Okadaic acid uncouples myosin light chain phosphorylation and tension in smooth muscle

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Tracheal smooth muscle precontracted with carbachol relaxes upon the addition of 3 μM okadaic acid. Although cytosolic Ca^{2+} concentrations decrease, myosin light chain remains highly phosphorylated (50%). In smooth muscle treated with carbachol alone or carbachol plus okadaic acid ^{32}P is incorporated into a single peptide on myosin light chain which corresponds to the site phosphorylated by myosin light chain kinase. Treatment with okadaic acid alone does not result in myosin light chain phosphorylation or tension development. These results suggest that a cellular mechanism other than myosin light chain phosphorylation can regulate contractile tension.

Okadaic acid; Smooth muscle relaxation; Myosin light chain phosphorylation

1. INTRODUCTION

The phosphorylation of the regulatory light chain of myosin (MLC) by Ca^{2+}/calmodulin-dependent MLCK is a primary step in the initiation of smooth muscle contraction. Phosphorylation of MLC increases the actin-activated myosin MgATPase activity and increases cross-bridge cycling rates leading to tension development [1,2]. Dephosphorylation of MLC by a protein phosphatase results in relaxation. While myosin phosphorylation/dephosphorylation may be the dominant regulatory pathway involved in initiating smooth muscle contraction, others have proposed Ca^{2+} activation mechanisms involving thin-filament regulatory processes [3,4] which may coexist with the thick-filament regulatory scheme involving MLC phosphorylation. We report here that okadaic acid, a potent protein phosphatase inhibitor [5–7], relaxes tracheal smooth muscle precontracted with carbachol without affecting MLC phosphorylation. These findings raise the possibility that a phosphoprotein(s) other than phosphorylated MLC might be involved in regulation of tension maintenance in smooth muscle.

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Abbreviations: MLC, 20 kDa myosin light chain; OA, okadaic acid; MLCK, myosin light chain kinase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

2.1. *Tissue preparation*

Strips of smooth muscle (5–10 mg, wet weight) were prepared from bovine trachea as previously described [8]. Muscles, stretched to optimal length (L_o) for tension development, were clamped after described treatments with tongs cooled in liquid nitrogen. After ^{32}P-labeling, strips were quick-frozen by submersion in dichlorodifluoromethane chilled in liquid nitrogen. All frozen muscles were stored at −60°C prior to biochemical analyses.

2.2. *Cytosolic Ca^{2+} levels*

Cytosolic Ca^{2+} concentration $[Ca^{2+}]_{cyt}$ was measured simultaneously with muscle contraction as reported previously [7,9] with a fluorescent Ca^{2+} indicator, fura-2 [10]. Muscle strips were loaded with 5 μM acetoxymethyl ester of fura-2 for 3 h in the presence of 0.02% Cremophor EL at room temperature (23–25°C) and then placed in a tissue bath at 37°C. Each muscle strip was illuminated alternately (48 Hz) with 340 and 380 nm light, and emission was detected at 500 nm. The amount of the 500 nm fluorescence induced by excitation at 340 and 380 nm was measured, and the ratio of the fluorescence values ($R_{340/380}$) was calculated. The ratio was used as an indicator of $[Ca^{2+}]_{cyt}$ with values normalized to 0% in resting and 100% in high K^+-stimulated muscle, respectively.

2.3. *Myosin light chain phosphorylation*

Frozen muscle strips (weighing approximately 3–5 mg) were placed in 10% trichloroacetic acid in acetone partially frozen (slurry), allowed to warm to room temperature and then homogenized in 200 μl 10% trichloroacetic acid and 10 mM dithiothreitol. After centrifugation, the protein pellet was processed for urea/glycerol-PAGE and immunoblotting [11,12]. Relative amounts of non-phosphorylated and monophosphorylated light chain were quantified from densitometric scans of immunostained nitrocellulose blots.

2.4. *Two-dimensional phosphopeptide mapping*

Protein samples from ^{32}P-labelled tissues were subjected to SDS-PAGE [12]. Following staining with Coomassie blue, gels were subjected to autoradiography. The band corresponding to the 20 kDa

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MLC was excised and processed for phosphopeptide mapping as previously described [12].

2.5. *Drugs and chemicals*

The following drugs and chemicals were used: carbachol, atropine (Sigma Chemical Co., St. Louis, MO, USA), acetoxymethyl ester of fura-2, EGTA (Dojindo Laboratories, Kumamoto, Japan), and Cremophor EL (Nakarai Chemicals, Kyoto, Japan). Okadaic acid isolated from *Halichondria okadai* (donated by Dr Daisuke Uemura, Shizuoka University) was dissolved in 100% ethanol to a 5 mM stock.

3. RESULTS

Bovine tracheal smooth muscle contracted in the presence of 0.1 μM carbachol relaxed upon the addition of 3 μM okadaic acid (Fig. 1). The relaxation response was associated with a decrease in the intracellular free Ca^{2+} concentration by 93% (Fig. 1). However, the relaxation process was not associated with a decrease in MLC phosphorylation (Table I). With carbachol alone, the extent of MLC phosphorylation was 53%. With the addition of okadaic acid to carbachol-treated tissues, MLC phosphorylation remained elevated. Treatment with okadaic acid alone did not result in MLC phosphorylation or tension development. Tissues pretreated with okadaic acid contracted upon the addition of carbachol although the response was attenuated compared to treatment with carbachol alone. No additional prominent phosphoprotein(s) were detected in muscle strips precontracted with carbachol and relaxed with okadaic acid compared with tissues treated with carbachol alone (Fig. 2). Two-dimensional phosphopeptide mapping of

Table I

Effect of carbachol and okadaic acid on tension and myosin light chain phosphorylation in bovine tracheal smooth muscle strips

Condition	Fractional tension[a,b]	Myosin light chain phosphorylation[b]
Relaxed	0	6 ± 0.8
0.1 μM carbachol 10 min	1.0	53 ± 3
0.1 μM carbachol 25 min	1.0	51 ± 3
0.1 μM carbachol (10 min) + 3 μM OA 15 min	0.09 ± 0.04	50 ± 3
3 μM OA 15 min	0	0

[a] Fractional tension is tension normalized to that produced by 0.1 μM carbachol at 10 min

[b] Values shown are means ± SEM, for 8–10 muscle strips

MLC showed that the same site was phosphorylated in tissues treated with carbachol alone or carbachol plus okadaic acid (Fig. 2). The primary [^{32}P]phosphopeptide corresponded to MLC monophosphorylated at serine 19 by MLCK [8,12].

4. DISCUSSION

It has been generally accepted that the phosphorylation of MLC by Ca^{2+}/calmodulin-dependent MLCK is both obligatory and sufficient for the biochemical activation of smooth muscle contraction [1,2,13]. Recent physiological observations suggest that other regulatory mechanisms may modify the Ca^{2+} sensitivity of contractile tension in smooth muscle [14–16]. Since

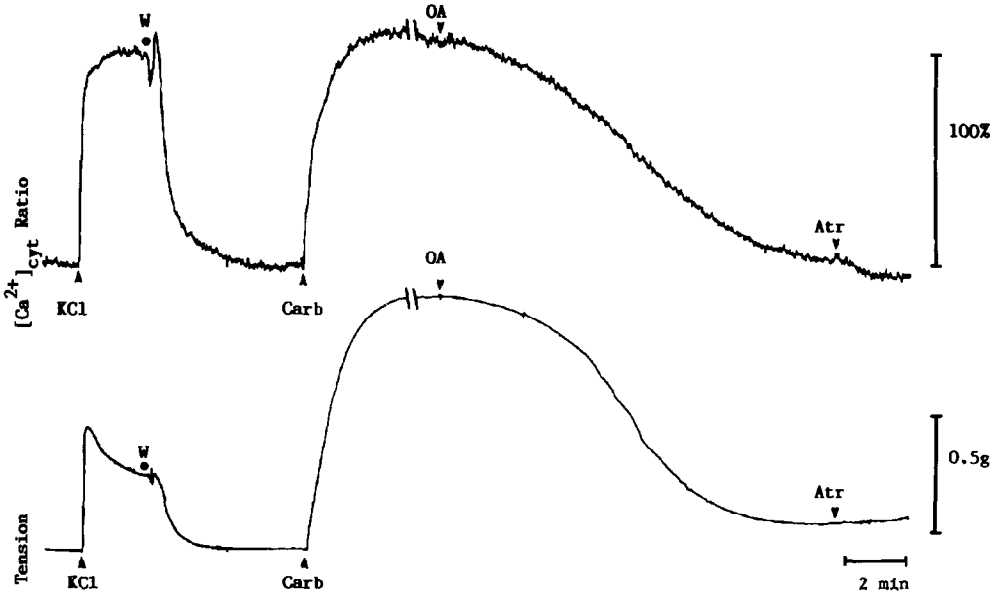

Fig. 1. Effect of okadaic acid (OA) on $[Ca^{2+}]_{cyt}$ and tension in bovine tracheal smooth muscle. Muscles were first contracted in 72.7 mM KCl (made by replacing NaCl with KCl) and then with 0.1 μM carbachol (Carb). Carbachol remained thereafter. Atropine (Atr) at 1 μM final concentration was added as indicated after treatment with 3 μM okadaic acid.

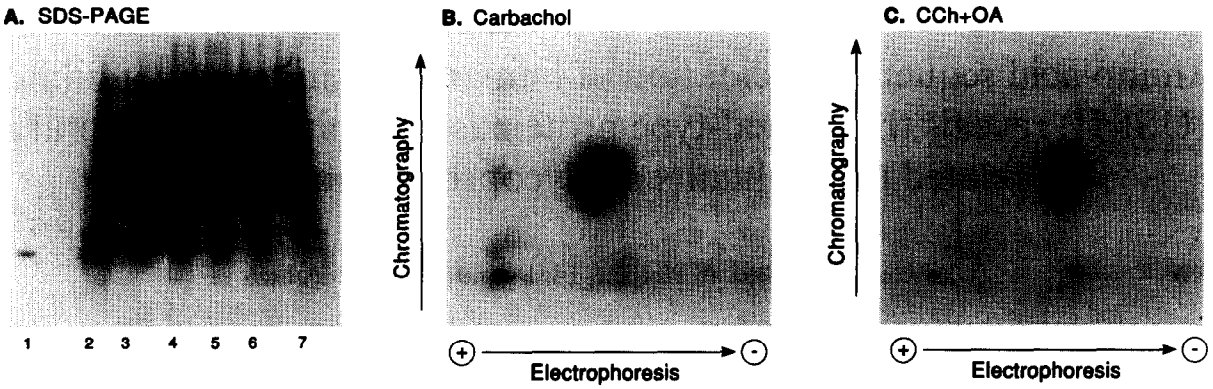

Fig. 2. (A) Autoradiogram of SDS-polyacrylamide gradient gel (3–20%) of homogenates from ^{32}P-labeled bovine tracheal smooth muscle (lane 1, purified [^{32}P]MLC; lane 2, 10 min CCh; lane 3, 25 min CCh; lanes 4 and 5, 10 min CCh + 15 min OA; lanes 6 and 7, 15 min OA alone). Two-dimensional phosphopeptide maps of MLC from tracheal smooth muscle treated with carbachol (B) or carbachol plus okadaic acid (C).

the protein phosphatase inhibitor okadaic acid (3 μM) relaxes precontracted tracheal smooth muscle with no significant decrease in the extent of MLC phosphorylation, it is reasonable to postulate the existence of another biochemical mechanism that is regulating smooth muscle contraction. The maintenance of MLC phosphorylation is probably due to inhibition of the protein phosphatase that dephosphorylates this protein. Relaxation could be mediated by the decrease in $[Ca^{2+}]_{cyt}$ resulting in inhibition of phosphorylated myosin crossbridge interactions with actin, potentially mediated by thin filament regulatory proteins such as calponin or caldesmon [3,4,17]. The decrease in $[Ca^{2+}]_{cyt}$ may be due to inhibition of a protein phosphatase that dephosphorylates proteins involved in Ca^{2+} influx. Alternatively, okadaic acid treatment could lead to phosphorylation of a contractile regulatory protein in addition to MLC. In either case, this raises the possibility that a phosphoprotein may be involved in the secondary regulation of smooth muscle relaxation. Our findings indicate that MLC phosphorylation is not the only mechanism involved in the regulation of tension maintenance in smooth muscle.

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