^{2+}-activated tension (2.6 ± 0.7%), while a significant reduction in tension (14.9 ± 3.4%) was observed in the presence of caffeine (10 mM). In slow-twitch fibres, the effect of heparin on maximal Ca^{2+}-activated tension (13.6 ± 1.9%) was slightly increased in the presence of caffeine (18.9 ± 3.6%).

Furthermore, in both types of muscles, the effect of heparin (1200 μg/ml) in the presence of caffeine was not reversible. Indeed, after the return to control conditions, the maximal Ca^{2+}-activated tension remained reduced in fast and slow-twitch fibres (12.6 ± 2.8 and 14.8 ± 1.6%, respectively).

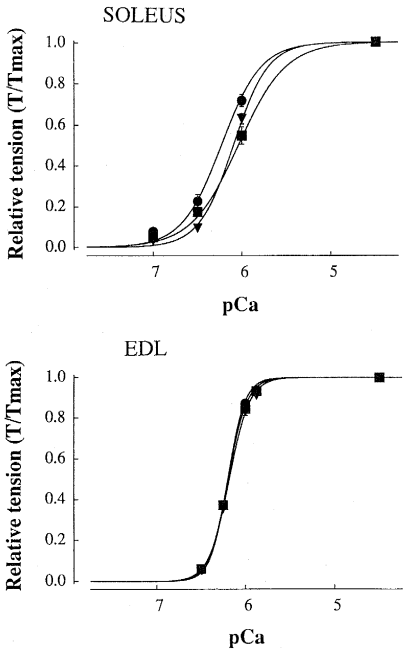

Fig. 3. Effects of various concentrations of heparin on the myofibrillar Ca^{2+} sensitivity of slow and fast Triton-skinned fibres from skeletal muscle. Isometric tension-pCa ($-\log[Ca^{2+}]$) relationships in twitch fibres in the absence (●) or presence of heparin: 400 (▼) and 1200 (■) μg/ml. Tension-pCa curves were obtained with slow-twitch and fast-twitch fibres. Force is expressed as the percentage of maximal tension at pCa 4.5 for each concentration of heparin tested. Curves were fitted by the modified Hill equation (see text). Temperature 22°C.

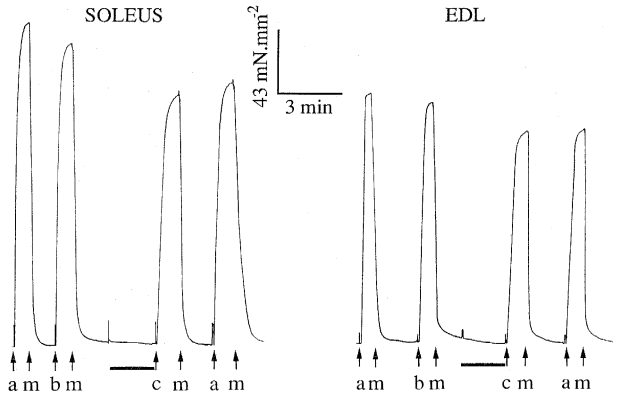

Fig. 4. Effect of caffeine (10 mM) and of caffeine with heparin (1200 μg/ml) on maximal Ca^{2+}-activated tension (pCa 4.5) in slow and fast Triton-skinned fibres. Solution changes are indicated by arrows. a = pCa 4.5; b = presence of caffeine; c = addition of caffeine after application of heparin for 2 min; m = pCa 9.0. Horizontal bars indicate the application of heparin during 2 min.

4. Discussion

The present work demonstrates that heparin (400 or 1200 μg/ml), as compared to the control, induced a decrease in the amplitude of the caffeine contracture in both edl and soleus skinned fibres. These results may have been due to direct inhibition of caffeine-induced Ca^{2+} release by heparin. In skeletal muscle, heparin, an inhibitor of $InsP_3$ receptors, could block ryanodine receptors. However, previous reports do not favour this possibility. Heparin has been shown to inhibit $InsP_3$-induced Ca^{2+} release specifically in smooth muscle (Kobayashi et al., 1988) and to block $InsP_3$ receptors and activate ryanodine receptors in skeletal muscle (Bezprozvanni et al., 1993). Heparin might also block caffeine-induced Ca^{2+} release by binding to $InsP_3$ receptors. However, this suggests that caffeine not only acts on ryanodine-sensitive receptors but also on $InsP_3$ receptors by releasing Ca^{2+}. In smooth muscle as well as in non-muscle cells, caffeine was unable to release Ca^{2+} from $InsP_3$ pools even though it bound to $InsP_3$ receptors (Parker and Ivorra, 1991; Missiaen et al., 1992; Hirose et al., 1993). Thus, it is unlikely that the heparin-induced decrease of the caffeine contracture observed in this study was related to a change at the receptor level.

In contrast to smooth muscle where low concentrations of heparin (0.3–500 μg/ml) were used to block the $InsP_3$ receptors (Kobayashi et al., 1988; Kannan et al., 1996), higher concentrations were needed in skeletal muscle to inhibit the $InsP_3$-induced Ca^{2+} release. Particularly, Lopez and Terzic (1996) have shown that in frog skeletal muscle heparin inhibits $InsP_3$-induced Ca^{2+} release at a half-maximal concentration of 1720 μg/ml. Then, in the present study, two high concentrations of heparin (400 and 1200

μg/ml) were chosen in the range of concentrations (200–10 000 μg/ml) that were previously reported to block $InsP_3$-induced Ca^{2+} release in skeletal muscle (Lopez and Terzic, 1996). The $InsP_3$ receptors could play a role less important in skeletal muscle than in smooth muscle in the regulation of intracellular Ca^{2+} activity. The previously noticed absence of an effect of heparin at a low concentration (Pape et al., 1988; Lamb et al., 1994) could be related to a less important role of $InsP_3$ receptors in skeletal muscle than in smooth muscle. Contrary to that was previously reported (Parker and Ivorra, 1991; Missiaen et al., 1992; Hirose et al., 1993), this assumption implies that caffeine acts on ryanodine receptors as well as on $InsP_3$ receptors by releasing Ca^{2+}. Another possibility would be that the $InsP_3$ receptors in skeletal muscle are not blocked by heparin at low concentrations because binding sites are pharmacologically different from those present in the other tissues, as previously suggested by Rojas and Jaimovich (1990). However, by studying caffeine contractures, the present experimental approach does not allow us to see whether heparin acts differently on the $InsP_3$ receptors in skeletal muscle and in smooth muscle.

In slow-twitch skinned fibres, heparin decreased the maximal Ca^{2+}-activated tension and the Ca^{2+} sensitivity of contractile proteins (pCa_{50}) (Tables 2 and 3). The decrease in maximal Ca^{2+}-activated tension was more pronounced when caffeine was present in the activating solution. The effect of heparin on the 10 mM caffeine contracture in soleus muscle was determined by comparing the amplitude of the maximal Ca^{2+}-activated tension and that of the contracture. In slow-twitch fibres, the amplitude of the 10 mM caffeine contracture was 65.4 ± 5.4% of the maximal Ca^{2+}-activated tension (see Section 3). From the tension-pCa curve (Fig. 3), maximal tension induced by 10 mM of caffeine was estimated to correspond to the tension achieved at approximately pCa 6.0. Heparin (1200 μg/ml) decreased the tension at pCa 6.0 by 33.1% and the amplitude of the caffeine contracture by 18.9% (see Section 3). Thus, it seems likely that the effects of heparin on the caffeine response in soleus muscle were mainly due to its action on contractile proteins.

In fast-twitch fibres, heparin produced a slight effect on the maximal Ca^{2+}-activated tension (2.6% decrease) but no significant effect on the Ca^{2+} sensitivity of the contractile apparatus. These results differ from those of Lamb et al. (1994), who showed that 500 μg/ml of heparin decreased maximal Ca^{2+}-activated tension by 66% in rat edl muscle without affecting the Ca^{2+} sensitivity of myofilaments. This difference could reflect the use of low-molecular-weight heparin (6000 g/mol) in our study and a high-molecular-weight form (500–25 000 g/mol) by Lamb et al. (1994). The effect of heparin applied before achievement of maximal Ca^{2+}-activated tension was potentiated when caffeine was present in the activating solution. Heparin (1200 μg/ml) or caffeine (10 mM) alone induced a decrease in maximal Ca^{2+}-activated tension of 2.6 or 2.4%, respectively. This decrease reached 14.9% when heparin and caffeine (10 mM) were combined. As in soleus muscle, the maximal tension and the amplitude of the caffeine contracture were compared. In the presence of caffeine, both parameters were decreased to a similar extent (17.6 and 14.9%, respectively) after application of heparin (1200 μg/ml). Since the amplitude of the 10 mM caffeine contracture represented 82.4 ± 3.1% of the maximal Ca^{2+}-activated tension (see Section 3), it would appear that the heparin-induced decrease in the amplitude of the caffeine contracture was due to a desensitising effect of heparin and caffeine on myofilaments rather than to a change in caffeine-induced Ca^{2+} release.

The present study shows that in slow- and fast-twitch fibres, the effect of heparin on the caffeine contracture was not reversible while its effect on the maximal Ca^{2+}-activated tension was almost reversible. In the presence of caffeine, the effect of heparin on the maximal Ca^{2+}-activated tension was not reversible. So, it seems that caffeine changes the reversibility of the effect of heparin on the contractile proteins. Furthermore, in soleus and edl muscles, the desensitising effect of heparin and caffeine on myofilaments may have been due to a lower affinity of Ca^{2+} for contractile proteins, particularly for troponin C or to some aspect of cross-bridge interactions following Ca^{2+} binding to troponin C.

It may be concluded that heparin decreased the amplitude of the caffeine contracture and maximal Ca^{2+}-activated tension. However, as heparin acts mainly on myofibrillar responsiveness, it is rather unlikely that the heparin-induced decrease in the amplitude of the caffeine contracture was due to an inhibitory effect on Ca^{2+} release from ryanodine-sensitive Ca^{2+} pools. Depending on whether caffeine was present or not, heparin had a different effect on the properties of contractile protein in fast- and slow-twitch muscles. Thus, due care must be taken in interpreting results obtained for fast- and slow-twitch fibres when heparin or heparin and caffeine are used to investigate the role of $InsP_3$ receptors in the excitation-contraction coupling of mammalian skeletal muscles.

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