siRNA knock down of casein kinase 2 increases force and cross-bridge cycling rates in vascular smooth muscle

Elaine M. Smolock, Tanchun Wang, Jocelyn K. Nolt, and Robert S. Moreland

Department of Pharmacology and Physiology and Department of Biochemistry and Molecular Biology, Drexel University College of Medicine, Philadelphia, Pennsylvania

Submitted 20 June 2006; accepted in final form 18 September 2006

Smolock EM, Wang T, Nolt JK, Moreland RS. siRNA knock down of casein kinase 2 increases force and cross-bridge cycling rates in vascular smooth muscle. Am J Physiol Cell Physiol 292: C876–C885, 2007. First published September 20, 2006; doi:10.1152/ajpcell.00343.2006.—Contraction of smooth muscle involves myosin light chain (MLC) kinase catalyzed phosphorylation of the regulatory MLC, activation of myosin, and the development of force. However, this cannot account for all aspects of a smooth muscle contraction, suggesting that other regulatory mechanisms exist. One potentially important technique to study alternative sites of contractile regulation is the use of small interfering RNA (siRNA). The goal of this study was to determine whether siRNA technology can decrease the levels of a specific protein and allow for the determination of how that protein affects contractile regulation. To achieve this goal, we tested the hypothesis that casein kinase 2 (CK2) is part of the complex regulatory scheme present in vascular smooth muscle. Using intact strips of swine carotid artery, we determined that siRNA against CK2 produced a tissue that resulted in a ~60% knockdown after 4 days in organ culture. Intact strips of vascular tissue depleted of CK2 produced greater levels of force and exhibited an increased sensitivity to all stimuli tested. This was accompanied by an increase in cross-bridge cycling rates but not by a change in MLC phosphorylation levels. α-Toxin-permeabilized vascular tissue depleted of CK2 also showed an increased sensitivity to calcium compared with control tissues. Our results demonstrate that siRNA is a viable technique with which to study regulatory pathways in intact smooth muscle tissue. Our results also demonstrate that CK2 plays an important role in the mechanism(s) responsible for the development of force and cross-bridge cycling by a MLC phosphorylation-independent pathway.

myosin light chain phosphorylation; shortening velocity; α-toxin permeabilization; swine carotid artery; caldesmon

THE PRIMARY PATHWAY for the initiation of a vascular smooth muscle contraction involves Ca2+-calmodulin-dependent myosin light chain (MLC) kinase catalyzed phosphorylation of the Ser19 residue on the 20-kDa MLC (22). However, this cascade of cellular events cannot account for all known properties of smooth muscle contractile regulation. For example, the tonic maintenance of force is not supported by proportional levels of MLC phosphorylation (37), near-maximal levels of force can be developed in the complete absence of an increase in levels of MLC phosphorylation (8, 49), and loss or displacement of the thin filament protein, caldesmon, produces stimulation-independent, active cross-bridge cycling (7, 23). Results such as these have drawn attention to other potential mechanisms for the regulation, or at least the modulation, of smooth muscle contraction. In particular, proteins associated with the thin filaments of smooth muscle have been targeted for study.

The two most widely studied thin filament proteins as potential regulators of smooth muscle contraction are caldesmon and calponin. Both are inhibitors of actin-activated myosin ATPase activity, and the inhibition can be reversed biochemically by either high levels of Ca2+ and calmodulin or phosphorylation by a number of kinases (10, 11, 17, 19, 53). Unfortunately, with only a few notable exceptions, no clear role for these proteins has been shown in the regulation of smooth muscle contraction. Additionally, there is limited information as to which kinase, if any, catalyzes calponin or caldesmon phosphorylation in intact smooth muscle tissue (1, 12).

We have previously shown that decreasing caldesmon levels by ~85% produces a vascular tissue that exhibits active cross-bridge cycling in the absence of stimulation (7). However, we do not know what or even if an endogenous pathway exists for the “disinhibition” of caldesmon’s effects on myosin ATPase activity. Adam and Hathaway (1) have presented compelling evidence to suggest that the p42/p44 mitogen-activated protein (MAP) kinases could account for ~40% of stimulation-induced increases in caldesmon phosphorylation. Consistent with these findings, we and others (13, 25) have shown that inhibition of cellular MAP kinase activity decreases but does not abolish caldesmon phosphorylation.

Therefore, what is the identity of the other kinases that are potentially important in the regulation of a smooth muscle contraction? In addition to the potential role for MAP kinase activity (1, 5, 11) the other kinases that have been proposed to phosphorylate caldesmon include Ca2+-calmodulin-dependent protein kinase II (CaM kinase II), (21), protein kinase C (PKC), (20, 47), cdc42 kinase (30), and casein kinase 2 (CK2) (2, 45, 51). In this present study, we have aimed our investigations at the potential role of CK2 in the contraction of vascular smooth muscle.

CK2 is a ubiquitous Ser/Thr protein kinase that consists of three subunits: two catalytic subunits (α and α′) and a regulatory subunit (β) (42). CK2 has been shown to phosphorylate caldesmon on Ser26, Ser73, and Thr83 (51, 54), resulting in a 50% decrease in caldesmon binding to myosin (2). However, few studies have addressed any physiologically relevant ramifications of CK2 activation.

The goal of this study was to attempt to rectify this lack of information by determining the effects of decreasing CK2 expression in an intact vascular smooth muscle preparation. To achieve this goal, we used small interfering RNA (siRNA)
technology to knock down CK2 levels in intact strips of vascular smooth muscle. Based on the information presented above, the hypothesis we tested was that decreasing CK2 expression in an intact smooth muscle tissue will result in decreased contractile properties of the tissue, including force development and cross-bridge cycling rates. Surprisingly, we found that depression of CK2 expression significantly enhanced stimulation-induced levels of both force and cross-
bridge cycling rates, independent of any change in levels of MLC phosphorylation. Therefore, our results suggest that CK2 is an important modulator of smooth muscle contraction but does not act by dysinhibition of caldesmon. Instead, we propose that actinin-associated LIM protein may be the protein regulated by CK2 that results in a decrease in contractility. Finally, our results demonstrate that siRNA technology is a viable approach for studying functionality in intact smooth muscle.

MATERIALS AND METHODS

Tissue preparation. Swine carotid arteries were obtained from a local slaughterhouse and transported to the laboratory in ice-cold physiological salt solution (PSS). PSS contained (in mM) 140 NaCl, 4.7 KCl, 1.2 MgSO4, 1.6 CaCl2, and 1.2 Na2HPO4. Arteries were cleaned of excess fat and connective tissue and then dissected free of both intimal and adventitial layers, leaving a thin medial layer for experimentation. Strips of the intact medial swine carotid artery were cut (7 × 0.7 mm) and were stored in PSS at 4°C.

Introduction of siRNA and organ culture. Medial strips of swine carotid artery were subjected to a chemical loading protocol originally described by Morgan and Morgan (35) for the introduction of aceto
quin into intact smooth muscle tissue and similar to that previously described by our laboratory (7) for the introduction of antisense oligonucleotides into intact strips of smooth muscle.

The carotid strips were incubated on ice in 1.5-ml Eppendorf tubes (4 strips per 1 ml per tube) with agitation for 90 min in a solution containing the following (in mM): 10 EGTA, 120 KCl, 2 MgCl2, 5 ATP, and 20 HEPES (pH 6.8). The strips were then transferred to a solution containing (in mM) 120 KCl, 2 MgCl2, 5 ATP, and 20 HEPES (pH 6.8) and agitated on ice for 30 min. The strips were then divided into three groups, vehicle (scrambled siRNA), and experimental (siRNA against CK2), and incubated on ice for 90 min while agitated in the same solution (vehicle) or in the same solution with either siRNA against CK2 or the scrambled siRNA. The final concentration of siRNA used was 200 nM as suggested by the vendor. All strips were then incubated for 30 min on ice, with agitation in a similar solution containing either vehicle or the appropriate 200 nM siRNA, except the MgCl2 concentration was increased to 10 mM. Following this incubation, the strips were washed twice with 500 μl of a solution composed of the following (in mM): 140 NaCl, 5 KCl, 10 MgCl2, 5.6 D-glucose, and 2 HEPES (pH 6.8). Strips were then incubated for 30 min at room temperature with agitation in the same solution with 200 nM siRNA added to the experimental and control tubes. At the end of this incubation, all strips were introduced to increasing CaCl2 concentrations every 15 min, from 0.001, 0.01, and 0.1 mM, to a final concentration of 1.6 mM. The strips were then soaked in filter-sterilized PSS for 30 min at room temperature.

The strips were placed four to a well in a six-well culture plate. Each well was coated with a polymerized silicone elastomer and then filled with 5 ml of sterile PSS. The strips were mounted onto the elastomer using 0.2-mm-wide, sterilized stainless-steel pins. The pins were positioned at the corners of the strips such that the strips were gently stretched when lifted 2–3 mm above the elastomer. The PSS was then removed by vacuum suction and replaced with 5 ml of serum-free medium composed of DMEM/F-12, 100 U/ml penicillin-G, 100 μg/ml streptomycin, 35 mg/ml l-ascorbic acid, 200 μg/ml l-glutamine, and 1× insulin-transferrin-selenium. The wells mounted with the siRNA-loaded strips also received 200 nM siRNA. The strips were maintained at 37°C with 5% CO2 for a maximum of 6 days after mounting. The medium was changed daily, preceded by a 10-min wash with sterile PSS, stimulation with 110 mM sterile KCl-PSS (equimolar substitution for NaCl) for 10 min, and a second wash in sterile PSS for 10 min, and then strips were incubated in 5 ml of serum-free medium overnight.

Immunoblots of CK2. Strips of swine carotid artery were removed from culture, incubated in PSS warmed to 37°C for 10 min, rinsed in acetone, and then completely air dried. The strips were then homogenized in a 1% SDS, 10% glycerol, and 1 mM DTT solution with glass/glass homogenizers. Samples were centrifuged for 5 min, assayed for protein concentration, and then subjected to one-dimen
sional electrophoresis on a 12% SDS gel and transferred to a nitro
cellulose membrane. After transfer the membranes were blocked in 3% milk-phosphate-buffered saline (PBS) for 60 min at room temper
ature and incubated overnight at 4°C in 1% milk-PBS, containing primary antibody (4 μg/ml) directed against CK2. The membranes were briefly washed twice in double-deionized water and then incubated in 1% milk-PBS, containing a goat anti-rabbit secondary anti
body (1:5,000) for 90 min at room temperature. The membranes were washed twice in double-deionized water and incubated in an enhanced chemiluminescence solution for 1 min and then developed. After being developed, the membranes were washed in 0.05% Tween-PBS for 20 min and then stripped for 30 min, washed again in 0.05% Tween-PBS for 20 min, and then blocked in 3% milk-PBS for 1 h. The membranes were incubated in 1% milk-PBS containing either actin (1:2,500,000, clone no. 1A4) or glyceraldehyde phosphate de
hydrogenase (GAPDH) (1:20,000) primary antibody overnight at 4°C. The membranes were washed twice, each for 10 min in PBS and 10 min in 0.05% Tween-PBS, and then incubated in 1% milk-PBS containing sheep anti-mouse secondary antibody (1:10,000) for 2 h. The washes were repeated, and the membranes were incubated in enhanced chemiluminescence solution for 1 min and then developed.

Interferon-γ ELISA. After 4 days of culture (the optimal day as determined by immunoblotting for CK2 content), strips were removed and homogenized as described above. The homogenate was subjected to an ELISA assay specific for porcine interferon-γ. The assay was performed precisely according to the protocol and guidelines sug
gested by the vendor.

Isometric force development. On day 4 strips were removed from culture and allowed to soak in PSS warmed to 37°C for 10 min. The strips were then suspended between a Grass model FT.03 force transducer and a stationary clip in water-jacketed organ baths, and isometric force was recorded by well-described standard techniques (7, 8, 12, 13).

Isotonic shortening velocity. On day 4, strips were removed from culture and mounted between a clip attached to a micrometer on one end for control of initial muscle length and an aluminum foil tube connected to a Cambridge Technology model 300H servo-lever in
terfaced to a Linux-based microcomputer equipped with custom software to control length and force, as well as to fit the collected data for analysis of shortening velocity. Strips were incubated in 60 ml of PSS at 37°C, aerated with 100% O2, stretched to ~2 g of force, and allowed to equilibrate for ~90 min. After equilibration, the strips were stimulated with 110 mM KCl-PSS several times until a stable force was achieved. Maximal velocity of shortening was measured at various times during a contraction in response to 30 μM histamine, the agonist that produces the greatest amount of force in this tissue. After the addition of histamine, the strips were subjected to a series of rapid isotonic releases to 5, 10, 15, 20, and 25% of the force at time of release. The change in length during each force clamp was stored on the computer and the change in length vs. time at 100 ms after the release was used as the isotonic shortening velocity in muscle lengths per second. Linearization of the force-velocity equation using the
results from the five force clamps was used to extrapolate velocity at zero load and therefore approximate the maximal velocity of shortening, $V_c$.

MLC phosphorylation. After 4 days of culture the strips were placed in PSS warmed to $37^\circ$C and mounted for isometric force recording. At various time points of stimulation, the strips were rapidly frozen in a dry ice-acetone slurry, containing 6% trichloroacetic acid and 10 mM DTT. The strips were then slowly thawed to room temperature, rinsed in acetone, air dried, and then subjected to homogenization in a 1% SDS, 10% glycerol, and 1 mM DTT solution using glass/glass homogenizers and then subjected to two-dimensional gel electrophoresis and transferred to nitrocellulose membranes for quantification of MLC phosphorylation as previously described (32).

$\alpha$-Toxin permeabilization. On day 4 of culture, the strips were placed in PSS warmed to room temperature and then mounted on a Muscle Research Station (Scientific Instruments, Heidelberg, Germany) between a force transducer and a stable clip in a 300-μl cuvette and allowed to equilibrate for at least 90 min as previously described (57). The tissues were then subjected to $\alpha$-toxin permeabilization by previously published methods (32).

$[^{\gamma-32P}]$ATP incorporation and phosphor-imaging. To measure $^{32}$P incorporation into proteins in the control and experimental strips, the strips were subjected to Triton X-100 detergent skinning in conjunction with $[^{\gamma-32P}]$ATP. On day 4 of culture, strips were placed in PSS at room temperature for 10 min. The strips were then placed in a Ca$^{2+}$-free PSS solution containing 1 mM EGTA for 10 min, after which 100 μM histamine was added for 30 min. The strips were then incubated for 60 min in a 0.5% Triton X-100 skinning solution (in mM) 5 EGTA, 20 imidazole (pH 6.8), 50 potassium acetate, 1 DTT, and 150 sucrose. All strips were then incubated for 15 min in a high-EGTA relaxing solution containing (in mM) 5 EGTA, 20 imidazole (pH 6.8), 50 potassium acetate, 1 DTT, 6 MgCl$_2$, and 6 ATP. The strips were then incubated with 0.2 μCi/ml $[^{\gamma-32P}]$ATP for 15 min in a low-EGTA relaxing solution containing (in mM) 0.1 EGTA, 20 imidazole (pH 6.8), 50 potassium acetate, 1 DTT, 6 MgCl$_2$, and 6 ATP. The strips were then stimulated for 20 min with a Ca$^{2+}$-contracting solution (either 0, 0.3, 1.0, or 3.0 μM) containing (in mM) 5 EGTA, 20 imidazole (pH 6.8), 50 potassium acetate, 1 DTT, 1 MgCl$_2$, and 4 mM ATP spiked with 0.2 μCi/ml $[^{\gamma-32P}]$ATP. Tissue strips were then rapidly frozen in a dry ice-acetone slurry containing 6% trichloroacetic acid and 10 mM DTT. The strips were then slowly thawed to room temperature, rinsed in acetone, air dried, and then subjected to homogenization in a solution of 1% SDS, 10% glycerol, and 1 mM DTT with glass/glass homogenizers.

To determine cellular targets specific for CK2-catalyzed phosphorylation, a tissue strip was subjected to the Triton X-100 detergent skinning protocol described above and then immediately homogenized on ice by three 5-s bursts of a Brinkmann Polytron. The tissues were homogenized at a concentration of 50 mg tissue wet weight/ml and in a solution containing (in mM) 0.1 EGTA, 20 imidazole (pH 6.8), 50 potassium acetate, 1 DTT, and 6 MgCl$_2$. We have previously demonstrated that this provides a cellular lysate that allows for the measurement of intracellular kinase activity and phosphoprotein analysis (41). Following homogenization, 0.5 ml of the homogenate was incubated for 15 min with 0.2 μCi/ml $[^{\gamma-32P}]$ATP. Activated CK2 (10,000 units) was then added to the homogenate for 20 min followed by addition of ice-cold 40% trichloroacetic acid to a final concentration of 5%.

All samples were centrifuged for 5 min, assayed for protein concentration, and subjected to two-dimensional gel electrophoresis using a slight modification of our standard protocol for MLC phosphorylation as described above. The modifications made to the isoelectric focusing dimension were an increase in gel length to 16 cm, a change in ampholytes to 6.7% pH 3–10 and 0.76% pH 5–6, an increase in protein loading to 150 μg of protein, and an increase in volt-hours such that the gels were run for 17 h at 950 V at 18°C. The modifications made to the second dimension were that a 10% SDS large format gel was used, and the entire length of the isoelectric focusing gel was run at 45 mA/gel at 4°C. The gels were then silver-stained, dried using a Bio-Rad model 543 gel dryer, and exposed in a phosphor-screen from Amersham Biosciences/GE Healthcare for 36 h. The phosphor-screens were then scanned using a Storm scanner and imaging software.

Materials and statistics. siRNA against CK2, scrambled siRNA, CK2 primary antibody, and CK2-positive controls were obtained from Upstate Biotechnology. Actin primary and goat anti-rabbit secondary antibodies, ATP, creatine phosphate, histamine, EGTA, Triton X-100, and silver stain kits were obtained from Sigma. Culture media was obtained from Mediatech, and media antibiotics and reagents were obtained from Gibco. The porcine interferon-γ ELISA kit and Western blot stripping buffer were purchased from Pierce Endogen. α-Toxin was purchased from List Biologicals. Activated CK2 was purchased from New England Biolabs. $[^{\gamma-32P}]$ATP was purchased from PerkinElmer Life and Analytical Sciences. All protein assay and electrophoretic chemicals and materials were obtained from Bio-Rad. Sheep anti-mouse secondary antibody, Western blotting detecting reagents, Hyperfilm, and colloidal gold were all purchased from Amersham Biosciences. GAPDH primary antibody was purchased from Advanced Immunochemical. All other reagents were obtained from Fisher Scientific and were analytical grade or better.

All values are means ± SE, with “n” representing the number of vascular preparations. Statistical significance was determined using the Student’s $t$-test for unpaired values. $P < 0.05$ was taken as significant.

RESULTS

siRNA-induced downregulation of CK2. Strips of swine carotid artery were cultured for 2, 4, and 6 days and then processed for measurement of CK2 content as shown in a representative Western blot in Fig. 1A. The $\alpha'$ catalytic subunit of CK2 was decreased after 2 and 4 days of culture in the presence of siRNA. The $\alpha'$ catalytic subunit of CK2 is the subunit that is required for CK2-catalyzed phosphorylation and is the band that corresponds to the positive control. Four days of culture in the presence of the siRNA produced the greatest decrease in CK2 expression. Six days of culture in the presence of siRNA resulted in a surprising increase in CK2 expression to levels that exceeded that in the 6-day vehicle strips. With 4 days of culture generating the greatest amount of downregulation, a standard loading curve with incremental amounts of protein was generated. CK2 content in vehicle, control (scrambled siRNA), and experimental (siRNA against CK2) tissue strips is shown in Fig. 1B.

All subsequent studies were performed on strips cultured for 4 days in the presence of the siRNA against CK2. Several blots of the type shown in Fig. 1, A and B, were scanned for CK2 content and normalized to actin content. The averaged results are shown in Fig. 1C. After 4 days of culture in the presence of siRNA, CK2 levels are decreased by ~60% of the levels present in vehicle strips.

To ensure that actin content was not affected by siRNA introduction or by culturing, GAPDH was also used as a loading control (data not shown). No change in GAPDH was shown, demonstrating that there were no significant changes in the proteins used for normalization and that CK2 content was in fact downregulated as a result of the siRNA targeted against CK2. Additionally, it has been shown that the introduction of siRNA into some cell lines can nonspecifically result in the upregulation of interferon-induced genes with a resultant increase in cell apoptosis (24, 36). To ensure that the siRNA had
no effect on interferon levels and therefore apoptosis, an ELISA was performed to specifically measure porcine levels of interferon-γ. There were no measurable differences between vehicle, scrambled siRNA, or siRNA against CK2 tissue strips on interferon-γ levels (data not shown), indicating that any physiological results obtained following culture were not a consequence of the potential toxic effects of siRNA.

**Isometric force development.** After 4 days of culture, vehicle- and siRNA-exposed vascular strips were mounted for measurement of isometric force and subjected to the cumulative addition of KCl, histamine, or serotonin. The results of these experiments are shown in Fig. 2. All results shown are the values of stress (force/cross-sectional) attained at each concentration of stimuli. Concentration response curves to the cumulative addition of KCl (10–110 mM) are shown in Fig. 2A, to the cumulative addition of histamine (0.1–30 μM) in Fig. 2B, and to the cumulative addition of serotonin (0.01–10 μM) in Fig. 2C. Consistent with previous work done by Singer (43) contractions in response to KCl and serotonin were tonic and well maintained. However, the contractions in response to histamine were more phasic in nature, falling to a stable plateau after achieving peak values. The stress values for histamine-induced contractions in Fig. 2B are peak values of stress, whereas those in Fig. 2D are steady-state values of stress. The CK2-depleted tissues produced significantly more stress in response to all three stimuli. After 6 days of culture in the presence of siRNA, the responses to the cumulative addition of all three stimuli were not significantly different than those produced by the vehicle-cultured strips, suggesting that “overexpression” of CK2 has no enhancing effect on contractility. Our results do, however, suggest that a decrease in CK2 expression increases the force of contraction in response to either membrane depolarization or agonist activation.

**MLC phosphorylation.** MLC phosphorylation is one of the primary activators of a smooth muscle contraction and determinate of peak levels of stress developed (22). Thus it is logical to predict that if culturing smooth muscle in the presence of siRNA against CK2 significantly increases the levels of stress developed by the vascular strip, then MLC phosphorylation may be similarly elevated. To determine whether this is correct, vehicle- and siRNA-exposed tissues were removed from culture on day 4, mounted for isometric force recording, and contracted with 1 μM histamine. Histamine is the most potent and reliable agonist for the swine carotid artery and...
therefore was used to initiate force production. The tissues were frozen at various time points during the contraction and processed for quantitation of MLC phosphorylation levels as described in MATERIALS AND METHODS. The results of these experiments are shown in Fig. 3. There were no significant differences in MLC phosphorylation levels in the siRNA-exposed tissues compared with the vehicle strips at any point during the course of the contraction except for the longest time point measured at 20 min, which was significantly lower than vehicle values. The lack of a difference in stimulation-induced MLC phosphorylation levels in CK2-depleted tissues compared with knockdown tissues was not due to the sensitivity of the assay, as very small changes in MLC phosphorylation can be reliably and reproducibly measured (11, 12, 15, 32, 43). This result suggests that the higher levels of stress in the CK2-depleted muscle strips were not the result of elevations in levels of MLC phosphorylation. In fact, although not statistically significant, there was a trend at every time point measured for MLC phosphorylation values in siRNA-treated tissues to be lower than the values in vehicle tissues, even though stress values were significantly greater.

Isotonic shortening velocity. The maximal velocity of shortening has been proposed to be linearly related to the levels of MLC phosphorylation, at least in the swine carotid artery (15). Although levels of MLC phosphorylation were not on average different in siRNA-exposed strips compared with vehicle, other factors such as thin filament regulatory proteins can impact actin-activated myosin ATPase activity and therefore shortening velocity (3, 52, 55). For this reason and to provide a more complete characterization of the CK2-depleted tissue, we measured maximal velocity of shortening. Vehicle- and siRNA-exposed tissues were removed from culture on day 4 and contracted with 30 μM histamine. At various times during the contraction the strips were subjected to a series of isotonic quick releases as described in MATERIALS AND METHODS to provide an estimate of the maximal velocity of shortening at zero load. The results of these estimates are shown in Fig. 4. At every time point measured during the histamine-induced con-
traction, the values for maximal velocity of shortening are significantly higher in tissues depleted of CK2 compared with vehicle tissues. This suggests that CK2 may be either important in the determination of the isoform of myosin present in the smooth muscle cell or involved in inhibition of actin-activated myosin ATPase activity.

α-Toxin-permeabilized tissue studies. Studies performed on intact tissues provide information on the physiological response of the muscle cell to stimulation. However, it is difficult to determine whether any change in the magnitude of stress developed is the result of an increase in activator calcium or a change in sensitivity of the myofilaments. The use of the α-toxin-permeabilized preparation alleviates this problem by allowing one to measure the development of force under controlled calcium conditions. Vehicle- and siRNA-exposed tissues were removed from culture on day 4, as well as strips that were not cultured, mounted for isometric force recording, and subjected to α-toxin permeabilization. After the muscle strips were permeabilized, they were subjected to the cumulative addition of Ca2+. The results of these experiments are shown in Fig. 5. The force of contraction at each Ca2+ concentration ([Ca2+]i) was normalized to that produced in response to 3.0 μM Ca2+. The data shown in Fig. 5 indicate that decreased levels of CK2 in the vascular strips significantly increases the sensitivity to Ca2+, or at least the threshold [Ca2+]i, at which contraction is initiated.

[γ-32P]ATP incorporation and phosphor-imaging. The results shown thus far demonstrate that siRNA technology can be used to decrease content of a specific protein in intact vascular tissue and therefore not only in isolated or cultured cells. Moreover, the results shown thus far also demonstrate that the loss of CK2 has significant effects on the mechanical and biochemical characterization of a vascular smooth muscle contraction. The results do not, however, suggest a potential cellular target for CK2 and therefore a possible mechanism responsible for the proposed CK2-dependent decreases in cross-bridge cycling, calcium sensitivity, and force development. The approach we took to obtain information that at least was suggestive of a potential cellular target and mechanism was to determine the identity of CK2-catalyzed phosphorylated proteins in the swine carotid artery. The simplest preparation to use for this approach is the Triton X-100 detergent-skinned fiber. This preparation allows the direct introduction of

---

**Fig. 3.** Myosin light chain (MLC) phosphorylation levels in swine carotid artery strips after 4 days of organ culture. Vehicle (●) and CK2-depleted (○) swine carotid artery strips were stimulated with 1 μM histamine and then processed for quantitation of MLC phosphorylation levels. Although only the 20 min MLC phosphorylation value was significantly lower in siRNA-treated strips compared with vehicle, all MLC phosphorylation values in the CK2-depleted tissues tended to be lower than the values for the vehicle strips. Values are means ± SE for at least 6 determinations. *Statistically different from control at P < 0.05.

**Fig. 5.** Cumulative calcium dose response curves in α-toxin-permeabilized swine carotid artery strips subjected to 4 days of organ culture. Vehicle (●) and CK2-depleted (○) swine carotid artery strips were stimulated by the addition of 30 μM histamine and subjected to isotonic quick releases at various time points during the contraction. Tissue strips depleted of CK2 exhibited significantly greater values of maximal shortening velocity (V0), expressed in muscle lengths per second, compared with vehicle strips. Values are means ± SE for at least 4 determinations. *Statistically different from control at P < 0.05.
[\gamma^{32}\text{P}]\text{ATP} into the cytoplasm without the problems of ecto-ATPase activity, which can break down the labeled ATP, that is present in \textit{\alpha}-toxin-permeabilized preparations (50), or the need to label cellular pools of ATP by incubation for long periods of time in \textit{\nu}^{32}\text{P} as is required with intact tissue (6).

The first requirement to be addressed in these experiments is to verify the presence of CK2 in the Triton X-100 detergent-skinned fiber. Although we have not found the need to add back small proteins such as calmodulin to the Triton X-100 detergent-skinned fiber (32), it is possible that CK2 could be lost during the skinning process. However, we found that CK2 content was not lost in our skinned tissues (data not shown), therefore providing the basis for determining CK2-catalyzed phosphorylation of proteins in this preparation.

We measured CK2-catalyzed phosphoproteins in Triton X-100 detergent-skinned vehicle tissues and in siRNA CK2 knockdown tissues. To determine which cellular proteins may be regulated by CK2, we measured phosphoprotein levels in a cellular homogenate of the vascular tissues stimulated by constitutively active CK2. Figure 6 shows the phosphor-image scans of the two-dimensional gels that resulted from these experiments. Figure 6A shows the results using a tissue homogenate stimulated by constitutively active CK2. The arrow in Fig. 6A points to the primary protein phosphorylated by the addition of constitutively active CK2, which is also phosphorylated by calcium stimulation of vehicle tissues.

Figure 6B shows the results using a Triton X-100 detergent-skinned vehicle tissue strip stimulated by the addition of 1 \text{\mu}M \text{Ca}^{2+} in the presence of [\gamma^{32}\text{P}]\text{ATP} for 20 min. Tissues were homogenized and then subjected to two-dimensional gel electrophoresis, silver staining, and exposed to a phosphor-imager screen. Arrow denotes the position of the primary protein phosphorylated by the addition of constitutively active CK2, which is also phosphorylated by calcium stimulation of vehicle tissues.

Figure 6C shows the results using a Triton X-100 detergent-skinned CK2 knockdown tissue strip stimulated by the addition of 1 \text{\mu}M \text{Ca}^{2+} in the presence of [\gamma^{32}\text{P}]\text{ATP}. The arrow in Fig. 6C points to the same phosphoprotein as was identified in...
Fig. 6, A and B; although in the CK2 knockdown tissue, $^{32}$P incorporation appears to be significantly depressed.

By measuring the pH of the isoelectric focusing dimension (pI) and the molecular weight (MW) of the SDS dimension of the gels, we determined that the phosphoprotein identified in Fig. 6 has an apparent pI of 6.8 and a MW of 44,000. We used the TagIdent site (http://www.expasy.org/tools/tagident.html) on the ExPASy Molecular Biology Server (9) to request the identity of possible proteins of similar pI and MW. Using the values of $6.8 \pm 0.25$ for pI and $44,000 \pm 8,800$ for MW in the pig, TagIdent provided 29 potential proteins. Eleven of these potential proteins were also identified using CK2 as a cosearch term on PubMed (http://www.pubmed.gov). These 11 proteins are listed in Table 1.

### DISCUSSION

Smooth muscle contractile activation via the thick-filament-based MLC phosphorylation system is well accepted as the primary mechanism for the initiation of the development of force or shortening. However, this mechanism cannot account for all known aspects of a smooth muscle contraction. Therefore, in addition to the established role of the thick-filament-based regulatory system, numerous investigators have presented evidence supporting the presence of a thin-filament-based regulatory system (reviewed in Refs. 18 and 34). Although the thin filament protein calponin has received some attention in terms of its potential for regulation or modulation during physiologically relevant stimulation, the list of kinases and this inhibition is reversed by high levels of Ca$^{2+}$ and calmodulin or by phosphorylation. An interesting dilemma arises, however, when one attempts to speculate as to which of the numerous kinases shown in biochemical assays to reverse thin-filament-protein-based inhibition actually function in vivo during physiologically relevant stimulation. The list of kinases is extensive and includes one kinase that has received little attention in terms of its potential for regulation or modulation of a smooth muscle contraction, that being CK2. Thus we initiated this current study to determine whether we could use siRNA technology to decrease CK2 expression and therefore be able to examine a potential role of this enzyme in contractile regulation.

#### Table 1. Results from TagIdent on the ExPASy Molecular Biology Server

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annexin A1</td>
<td></td>
</tr>
<tr>
<td>Beta2-adrenergic receptor</td>
<td></td>
</tr>
<tr>
<td>Cellular tumor antigen p53</td>
<td></td>
</tr>
<tr>
<td>Citrate synthase</td>
<td></td>
</tr>
<tr>
<td>Creatine kinase M-type</td>
<td></td>
</tr>
<tr>
<td>Mothers against decapentaplegic homolog 3</td>
<td></td>
</tr>
<tr>
<td>NADH ubiquinone</td>
<td></td>
</tr>
<tr>
<td>NF-kappa-B inhibitor-like protein 1</td>
<td></td>
</tr>
<tr>
<td>PDZ and LIM domain protein 3</td>
<td></td>
</tr>
<tr>
<td>Plasminogen activator inhibitor 1 precursor</td>
<td></td>
</tr>
<tr>
<td>Secretory carrier-associated membrane protein 1</td>
<td></td>
</tr>
</tbody>
</table>

Results from TagIdent on The ExPASy Molecular Biology Server (Ref. 9; http://www.expasy.org/tools/tagident.html) as potential proteins identified on the 2-dimensional phosphor-imager scans shown in Fig. 6. Input values for the search were pI = 6.8 ± 0.25; MW = 44,000 ± 8,800; species = pig. Twenty-nine total proteins were identified, of which 11 were also identified on PubMed (http://www.pubmed.gov) using CK2 as a cosearch term.

As stated above, one of the major goals of this study was to demonstrate whether siRNA technology can be successfully used in the swine carotid artery smooth muscle tissue to decrease protein expression. Most work in the field using siRNA technology has used cell culture and has not utilized intact tissue strips (27, 46, 48). Because our overall interest is to study the contractile properties of smooth muscle, we prefer to use tissue rather than cultured cells, which typically undergo phenotypic modulation to a noncontractile phenotype. Culturing of intact tissue strips allows for maintenance of the contractile phenotype (7, 16).

The chemical loading technique first described by Morgan and Morgan (35) was used in our lab for the introduction of antisense oligonucleotides into the swine carotid arterial tissue (7) and was used in this study to introduce siRNA against CK2 into the swine carotid strips. Our results demonstrate that this technique in conjunction with tissue culture is a viable means for introducing siRNA into the smooth muscle tissue preparation while maintaining the smooth muscle cells in a contractile state. We also show that there is a surprisingly rapid time frame for siRNA mediated knock down of CK2 expression. Four days of culture following chemical loading resulted in the greatest decrease in CK2 expression. At 6 days of culture, there was an apparent rebound of CK2 expression with levels exceeding that of the control cultured strips. We did not determine whether this was due to degradation of the siRNA or loss of siRNA inhibition. We were able to use this phenomenon, however, to show that the effects on contraction due to the loss of CK2 were returned to normal levels when CK2 expression increased. Overexpression of CK2 after 6 days of contraction did not result in a larger decrease in contractility compared with control tissues suggesting that normal CK2 levels are saturating in terms of functionality. Importantly, the tissue culture in the presence of siRNA against CK2 did not alter the expression of other cellular proteins including actin and GAPDH. Additionally, exposure to siRNA did not increase interferon levels, and a scrambled siRNA had no effect on levels of CK2. Therefore, we believe we have shown that the use of siRNA technology in the intact smooth muscle tissue provides an efficient means for downregulating protein expression.

The second major goal of this study was to examine the potential role of CK2 in the regulation of smooth muscle contraction by determining the changes that occur following downregulation of expression levels. Based on the literature, a reasonable hypothesis would be that a decrease in CK2 levels would reduce contractility. This would result if CK2 is an endogenous caldesmon kinase, as has been suggested by Vorotnikov et al. (51). Our results, however, do not support this hypothesis and instead demonstrate that depletion of CK2 significantly increases force development and actin-activated myosin ATPase activity as assessed by estimation of shortening velocity. Consistent with our results is the finding of Sutherland and colleagues (45), who demonstrated that CK2-catalyzed phosphorylation of caldesmon did not reverse the inhibition of actin-activated myosin ATPase activity. CK2 phosphorylates caldesmon on Ser$^{73}$, which is within the myosin binding domain (54), and therefore CK2-catalyzed caldesmon phosphorylation decreases myosin binding.

If CK2 is not acting via caldesmon, then are there other potential sites of action? The simplest explanation for an...
increase in force is an increase in intracellular calcium concentration and levels of MLC phosphorylation. This simple explanation is, however, not supported by our results. Although not perfect, MLC phosphorylation levels during a KCl- and histamine-induced contraction can be used as a qualitative measure of intracellular calcium levels. Force development in the CK2-depleted strips in response to KCl and histamine stimulation was elevated compared with vehicle strips. However, MLC phosphorylation levels tended to be lower compared with vehicle tissues. This was not due to a lack of sensitivity in the assay to measure MLC phosphorylation, as small changes in MLC phosphorylation levels can be quantified using two-dimensional gel electrophoresis (12, 15, 32, 43). Moreover, the calcium sensitivity of force development in the α-toxin-permeabilized CK2-depleted strips, in which calcium levels can be held constant, was increased compared with vehicle tissues. These two facts argue against CK2 depletion increasing levels of activator calcium.

CK2 is a highly ubiquitous protein present in most if not all cell types. CK2 has been shown to be involved in noncontractile smooth muscle events such as adhesion (28, 44), but contractile effects of this enzyme are not as clear. Our measurement of 32P incorporation in the CK2 knockout tissues (Fig. 6) provided information on potential cellular targets of CK2 and therefore potential mechanisms of action resulting in enhanced contractility. We identified 11 proteins that fit the pI CK2 and that have also been shown to be substrates for CK2 (Table 1). Of these 11 proteins we identified one that has also been proposed to have a role in smooth muscle contraction. PDZ and LIM domain protein 3 is also known as actinin-associated LIM protein. Actinin-associated LIM protein has been shown to be upregulated during differentiation of smooth muscle into a contractile phenotype (40). Deficits in actinin-associated LIM protein in cardiac muscle have been shown to result in diminished ventricular contractility (29, 39). α-Actinin has recently been shown to be directly involved in and necessary for the development of active force, at least in airway smooth muscle (56). The role of α-actinin is to associate with β-integrin complexes and allow transmission of the force developed by the contractile filaments to the extracellular matrix. It is therefore interesting to speculate that CK2-dependent phosphorylation of actinin-associated LIM protein alters α-actinin association with the integrin complexes and decreases the transmission of force, resulting in a decrease in measured contractility. Knock down of CK2 would then decrease actinin-associated LIM protein phosphorylation levels, increase α-actinin binding to the integrin complexes, and increase contractile force. Therefore, a potentially interesting experiment to test this hypothesis in future studies would be to use siRNA technology to knock down CK2 in one set of tissues, actinin-associated LIM protein in another set of tissues, and both actinin-associated LIM protein and CK2 in a third set of tissues. If our hypothesis is correct, then knock down of CK2 in tissues without actinin-associated LIM protein would have no effect.

In summary, we have shown that siRNA technology can be a useful technique with which to study the function of specific proteins in intact vascular tissue. Moreover, we have shown that depletion of CK2 significantly increases the magnitude and sensitivity of contraction in both intact and α-toxin-permeabilized vascular preparations. This increase in magnitude and sensitivity of contraction is not accompanied by a concomitant change in MLC phosphorylation levels but is accompanied by an increase in cross-bridge cycling rates. Our results are not consistent with an effect of CK2 depletion on activator calcium concentrations. Instead, we propose that CK2 is a physiologically relevant regulator of smooth muscle contraction. We offer the hypothesis that CK2-catalyzed phosphorylation of actinin-associated LIM protein decreases α-actinin binding to integrin complexes, which in turn decreases force development.

GRANTS

This study was funded, in part, by National Institutes of Health Grants HL-37956 (to R. S. Moreland), DK-57252 (R. S. Moreland), and HL-67401 (to J. T. Nickels, Department of Biochemistry and Molecular Biology). E. M. Smolock is the recipient of an American Heart Association Predoctoral Fellowship from the Pennsylvania/Delaware Affiliate.

REFERENCES

What is special about smooth muscle? The significance of filament-associated proteins.


