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PROLONGED RELAXATION OF DETERGENT-SKINNED SMOOTH MUSCLE INVOLVES DECREASED ENDOGENOUS PHOSPHATASE ACTIVITY

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ABSTRACT. Since contraction of smooth muscle involves Ca^{2+}-dependent phosphorylation of the 20 Kd myosin light chains, changes in endogenous phosphatase activity may participate in regulating smooth muscle contractility. We found that detergent-skinned fibers from 7 of 10 chicken gizzards studied were characterized by relatively high endogenous light chain phosphatase activity (23 mU/mg protein) and rapid relaxation ($t_{1/2}$ = 1-3 min) in the absence of Ca^{2+} ($<10^{-8}$M). In contrast, skinned fibers from 3 of the gizzards exhibited very low phosphatase activity (3 mU/mg protein) and markedly prolonged relaxation ($t_{1/2}$ = 50-200 min). However, such slow relaxing fibers were converted to a form resembling rapidly relaxing fibers ($t_{1/2}$ = 4-10 min) when an aortic polycation-modulable phosphatase was included in the incubation medium. Moreover this phosphatase-enhanced relaxation was associated with dephosphorylation of the light chains. Maximal isometric force (1 mN) and light chain phosphorylation (0.8 mol PO_4/mol light chain) were similar in slowly and rapidly relaxing fibers. Thus, the two populations of skinned fibers, though dramatically different with respect to phosphatase activity and relaxation time, appeared to be very similar in terms of Ca^{2+}-dependent contraction. These findings strongly suggest that prolonged relaxation of smooth muscle of the kind noted in this study, and perhaps in hypertensive or aging vascular smooth muscle, may reflect decreased endogenous phosphatase activity.

INTRODUCTION. Contraction of a variety of smooth muscles, including the smooth muscle of chicken gizzard, involves Ca^{2+}-dependent phosphorylation of the 20 Kd myosin light chains (1,2). Accordingly, dephosphorylation of the light chains by protein phosphatase should suppress actin-myosin interaction and enhance relaxation of smooth muscle. Recently, we identified an aortic protein phosphatase which dephosphorylated aortic native myosin, myocardial myosin light chains, and phosphorylase a (3). This multisubstrate phosphatase was called polycation modulable (PCM-) phosphatase because its activity in vitro was subject to modulation by polycationic effectors such as lysine-rich histone-H_1 or polylysine (4-6). The PCM-phosphatase inhibited actin-myosin interaction in aortic native actomyosin and accelerated relaxation of detergent-skinned porcine carotid arterial smooth muscle (3,7). However, the extent to which relaxation was correlated to light chain phosphorylation was not determined.

In this study, we exploited the availability of two apparently different populations of chicken gizzard. Detergent-skinned fibers from one population were characterized by rapid relaxation in the absence of Ca^{2+}, whereas relaxation of the second population was prolonged by 25-50 fold. In this communication we show that prolonged relaxation of slow relaxing fibers is probably ascribable to reduced endogenous phospha-

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tase activity and that relaxation can be normalized by addition of exogenous PCM-phosphatase. To our knowledge, this is the first report showing that prolonged relaxation of smooth muscle reflects a biochemical defect involving a deficiency in either the amount or activity of endogenous phosphatase. These findings suggest the interesting possibility that defects in phosphatase activity may contribute to prolonged relaxation of smooth muscle noted in pathological conditions such as hypertension (8).

MATERIALS AND METHODS. Skinned fiber bundles were prepared from fresh chicken gizzards as described earlier (7) using a method adapted from Gordon (9). Smooth muscle strips (1.5 cm x 2 mm) were incubated at 0°C for 30 min in a solution containing 20 mM imidazole pH 7.4, 50 mM KCl, 5 mM EGTA, and 150 mM sucrose followed by 4 hr in the same solution containing 1% triton X-100 and 0.5 mM dithioerythritol (DTE). The strips were transferred to 20 mM imidazole pH 7.4, 4 mM EGTA, 10 mM $MgCl_2$, 7.5 mM ATP, 1 mM NaN_3, 0.5 mM DTE and 50% glycerol.

Skinned fiber bundles (5-7 mm long, 100-200 μm thick) were mounted horizontally between a glass post attached to an isometric force transducer and a fixed micrometer drive. The bundle was stretched by about 5% to a resting tension of 0.1-0.3 mN and bathed in 250 μl of a "relaxing solution" containing 20 mM imidazole pH 7.0, 4 mM EGTA, 7.5 mM ATP, 10 mM $MgCl_2$, 1 mM NaN_3, 2 mM DTE, 0.5 μM calmodulin, and an ATP regenerating system consisting of 10 mM phosphocreatinine and 10 U/ml of creatine phosphokinase. The concentration of Ca^{2+} was $<10^{-8}$M and the temperature of the solution was 25°C. Maximal contraction was elicited by transferring the fiber to a "contracting solution" of similar composition wherein EGTA was partially replaced with Ca-EGTA to provide the desired Ca^{2+} (1.25×10^{-5}M). The concentration of Ca^{2+} was adjusted by using an apparent dissociation constant of $4.2 \times 10^{7}M^{-1}$ for EGTA at pH 7.0 correcting the fact that pH refers to H^+ activity rather than concentration (3,7,10).

The time required for 50% relaxation was determined graphically (10). Extent of light chain phosphorylation was assessed by 2-dimensional electrophoresis (11-13). At the desired time during contraction (1.25×10^{-5}M Ca^{2+}) or relaxation ($<10^{-8}$M Ca^{2+}) the fibers were immersed in ice cold 15% trichloracetic acid for 15 min, rinsed in 20 mM imidazole pH 7.0 and homogenized in 200 μl 10 mM DTE, 50 mM phosphate buffer pH 7.5, and 1% sodium dodecylsulfate (SDS). This arrests endogenous enzyme activities so that spurious changes in light chain phosphorylation are avoided during further processing of the sample for analysis (11). Samples were subjected to isoelectric focusing in the first dimension (12), and then to SDS electrophoresis in the second dimension (11,13, 15).

Experiments were performed in the presence and absence of aortic PCM-phosphatase. The phosphatase was purified by procedures including ion exchange chromatography on DEAE-Sephadex, precipitation with 30-50% $(NH_4)_2SO_4$, gel filtration on Ultrogel AcA-34, and chromatography on polylysine-Sepharose (3-7). The specific activity of the enzyme was 1,600 U/mg when phosphorylated myocardial light chains were substrate and 300 U/mg when phosphorylase a was substrate. One unit (U) of phosphatase activity is that amount of enzyme which releases 1 nmol ^{32}P/min at 30°C from the substrate tested. In accord with previous studies (3-6) the aortic phosphatase was active in the absence of either Mg^{2+}, Mn^{2+}, or Co^{2+} and was free of calmodulin, calmodulin binding proteins, light chain kinase and cAMP-dependent kinase. Phosphorylase phosphatase activity was stimulated 5-7 fold by 40 nM polylysine (Mr = 13,000) whereas light chain phosphatase activity was abolished (5,6). Endogenous phosphatase activity of skinned fibers was determined using phosphorylated myocardial light chains as substrate (3-7). Results are given as means ± 1 SEM, and statistical significance of differences between measured parameters was assessed with Student's t-test.

RESULTS AND DISCUSSION. Two types of skinned fibers were distinguishable from each other with respect to time required for relaxation following removal of Ca^{2+} from the incubation medium (Fig. 1). The predominant type, obtained from 7 of 10 gizzards, was characterized by a rapid rate of relaxation in low Ca^{2+} ($<10^{-8}$M). The $t_{1/2}$ for relaxation of fibers of this kind was 2 ± 0.4 min (n=10). In contrast, the $t_{1/2}$ for relaxation of skinned fibers prepared from each of 3 other gizzards was 25 to 50 fold greater ranging from 50 to 200 min (n=5). However, when aortic PCM-phosphatase was added to the incubation medium, relaxation of slow fibers was markedly enhanced (Fig. 1, top). For example, the $t_{1/2}$ for relaxation of such fibers was reduced to 9.2 ± 0.4

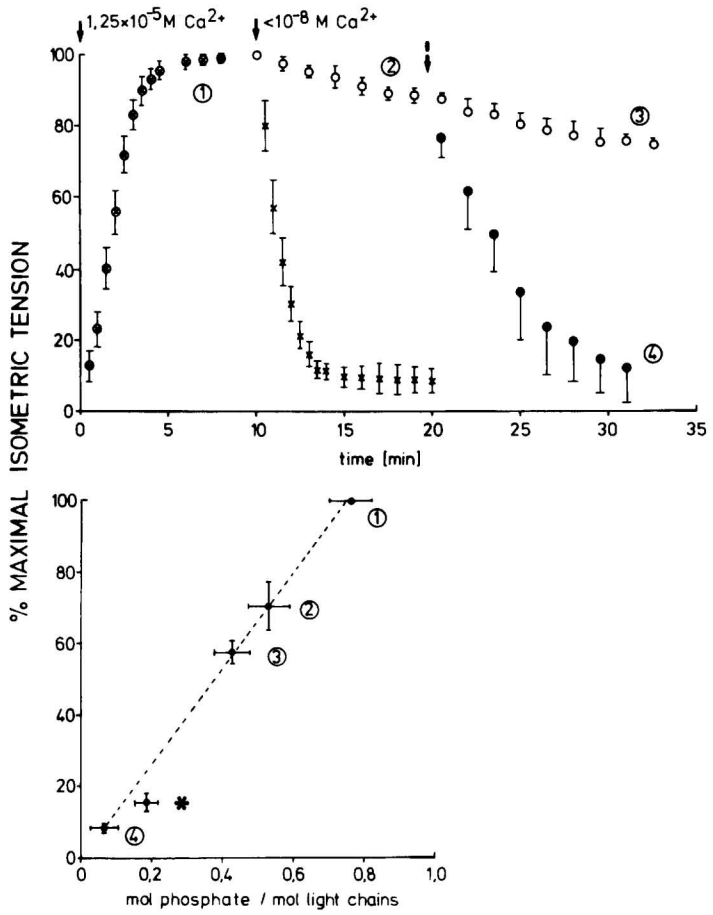

Fig. 1. The top panel shows the time course for isometric contraction and relaxation of 16 detergent-skinned fiber bundles from fast and slow gizzards. Maximal contraction was elicited for 10 min in 1.25 x 10^{-5}M Ca^{2+} (first arrow). Since maximal isometric tension (1.02 ± 0.41 mN) and rate of contraction were essentially the same for both types of fiber corresponding, data points (⊠) were combined. Relaxation, induced by lowering Ca^{2+} to $<10^{-8}$M (second arrow), of fast fibers (X, n=6) was virtually complete within 2 min, whereas less than 10% relaxation occurred in slow fibers during the same interval (O, n=10). Addition of aortic PCM-phosphatase (interrupted arrow, 12.8 U/ml) to half of the slow fibers studied resulted in rapid and pronounced relaxation (●). In contrast, isometric tension remained high in slow fibers which were not exposed to PCM-phosphatase (O). Each point is the mean value for the number of fibers previously indicated and small vertical bars represent 1 SEM. 1, 2, 3, and 4 indicate times at which analogous fibers were processed for determination of myosin light chain phosphorylation.

The bottom panel shows the relationship between isometric tension attained and light chain phosphorylation in 25 detergent-skinned fibers from 3 slow gizzards. In accordance with the time course illustrated in the top panel, fibers were analyzed after 10 min of incubation in $<10^{-8}$M Ca^{2+} (•); following maximal contraction in 1.25 x 10^{-5}M Ca^{2+} (1); 10 min after transfer of maximally contracted fibers to relaxing solution of $<10^{-8}$M Ca^{2+} (2); 30 min after transfer of maximally contracted fibers to relaxing solution (3); after phosphatase-induced (12.8 U/ml) relaxation of contracted fibers (4). Five fibers were included in each group. Small vertical bars show 1 SEM for isometric tension, whereas horizontal bars show 1 SEM for extent of phosphorylation.

min in the presence of 6.4 U phosphatase/ml (n=4). Moreover, the relaxant effect of PCM-phosphatase was concentration dependent: increasing the concentration of phosphatase to 12.8 U/ml produced a further decrease in $t_{1/2}$ to 4.2 ± 0.3 min (n=5, p <0.005 with respect to 6.4 U/ml).

The apparent conversion of slowly relaxing fibers (SRF) to a form resembling rapidly relaxing fibers (RRF) during incubation with PCM-phosphatase suggested that the slow fibers contained significantly lower phosphatase activity than RRF. This hypothesis was confirmed in that the endogenous phosphatase activity of rapid fibers (23 mU/mg protein) measured against phosphorylated myocardial myosin light chains was about 8 fold greater than the activity of slow fibers (3 mU/mg protein). This difference in phosphatase activity cannot be ascribed to procedural differences because rapidly and slowly relaxing fibers were skinned, stored, and studied under the same conditions. Conceivably, low endogenous phosphatase activity might reflect a biochemical lesion underlying slow relaxation of smooth muscle which is reportedly manifest in pathophysiological conditions. It will be interesting to determine whether or not prolongation of relaxation noted in aging (14) or hypertensive vascular smooth muscle (8) is associated with decreased endogenous phosphatase activity.

We also found that the level of isometric tension attained in SRF was correlated to the extent of myosin light chain phosphorylation (Fig. 1, bottom). The level of phosphorylation attained during maximal contraction (0.8 ± 0.06 mol phosphate/mol light chain, n=5), though similar to values obtained with skinned porcine carotid artery (11,15), was about 2-3 fold greater than values reported earlier for skinned gizzard fibers (19). This apparent disparity may be due to procedural differences (e.g., methods for skinning fibers and/or arresting endogenous phosphatase activity to prevent dephosphorylation during analysis).

A linear relationship was obtained between isometric tension and light chain phosphorylation (Fig. 1). This result is consistent with findings of Chacko and Rosenfeld (17) which indicated that actin-activated myosin ATPase activity of myosin purified from porcine pulmonary artery was directly proportional to light chain phosphorylation. The result also in agrees with a report by Silver and Stull (18) showing that isometric force and light chain phosphorylation were linearly related in living bovine tracheal smooth muscle. However, all of these findings differ from results initially reported by Persechini and Hartshorne (22) which showed that phosphorylation of gizzard light chains proceeds in an ordered fashion. In this situation a nonlinear relationship is obtained between myosin ATPase activity and light chain phosphorylation. Sigmoidal relationships between phosphorylation and actin-myosin interaction were also obtained in recent studies performed in this laboratory with aortic native actomyosin (7) and skinned porcine carotid artery (11). The reason(s) for these disparities is unknown and further discussion in the absence of additional data is probably premature.

The major points developed from the analysis given in Fig. 1 are that (a) prolonged maintenance of isometric tension by SRF in the absence of Ca^{2+} ($<10^{-8}$M) is associated with persistent high levels of light chain phosphorylation, and (b) prompt relaxation produced in response to PCM-phosphatase is associated with dephosphorylation of the light chains. These observations reinforce the conclusion that prolongation of isometric tension in SRF is ascribable to an apparent deficiency of endogenous phosphatase. This deficiency may reflect decreases in enzyme content, and/or decreases in expressed enzyme activity possibly resulting from alterations of interactions between phosphatase and cationic effectors (5,6).

In the past we tended to regard SRF as technically poor preparations. It is likely that other scientists encountering similar situations reacted in the same fashion (4). However, the present findings suggest that such fibers may reflect naturally occurring pathophysiologic conditions. Our findings are compatible with the hypothesis that alterations in expressed phosphatase activity (i.e., SRF vs. RRF) may participate in modulating contractile responses in smooth muscle.

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