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Rapid myosin phosphorylation transients in phasic contractions in chicken gizzard smooth muscle

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In *intact* smooth muscle strips from chicken gizzard, electrical stimulation and carbachol elicited brief, phasic contractions which were associated with a very rapid, transient phosphorylation of the 20 kDa myosin light chains. The phosphorylation transients reached their peak after 3 s and 6 s and preceded that of force. Phosphorylation was not significantly different from basal levels after 10 s and 30 s while force still amounted to 50% of the peak value. The rate of tension decline could be increased by cessation of stimulation or by addition of atropine, even at apparently basal phosphorylation levels suggesting a phosphorylation independent regulation.

Smooth muscle; Myosin light chain phosphorylation; Smooth muscle mechanics; (Chicken gizzard, intact)

1. INTRODUCTION

The reversible phosphorylation of the 20 kDa light chain of myosin (LC) is considered to be the primary regulatory mechanism of smooth muscle contraction. Although a great body of biochemical data in support of this hypothesis has been obtained in skinned fibres, and isolated contractile and regulatory proteins from chicken gizzard (reviewed in [1]), its physiological relevance has not yet been tested in the *intact* smooth muscle of chicken gizzard. We, therefore, report on the temporal relation between force and LC phosphorylation in *intact* smooth muscle strips from chicken gizzard. There is a rapid phosphorylation transient preceding the force transient, i.e. phosphorylation already declines while force still rises, and is basal at elevated tension levels. A dissociation between force and phosphorylation has previously been found in skinned chicken gizzard [2].

2. MATERIALS AND METHODS

2.1. *Tissue preparation and experimental conditions*

Chicken gizzards were collected at a local farm immediately after slaughter and immersed in oxygenated, ice-cold physiological saline solution (PSS). Muscle strips of approximately 15 mm length and 0.4–0.5 mm diameter were cut out of the superficial circumferential muscle layer and mounted vertically between a force transducer (Statham UC2 green cell) attached to a micrometer drive, and a fixed stainless steel rod. They were incubated in PSS in a water jacketed organ bath maintained at 37°C. After stretching to a passive force of 5 mN the muscle was allowed to equilibrate for 2 h at 37°C in PSS, modified from [3], containing (in mM): NaCl 150, KCl 5, $MgCl_2$ 2, $CaCl_2$ 5, Hepes 24 (pH 7.4 at 37°C) and glucose 10, which was gassed with 100% oxygen. The preparation was stimulated several times with carbachol (3 μM) until stable and reproducable contractions were obtained. Muscle length was readjusted so that passive force was maintained at 10% of total force. Stimulation with 3 μM carbachol elicited 85% of maximal force obtained with 30 μM carbachol.

For electrical stimulation, rectangular pulses (1 ms, 10–15 V at 30 Hz) were applied by parallel platinum electrodes (1×10 mm, approximately 6 mm apart). Voltage was adjusted so that force development was comparable to the contractions elicited by carbachol (3 μM).

For the mechanical experiments, the muscle was mounted between the force transducer and a length step generator (Ling-Dynamics 101 Vibrator) that allowed one to impose quick length changes with a ramp time of less than 10 ms. The force and length signals were recorded with a digital oscilloscope (Nicolet 1090 Explorer) at a frequency of 1 kHz.

2.2. *Analysis of LC phosphorylation*

At selected times the muscle strips were frozen within 60 ms by clamping them between two brass-bolts precooled to −100°C with liquid nitrogen. The frozen strips were stored in a 10% (w/v) TCA/acetone solution at −80°C for at least 48 h. They were allowed to warm to room temperature in this solution over a period of 1 h [4], and were further processed for 2D-gel electrophoresis, as described previously [5]. The relative amounts of phosphorylated and non-phosphorylated myosin light chains were determined by densitometry scans of the Coomassie stained gels.

2.3. *Statistics*

The data were expressed as the mean ± SE. Student's *t*-test was used to determine significant differences between two population means and a *P*-value of 0.05 or less was considered statistically significant.

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Abbreviations: TCA, trichloroacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MLCK, myosin light chain kinase

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3. RESULTS

In *intact* smooth muscle strips from chicken gizzard, carbachol and electrical field stimulation elicited only phasic contractions without a tonic component. The time course of tension and LC phosphorylation in the presence of 3 μM carbachol is given in fig.1. Time to peak tension was 14.0 ± 0.8 s and, in the continued presence of carbachol, active force was below 5% of peak force within 60 s. Once relaxation has proceeded to 70% of peak tension, the time course of relaxation may be described by a single exponential with a rate constant of 0.12 ± 0.004 s^{-1} (n = 10). Tension development was preceded by LC phosphorylation which rose from the resting (0.04 ±0.01 mol P_i/mol LC) to the peak value (0.36 ± 0.03 mol P_i/mol LC) within 6 s and then declined while force was still rising. At 30 s, phosphorylation (0.05 ± 0.01 mol P_i/mol LC) was not significantly different from basal levels while force had only declined to 40% of the peak value (fig.1).

The time course of force decline depended on the continued presence of carbachol. Atropine (10^{-4} M), added either 18 or 30 s after onset of stimulation, increased the rate of relaxation 4–5-fold (0.54 ± 0.03 s^{-1} compared to 0.12±0.004 s^{-1} without atropine). Note, that atropine increased the relaxation rate even when phosphorylation was not significantly different from basal levels (fig.1, lower panel).

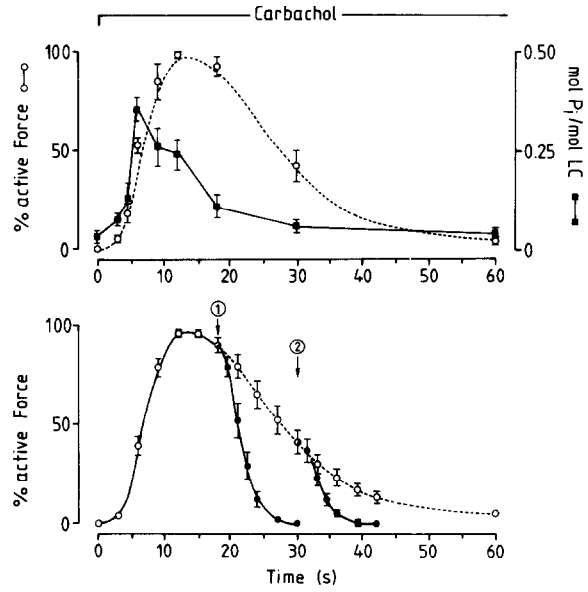

Fig.1. (Upper panel) Time course of force and LC phosphorylation in response to 3 μM carbachol. Force at the time of freezing was normalized in respect to the preceding contraction. Each point represents the mean value ± SE from 5 fibres taken from different animals. Peak force was 21 ± 2 N/cm² (n = 49), the broken line represents an average force tracing. (Lower panel) Effect of atropine on the time course of tension decline. First, a control contraction was induced with carbachol. In the following contraction, atropine (0.1 mM) was added either 18 s (arrow 1) or 30 s (arrow 2) after onset of stimulation with carbachol (n = 5). The broken line indicates the time course of tension decline of the control contraction (n = 10).

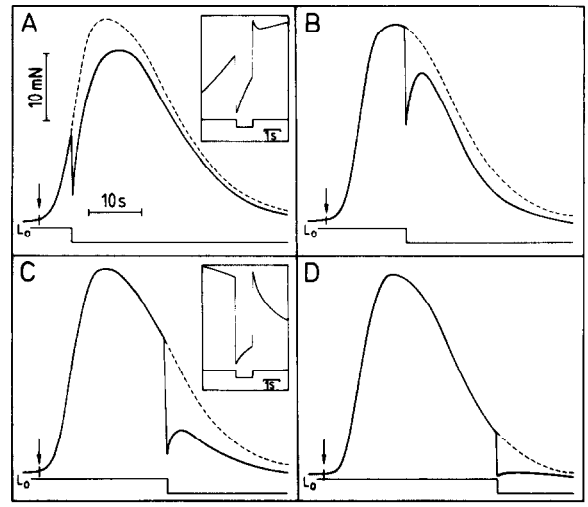

Fig.2. Tension transients following a quick release (5% L_o) applied at different times during carbachol induced contractions (carbachol was added at arrow). Note that the tension transient after the quick release is completely contained within the force envelope of a normally contracting and relaxing preparation (i.e. dotted line). Force can be restored by restretching the preparations to the initial length (insets in A and C).

At different times of the phasic contraction, the muscle strips were subjected to quick releases of 5% L_o to estimate the active state [6]. At corresponding tension levels, tension recovery following the quick release was slower (cf. fig.2A,C) and less complete during the falling phase than during the rising phase of the contraction (fig.2). Force could, however, be restored to the value existing before the release when the preparation was restretched to the initial length (fig.2A,C). During relaxation, the tension recovery was maximal after 3 s. This value, normalized to the peak value of isometric force, was taken as a measure of the apparent active state. Fig.3 shows the temporal relation between tension, phosphorylation and apparent active state while in fig.4, isometric tension and the apparent active state are shown as a function of LC phosphorylation. Note, that a given apparent active state may be associated with

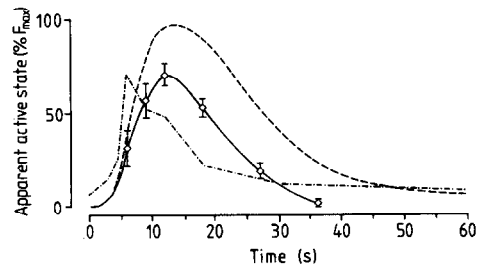

Fig.3. Time course of the apparent active state during carbachol-induced contractions. The tension recovered 3 s after onset of the release (5% L_o) expressed in percent of peak force was taken as a measure of the apparent active state (n = 5 - 6). Force (– – –), phosphorylation (– · – · –), data taken from fig.1.

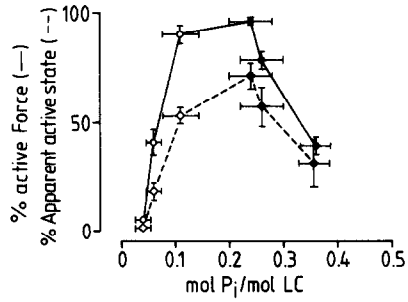

Fig.4. Relation between LC phosphorylation and force (○,●) or apparent active state (◇,◆). Closed symbols, rising phase; open symbols, falling phase of the contraction. Data replotted from figs 1 and 3.

both low and high LC phosphorylation and is, thus, not uniquely correlated with phosphorylation. During the rising phase of the contraction, force and apparent active state nearly superimposed while during the declining phase the apparent active state was lower than force.

In electrically stimulated preparations, both the tension and the phosphorylation transient occurred even more rapid. Nevertheless, a similar dissociation between force and phosphorylation was observed. Phosphorylation reached its peak between 3 s and 4 s (0.26 ± 0.02 mol P_i/mol LC at 3 s, $n = 4$), and force within 5.5 s. Phosphorylation was basal after 10 s while force still amounted to 50% of the peak value. Resting force levels were reached after 20 s.

As in the case of carbachol induced contractions, the rate of tension decline depended on continuous stimulation. Cessation of electrical stimulation after peak force had been reached increased the rate of tension decline from 0.26 ± 0.02 to 1.01 ± 0.04 s^{-1} ($n = 5$).

4. DISCUSSION

Phasic contractions in *intact* smooth muscle strips from chicken gizzard were associated with a rapid phosphorylation transcient, the peak preceding the peak of tension. Phosphorylation levels were basal as early as 10 s after beginning of electrical stimulation. A similar rapid phosphorylation transient was observed in phasic contractions of the ileum, where phosphorylation was close to basal after 30 s [7]. During the declining phase of the contraction, force and phosphorylation dissociated as has previously been described in tonic contractions of different types of smooth muscle (reviewed in [8]).

At phosphorylation levels indistinguishable from basal, there is virtually no force recovery following a quick release suggesting that, in this case, tension is maintained by non-cycling crossbridges. Most interestingly, addition of atropine at this point leads to a 4–5-fold increase in the rate of relaxation. This suggests that the net detachment of dephosphorylated crossbridges may be regulated independent of changes in the apparent LC phosphorylation, and would require a second regulatory mechanism. Net detachment could be increased either by increasing the apparent detachment rate constant, g, and/or inhibiting the reattachment of dephosphorylated crossbridges. The detachment rate constant could be affected by regulation of the product release from $AM \cdot ADP \cdot P_i$, for instance, by caldesmon, which slows the rate of product release and, in addition, increases the affinity of smooth muscle HMM to actin (reviewed in [9]). Reattachment of dephosphorylated crossbridges, due to a cooperative interaction between phosphorylated and non-phosphorylated crossbridges, has also been postulated [7,10]. Such a cooperative interaction might also explain the increase in force while phosphorylation is already declining.

An alternative explanation is the latch hypothesis of Murphy and coworkers [11] which postulates LC phosphorylation as the sole regulatory mechanism. At low phosphorylation, tension is maintained by latch-bridges which have a low detachment rate compared to phosphorylated crossbridges, and which are generated by the dephosphorylation of attached crossbridges. Thus, at low or even basal LC phosphorylation there has to be an elevated phosphorylation turnover which we cannot exclude in our experiments if, in the continued presence of carbachol, MLCK activity is suprabasal. Antagonizing the action of carbachol by atropine would decrease the MLCK activity to basal levels thereby preventing the replacement of detaching latch-bridges by new ones. The relaxation rate in the presence of atropine would then reflect the detachment rate of latch-bridges which, however, would have to be much higher than proposed by Hai and Murphy [11]. If one assumes that the detachment rate of latch-bridges is not regulated, then about 3 out of 4 detaching latch-bridges have to be replaced by new ones in order to allow for the 5-fold lower relaxation rate without atropine. This requires a high phosphorylation turnover and a high ATP utilization not related to actin-myosin interaction. Therefore, regulation of the detachment rate of dephosphorylated crossbridges would obviously be more economical.

In any case, this is the first study of the temporal relation between phosphorylation and force in the *intact* smooth muscle of chicken gizzard showing a dissociation between force and phosphorylation during phasic contractions. Most interestingly, the rate of relaxation is regulated at apparently basal MLC phosphorylation. Energetic measurements might allow to decide whether this is due to a regulation of the detachment rate of dephosphorylated crossbridges.

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