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Increased Phosphorylation of Myosin Light Chain Kinase After an Increase in Cyclic AMP in Intact Smooth Muscle

Abstract. *The role of cyclic adenosine monophosphate–mediated phosphorylation of myosin light chain kinase in relaxing smooth muscle was examined. The kinase was immunoprecipitated from tissue extracts and the phosphate content was determined. The addition of forskolin to resting or methacholine-contracted muscles resulted in an increase in myosin light chain kinase phosphorylation and a relaxation of contracted muscles. These findings suggest that phosphorylation of myosin light chain kinase is one of the reactions in the process by which cyclic adenosine monophosphate causes relaxation of smooth muscle.*

Myosin phosphorylation is essential for smooth muscle contraction (*1–3*). The mechanisms that regulate smooth muscle relaxation, however, are not known. In recent in vitro experiments phosphorylation by adenosine 3′,5′-monophosphate (cyclic AMP)–dependent protein kinase decreased the catalytic activity of myosin light chain kinase (MLCK) (*4*). Since β-adrenergic receptor stimulation increases cyclic AMP and causes relaxation of smooth muscles (*5*), it has been suggested that a decrease in MLCK activity after phosphorylation and the subsequent dephosphorylation of myosin by phosphatases (*1, 2*) could be part of the mechanism of cyclic AMP–mediated relaxation. However, indirect experiments on the state of MLCK phosphorylation in intact smooth muscle have provided contradictory results (*6*). Therefore we performed experiments to correlate MLCK phosphate content with cyclic AMP levels, myosin phosphate content, and tension in intact canine tracheal smooth muscle treated with pharmacological agents.

Resting (noncontracted) tracheal muscles incubated with ^{32}P (Fig. 1) were frozen after being treated with forskolin, an agent that increases cyclic AMP (*7*) and relaxes smooth muscles (Table 1). The frozen muscles were homogenized in boiling sodium dodecyl sulfate (SDS) and MLCK was immunoprecipitated from the SDS extracts with affinity-purified antibodies to turkey gizzard MLCK (*8*). Control experiments have demonstrated that the phosphate content of MLCK does not change during purification. Polyacrylamide gel electrophoresis in the presence of SDS (Fig. 1A) showed a single protein band with a molecular weight of about 155,000 in the immunoprecipitates. This value is in good agreement with that reported for MLCK purified from bovine stomach muscle (*9*). An autoradiograph of this gel (Fig. 1B) demonstrated that MLCK was phosphorylated in resting muscle and that phosphorylation increased on treatment with forskolin. In addition, it appeared that MLCK phosphorylation increased within 1 or 2 minutes after forskolin was added.

Experiments on the stoichiometry of phosphorylation were performed on tracheal muscles incubated with ^{33}P. The muscles were treated with various pharmacological agents and frozen (Table 1). MLCK was immunoprecipitated from half of each muscle (see legend to Fig. 1) and the extent of MLCK phosphorylation was determined (see legend to Table 1). ATP was purified from the other half of each muscle with affinity chromatography and high-performance liquid chromatography and the specific activity was determined. Control experiments demonstrated that the ^{33}P was in isotopic equilibrium with intracellular ATP within 60 minutes, that more than 90 percent of the purified ATP was labeled in the gamma position, and that the ATP was free of other nucleotides (*10*). The MLCK phosphorylation data were then compared with the data on cyclic AMP, myosin phosphate (PO_4), and relaxation.

Addition of forskolin to resting muscles (Table 1) caused significant increases in cyclic AMP (from 4.4 to 71.0 pmole per milligram of protein) and MLCK PO_4 (from 1.0 to 1.7 moles per mole of MLCK) and a slight but statistically significant decrease in myosin phosphorylation. Methacholine did not change the cyclic AMP content or the MLCK PO_4 content but did increase myosin phosphorylation and tension (*2*). Addition of atropine to methacholine-contracted muscles decreased both the myosin PO_4 content and tension to the resting levels. Addition of forskolin to methacholine-contracted muscles was accompanied by a significant decrease in myosin phosphate content and tension, although neither value returned to the resting level. Moreover, cyclic AMP was elevated and MLCK phosphorylation was near the maximum level of 2 moles of PO_4 per mole of MLCK when methacholine-contracted muscles were relaxed with forskolin.

The level of phosphorylation in resting tracheal muscle is surprising. We had anticipated a lower level of phosphorylation than the 1 mole of PO_4 per mole of MLCK measured in resting, untreated tracheal muscles. Torphy *et al.* (*11*) recently reported that as much as 24 percent of the protein kinase molecules activatable by cyclic AMP are active in resting canine tracheal smooth muscle. This level of active cyclic AMP–dependent protein kinase may be sufficient to maintain 50 percent of the available MLCK sites in the phosphorylated state.

The identity of the site on MLCK that is phosphorylated in resting tracheal smooth muscle is of interest. It is possible to phosphorylate two sites on turkey gizzard smooth muscle MLCK, and both sites apparently must be phosphorylated before there is a reduction in MLCK catalytic activity (*4*). Thus phosphorylation would be most effective in modulating MLCK activity if one site is phosphorylated on all MLCK molecules in resting muscle and both sites become phosphorylated after the addition of forskolin.

The presence of 1.7 ± 0.1 moles of PO_4 per mole of MLCK in resting muscle treated with forskolin suggests that a significant fraction of the MLCK molecules are phosphorylated at two sites.

Data from biochemical experiments (*4*) have demonstrated a significant reduction in MLCK activity at this level of phosphorylation and predict an attenuation of the contractile response when smooth muscles with a significant amount of diphosphorylated MLCK are stimulated with a contractile agonist. We have found an attenuation of the contractile response when forskolin-treated tracheal muscles are contracted with methacholine (*12*). It is possible that this attenuation of the contractile response is a reflection of decreased activity or a lower rate of activation of the phosphorylated MLCK (or both).

The increase in MLCK phosphorylation when methacholine-contracted muscles are treated with forskolin (Table 1) supports the hypothesis that MLCK phosphorylation is part of the mechanism of cyclic AMP–mediated relaxation of smooth muscles. The hypothesis is further supported by the observation that the increase in MLCK phosphorylation is seen only in forskolin-treated muscles and is accompanied by an increase in cyclic AMP (Table 1). There is no increase in MLCK phosphorylation or cyclic AMP when tracheal muscles are contracted with methacholine or when methacholine-contracted tracheal muscles are relaxed with atropine. Con-

Table 1. Cyclic AMP content, MLCK phosphorylation, myosin dephosphorylation, and relaxation of tracheal smooth muscle. Tracheal smooth muscle strips were prepared (*2*) and treated with $4 \times 10^{-5}M$ forskolin, $10^{-6}M$ methacholine, or $2 \times 10^{-7}M$ atropine, individually or sequentially, for various periods. Control muscles were not treated with pharmacological agents. Cyclic AMP content, myosin PO_4 content, and changes in tension were determined on muscle strips treated with the pharmacological agents and frozen with clamps cooled in liquid nitrogen. The muscles were broken in half while frozen and half of each muscle was assayed for myosin PO_4 content (*2*). Cyclic AMP was measured on the other half of each muscle by the automated radioimmunoassay method (*16*). Percent relaxation was calculated from the equation $(DT/DM)100$ where DT is the decrease in tension at 10 minutes after the addition of atropine or forskolin and DM is the increase in tension at 15 minutes after adding $10^{-6}M$ methacholine. Thus 100 percent relaxation indicates a return to the resting tension (before methacholine). The stoichiometry of MLCK phosphorylation was determined in separate pieces of tissue labeled with ^{33}P (see legend to Fig. 1). The muscles were treated with the various agents, frozen, and broken in half while frozen. ATP was purified from half of the muscle and the specific activity expressed as counts per minute per picomole of ATP. MLCK was immunoprecipitated from the other half of each muscle (see legend to Fig. 1) and subjected to electrophoresis on an SDS-polyacrylamide gel. The amount of MLCK in each immunoprecipitate and the extent of radioactive labeling were measured. Labeling was expressed as counts per minute per picomole of MLCK and the stoichiometry of phosphorylation was calculated by dividing this number by the specific activity of the ATP. Data (means ± standard error for at least six different tissue samples) were analyzed for statistical significance with the paired Student's *t*-test (*17*).

Treatment	Cyclic AMP (pmole/mg protein)	MLCK PO_4 (mole/mole)	Myosin PO_4 (mole/mole LC_{20})	Percent relaxation
None (control)	4.4 ± 0.2	1.1 ± 0.1	0.27 ± 0.01	
Forskolin (10 minutes)	71.0 ± 12.4*	1.7 ± 0.1*	0.23 ± 0.01†	
Methacholine (15 minutes)	3.2 ± 0.5	1.1 ± 0.14	0.45 ± 0.01*	
Methacholine (15 minutes) and atropine (10 minutes)	5.1 ± 1.5	1.3 ± 0.1	0.30 ± 0.02	100
Methacholine (15 minutes) and forskolin (10 minutes)	38.4 ± 3.1*	1.9 ± 0.15†	0.34 ± 0.01†	73 ± 4.6

*$P < 0.001$. †$P < 0.01$.

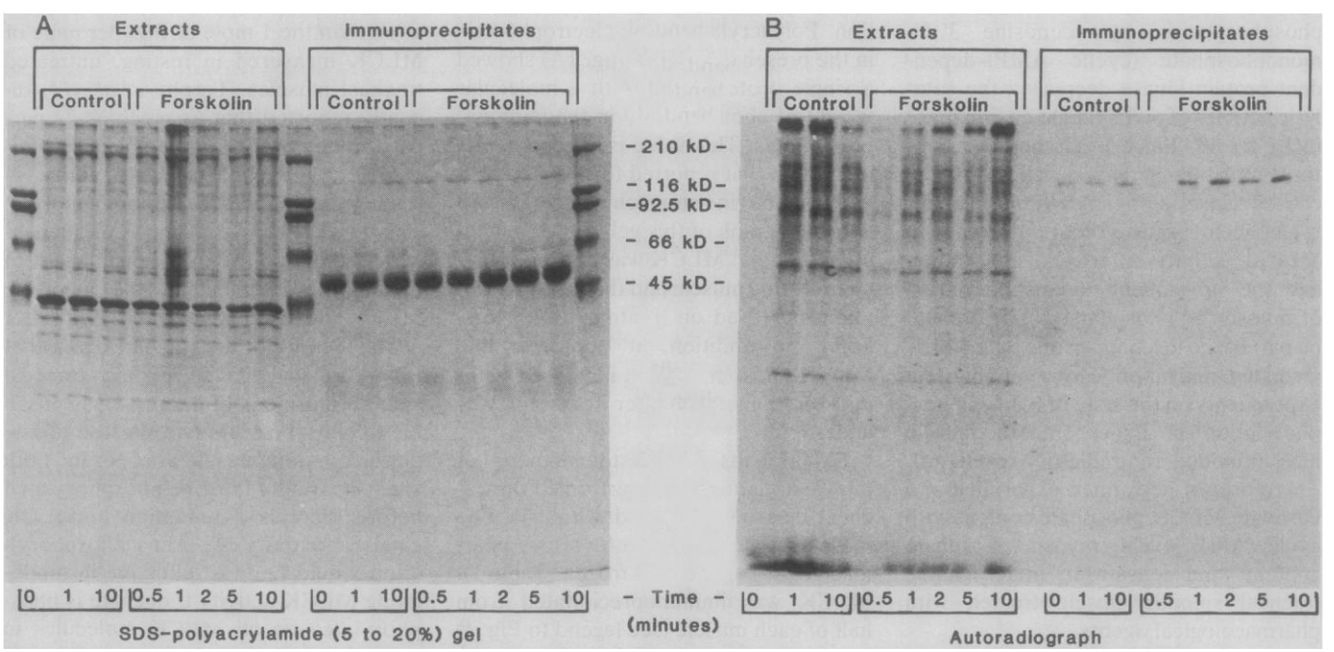

Fig. 1. (A and B) Myosin light chain kinase phosphorylation after treatment of tracheal smooth muscle with forskolin. Tracheal smooth muscles (*2*) were incubated at 37°C in 150 ml of PO_4-free Krebs-bicarbonate buffer (bubbled with 95 percent O_2 and 5 percent CO_2) containing 5 mCi of ^{32}P for 90 minutes. Control muscles and muscles treated with $4 \times 10^{-5}M$ forskolin were frozen at various times. The frozen muscles were homogenized in boiling 0.4 percent SDS and 40 m*M* tris (*p*H 7.5) with a ground glass homogenizer. The extract supernatants were collected by centrifugation, diluted with three volumes of 1.33 percent Triton X-100, 1.33 percent deoxycholate, 2.7*M* urea, 100 m*M* NaCl, 40 m*M* tris (*p*H 7.5), and incubated with 15 μg of affinity-purified antibodies to turkey gizzard MLCK (*8*) for 30 minutes at 4°C. The antigen-antibody complexes were precipitated with protein A–Sepharose, washed in 2*M* NaCl, 1 percent Triton X-100, 1 percent deoxycholate, 20 m*M* tris (*p*H 7.5), and eluted by boiling in SDS sample buffer. Muscle extracts and the eluted proteins were subjected to electrophoresis on a 5 to 20 percent polyacrylamide gradient gel in the presence of SDS, stained, destained, dried, and exposed to Kodak XTL-2 x-ray film; *kD*, kilodalton.

sequently, one can propose that forskolin treatment leads to an increase in cyclic AMP, activation of cyclic AMP–dependent protein kinase, phosphorylation of MLCK, dephosphorylation of myosin by phosphatases, and relaxation of tracheal smooth muscle.

However, an increase in cyclic AMP has been associated with a decrease in intracellular Ca^{2+} in smooth muscle (*13*). Since MLCK requires calcium-calmodulin for activity (*14*), relaxation could be due to inactivation of MLCK after a decrease in intracellular Ca^{2+}. Nevertheless, Kerrick and Hoar (*15*) have relaxed skinned smooth muscles contracted with high concentrations of calcium by adding the catalytic subunit of cyclic AMP–dependent protein kinase. This experiment suggests that it is possible to relax smooth muscles, presumably by a mechanism that involves MLCK phosphorylation, even in the presence of high intracellular calcium.

Finally, the data indicate that myosin is dephosphorylated when methacholine-contracted muscles are relaxed with atropine or forskolin (Table 1). These agents work by different mechanisms (*7*), and the fact that myosin is dephosphorylated after the addition of either agent suggests that myosin dephosphorylation may be essential for relaxation of smooth muscles.

In conclusion, these results show that MLCK is phosphorylated in intact tracheal smooth muscle, that MLCK phosphorylation increases when tracheal muscles are treated with forskolin, and that myosin dephosphorylation may be essential for relaxation of previously contracted muscles. Thus MLCK phosphorylation and myosin dephosphorylation appear to be part of the mechanism by which cyclic AMP causes relaxation of intact smooth muscles.

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Identification of an Infective Stage of *Leishmania* Promastigotes

Abstract. *Sequential development of* Leishmania *promastigotes from a noninfective to an infective stage was demonstrated for promastigotes growing in culture and in the sandfly vector. The generation of an infective stage was found to be growth cycle–dependent and restricted to nondividing organisms.*

Despite the importance of leishmaniasis among the parasitic diseases of man, our understanding of some elementary aspects of the developmental cycle of *Leishmania* parasites remains incomplete. Most *Leishmania* species are known to multiply as intracellular amastigotes in macrophages of their vertebrate host and as extracellular promastigotes in the midgut of their sandfly vector (*1*). A basic question remaining is whether or not sandfly promastigotes differentiate into an infective stage. If there is an infective stage of *Leishmania* in invertebrates, then, unlike other hemoflagellates, it does not appear to have a readily distinguishable morphological identity. Morphological differences between dividing midgut forms and those found anteriorly have been described (*2*); however, to our knowledge there has been no evidence that these changes reflect development of promastigotes into an infective stage. There is some

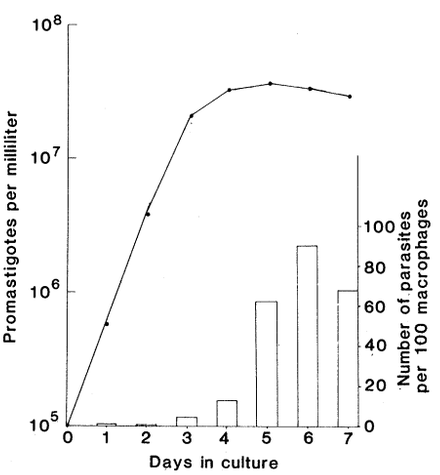

Fig. 1. Infectivity of cultured promastigotes, taken from various points in their growth curve, in mouse peritoneal macrophages in vitro. *Leishmania tropica* promastigotes (Friedlin strain NIH) were obtained from logarithmic phase cultures and inoculated (10^5 promastigotes per milliliter) into fresh medium containing medium 199 with Earle's balanced salts, 20 percent heat-inactivated fetal calf serum, 12 m*M* Hepes, 20 m*M* L-glutamine, and penicillin-streptomycin (50 μg/ml). One to seven days after initiation of the cultures, promastigotes were washed twice in medium 199, adjusted to a concentration of 5×10^6 promastigotes per milliliter, and used to infect adherent cultures of mouse peritoneal macrophages. Peritoneal cells were obtained from C57BL/6 mice (Jackson Laboratory), washed twice, and resuspended (2.5×10^6 cells per milliliter) in RPMI 1640 medium containing 10 percent fetal calf serum, 12 m*M* Hepes, 20 m*M* L-glutamine, and penicillin-streptomycin (50 μg/ml). Cells were plated in 0.4 ml on eight-chamber Lab-Tek tissue culture slides (Miles Laboratories) and allowed to adhere to the slides overnight at 37°C in 5 percent CO_2. Nonadherent cells were removed; adherent cells were infected with 10^6 promastigotes at an approximate ratio of two parasites per macrophage. After a 2-hour infection period at 35°C, free promastigotes were removed by repeated washings and cultures were incubated for up to 7 days at 35°C in 5 percent CO_2. Slides were fixed in absolute methanol for 30 minutes and stained with Diff-Quick solutions to make the intracellular amastigotes visible. For each culture over 500 cells were counted to determine the percentage of macrophages infected and the number of parasites per cell. The bars represent the total number of amastigotes per 100 macrophages present 3 days after infection with promastigotes obtained from the corresponding points in their growth curve.