# Contraction of myofibroblasts in granulation tissue is dependent on Rho/Rho kinase/myosin light chain phosphatase activity

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## ABSTRACT

During wound healing and fibrocontractive diseases fibroblasts acquire a smooth muscle cell-like phenotype by differentiating into contractile force generating myofibroblasts. We examined whether regulation of myofibroblast contraction in granulation tissue is dominated by $Ca^{2+}$-induced phosphorylation of myosin light chain kinase or by Rho/Rho kinase (ROCK)-mediated inhibition of myosin light chain phosphatase, similar to that of cultured myofibroblasts. Strips of granulation tissue obtained from rat granuloma pouches were stimulated with endothelin-1 (ET-1), serotonin, and angiotensin-II and isometric force generation was measured. We here investigated ET-1 in depth, because it was the only agonist that produced a long-lasting and strong response. The ROCK inhibitor Y27632 completely inhibited ET-1–promoted contraction and the phosphatase inhibitor calyculin elicited contraction in the absence of any other agonists, suggesting that activation of the Rho/ROCK/myosn light chain phosphatase pathway is critical in regulating in vivo myofibroblast contraction. Membrane depolarization with $K^+$ also stimulated a long-lasting contraction of granulation tissue; however, the amount of force generated was significantly less compared to ET-1. Moreover, $K^+$-induced contraction was inhibited by Y27632. These results are consistent with inhibition of myosin light chain phosphatase by the Rho/ROCK signaling pathway, which would account for the long-duration contraction of myofibroblasts necessary for wound closure.

Myofibroblasts are responsible for the generation of contractile force in granulation tissue and pathological contractures.[1–3] Granulation tissue that contains myofibroblasts generates measurable amounts of isometric force in response to specific agonists of smooth muscle (SM) cell contraction, such as serotonin, angiotensin-II (AT-II), and endothelin-1 (ET-1).[4–7] Previous studies have examined the effect these agonists have on myofibroblast force generation;[6,8] however, little has been done to examine the intracellular signaling pathways utilized by these agonists to promote the contraction of myofibroblasts in granulation tissue.

Myofibroblasts form stress fibers containing the major contractile elements actin and myosin II (for a review, see Tomasek et al.[2]). The activation of myosin II that allows its cyclic attachment to actin filaments involves myosin light chain phosphorylation by the $Ca^{2+}$-calmodulin-dependent myosin light chain kinase (MLCK). Dephosphorylation of myosin light chain by myosin light chain phosphatase (MLCP) inactivates myosin.[9] In fibroblasts and SM cells, stress fiber contraction is mediated by MLCK in response to intracellular $Ca^{2+}$ and is rapid and short in duration being terminated by MLCP.[10,11] In contrast, inactivation of MLCP via a Rho/Rho kinase (ROCK) mechanism leads to a sustained contraction.[10,12,13] Differentiated myofibroblasts exhibit SM cell features, such as the expression of SM α-actin in stress fibers,[14] raising the question of whether myofibroblast contraction is regulated similar to SM cells or to fibroblasts. We, and others, have recently demonstrated that

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|---|---|
| AT-II | Angiotensin-II |
| CPI-17 | 17 kDa protein kinase C-potentiated inhibitory protein of type I protein phosphatase |
| ET-1 | Endothelin-1 |
| MLCK | Myosin light chain kinase |
| MLCP | Myosin light chain phosphatase |
| MYPT1 | Myosin phosphatase target subunit-1 |
| ROCK | Rho/Rho kinase |
| SM | Smooth muscle |

myofibroblast contraction in vitro is regulated predominantly through MLCP inactivation via the Rho/ROCK pathway.[15,16] We hypothesize that the Rho/ROCK/MLCP pathway is the predominant regulatory mechanism for myofibroblast contractile activity in granulation tissue and that this can promote wound closure by sustained rather than rapid contraction.[2]

In this study we have used rat granuloma pouch granulation tissue[17,18] to evaluate the role of the Rho/ROCK/MLCP pathway in regulating myofibroblast contraction in vivo. We have found that ET-1 promotes significantly greater and longer-lasting force than either serotonin or AT-II; therefore, we confined our studies to the regulation of ET-1–promoted contraction of myofibroblasts in granulation tissue. ET-1–promoted contraction was partially inhibited by the MLCK inhibitor ML-7 and completely blocked by the broad spectrum kinase inhibitor H-7, suggesting that an alternative kinase to MLCK is critical for regulating the contraction of myofibroblasts in granulation tissue. The ROCK inhibitor Y27632 completely inhibited ET-1–promoted contraction, while the phosphatase inhibitor calyculin promoted contraction of myofibroblasts in granulation tissue. Membrane depolarization with $K^+$, which results in an influx of $Ca^{2+}$, promoted a long-duration contraction similar to ET-1; however, the amount of force generated was less than 35% of that generated by ET-1. Also similar to ET-1, $K^+$-induced contraction was inhibited by the ROCK inhibitor Y27632. These results are consistent with the hypothesis that the Rho/ROCK/MLCP pathway is critical in regulating myofibroblast contraction in granulation tissue and is responsible for the sustained force generation required for wound closure.

## MATERIALS AND METHODS

We have used the granuloma pouch model of granulation tissue to study the effects of several SM cell contraction-inducing and -inhibiting agents on the isometric force development by a myofibroblast-rich tissue. Granuloma pouches were induced in anesthetized male Sprague–Dawley rats (200–300 g) by injecting 20 mL of air into the dorsal subcutaneous tissue, followed by 1 mL of 1% croton oil in corn oil.[17,18] This treatment produces the formation of an abscess-like structure whose wall is rich in myofibroblasts.[5,7,19] Granuloma pouch granulation tissue was dissected 21 days postformation and transferred to Tyrode's solution (134 mM NaCl, 3.4 mM KCl, 2.3 mM $KH_2PO_4$, 0.30 mM $MgSO_4$, 2.8 mM $CaCl_2$, 7.7 mM D-glucose, and 16.1 mM $NaHCO_3$), bubbling with 95% $O_2$ and 5% $CO_2$. All experiments were performed following the University of Oklahoma-Health Sciences Center IACUC approved protocols.

### Isometric force measurement

Dissected granuloma pouch tissues were placed into gassed Tyrode's solution and cut into strips 5×15 mm in length. The ends of the strips were tied with 5-0 silk suture and immediately placed in tissue baths (Radnoti Glass Technology Inc., Monrovia, CA) containing 9 mL of Tyrode's solution bubbling with a 95% $O_2$ and 5% $CO_2$ mixture at 37 °C. One side of each strip was tied to an analog force transducer (Kent Scientific, Torrington, CT) and the other was tied to a stabilized glass rod in the tissue bath. The strips were allowed to rest for 1 hour, stretched three times to a tension of 1.5 g over 1 hour, and then allowed to rest for another hour before treatment. During this time tissue baths were washed every 10 minutes with fresh Tyrode's solution and continuously bubbled with a 95% $O_2$ and 5% $CO_2$ mixture. Measurements were made on an Apple Macintosh Quadra 650 using the Strawberry Tree Data Acquisition System (copyright 1987–89, Strawberry Tree Inc., Sunnyvale, CA). Tension was recorded every 2 seconds, and the peak resulting from each treatment was used to produce dose–response curves.

In a first series of experiments, maximal tension development was determined after cumulative stimulation with ET-1, AT-II, and serotonin (all from Bachem Inc., Torrance, CA) in concentrations ranging from $10^{-10}$ to $10^{-4}$ M. ET-1 promoted the strongest and longest duration contraction and was used in subsequent experiments at $10^{-7}$ M. The phosphatase inhibitor calyculin (BIOMOL Research Laboratories Inc., Plymouth Meeting, PA) was also tested for contraction. For membrane depolarization tissue strips were exposed to 60 mM $K^+$ solution. The following inhibitors were used to determine their effect on ET-1–promoted contraction: broad spectrum kinase inhibitor H-7 (1-(5-iso-quinolinylsulfonyl)-2-methylpiperazine; Sigma-Aldrich, St. Louis, MO),[20] specific MLCK inhibitor ML-7 (1-(5-chloronaphthalenesulfonyl)-1H-hexahydro-1,4-diazepine; BIOMOL Research Laboratories Inc.),[21] and ROCK inhibitor Y27632 (Calbiochem-Novabiochem Corp., San Diego, CA).[22] Granulation tissue strips were initially contracted with ET-1 followed by extensive washing for 1 hour and subsequent treatment with the inhibitor and a secondary contraction with ET-1. Percent inhibition was determined by comparing the secondary contraction that occurred in response to inhibitor plus ET-1 with the initial contraction that occurred in response to ET-1 alone. Tension peaks were determined and mean values calculated from three tissue strips per animal and at least three animals per experimental condition.

## RESULTS

Previous studies have shown that ET-1, AT-II, and serotonin promote contraction of granulation tissue.[4–7] In this study, we compared the ability of these agonists to promote contraction and the duration of force generation. All three substances promoted a dose-dependent contraction of granulation tissue, with approximately a twofold greater amount of force generated by ET-1 compared with AT-II and serotonin (Figure 1A). Serotonin (Figure 1B) and AT-II (Figure 1D) both elicited a rapid rise in force with maximum force generated within 5 minutes and force levels returned to baseline within 30 minutes of drug administration. In contrast, ET-1 elicited a slower rise in force requiring up to 15 minutes to reach maximum contraction (Figure 1C). This maximum force generation was maintained for up to 60 minutes, with a subsequent slow loss of force taking as long as 90 minutes to reach baseline. These results show that ET-1 promotes a contraction of much greater intensity and longer duration than either AT-II or

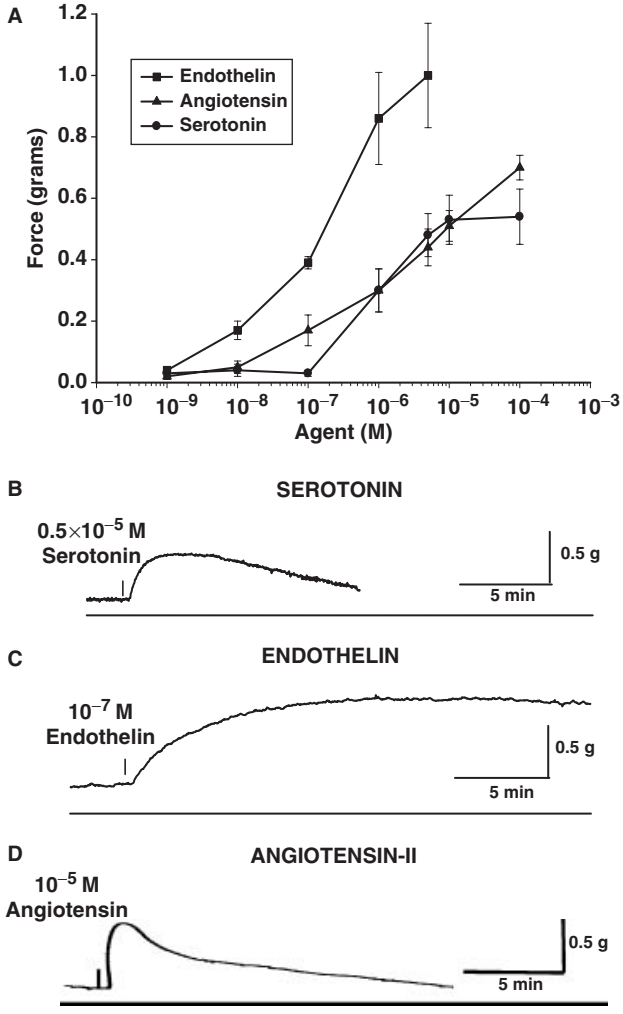


**Figure 1.** Generation of contractile force by granulation tissue in response to ET-1, AT-II and serotonin. Strips of 21-day granuloma pouch tissue were treated with ET-1, AT-II, and serotonin and force generation was recorded. (A) Dose–response curves were determined by measuring maximum contraction after application of contraction agonists; all agonists elicited a dose-dependent increase in force generation; however, ET-1 generated greater force than either AT-II or serotonin. (B) Serotonin promoted a rapid increase in force generation followed by a short plateau and a rapid fall in force. (C) ET-1 promoted a slow increase in force followed by a long plateau. (D) AT-II promoted a sharp increase in force generation, followed by a drop to approximately 70% within 2 minutes and a slow decrease thereafter.

serotonin; therefore, we examined regulation of force generation in ET-1–promoted contraction in this study.

To be certain that contraction of the granulation tissue strips was not the result of SM cell contraction in small vessels we compared the contraction of rat granuloma pouch tissue and bladder in response to specific SM cell agonists. Electrical field stimulation (5–40 kHz), norepinephrine ($10^{-5}$ μM), carbachol ($10^{-4}$ μM), and α-methyl ATP ($10^{-3}$ μM) promoted significant contraction of bladder; however, none of these treatments could promote greater than 5% of the contraction elicited by ET-1 ($10^{-7}$ M) on the same granulation tissue strip (not illustrated).

### Protein kinase inhibitors inhibit ET-1–promoted contraction of granulation tissue

Two protein kinase inhibitors were tested for their abilities to inhibit ET-1–promoted contraction of granulation tissue; H-7, a broad spectrum kinase inhibitor,[20] and ML-7, a specific inhibitor for MLCK.[21] We have previously demonstrated that both inhibitors inhibit contraction of cultured myofibroblasts.[15] H-7 was found to inhibit ET-1–promoted contraction in a dose-dependent manner by greater than 50% at 50 μM (Figure 2) and elicited relaxation of ET-1–contracted tissue (not illustrated). ML-7 inhibited ET-1–promoted contraction significantly less than H-7 (Figure 2), suggesting that an alternative kinase to MLCK is important in regulating myofibroblast contraction in granulation tissue.

### ROCK inhibitor Y27632 blocks ET-1–promoted granulation tissue contraction

The small GTPase Rho and its downstream mediator, ROCK, promote stress fiber assembly and increased contractility in cultured fibroblasts.[12,23] We have previously demonstrated that activation of Rho and ROCK is necessary for contraction of cultured myofibroblasts.[15] We utilized the ROCK inhibitor Y27632 to determine whether activation of ROCK is necessary for ET-1–promoted contraction of granulation tissue. Granulation tissue strips treated with Y27632 at concentrations greater than $10^{-7}$ M showed a reduced contractile response to ET-1 (Figure 3). Y27832 at $10^{-4}$ M decreased baseline tension and dramatically reduced ET-1-promoted force by greater than 80%. When added to granulation tissue in the phase of ET-1–promoted contraction, Y27632 caused relaxation at concentrations of $10^{-5}$ M and above (not illustrated). These results suggest that active ROCK is necessary for ET-1 to promote contraction of granulation tissue.

### Type I phosphatase inhibitor is sufficient to promote granulation tissue contraction

MLCP activity is reduced by ROCK-dependent phosphorylation of the myosin binding subunit resulting in increased force generation.[24] MLCP is a member of the family of type 1 phosphatases that can be inhibited by the phosphatase inhibitor calyculin,[24–26] which is sufficient to promote contraction of cultured myofibroblasts.[15] Granulation tissue contracted in response to the addition of $10^{-6}$ M calyculin (Figure 4). The amount of force generated in response to calyculin was greater than 50% of that elicited in response to $10^{-7}$ M ET-1 administered after washing out the calyculin. These results suggest that MLCP plays an important role in the regulation of granulation tissue contraction.

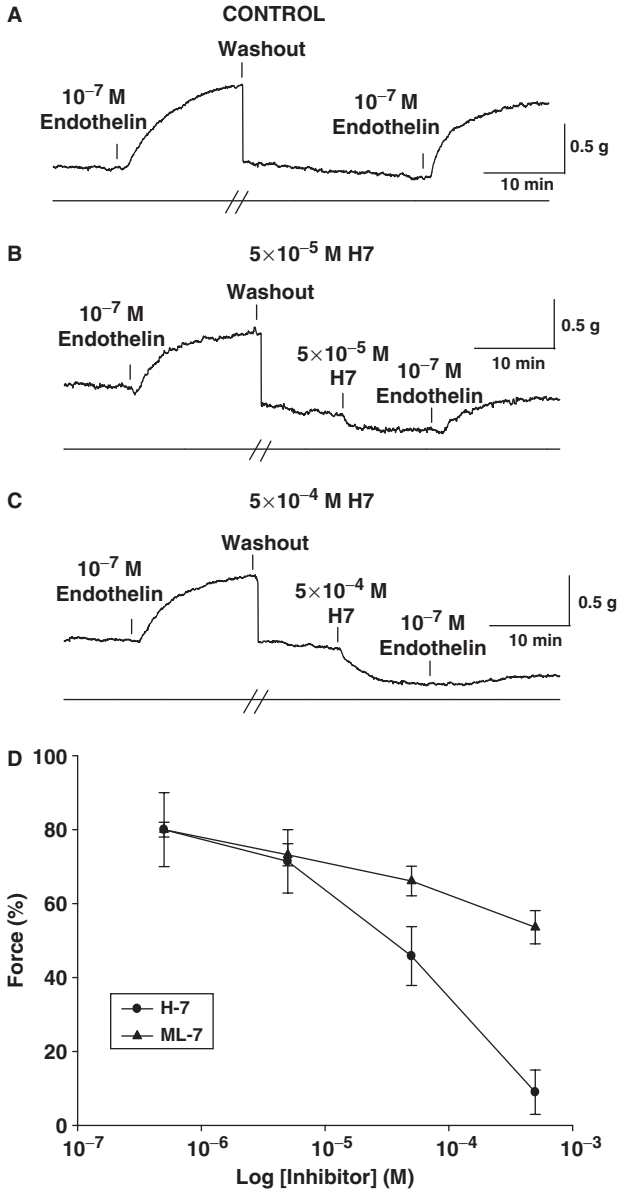


**Figure 2.** Kinase inhibitors H-7 and ML-7 inhibit ET-1-promoted force generation of granulation tissue. (A-C) Strips of 21-day-old granuloma pouch tissue were initially contracted with ET-1, extensively washed for 1 hour, subsequently treated with H-7, followed by a secondary contraction with ET-1. (A) Compared to the initial contraction in control experiments, secondary contraction with ET-1 is slightly lower; (B) this secondary contraction is dramatically reduced after treatment with $5\times10^{-5}$ M H-7; and (C) almost completely inhibited after treatment with $5\times10^{-4}$ M H-7. Moreover, baseline force generation drops immediately after addition of $5\times10^{-4}$ M H-7. (D) Dose–inhibition curves were determined for H-7 and ML-7 inhibition of ET-1–promoted contraction of 21-day granuloma pouch tissue strips. Granulation tissue was initially contracted with ET-1 ($10^{-7}$ M), subsequently washed for 1 hour, treated with inhibitors H-7 and ML-7 at different doses and stimulated a second time with ET-1 ($10^{-7}$ M). Percent force was determined by comparing the secondary contraction with the initial contraction.

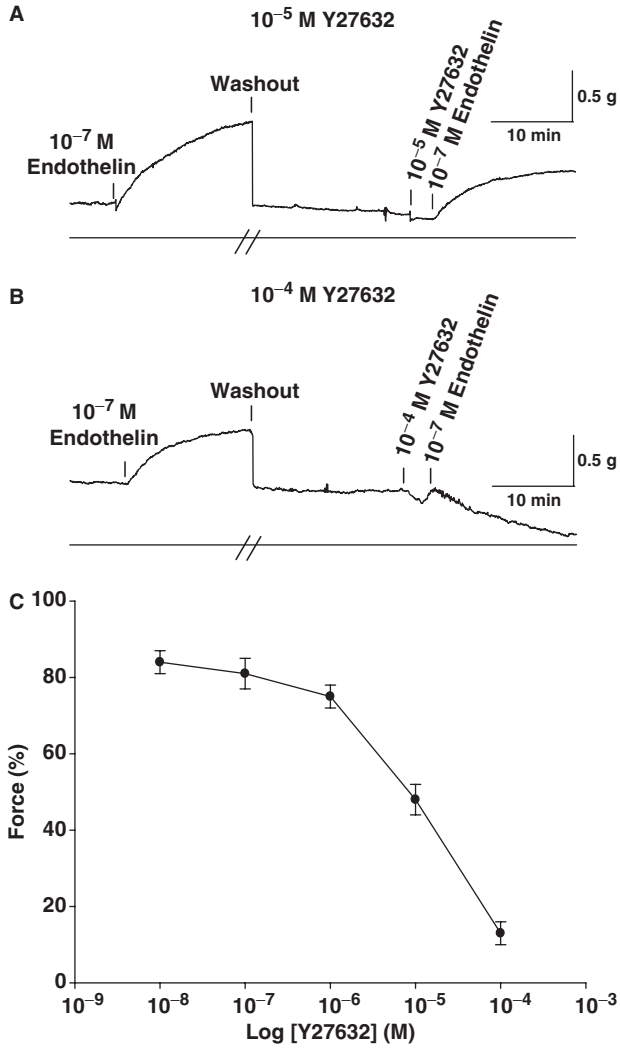


**Figure 3.** Y27632 inhibits ET-1-promoted force generation of granulation tissue. (A and B) Strips of 21-day-old granuloma pouch tissue were initially contracted with ET-1, extensively washed for 1 hour, subsequently treated with Y27632, followed by a secondary contraction with ET-1. Compared with control (see Figure 2A) the secondary contraction with ET-1 is dramatically reduced after treatment with (A) $10^{-5}$ M Y27632 and (B) almost completely inhibited at $10^{-4}$ M Y27632. (C) Dose–inhibition curve for Y27632 of ET-1–promoted contraction of 21-day granuloma pouch tissue strips. Granulation tissue strips were initially contracted with ET-1 ($10^{-7}$ M), subsequently washed for 1 hour, treated with the inhibitor Y27632 at different doses and stimulated a second time with ET-1 ($10^{-7}$ M). Percent force was determined by comparing the secondary contraction with the initial contraction.

### Effects of membrane depolarization on granulation tissue contraction

SM cell membrane depolarization with $K^+$ opens voltage gated $Ca^{2+}$ channels causing $Ca^{2+}$ influx to increase intracellular $Ca^{2+}$. Addition of $K^+$ to granulation tissue promoted generation of a long-duration contractile force,

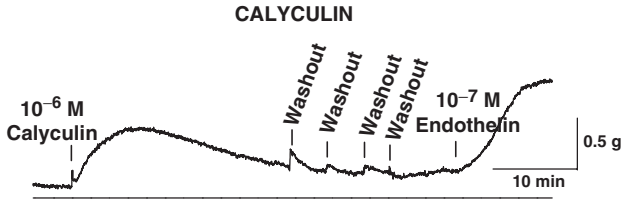


**Figure 4.** Calyculin promotes contraction of granulation tissue. Strips of 21-day-old granuloma pouch tissue were contracted with calyculin ($10^{-6}$ M) followed by extensive washing and subsequent addition of ET-1 ($10^{-7}$ M) to determine contractile potential of the strip. In the absence of any other contractile agonist, the phosphatase inhibitor calyculin elicited tissue contraction, which reached ~50% of contraction after secondary stimulation with ET-1.

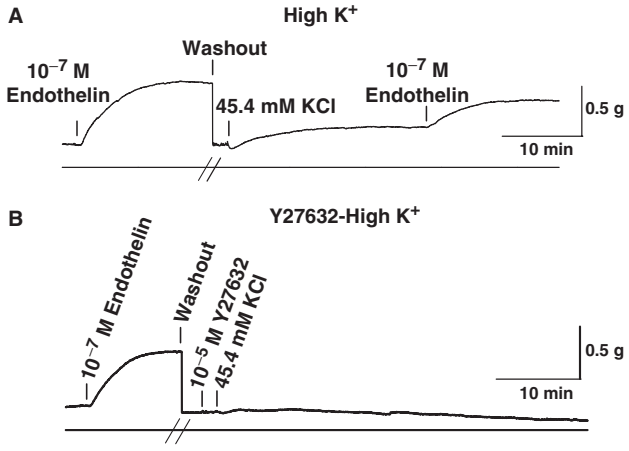


**Figure 5.** Force curves for $K^+$-promoted contraction of granulation tissue. (A) Strips of 21-day-old granuloma pouch tissue were initially contracted with ET-1 ($10^{-7}$ M) followed by extensive washing for 1 hour, subsequent treatment with $K^+$ and a secondary contraction with ET-1 ($10^{-7}$ M). (B) Strips of 21-day-old granuloma pouch tissue were initially contracted with ET-1 ($10^{-7}$ M) followed by extensive washing for 1 hour and subsequent treatment with Y27632 ($10^{-5}$ M) and a secondary contraction with $K^+$.

similar to ET-1; however, force generation was $30.1 \pm 8\%$ of that observed for ET-1 (Figure 5A). To determine whether $K^+$ promoted maximum contraction, ET-1 was added to granuloma pouch tissue on top of $K^+$ mediated contraction. ET-1–promoted additional contraction that reached approximately 80% the amount of force generated by the initial addition of ET-1 (Figure 5A); the same amount of contraction observed with a second addition of ET-1 to control cultures (Figure 2A). These results show that $K^+$ can promote only partial contraction compared with ET-1. To determine what role ROCK activity might play in the response of granulation tissue to $K^+$, the ROCK inhibitor Y27632 was added either before addition of $K^+$ or at the peak of $K^+$ promoted-force generation. The administration of $10^{-6}$ M Y27632 before $K^+$ reduced force generation by 18% (not illustrated), while $10^{-5}$ M Y27632 reduced the force generation by greater than 75% (Figure 5B). The administration of $10^{-5}$ M Y27632 after the tissue was contracted with $K^+$ resulted in a rapid relaxation (not illustrated). These results suggest that ROCK activity is necessary for granulation tissue contraction in response to increased intracellular $Ca^{2+}$ elicited by $K^+$.

## DISCUSSION

We previously proposed that the Rho/ROCK/MLCP pathway is the key regulator of myofibroblast contraction in vitro.[2,15] Here we examined whether the same mechanism is important in regulation of myofibroblast contraction of granuloma pouch granulation tissue. We selected to study the intracellular signaling pathways regulating ET-1–promoted myofibroblast contraction of granulation tissue because ET-1 promoted the strongest and longest lasting tissue contraction among the three effective agonists studied, serotonin, AT-II, and ET-1. The specific ROCK inhibitor Y27632 completely inhibited ET-1–promoted contraction of myofibroblasts in granulation tissue and caused complete relaxation of ET-1–promoted contraction. In contrast, the MLCK inhibitor ML-7 could only partially inhibit ET-1–promoted contraction. As previously shown for myofibroblasts in vitro,[15] the phosphatase inhibitor calyculin alone promoted contraction of myofibroblasts in granulation tissue. Increased intracellular $Ca^{2+}$ in response to membrane depolarization by $K^+$-promoted contraction that lasted as long as ET-1 stimulation but was significantly smaller; this response was inhibited by Y27632. These results show that while activation of MLCK by elevated intracellular $Ca^{2+}$ participates in myofibroblast force generation, simultaneous activation of the Rho/ROCK/MLCP pathway is essential to promote full granulation tissue contraction as illustrated schematically in Figure 6.

In SM and nonmuscle cells, contractile force generation by actomyosin interaction is regulated by phosphorylation of MLC[27] via specific protein kinases of which MLCK is the only one identified as regulated by $Ca^{2+}$. In this study we found that MLCK activity is mandatory for maximal contraction of granulation tissue myofibroblasts, because the MLCK-specific inhibitor ML-7 partially inhibits ET-1–promoted contraction. In addition, we found that increasing intracellular $Ca^{2+}$ by $K^+$ depolarization can promote myofibroblast contraction suggesting a $Ca^{2+}$-dependent regulation of contraction in granulation tissue myofibroblasts. Previous studies showed using isolated stress fibers from fibroblasts that contraction is stimulated by MLCK[10,11] and we have previously demonstrated a role for MLCK in contraction of myofibroblasts in vitro.[15] However, it should be emphasized that the level of contraction in response to increased intracellular $Ca^{2+}$ was only 30% of the contraction in response to ET-1 suggesting additional pathways regulating myofibroblast contraction.[28]

Our results showing that the phosphatase inhibitor calyculin can promote force generation in granulation strips are consistent with a possible $Ca^{2+}$-independent mechanism of force generation in myofibroblasts. $Ca^{2+}$-independent kinases have been shown to phosphorylate MLC, including ROCK,[29,30] integrin-linked kinase,[31] zipper-interacting protein kinase,[32,33] and citron kinase.[34] Whether

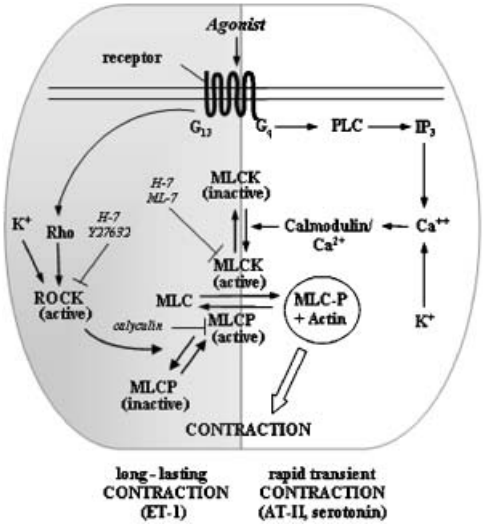


**Figure 6.** Model for regulation of two types of myofibroblast contraction in granulation tissue. (1) ET-1 activation of the Rho/Rho kinase (ROCK) pathway results in myosin light chain phosphatase (MLCP) inactivation and maintained phosphorylation of myosin light chain leading to a long-lasting contraction. This pathway is the main regulatory mechanism for long-lasting myofibroblast contraction important in wound closure (gray panel). (2) Serotonin and AT II can lead to phosphorylation of MLC by activation of MLCK through $Ca^{2+}$ release, promoting a rapid and transient contraction (white panel). Inhibitors and agonists used in this study are printed in this study are printed in italics.

these $Ca^{2+}$-independent kinases play a role along with MLCK in regulating myofibroblast contraction is currently unclear. We have previously found in cultured myofibroblasts that the ROCK inhibitor Y27632 could only partially inhibit calyculin promoted contraction suggesting that a kinase other than ROCK was important in promoting $Ca^{2+}$-independent contraction.[15] It will be important in future studies to determine the possible role of these $Ca^{2+}$-independent kinases in myofibroblast force generation.

A number of studies have shown the importance of MLC dephosphorylation by MLCP in regulating SM and nonmuscle cell contraction.[28] Inhibition of MLCP will increase myosin light chain phosphorylation levels and is a primary cause for $Ca^{2+}$-sensitization in SM cell contraction.[28] Phosphorylation of the 110 kDa noncatalytic myosin phosphatase target subunit (MYPT1) has been shown to be one of the major mechanisms that inhibits MLCP activity[9] and ROCK is the primary kinase responsible for phosphorylation of MYPT1.[24] Contraction of isolated fibroblast stress fibers appears to follow a similar ROCK–MLCP pathway.[10,12] Our results suggest that myofibroblast contraction in granulation tissue is also regulated by ROCK inhibition of MLCP. The ROCK inhibitor Y27632 can inhibit ET-1–promoted contraction of granulation tissue strips and the MLCP inhibitor calyculin can promote contraction.

An alternative mechanism by which MLCP may be inhibited is through the binding of CPI-17 (17 kDa PKC-potentiated inhibitory protein of type 1 protein phosphatase) to the 38 kDa catalytic subunit of MLCP.[35] The phosphorylation of CPI-17 results in its binding to the 38 kDa catalytic subunit of MLCP and thereby inhibition of MLCP activity. While originally identified as a substrate for protein kinase C, other kinases have been identified to phosphorylate CPI-17 including ROCK,[36] integrin-linked kinase,[37] protein kinase N[38] and zip-like kinase.[39] Inhibition of myofibroblast contraction by Y27632 could potentially block phosphorylation of CPI-17 resulting in continued activity of MLCP and decreased contraction. Whether inhibition of MLCP activity occurs via phosphorylation of MYPT1 or CPI-17 it would appear that ROCK is critical in decreasing the activity of MLCP and promoting myofibroblast contraction.

Membrane depolarization with $K^+$ was found to be able to promote contraction of granuloma pouch granulation tissue. Similar to the response to ET-1 this contraction was long in duration and could be inhibited by Y27632. There appear to be two possible explanations as to how increased cytosolic $Ca^{2+}$ in response to membrane depolarization could promote myofibroblast contraction. It is possible that increased $Ca^{2+}$ activates MLCK to a level where it can overcome the activity of MLCP resulting in contraction. Alternatively, in addition to activating MLCK-increased $Ca^{2+}$ could increase the activity of ROCK and subsequently decrease the activity of MLCP. Recently it has been shown that membrane depolarization-induced contraction of rat caudal arterial SM involves activation of ROCK.[40] Either of these alternatives could be operating in myofibroblasts in granuloma pouch granulation tissue. It is clear that membrane depolarization does not fully promote force generation, as addition of ET-1 to $K^+$-contracted tissue will increase force generation. Interestingly, we have found that excisional wound granulation tissue does not contract in response to membrane depolarization with $K^+$ (B. Hinz, unpublished results) suggesting differences in different populations of myofibroblasts. It has been shown that MLCK and MLCP activities vary among SMs[41] and it is very likely that the activities of these enzymes vary in different populations of myofibroblasts. It will be interesting to determine whether differences in $K^+$-induced contractions of different types of granulation tissues are due to differences in MLCK and/or MLCP activity in different populations of myofibroblasts.

Our results are consistent with two types of contraction in myofibroblasts; one being a rapid, transient contraction promoted by AT-II and serotonin and the other being a slow, long-lasting contraction promoted by ET-1. We have previously proposed that the function of myofibroblasts during granulation tissue contraction is to maintain a constant contractile force on the surrounding tissue while a new shorter matrix is deposited.[2] Previous studies have shown that increased $Ca^{2+}$ elicits a rapid, but transient contractile response in stress fiber[12]; not the type of long-term contraction needed to be generated by myofibroblasts during tissue reorganization. Presumably AT-II and serotonin function by increasing $Ca^{2+}$ and activating MLCK[42,43]; however, this remains to be determined for myofibroblasts in granulation tissue. In contrast, slow, long-term contraction of stress fibers results from activation of the Rho/ROCK pathway with subsequent inhibition of MLCP.[12] We demonstrate here that ET-1 promotes

the slow, long-term contraction of myofibroblasts in granulation tissue through the Rho/ROCK/MLCP pathway. Previously it has been shown that granuloma pouch myofibroblasts express both the $ET_A$ and the $ET_B$ ET-1 receptors; however, only the $ET_A$ receptor appears to be involved in promoting ET-1 stimulated contraction.[6] In SM cells ET-1 can promote long-lasting contraction by binding to the $ET_A$ receptor and utilizing $G_{13}$ to activate the Rho/ROCK pathway and subsequently inhibit MLCP.[44] Whether myofibroblasts in granulation utilize this same pathway needs to be determined. We propose in myofibroblasts in granulation tissue that activation of the Rho/ROCK pathway with subsequent inhibition of MLCP is essential to promote the slow, long-duration contraction necessary for myofibroblast function during wound healing.

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## REFERENCES

1. Grinnell F. Fibroblasts, myofibroblasts, and wound contraction. *J Cell Biol* 1994; 124: 401–4.
2. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA. Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol* 2002; 3: 349–63.
3. Serini G, Gabbiani G. Mechanisms of myofibroblast activity and phenotypic modulation. *Exp Cell Res* 1999; 250: 273–83.
4. Majno G, Gabbiani G, Hirschel BJ, Ryan GB, Statkov PR. Contraction of granulation tissue in vitro: similarity to smooth muscle. *Science* 1971; 173: 548–50.
5. Appleton I, Tomlinson A, Chander CL, Willoughby DA. Effect of endothelin-1 on croton oil-induced granulation tissue in the rat. A pharmacologic and immunohistochemical study. *Lab Invest* 1992; 67: 703–10.
6. Goto T, Yanaga F, Ohtsuki I. Studies on the endothelin-1-induced contraction of rat granulation tissue pouch mediated by myofibroblasts. *Biochim Biophys Acta* 1998; 1405: 55–66.
7. Hinz B, Mastrangelo D, Iselin CE, Chaponnier C, Gabbiani G. Mechanical tension controls granulation tissue contractile activity and myofibroblast differentiation. *Am J Pathol* 2001; 159: 1009–20.
8. Watson S, Burnside T, Carver W. Angiotensin II-stimulated collagen gel contraction by heart fibroblasts: role of the AT1 receptor and tyrosine kinase activity. *J Cell Physiol* 1998; 177: 224–31.
9. Hartshorne DJ, Ito M, Erdodi F. Myosin light chain phosphatase: subunit composition, interactions and regulation. *J Muscle Res Cell Motil* 1998; 19: 325–41.
10. Katoh K, Kano Y, Amano M, Kaibuchi K, Fujiwara K. Stress fiber organization regulated by MLCK and Rho-kinase in cultured human fibroblasts. *Am J Physiol Cell Physiol* 2001; 280: C1669–79.
11. Burridge K, Chrzanowska-Wodnicka M. Focal adhesions, contractility, and signaling. *Annu Rev Cell Dev Biol* 1996; 12: 463–518.
12. Katoh K, Kano Y, Amano M, Onishi H, Kaibuchi K, Fujiwara K. Rho-kinase–mediated contraction of isolated stress fibers. *J Cell Biol* 2001; 153: 569–84.
13. Kawano Y, Fukata Y, Oshiro N, Amano M, Nakamura T, Ito M, Matsumura F, Inagaki M, Kaibuchi K. Phosphorylation of myosin-binding subunit (MBS) of myosin phosphatase by Rho-kinase in vivo. *J Cell Biol* 1999; 147: 1023–38.
14. Darby I, Skalli O, Gabbiani G. Alpha-smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing. *Lab Invest* 1990; 63: 21–9.
15. Parizi M, Howard EW, Tomasek JJ. Regulation of LPA-promoted myofibroblast contraction: role of Rho, myosin light chain kinase, and myosin light chain phosphatase. *Exp Cell Res* 2000; 254: 210–20.
16. Anderson S, DiCesare L, Tan I, Leung T, SundarRaj N. Rho-mediated assembly of stress fibers is differentially regulated in corneal fibroblasts and myofibroblasts. *Exp Cell Res* 2004; 298: 574–83.
17. Selye H. On the mechanism through which hydrocortisone affects the resistance of tissue to injury. *J Am Med Assoc* 1953; 152: 1207–13.
18. Gabbiani G, Hirschel BJ, Ryan GB, Statkov PR, Majno G. Granulation tissue as a contractile organ. A study of structure and function. *J Exp Med* 1972; 135: 719–34.
19. Gabbiani G, Ryan GB, Majno G. Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. *Experientia* 1971; 27: 549–50.
20. Volberg T, Geiger B, Citi S, Bershadsky AD. Effect of protein kinase inhibitor H-7 on the contractility, integrity, and membrane anchorage of the microfilament system. *Cell Motil Cytoskeleton* 1994; 29: 321–38.
21. Zhong C, Kinch MS, Burridge K. Rho-stimulated contractility contributes to the fibroblastic phenotype of Ras-transformed epithelial cells. *Mol Biol Cell* 1997; 8: 2329–44.
22. Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, Tamakawa H, Yamagami K, Inui J, Maekawa M, Narumiya S. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* 1997; 389: 990–4.
23. Ridley AJ, Hall A. The small GTP-binding protein Rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* 1992; 70: 389–99.
24. Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, Yamamori B, Feng J, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science* 1996; 273: 245–8.
25. Ishihara H, Martin BL, Brautigan DL, Karaki H, Ozaki H, Kato Y, Fusetani N, Watabe S, Hashimoto R, Uemura D. Calyculin A and okadaic acid: inhibitors of protein phosphatase activity. *Biochem Biophys Res Commun* 1989; 159: 871–7.
26. Shimizu H, Ito M, Miyahara M, Ichikawa K, Okubo S, Konishi T, Naka M, Tanaka T, Hirano K, Hartshorne DJ. Characterization of the myosin-binding subunit of smooth muscle myosin phosphatase. *J Biol Chem* 1994; 269: 30407–11.

27. Hartshorne DJ. Biochemistry of the contractile process in smooth muscle. In: Johnson LR, editor *Physiology of the gastrointestinal tract*. New York: Raven Press, 1987: 423–82.
28. Hirano K, Hirano M, Kanaide H. Regulation of myosin phosphorylation and myofilament Ca(2+) sensitivity in vascular smooth muscle. *J Smooth Muscle Res* 2004; 40: 219–36.
29. Chihara K, Amano M, Nakamura N, Yano T, Shibata M, Tokui T, Ichikawa H, Ikebe R, Ikebe M, Kaibuchi K. Cytoskeletal rearrangements and transcriptional activation of c-fos serum response element by Rho-kinase. *J Biol Chem* 1997; 272: 25121–7.
30. Amano M, Chihara K, Nakamura N, Fukata Y, Yano T, Shibata M, Ikebe R, Kaibuchi K. Myosin II activation promotes neurite retraction during the action of Rho and Rho-kinase. *Genes Cells* 1998; 3: 177–88.
31. Deng JT, Van Lierop JE, Sutherland C, Walsh MP. Ca2+-independent smooth muscle contraction. A novel function for integrin-linked kinase. *J Biol Chem* 2001; 276: 16365–73.
32. Murata-Hori M, Suizu F, Iwasaki T, Kikuchi A, Hosoya H. ZIP kinase identified as a novel myosin regulatory light chain kinase in HeLa cells. *FEBS Lett* 1999; 451: 81–4.
33. Niiro N, Ikebe M. Zipper-interacting protein kinase induces Ca(2+)-free smooth muscle contraction via myosin light chain phosphorylation. *J Biol Chem* 2001; 276: 29567–74.
34. Yamashiro S, Totsukawa G, Yamakita Y, Sasaki Y, Madaule P, Ishizaki T, Narumiya S, Matsumura F. Citron kinase, a Rho-dependent kinase, induces di-phosphorylation of regulatory light chain of myosin II. *Mol Biol Cell* 2003; 14: 1745–56.
35. Eto M, Ohmori T, Suzuki M, Furuya K, Morita F. A novel protein phosphatase-1 inhibitory protein potentiated by protein kinase C. Isolation from porcine aorta media and characterization. *J Biochem (Tokyo)* 1995; 118: 1104–7.
36. Koyama M, Ito M, Feng J, Seko T, Shiraki K, Takase K, Hartshorne DJ, Nakano T. Phosphorylation of CPI-17, an inhibitory phosphoprotein of smooth muscle myosin phosphatase, by Rho-kinase. *FEBS Lett* 2000; 475: 197–200.
37. Deng JT, Sutherland C, Brautigan DL, Eto M, Walsh MP. Phosphorylation of the myosin phosphatase inhibitors, CPI-17 and PHI-1, by integrin-linked kinase. *Biochem J* 2002; 367: 517–24.
38. Hamaguchi T, Ito M, Feng J, Seko T, Koyama M, Machida H, Takase K, Amano M, Kaibuchi K, Hartshorne DJ, Nakano T. Phosphorylation of CPI-17, an inhibitor of myosin phosphatase, by protein kinase N. *Biochem Biophys Res Commun* 2000; 274: 825–30.
39. MacDonald JA, Eto M, Borman MA, Brautigan DL, Haystead TA. Dual Ser and Thr phosphorylation of CPI-17, an inhibitor of myosin phosphatase, by MYPT-associated kinase. *FEBS Lett* 2001; 493: 91–4.
40. Mita M, Yanagihara H, Hishinuma S, Saito M, Walsh MP. Membrane depolarization-induced contraction of rat caudal arterial smooth muscle involves Rho-associated kinase. *Biochem J* 2002; 364: 431–40.
41. Gong MC, Cohen P, Kitazawa T, Ikebe M, Masuo M, Somlyo AP, Somlyo AV. Myosin light chain phosphatase activities and the effects of phosphatase inhibitors in tonic and phasic smooth muscle. *J Biol Chem* 1992; 267: 14662–8.
42. Touyz RM, Berry C. Recent advances in angiotensin II signaling. *Braz J Med Biol Res* 2002; 35: 1001–15.
43. Nickenig G, Geisen G, Vetter H, Sachinidis A. Characterization of angiotensin receptors on human skin fibroblasts. *J Mol Med* 1997; 75: 217–22.
44. Hersch E, Huang J, Grider JR, Murthy KS. Gq/G13 signaling by ET-1 in smooth muscle: MYPT1 phosphorylation via ETA and CPI-17 dephosphorylation via ETB. *Am J Physiol Cell Physiol* 2004; 287: C1209–18.