Inhibition of zipper-interacting protein kinase function in smooth muscle by a myosin light chain kinase pseudosubstrate peptide

Eikichi Ihara, Elena Edwards, Meredith A. Borman, David P. Wilson, Michael P. Walsh, and Justin A. MacDonald

Smooth Muscle Research Group and Department of Biochemistry and Molecular Biology, University of Calgary Faculty of Medicine, Calgary, Alberta, Canada

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Ihara E, Edwards E, Borman MA, Wilson DP, Walsh MP, MacDonald JA. Inhibition of zipper-interacting protein kinase function in smooth muscle by a myosin light chain kinase pseudosubstrate peptide. Am J Physiol Cell Physiol 292: C1951–C1959, 2007. First published January 10, 2007; doi:10.1152/ajpcell.00434.2006.—As a regulator of smooth muscle contractility, zipper-interacting protein kinase (ZIPK) appears to phosphorylate the regulatory myosin light chain (RLC20), directly or indirectly, at Ser19 and Thr18 in a Ca\(^{2+}\)-independent manner. The calmodulin-binding and autoinhibitory domain of myosin light chain kinase (MLCK) shares similarity to a sequence found in ZIPK. This similarity in sequence prompted an investigation of the SM1 peptide, which is derived from the autoinhibitory region of MLCK, as a potential inhibitor of ZIPK. In vitro studies showed that SM1 is a competitive inhibitor of a constitutively active 32-kDa form of ZIPK with an apparent K\(_i\) value of 3.4 \(\mu\)M. Experiments confirmed that the SM1 peptide is also active against full-length ZIPK. In addition, ZIPK autophosphorylation was reduced by SM1. ZIPK activity is independent of calmodulin; however, calmodulin suppressed the in vitro inhibitory potential of SM1, likely as a result of nonspecific binding of the peptide to calmodulin. Treatment of ileal smooth muscle with exogenous ZIPK was accompanied by an increase in RLC20 diphosphorylation, distinguishing between ZIPK [and integrin-linked kinase (ILK)] and MLCK actions. Administration of SM1 suppressed steady-state muscle tension developed by the addition of exogenous ZIPK to Triton-skinned rat ileal muscle strips with or without calmodulin depletion by trifluoperazine. The decrease in contractile force was associated with decreases in both RLC20 mono- and diphosphorylation. In summary, we present the SM1 peptide as a novel inhibitor of ZIPK. We also conclude that the SM1 peptide, which has no effect on ILK, can be used to distinguish between ZIPK and ILK effects in smooth muscle tissues.

inhibitory peptide; calcium sensitization

SMOOTH MUSCLE CONTRACTION is a dynamic and highly regulated process (12, 20). The contraction and relaxation of smooth muscle depends mainly on the intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) and the level of phosphorylation of the myosin regulatory light chain (RLC20). Contractile stimuli that cause an increase in [Ca\(^{2+}\)]\(_i\), result in Ca\(^{2+}\) binding to calmodulin, activation of myosin light chain kinase (MLCK) (7), and stimulation of RLC20 phosphorylation, which results in cross-bridge cycling and force development. Because the phosphorylation level of RLC20 is determined by the opposing activities of MLCK and myosin light chain phosphatase (MLCP) (13, 19), the Ca\(^{2+}\) sensitivity of contraction can be affected by a change in the MLCK-to-MLCP activity ratio. MLCP function can be regulated by G protein-coupled signaling pathways (27, 36, 37, 40), leading to increases in both RLC20 phosphorylation and contractile force development in smooth muscle without any changes in [Ca\(^{2+}\)]\(_i\) (37), i.e., Ca\(^{2+}\) sensitization, via phosphorylation of the MLCP inhibitor protein CPI-17 and/or the myosin targeting subunit, MYPT1, of MLCP. Ca\(^{2+}\)-independent contraction can also be elicited by inhibition of MLCP activity with microcystin, for example (42). The resulting contraction is slow compared with the Ca\(^{2+}\)-induced contraction; however, a comparable steady-state tension level is achieved. Treatment with phosphatase inhibitors unmasks endogenous Ca\(^{2+}\)-independent RLC20 kinase activity that is associated with the contractile machinery. Similar results have been obtained with a number of different smooth muscle tissues and other type 1 phosphatase inhibitors, suggesting a common mechanism (18, 34). Recent studies indicate that several protein kinases, acting either by inhibition of MLCP or by Ca\(^{2+}\)-independent phosphorylation of RLC20, may contribute to Ca\(^{2+}\) sensitization (37).

Zipper-interacting protein kinase (ZIPK) is a Ser/Thr protein kinase that has been linked to the regulation of smooth muscle contraction (2, 30, 32, 33) and other cellular processes, including cell motility (27) and cell death (23, 25). ZIPK is able to phosphorylate nonmuscle myosin light chains (27). This phosphorylation is known to cause reorganization of the actin cytoskeleton, which could explain some of the cellular phenotypes observed with ZIPK overexpression (e.g., detachment from the matrix and cell rounding). ZIPK can phosphorylate RLC20 at Ser19 and Thr18 in a Ca\(^{2+}\)-independent manner (2, 32, 33). In addition, ZIPK has been proven to be involved in the regulation of MLCP. It was shown to associate with and phosphorylate MYPT1 in rabbit ileum (30). The addition of constitutively active, recombinant ZIPK to permeabilized smooth muscle causes profound Ca\(^{2+}\) sensitization through inhibition of MLCP activity via phosphorylation of endogenous MYPT1 at an inactivating site, Thr95 (chicken sequence) (2, 30). More recently, ZIPK was demonstrated to be the major MYPT1-associated kinase in aortic smooth muscle cells (6, 21). However, it is unclear from these studies whether the sensitizing effects of ZIPK are solely due to the direct phosphorylation of RLC20 or the inhibition of MLCP via phosphorylation of MYPT1.

The addition of the protein phosphatase-1 and protein phosphatase-2A phosphatase inhibitor microcystin to smooth muscle in the absence of Ca\(^{2+}\) elicits a slow, sustained contractile...
response (9, 42). This Ca\textsuperscript{2+}-independent contraction correlates with phosphorylation of RLC20 at Ser19 and Thr18 (42). Since MLCK is absolutely dependent on Ca\textsuperscript{2+} and, at physiological levels of the kinase, is specific for phosphorylation at Ser19 (16, 42), the kinase responsible is clearly not MLCK. Several other protein kinases are able to phosphorylate RLC20 in vitro (1, 2, 4, 5, 17, 20, 26, 32, 38, 39, 44); however, in most cases, RLC20 phosphorylation is restricted to Ser19. For these and other reasons, only integrin-linked kinase (ILK) (43) and ZIPK have emerged as bona fide candidates for the Ca\textsuperscript{2+}-independent diphosphorylation of RLC20 at Ser19 and Thr18 in smooth muscle. Differential kinase inhibition has been used in an attempt to distinguish between the effects of ZIPK and ILK in rat cardiac muscle. We recently demonstrated that direct inhibition of MLCP by microcystin in the absence of Ca\textsuperscript{2+} unmasked ILK activity (43). Further application of a battery of protein kinase inhibitors indicated that ILK was solely responsible for direct RLC20 diphosphorylation in this vascular smooth muscle. This raises the possibility that the involvement of ZIPK in the regulation of RLC20 phosphorylation and smooth muscle contraction may be indirect, e.g., via phosphorylation of MYPT1 and inhibition of MLCP.

It has become clear that specific inhibitors of ILK and ZIPK will be required for the determination of their physiological role(s) in smooth muscle contractility. ZIPK shares significant sequence similarity with members of the Ca\textsuperscript{2+}/calmodulin-dependent protein kinase (CaMK) family. In fact, ZIPK was identified in a cDNA library screen using the MLCK catalytic domain as a probe (32). The autoinhibitory domains of protein kinases in the CaMK family [e.g., smooth muscle (sm)MLCK, skeletal muscle (sk)MLCK, and CaMKII] have been well characterized (24) and share similarity to a sequence found in ZIPK. These autoinhibitory regions in ZIPK and smMLCK have pseudosubstrate characteristics; that is, they have a number of basic residues in common with the sequence surrounding the primary phosphorylation site (Ser19) of RLC20. The sequence similarities between ZIPK and the other members of the CaMK family of kinases prompted us to investigate whether the previously characterized SM1 peptide (42), isolated from the autoinhibitory region of MLCK, would function as an inhibitor of ZIPK. Here, we describe a series of experiments that characterize the inhibition of ZIPK by SM1 peptide.

**EXPERIMENTAL PROCEDURES**

**Materials.** All chemicals were reagent grade unless otherwise indicated. [γ-\textsuperscript{32}P]ATP and trifluoperazone (TFP) were purchased from ICN Biomedical (Aurora, OH). Triton X-100 was obtained from Sigma (St. Louis, MO); thrombin, A23187, and anti-ZIPK polyclonal antibody were from Calbiochem (San Diego, CA); and Precision Protease and anti-rabbit IgG coupled to horseradish peroxidase were from GE Healthcare (Piscataway, NJ). SM1, scSM1, and cysSM1 peptides were produced by the University of Calgary Protein Synthesis Facility (Calgary, AB, Canada), confirmed by amino acid analysis, and shown to be >95% pure by analytical HPLC. SM1 (sequence: AKKLSDKRMKKYMAKRWKGT) is a synthetic peptide inhibitor of MLCK that corresponds to the autoinhibitory domain of smMLCK (residues 783–804 of chicken gizzard MLCK) (42). The scSM1 peptide has a scrambled sequence and serves as the control for the SM1 peptide. The cysSM1 peptide was identical to SM1 except for the presence of an NH\textsubscript{2}-terminal cysteine residue for coupling to an iodoacetetyl-activated agarose resin. The RLC substrate peptide (KKKRQATSNVF) containing Lys10 to Phe22 of RLC20 was synthesized by Biomolecules Midwest (St. Louis, MO).

**Protein purifications.** Myosin regulatory light chains (RLC20) were purified from chicken gizzard (42). Calmodulin was purified from bovine testis (10). A constitutively active form of ZIPK, GST-ZIPK\textsuperscript{(1–320)}, was expressed in Escherichia coli and purified with glutathione-Sepharose as previously described (2). Because the catalytic characteristics of ZIPK\textsuperscript{(1–320)} are essentially the same as those of the purified native 32-kDa enzyme from smooth muscle tissue (30), we used the recombinant catalytic fragment in this study. The GST moiety was cleaved from the recombinant protein using Precision Protease according to the manufacturer’s instructions. Rat kidney GST-MYPT1\textsuperscript{(1–658)} was expressed and purified as previously described (14). The GST moiety was cleaved from the recombinant MYPT1 protein using thrombin.

**Protein kinase assay.** The phosphorylation of RLC20 and RLC substrate peptide by ZIPK was measured with a standard assay at 25°C in a final volume of 50 μl. The kinase (0.1 μg) was diluted in 25 mM HEPES, pH 7.4, 2.5 mM MgCl\textsubscript{2}, and 200 μM [γ-\textsuperscript{32}P]ATP [20,000 counts min\textsuperscript{-1} (cpm) mol\textsuperscript{-1}] with 40 μM RLC20 protein or 100 μM RLC peptide substrate. SM1 or scSM1 was present where indicated. Reactions were initiated by the addition of ATP solution (ATP and MgCl\textsubscript{2}) and terminated after 15 min. Phosphorylation time courses were linear with respect to time and protein concentration under these conditions as determined in preliminary experiments. Reactions were stopped at the indicated times by addition of 20 mM H\textsubscript{2}PO\textsubscript{4} (50 μl). Reaction mixtures (100 μl) were spotted onto phosphocellulose P81 paper, which was washed three times with 20 mM H\textsubscript{2}PO\textsubscript{4} and placed in 1.5 ml Eppendorf tubes. \textsuperscript{32}P incorporation was determined by scintillation counting.

**Standard assay conditions were used to determine ZIPK phosphorylation of MYPT1\textsuperscript{(1–658)} at Thr627 (corresponding to Thr695 in the chicken sequence).** SM1 or scSM1 peptide was present where indicated. Kinase reactions were terminated after 15 min by addition of 10 μl of Laemml sample buffer. After being boiled for 5 min, the protein samples were resolved by SDS-PAGE (10% acrylamide). Densitometry of phosphorylated MYPT1 protein (0.2 mg) was carried out with a Storm Phosphorimagerr (GE Healthcare).

ZIPK autophosphorylation was measured by incubation of ZIPK (0.1 μg) with 1 mM MgCl\textsubscript{2} and 100 μM [γ-\textsuperscript{32}P]ATP (100,000 cpmmol\textsuperscript{-1}) in the absence of protein substrate. Incubations were terminated after 1 h by addition of 10 μl of Laemmli sample buffer. After being boiled for 5 min, the ZIPK samples were resolved by SDS-PAGE (10% acrylamide). ZIPK phosphorylation was detected by phosphorimaging analysis.

In experiments to determine the effect of SM1 on the apparent \textit{Km} for RLC20 and \textit{Vmax}, ZIPK was assayed with 0, 1, 2.5, 5, or 7.5 μM SM1 in 25 mM HEPES, pH 7.4, 2.5 mM MgCl\textsubscript{2}, 200 μM ATP, and a range of RLC20 concentrations (i.e., 0–500 μM). Reactions were initiated by the addition of ZIPK. Incubations were carried out at 25°C and sampled after 3 min. This time point was determined to be in the linear range for all assays. Incorporation of \textsuperscript{32}P into RLC20 protein substrate was monitored with P81 paper as described above. In experiments to determine the effect of Ca\textsuperscript{2+} and calmodulin on the inhibitory potential of SM1, the incorporation of \textsuperscript{32}P into synthetic RLC peptide (100 μM) by ZIPK was measured under three conditions: 1) with 5 mM EGTA alone; 2) with 0.1 mM CaCl\textsubscript{2} and 15 μM calmodulin; or 3) with 0.1 mM CaCl\textsubscript{2}, 15 μM calmodulin, and 5 mM EGTA. \textsuperscript{32}P incorporation into RLC peptide was monitored with P81 paper as described above.

**Kinetic analysis and statistics.** Kinetic constants (\textit{Km} and \textit{Vmax}) were determined from the Henri-Michaelis-Menten equation using a nonlinear least squares regression computer program (36). The concentrations of inhibitors that decrease enzyme velocity by 50% (\textit{IC}\textsubscript{50} values) were determined using computer-generated plots of \textit{Vmax}/\textit{V}_{\text{inhibitor}} vs. [inhibitor] (3). Values represent means ± SE, and \textit{n}
is the number of experiments; a Student's t-test was performed where
indicated.

Cell culture, transfection, and expression of full-length ZIPK. Full-length human ZIPK was amplified by PCR using the following
primers (which contain BamHI and XhoI sites, respectively): forward
primer, 5'-AGTCggatccTCCACGTTCAGGCAGGAGGAC; and re-
verse primer, 5'-CAGTctcgagCTAGCGCAGCCCGCACTCCAC. The PCR product was digested with BamHI and XhoI and ligated into
the corresponding sites in a pcDNA3.1/H11001 expression vector (Invitro-
gen) into which the Myc tag had been previously inserted. This
procedure encoded a Myc tag with the NH2-terminal sequence
MAEQKLISEEDL preceding ZIPK. Human embryonic kidney 293
(HEK293) cells were maintained in Dulbecco's modified Eagle's
medium (Invitrogen) supplemented with 10% bovine calf serum.

Fig. 1. Comparison of primary sequences of known and putative “autoinhibitory” regions with SM1 synthetic peptide. Clustal W (top) or Multalin (bottom)
multiple sequence alignment programs were used to align human sequences of zipper-interacting protein kinase (ZIPK; NP_001339), calcium/calmodulin-
dependent protein kinase I (CaMK1; Q14012), smooth muscle myosin light chain kinase (smMLCK; Q15746), calcium/calmodulin-dependent protein kinase II
(CaMKII; NP_057065), and skeletal muscle myosin light chain kinase (skMLCK; Q9H1R3). The SM1 peptide sequence that corresponds to the autoinhibitory
domain of smMLCK is underlined. Identical residues in all 5 sequences are indicated by an asterisk (*). Conserved and semi-conserved substitutions are indicated
by a colon (:) and period (.), respectively. Clustal W and Multalin are available at http://www.ebi.ac.uk/clustalw/index.html and http://prodes.toulouse.inra.fr/
multalin/multalin.html, respectively.

The PCR product was digested with BamHI and XhoI and ligated into
the corresponding sites in a pcDNA3.1+ expression vector (Invitrogen) into
which the Myc tag had been previously inserted. This procedure encoded a Myc tag with the NH2-terminal sequence
MAEQKLISEEDL preceding ZIPK. Human embryonic kidney 293
(HEK293) cells were maintained in Dulbecco's modified Eagle's
medium (Invitrogen) supplemented with 10% bovine calf serum.

Fig. 2. SM1 peptide effects on ZIPK in vitro. Purified recombinant ZIPK(1–320) was assayed
with regulatory myosin light chain (RLC20; A) or recombinant MYPT1(1–658) (B) as substrates
in the presence of the indicated concentrations of SM1 or scSM1 synthetic peptide. The results
are expressed relative to control in the absence of SM1 (■) or scSM1 (●) peptide. C: inhibition
of ZIPK autophosphorylation by SM1 peptide. Purified recombinant ZIPK(1–320) was incubated with
0.1 mM ATP, 1 mM MgCl2, [γ-32P]ATP (300,000 counts/min/μmol), and
25 mM HEPES, pH 7.2, for 1 h in the presence of increasing concentrations of SM1 peptide.
The autoradiogram is representative of 3 independent experiments. D: effect of calmodulin
on the concentration-dependent inhibition of ZIPK(1–320) by SM1. The results are expressed
relative to control in the absence of SM1. Purified recombinant ZIPK(1–320) was assayed in the presence of 5 mM EGTA (●), 0.1 mM CaCl2 and 15 μM calmodulin (●), or 0.1 mM CaCl2, 15 μM calmodulin, and 5 mM EGTA (●). Error bars indicate SE (n = 4). The absence of error bars indicates that they are smaller than the symbols.
Tissue preparation and force measurement. Ileum was removed from rats anesthetized and euthanized according to protocols approved by the University of Calgary Animal Care and Use Committee. Sheets of longitudinal ileal smooth muscle were dissected and cut into small strips (200 μm x 2 mm). For force measurement, muscle strips were tied with silk monofilaments to the tips of two fine wires. One wire was fixed, and the other was connected to a force transducer (SensoNor, AE801). The strip was mounted in a well on a stir plate to allow rapid solution exchange. Strips were stretched to 1.3 times resting length. Muscle strips were permeabilized by incubation with 0.1% Triton X-100 for 20 min at room temperature in an intracellular solution containing 1 mM EGTA and no added Ca^{2+} (G1), with 10 μM A23187 added for the final 10 min to deplete intracellular Ca^{2+} stores. In some experiments, calmodulin was then removed by treatment with 0.2 mM TFP for 20 min in pCa 4.5 solution. The muscle strips were washed in Ca^{2+}-free solution (G10) and then incubated for 30 min in the presence of recombinant ZIPK(1–320). The composition of the G1 solution was as follows (in mM): 10 creatine phosphate (Na2CP), 5.16 adenosine triphosphate (Na2ATP), 7.31 magnesium methanesulfonate (MgMS2), 74.1 potassium methanesulfonate (KMS), and 1

Transfections were performed using FuGene 6 transfection reagent (Roche) according to the manufacturer’s instructions. HEK293 cells were transfected with ZIPK DNA and incubated for 48 h.

Association of ZIPK with SM1-agarose. The cysSM1 peptide was covalently coupled to SulfoLink agarose (Pierce, Rockford, IL) according to the manufacturer’s instructions. A control agarose resin was also produced by omission of cysSM1 peptide during the coupling procedure. HEK293 cells were harvested and homogenized in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 1% NP-40, 1 mM dithiothreitol (DTT), 2 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 100 μg/ml pepstatin A. Cell lysates were clarified by centrifugation for 15 min at 13,000 g. The supernatant was removed and diluted with 25 mM Tris, pH 7.5, to give a final [NaCl] of 50 mM. The cell lysate, containing full-length ZIPK, was incubated with SM1-agarose or control resin for 1 h at 4°C. The resins were then washed extensively with 25 mM Tris (pH 7.5) buffer before elution of ZIPK with increasing [NaCl].
ethylene-bis-(oxyethylenenitrilo) tetraacetic acid (K2EGTA). The composition of the G10 solution was as follows (in mM): 10 Na2CP, 5.14 Na2ATP, 7.92 MgMS2, 46.6 KMS, and 10 K2EGTA. The composition of the CaG solution was as follows (in mM): 10 Na2CP, 5.14 Na2ATP, 7.25 MgMS2, 47.1 KMS, 10 and K2CaEGTA. Desired free Ca2+ levels (expressed as pCa) were obtained by mixing G10 and CaG solutions. All contractile measurements were carried out at room temperature (23°C).

Determination of RLC20 phosphorylation. Contractile responses were halted by immersion of ileal muscle strips in a dry ice-acetone solution containing 10% (vol/vol) trichloroacetic acid. The muscle strips were washed with a 10 mM DTT-acetone solution and lyophilized overnight. Proteins were extracted in a buffer containing 8 M urea, 1 M thiourea, and 10 mM DTT and separated by urea/glycerol PAGE. Western blotting using an antibody to RLC20 was carried out as described previously (42). The stoichiometry of RLC20 phosphorylation was calculated from the following equation: mol of Pi/mol RLC20 = (y + 2z)/(x + y + z), where x, y, and z are the signal intensities of unphosphorylated and mono- and di-phosphorylated RLC20 bands, respectively.

RESULTS

Identification and characterization of the autoinhibitory domain of ZIPK. Experimental results (11, 15, 22) suggest that an autoinhibitory region is present in the COOH-terminal portion of ZIPK. Expression of a truncated form of ZIPK lacking the COOH terminus results in a constitutively active enzyme (30). The site for a putative autoinhibitory domain within the ZIPK sequence was localized between amino acids 273 and 342 (25). On the basis of knowledge of other autoinhibitory domains in the CaMK family and an alignment of this region of ZIPK with sequences that span known autoinhibitory domains of smMLCK, skeletal muscle MLCK, CaMKII, and CaMKI (Fig. 1), the most likely site for a ZIPK autoinhibitory domain is a region that is rich in basic amino acids between kinase subdomains X and XI in the COOH-terminal portion of the protein. This region of ZIPK shares sequence similarity with the other CaMK family members and, accordingly, with the SM1 peptide. The results of this alignment suggest that SM1 could have inhibitory potential against ZIPK.

In vitro effects of SM1 peptide on ZIPK. SM1 was found to inhibit RLC20 phosphorylation by recombinant ZIPK(1–320) with an IC50 value of 3.8 ± 0.1 μM (Fig. 2A). Interestingly, SM1 is a weaker inhibitor of MYPT1 phosphorylation by ZIPK, with an IC50 value approximating 20 μM (Fig. 2B). The scSM1 peptide was found to have minor inhibitory effects on the phosphorylation of RLC20 and MYPT1 by ZIPK(1–320) but only at substantially higher concentrations. As shown in Fig. 2C, ZIPK autophosphorylation was also significantly reduced in the presence of SM1 peptide. The concentration of SM1 required for inhibition of ZIPK autophosphorylation was within the range needed to inhibit phosphorylation of the RLC20 protein substrate. In contrast to this result, the autophosphorylation of smMLCK is unaffected by the SM1 peptide (42).

![Fig. 5. Effects of SM1 on Ca2+-independent, ZIPK-induced contraction of Triton-skinned rat ileum.](http://ajpcell.physiology.org/)

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**Effect of Ca\(^{2+}\) and calmodulin on peptide inhibitor potency.** In a previous study, Kemp et al. (24) found that a peptide corresponding to the region between residues 480 and 501 of smMLCK (analogous to the SM1 peptide) serves both as a calmodulin and as a substrate antagonist. To define more precisely the potential effect of calmodulin on ZIPK inhibition by SM1, the inhibitory potential of the peptide was determined in the presence and absence of Ca\(^{2+}\) and calmodulin (Fig. 2D). RLC protein substrate was phosphorylated equally well by ZIPK in the presence and absence of Ca\(^{2+}\) and/or calmodulin. Similar results were obtained when the synthetic RLC peptide was used as substrate. These results confirm that ZIPK activity is independent of calmodulin, as has been previously reported (32). When Ca\(^{2+}\) and calmodulin were added to the ZIPK assay, the inhibitory potency of SM1 was significantly reduced (Fig. 2D). These results are consistent with the previously reported calmodulin-binding characteristics of the SM1 peptide (24, 42). Under these conditions, Ca\(^{2+}\) binding to calmodulin is expected to induce conformational changes with exposure of hydrophobic pockets for the interaction with target proteins, in this case with synthetic SM1 peptide (28). However, the suppression of SM1 inhibitory potential by calmodulin was not dependent on Ca\(^{2+}\), since the addition of EGTA to the assay had no apparent effect. The data suggest that calmodulin effects on SM1 inhibitory potential are likely a result of nonspecific binding of the peptide to calmodulin. There is some supportive evidence for an interaction between apocalmodulin and a peptide corresponding to the calmodulin-binding domain of smMLCK at low ionic strength (41).

**SM1 peptide is active against full-length ZIPK.** Given that previous reports have suggested that the 54-kDa full-length form of ZIPK is present in smooth muscle (6) and that the catalytic activity of the full-length and catalytic fragment may be different (33), we performed additional experiments to confirm that the SM1 peptide is also active against full-length ZIPK. Myc-tagged full-length ZIPK (MYC-FL-ZIPK) was immunoprecipitated from HEK293 cells. The activity of MYC-FL-ZIPK was potently inhibited by the addition of SM1 peptide (Fig. 3A). In addition, SM1-agarose recovered recombinant ZIPK(1–320) as well as MYC-FL-ZIPK (Fig. 3B). Control agarose was not effective in isolating ZIPK from either source. These results indicate that full-length ZIPK exists in a conformation that is able to interact with the SM1 peptide.

The inhibition of ZIPK activity toward RLC20 by SM1 was investigated as a function of varying RLC20 protein concentration to determine the nature of the inhibition. In the absence of SM1 peptide, ZIPK(1–320) was found to have a $K_m$ of 59.4 ± 2.5 μM and a $V_{max}$ of 0.087 ± 0.004 μmol·min\(^{-1}\)·mg\(^{-1}\). Similar kinetic values were obtained in the presence of calmodulin (data not shown), indicating that calmodulin does not alter the kinetics of ZIPK-catalyzed phosphorylation of RLC20. The experimentally determined kinetic values for ZIPK(1–320) are similar to those reported for smMLCK in the absence of calmodulin (43 μM; $V_{max}$, 0.02 μmol·min\(^{-1}\)·mg\(^{-1}\)) (24), although the addition of calmodulin to smMLCK decreased the $K_m$ by ~50% and increased the $V_{max}$ by 500-fold (24). For ZIPK(1–320), the addition of SM1 increased the $K_m$ without having an observable effect on the $V_{max}$. A double-reciprocal plot of SM1 inhibition (Fig. 4A) confirmed that the peptide exhibits competitive inhibition kinetics with RLC20 as substrate. A secondary plot (Fig. 4B) of apparent $K_m$ as a function of [SM1] was linear and produced an apparent inhibitor constant ($K_i$) value of 3.4 ± 0.7 μM.

**Effects of SM1 on Ca\(^{2+}\) sensitization and RLC20 phosphorylation induced by ZIPK administration to smooth muscle.** The observation that SM1 could inhibit ZIPK activity in vitro suggests that this synthetic peptide could be used to investigate the role of ZIPK in smooth muscle. Therefore, as a first approach, we tested the ability of SM1 peptide to block smooth muscle contraction induced by addition of exogenous ZIPK. Rat ileal strips were mounted on a force transducer and Triton-skinned (0.1%) for 20 min. Following addition of pCa 4.5 to establish the maximal contractile tension, the muscle strips were washed in Ca\(^{2+}\)-free solution (G10) and then incubated for 30 min in the presence of recombinant ZIPK(1–320). As shown in Fig. 5A, incubation with ZIPK evoked a Ca\(^{2+}\)-independent contraction that developed an initial tension at 1 min of 14.3 ± 1.9% ($n = 6$) of the pCa 4.5-induced contraction (i.e., the maximal force; $F_{max}$) and a sustained tension at 30 min that was equivalent to 27.5 ± 1.9% ($n = 6$) of the $F_{max}$. These results
are similar to our previous reports of ZIPK action in rabbit ileal smooth muscle (2). Under Ca\(^{2+}\)-free conditions (G10), muscle tension was not affected by the addition of SM1 (100 μM) when the peptide was added in the absence of exogenous ZIPK\(^{1–320}\) (Fig. 5B). When exogenous ZIPK\(^{1–320}\) was added to muscle strips that had been preincubated with SM1, a reduction in the initial (i.e., 1 min) force generation was evident (Fig. 5C). In control experiments, the scSM1 peptide did not alter ZIPK-induced contraction when used at similar concentrations (Fig. 5D). The muscle tension developed by ZIPK addition was significantly inhibited by the addition of SM1 peptide. SM1 reduced the initial muscle tension to 3.1 ± 1.9% (n = 6) of \(F_{\text{max}}\) and the steady-state tension to 9.7 ± 3.1% of \(F_{\text{max}}\) (Fig. 5E).

The addition of pCa 4.5 solution to Triton-skinned ileal muscle elicited an increase exclusively in monophosphorylated RLC\(_{20}\), while treatment with recombinant ZIPK\(^{1–320}\) in the absence of Ca\(^{2+}\) elicited both mono- and diphosphorylation of RLC\(_{20}\) (Fig. 6A). The decrease in contractile force observed with SM1 was associated with a significant reduction in RLC\(_{20}\) phosphorylation in Triton-skinned muscle strips. The amount of monophosphorylated RLC\(_{20}\) declined from 21.1 ± 2.6 to 13 ± 1.4% of total RLC\(_{20}\), and the amount of diphosphorylated RLC\(_{20}\) declined from 23.7 ± 2.2 to 11.2 ± 1.8% of total RLC\(_{20}\) (Fig. 6B). A corresponding increase in the amount of unphosphorylated RLC\(_{20}\) was also observed with application of SM1 peptide. The RLC\(_{20}\) phosphorylation stoichiometries were determined to be 0.02 ± 0.01 mol P/mol RLC\(_{20}\) (G10), 0.24 ± 0.04 mol P/mol RLC\(_{20}\) (pCa 4.5 solution), 0.65 ± 0.03 mol P/mol RLC\(_{20}\) (exogenous ZIPK addition), and 0.37 ± 0.02 mol P/mol RLC\(_{20}\) (exogenous ZIPK and SM1 peptide).

Suspecting that endogenous calmodulin could reduce the potency of SM1 inhibition in situ, we undertook similar experiments with ileal muscle strips that had been pretreated with TFP to deplete calmodulin from the ileal tissues. Treatment of the tissue with TFP at the peak of a Ca\(^{2+}\)-induced contraction relaxed the Ca\(^{2+}\)-dependent contraction (Fig. 7A) but did not affect the Ca\(^{2+}\)-independent, microcystin-induced contraction. ZIPK was still able to elicit a Ca\(^{2+}\)-independent contraction in TFP-treated ileum. Interestingly, the contractile response to exogenous ZIPK was 1.9-fold greater in TFP-treated ileum than in tissue that had been Triton-skinned in the absence of TFP (Fig. 7B). SM1 treatment (100 μM) reduced the initial ZIPK-induced force generation from 24.5 ± 3.9% (n = 5) to 12.2 ± 2.5% (n = 5) of \(F_{\text{max}}\) (Fig. 7C). The steady-state tension developed after 30 min by ZIPK was also significantly inhibited by SM1, with contractile force dropping from 52.3 ± 4.6 to 22.6 ± 6.1% of \(F_{\text{max}}\) (Fig. 7D). The addition of recombinant ZIPK\(^{1–320}\) to TFP-treated muscle also induced Ca\(^{2+}\)-independent mono- and diphosphorylation of RLC\(_{20}\) (Fig. 6A). A significant reduction in RLC\(_{20}\) phosphorylation (Fig. 6B) occurred in the presence of SM1. The amount of monophosphorylated RLC\(_{20}\) declined slightly from 23.0 ± 1.1 to 18.5 ± 0.8% of total RLC\(_{20}\). Again, SM1 had significant effects on RLC\(_{20}\) diphosphorylation, declining from 45.5 ± 3.3 to 27.5 ± 1.8% of total RLC\(_{20}\) (Fig. 6B). The RLC\(_{20}\) phosphorylation stoichiometries for TFP-treated smooth muscle were 1.14 ± 0.06 mol P/mol RLC\(_{20}\) (exogenous ZIPK addition) and 0.73 ± 0.03 mol P/mol RLC\(_{20}\) (exogenous ZIPK and SM1 peptide).

**DISCUSSION**

Our current understanding of the regulation of muscle and nonmuscle myosin II phosphorylation is limited by a paucity of information pertaining to protein kinases that are responsible for Ca\(^{2+}\)-independent phosphorylation of RLC\(_{20}\). Currently, it seems most likely that ILK, ZIPK, or some combination of the two kinases is responsible for Ca\(^{2+}\)-independent RLC\(_{20}\) phosphorylation (43). Unfortunately, specific molecules for the inhibition of ZIPK and ILK have yet to be developed. To
complicate the issue, many small molecule inhibitors that act on MLCK (i.e., ML-9, ML-7, and wortmannin) can also inhibit ZIPK at similar concentrations (2, 33). The nonselectivity observed for these purine analogs is likely a consequence of the high degree of sequence and structural conservation within the ATP-binding pocket of the CaMK family members. We believe that the design of a synthetic inhibitory peptide will prove to be the best approach for selective inhibition of ZIPK in smooth muscle. This peptide could be used in a similar manner to the potent and highly selective peptide inhibitor PKI(5–22) of cAMP-dependent protein kinase (PKA) (8). A ZIPK-specific inhibitor peptide would be an important tool for the delineation of ZIPK physiological function in smooth muscle contraction and other cellular events where ZIPK has been implicated (e.g., apoptosis).

Sequence comparison and kinetic studies described here suggest that ZIPK possesses a pseudosubstrate sequence that could act as an autoinhibitory region. The calmodulin-binding region of smMLCK has been shown to resemble a pseudosubstrate domain (24), and several key specificity requirements of this pseudosubstrate domain have been identified from structure/function studies. The spatial arrangement of basic residues present in the RLC20 substrate is remarkably similar to the arrangement found in the pseudosubstrate domain within the MLCK sequence. The specific alignment BXXBBB (where B is a basic residue R/K and X is any amino acid) within a cluster of basic residues was found to be important for the competitive substrate inhibitor. There are two such groups of basic residues (i.e., 276KAIRRR281 and 290RKPERRR296) within the ZIPK sequence (Fig. 1) that may provide the key structure/function determinants for autoinhibition. The SM1 synthetic peptide derived from the smMLCK autoinhibitory region inhibited both ZIPK autophosphorylation and ZIPK phosphorylation of RLC20 and MYPT1 substrate proteins in in vitro assays. Likewise, the SM1 peptide diminished the Ca2+-sensitizing effects of exogenous ZIPK in isolated smooth muscle strips, leading to a reduction in mono- and diphosphorylated RLC20 levels. It is likely that the competitive inhibitory potency of the smMLCK peptides toward ZIPK is a result of sequence conservation with the pseudosubstrate sequence in the putative ZIPK autoinhibitory domain.

In the original paper that identified ZIPK as an MLCP-associated kinase (30), treatment of isolated smooth muscle with the Ca2+-sensitizing agonist carbachol elicited phosphorylation and activation of ZIPK. Since this report, complex phosphorylation events have been shown to govern both the enzymatic activity and cellular localization of ZIPK. Several regulatory sites of phosphorylation (11, 15, 30, 35) have been identified by in vivo isotope labeling, mass spectrometry, and site-directed mutagenesis. There is still debate regarding the specific function of these phosphorylation sites in vivo; however, it appears that Thr180, Thr225, Thr265, and Thr300 are involved in the regulation of enzymatic activity. Interestingly, the Thr300 residue is located within the region of ZIPK that aligns with the pseudosubstrate region of smMLCK. Phosphorylation of this residue was shown to impact ZIPK enzymatic activity (35) and subcellular targeting (11). Thus phosphorylation of the Thr300 residue may exert conformational changes on the ZIPK structure to promote the removal of the autoinhibitory domain from the substrate-binding region. More direct studies will be required to assess the interaction between ZIPK autophosphorylation events and autoinhibition to determine the biological significance of these ideas and their relevance to the in vivo regulation of ZIPK in smooth muscle.

Synthetic peptides corresponding to both the skeletal and smooth muscle calmodulin-binding regions of MLCK are potent, competitive inhibitors of smMLCK (24). The inhibitory potency of the SM1 peptide toward ZIPK compares well with the K_i and IC50 values determined for similar peptides against smMLCK (24). Ultimately, the inhibitory potency of synthetic peptides is dependent on specific structural features contained within the substrate-binding pocket. The integration of the calmodulin-binding domain with the autoinhibitory domain in smMLCK provides a mechanism whereby calmodulin activation of smMLCK relieves autoinhibition. ZIPK, a calmodulin-independent enzyme, does not possess an integrated calmodulin-binding and autoinhibitory domain, and, therefore, the molecular interactions necessary for SM1 inhibition are likely different for this kinase. Our in vitro studies suggested that nonspecific interactions between calmodulin and SM1 could reduce the inhibitory potency in situ. We addressed this concern by analyzing SM1 effects in smooth muscle preparations with and without calmodulin depletion by TFP. While TFP treatment potentiates Ca2+-sensitization induced by ZIPK, our results indicate that SM1 can be used as an effective inhibitor of ZIPK activity under Ca2+-free conditions in smooth muscle, independent of the presence of calmodulin. Thus we contend that SM1 is suitable for use as an inhibitor of ZIPK function in smooth muscle contraction; the peptide, which does not inhibit ILK activity, can be used to distinguish between ZIPK and ILK effects in smooth muscle tissues. We also suggest that care be exercised when interpreting data from experiments that use SM1 and similar peptides for the inhibition of MLCK activity.

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Present address for D. P. Wilson: Discipline of Physiology, School of Molecular and Biomedical Science, University of Adelaide, Adelaide 5005, South Australia.

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REFERENCES


Hartshorne DJ.

Ikebe M, Hartshorne DJ, Elzinga M.

Haystead CM, Gailly P, Somlyo AP, Somlyo AV, Haystead TAJ.

Ikebe M, Hartshorne DJ, Elzinga M.

Kamm KE, Stull JT.

Kawai T, Matsumoto M, Takeda K, Takeda K, Sanjo H, Akira S.

Karim SM, Rhee AY, Given AM, Faulx MD, Hoit BD, Brozovich FV.


Endo A, Surks HK, Mochizuki S, Mochizuki N, Mendelsohn ME.

Gong MC, Cohen P, Kitazawa T, Ikebe M, Masuo M, Somlyo AP, Somlyo AV.


