Ca\textsuperscript{2+}-independent contraction of longitudinal ileal smooth muscle is potentiated by a zipper-interacting protein kinase pseudosubstrate peptide

Eikichi Ihara, Lori Moffat, Meredith A. Borman, Jennifer E. Amon, 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, Moffat L, Borman MA, Amon JE, Walsh MP, MacDonald JA. \(\text{Ca}^{2+}\) -independent contraction of longitudinal ileal smooth muscle is potentiated by a zipper-interacting protein kinase pseudosubstrate peptide. Am J Physiol Gastrointest Liver Physiol 297: G361–G370, 2009. First published June 18, 2009; doi:10.1152/ajpgi.00112.2009.—As a regulator of smooth muscle contraction, zipper-interacting protein kinase (ZIPK) can directly phosphorylate the myosin regulatory light chains (LC\textsubscript{20}) and produce contractile force. Synthetic peptides (SM-I and AV25) derived from the autoinhibitory region of smooth muscle myosin light chain kinase can inhibit ZIPK activity in vitro. Paradoxically, treatment of Triton-skinned ileal smooth muscle strips with AV25, but not SM-I, potentiated \(\text{Ca}^{2+}\)-independent, microcystin- and ZIPK-induced contractions. The AV25-induced potentiation was limited to ileal and colonic smooth muscles and was not observed in rat caudal artery. Thus the potentiation of \(\text{Ca}^{2+}\)-independent contractions by AV25 appeared to be mediated by a mechanism unique to intestinal smooth muscle. AV25 treatment elicited increased phosphorylation of LC\textsubscript{20} (both Ser-19 and Thr-18) and myosin phosphatase-targeting subunit (MYPT1, inhibitory Thr-697 site), suggesting involvement of a \(\text{Ca}^{2+}\)-independent LC\textsubscript{20} kinase with coincident inhibition of myosin phosphatase. The phosphorylation of the inhibitor of myosin phosphatase, CPI-17, was not affected. The AV25-induced potentiation was abolished by pretreatment with staurosporine, a broad-specificity kinase inhibitor, but specific inhibitors of Rho-associated kinase (ROK), integrin-linked kinase (ILK), or zipper-interacting protein kinase (ZIPK) (17). As a contributor to \(\text{Ca}^{2+}\) sensitization in smooth muscle, ZIPK can mediate inhibition of MLCP, either directly by phosphorylation of the myosin phosphatase-targeting subunit of MLCP (MYPT1) (3, 20) or indirectly via phosphorylation of a PKC-potentiated phosphatase inhibitor protein-17 kDa (CPI-17) (21). In addition, ZIPK can directly phosphorylate LC\textsubscript{20} at both Ser-19 and Thr-18 in a \(\text{Ca}^{2+}\)-independent manner (3, 22).

Although ZIPK has been linked to the regulation of smooth muscle contraction, it is unclear whether its role in these processes is attributable solely to the direct phosphorylation of LC\textsubscript{20} or the inhibition of MLCP via phosphorylation of MYPT1. A specific inhibitor of ZIPK would be an important tool for the delineation of its physiological function in smooth muscle contraction. Unfortunately, small molecule inhibitors that are selective for ZIPK have yet to be developed. Small molecule inhibitors of ZIPK (e.g., ML-9, ML-7, staurosporine, and wortmannin) (2) are also active against a number of other protein kinases found in smooth muscle. The nonselectivity is presumed to be a consequence of the high degree of sequence and structural conservation within the ATP binding pockets of protein kinases (11). We previously hypothesized that a synthetic peptide modeled on the autoinhibitory domain of ZIPK would be an appropriate molecule for the selective inhibition of ZIPK (16). Our first approach was to consider a synthetic peptide derived from the autoinhibitory domain of smooth muscle MLCK, the so-called SM-I peptide (sequence: AKKLSDKMRMKKAMYRKKWQTKGT). The autoinhibitory region of smooth muscle MLCK has been well characterized (6, 26) and has significant similarity to sequence found in the putative autoinhibitory domain of ZIPK (16). We demonstrated that SM-I inhibits ZIPK activity in vitro; furthermore, SM-I blocked \(\text{Ca}^{2+}\)-independent contraction of Triton-skinned rat ileal smooth muscle strips induced by application of exogenous, recombinant ZIPK (16). During the course of these studies, we examined a related synthetic peptide inhibitor of MLCK, AV25 (sequence: AKKLSDKMKKYMARRKWQTKG). Interestingly, AV25 potentiated \(\text{Ca}^{2+}\)-independent, microcystin (phosphatase inhibitor)-induced contraction in rat ileal smooth muscle although our initial in vitro evidence suggested that AV25 functions as an inhibitor of ZIPK (30). This AV25-induced potentiation was limited to ileal and colonic smooth muscles and was not observed in rat caudal artery (29, 30).

Smooth Muscle Research Group and Dept. of Biochemistry and Molecular Biology, Univ. of Calgary, Faculty of Medicine, 3330 Hospital Drive N.W., Calgary, AB, T2N 4N1, Canada (e-mail: jmacdo@ucalgary.ca).

Address for reprint requests and other correspondence: J. A. MacDonald, Smooth Muscle Research Group and Dept. of Biochemistry and Molecular Biology, Univ. of Calgary, Faculty of Medicine, 3330 Hospital Drive N.W., Calgary, AB, T2N 4N1, Canada (e-mail: jmacdo@ucalgary.ca).

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Thus the potentiation of Ca$^{2+}$-independent contraction by AV25 appeared to be mediated by a mechanism unique to intestinal smooth muscle beds. The objective of the present study was to elucidate the biochemical basis for AV25-induced potentiation of rat ileal smooth muscle contraction. We postulated that the potentiating effect of AV25 was governed by unique signaling pathways that could selectively contribute to Ca$^{2+}$ sensitization of intestinal smooth muscle.

**MATERIALS AND METHODS**

**Materials.** All chemicals were reagent grade unless otherwise indicated. Triton X-100, β-escin, PD98059, SB203580, and GF109203x were obtained from Sigma (St. Louis, MO). A23187 was from Calbiochem (San Diego, CA). [γ-32P]-ATP and trifluoperazine were purchased from ICN Biomedical (Aurora, OH). Microcystin was obtained from Alexis Biochemical (San Diego, CA). AV25 peptide was produced by the University of Calgary Peptide Synthesis Facility (Calgary, AB, Canada), confirmed by amino acid analysis and shown to be >95% pure by analytical HPLC. AV25 corresponds to the autoinhibitory domain of smooth muscle MLCK (residues 783–807 of chicken gizzard MLCK) with three amino acid substitutions: Trp800 was replaced by Leu to attenuate calmodulin binding, and Ser787 and Thr803 were replaced by Ala to remove potential phosphorylation sites in the peptide. The cysAV25 peptide was identical to AV25 except for the presence of an NH2-terminal cysteine residue. Monoclonal antibody specific for MYPT1 phosphoThr-697 (anti-[phosphoThr-697]-MYPT1; rat numbering) was purified as previously described (30). Polyclonal MYPT1 antibody generated against the NH2-terminal fragment of rat MYPT1 (30) was a gift from Dr. Timothy Haystead (Duke University, Durham, NC) and was used to quantify total MYPT1 levels. Polyclonal antibodies specific for total CPI-17, phosphorylated CPI-17 (anti-[phosphoThr-38]-CPI-17), and ZIPK were purchased from Upstate (Charlottesville, VA). Monoclonal antibodies specific for ILK and myc-tag were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling (Beverly, MA), respectively. LC20 was purified from chicken gizzard (29).

**Generation and expression of recombinant ZIPK constructs.** Constitutively active glutathione S transferase (GST)-ZIPK$^{1-320}$ protein was produced as previously described (3). Kinase dead ZIPK$^{1-320}$D161A [henceforth called KD-ZIPK$^{1-320}$] protein was amplified by PCR to introduce a 5′ myc-tag using the following primers (which contain BamHI and NotI sites, respectively): forward primer 5′-GGCGGGATCCGACAAACAAACTTATTTCTGAAGAAGATCGATGTCACGGTCAGGAGAGC-′3′; and reverse primer 5′-GGCGGCGGCCGC-GCCTATTTGAGAAGCGATCGGACAGG-′3′. The PCR product was digested with BamHI and NotI and ligated into the corresponding sites in a pGEX-6P1 expression vector (GE Healthcare, Piscataway, NJ). This procedure encoded a myc-tag (MAEQLKISEEDL) immediately preceding the NH2-terminal sequence of ZIPK. Recombinant ZIPK GST-fusion proteins were expressed in Escherichia coli and purified with glutathione-Sepharose. The GST moiety was cleaved from the recombinant proteins using PreScission Protease (GE Healthcare Bio-Sciences) according to the manufacturer’s instructions.

**Tissue preparation and force measurement of longitudinal smooth muscle strips.** Ileum was removed from rats anaesthetized and euthanized, mono- and diphosphorylated forms of LC20 were performed as previously described (16, 29). Contractile responses were halted by immersion of ileal muscle strips in a dry-ice/acetone solution containing 10% (wt/vol) TCA. The muscle strips were washed with a 10 mM DTT/acetone solution and lyophilized overnight. Muscle proteins were extracted in a buffer containing 8 M urea, 1 M thiourea, 10 mM 150 NaCl, 4 KCl, 2 calcium methanesulfonate (CaMS2), 1 magnesium methanesulfonate (MgMS2), 5.5 glucose, and 5 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, pH 7.3. Muscle strips were permeabilized with 50 μM β-escin for 40 min or “skinned” with 0.1% Triton X-100 for 20 min in an intracellular solution (G10) containing 10 mM EGTA and no added Ca$^{2+}$. A23187 (10 μM) was added for the final 10 min to deplete intracellular Ca$^{2+}$ stores. Maximal contraction was obtained with pCa 4.5 solution before and after the protocol in all experiments. Free Ca$^{2+}$ levels (expressed as pCa) were obtained by mixing G10 and CaG solutions to achieve the desired Ca-GTA/EGTA ratio. The composition of CaG solution was (in mM) 30 potassium piperazine-1,4-bis(2-ethanesulfonic acid) (K2PIPES), 10 sodium creatine phosphate (Na2CP), 5.14 sodium adenosine 5′-triphosphate (Na2ATP), 7.25 MgMS2, 47.1 potassium methanesulfonate (KMS), and 10 K2CaEGTA. The force levels obtained with relaxing solution (pCa 9 or G10) and pCa 4.5 were designated as 0% and 100%, respectively. All contractile measurements were carried out at room temperature (23°C).

**Western blot analysis of MYPT1 and CPI-17 phosphorylation.** Rat ileal smooth muscle was dissected into squares (5 × 5 mm) and mounted in a silicone-bottom dish. The muscles were permeabilized and washed as described above. To test the phosphorylation of MYPT1, ileum was treated with vehicle or AV25 before to the application of microcystin. Tissue was flash frozen in liquid N2 and homogenized using a glass-to-glass, hand-operated homogenizer with 10 volumes of sample buffer containing 1% SDS, 30 mM Tris-HCl, pH 6.8, 12.5% (vol/vol) glycerol and 4-amidinophenyl methanesulfonyl fluoride. Homogenates were resolved on 12% SDS-polyacrylamide gels and transferred to PVDF membranes in 25 mM Tris-HCl, 192 mM glycine, 20% (vol/vol) methanol at 110 V for 60 min at 4°C. Nonspecific binding sites were blocked with 5% (wt/vol) nonfat dry milk in 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% (vol/vol) Tween-20 (TBST). The blots were washed and incubated for 1 h with primary antibody (1:1,000 dilution of total-MYPT1 antibodies and 1:500 dilution of phospho-MYPT1 antibody) in TBST supplemented with 1% (wt/vol) nonfat dry milk. The blots were washed and then incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000 dilution) in TBST with 1% (wt/vol) nonfat dry milk. Blots were developed with enhanced chemiluminescence (GE Healthcare Bio-Sciences). The bands were quantified by densitometry, and the relative phosphorylation levels of MYPT1 were expressed as a function of the total MYPT1 protein.

To examine the phosphorylation of CPI-17, aliquots of the homogenate were resolved on 18% SDS-polyacrylamide gels and transferred to PVDF membrane in 10 mM sodium cyclohexylaminopropane sulfonic acid (pH 11) and 10% (vol/vol) methanol at 50 mA for 4 h at 4°C. Nonspecific binding sites were blocked with 1:5-Block solution (Applied Biosystems, Foster City, CA) in TBST overnight at 4°C. Blots were incubated with primary antibody for total or phosphoThr38-CPI-17 (1:2,500 dilution) in 1% (wt/vol) nonfat dry milk in TBST for 1 h. The blots were then washed and incubated with HRP-conjugated secondary antibody (1:50,000 dilution) in 1% (wt/vol) nonfat dry milk in TBST for 1 h at 20°C. The membrane was developed with Supersignal West Femto enhanced chemiluminescence (Pierce Chemical, Rockford, IL). Bands were quantified by densitometry, and the relative phosphorylation levels of CPI-17 were calculated as a function of the total CPI-17 protein.

**Measurement of LC20 phosphorylation.** Urea/glycerol gel electrophoresis and Western blotting to separate and quantify unphosphorylated, mono- and diphosphorylated forms of LC20 were performed as previously described (16, 29). Contractile responses were halted by immersion of ileal muscle strips in a dry-ice/acetone solution containing 10% (wt/vol) TCA. The muscle strips were washed with a 10 mM DTT/acetone solution and lyophilized overnight. Muscle proteins were extracted in a buffer containing 8 M urea, 1 M thiourea, 10 M 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, pH 7.3. Muscle strips were permeabilized with 50 μM β-escin for 40 min or “skinned” with 0.1% Triton X-100 for 20 min in an intracellular solution (G10) containing 10 mM EGTA and no added Ca$^{2+}$. A23187 (10 μM) was added for the final 10 min to deplete intracellular Ca$^{2+}$ stores. Maximal contraction was obtained with pCa 4.5 solution before and after the protocol in all experiments. Free Ca$^{2+}$ levels (expressed as pCa) were obtained by mixing G10 and CaG solutions to achieve the desired Ca-GTA/EGTA ratio. The composition of CaG solution was (in mM) 30 potassium piperazine-1,4-bis(2-ethanesulfonic acid) (K2PIPES), 10 sodium creatine phosphate (Na2CP), 5.14 sodium adenosine 5′-triphosphate (Na2ATP), 7.25 MgMS2, 47.1 potassium methanesulfonate (KMS), and 10 K2CaEGTA, and the composition of G10 solution was (in mM) 30 K2PIPES, 10 Na2CP, 5.14 Na2ATP, 7.92 MgMS2, 46.6 KMS, and 10 K2CaEGTA. The force levels obtained with relaxing solution (pCa 9 or G10) and pCa 4.5 were designated as 0% and 100%, respectively. All contractile measurements were carried out at room temperature (23°C).
**RESULTS**

**Effects of AV25 on ZIPK activity in vitro.** We have previously demonstrated that a synthetic peptide (SM-1) derived from the autoinhibitory region of smooth muscle MLCK residues 783–804 of chicken gizzard MLCK) inhibits ZIPK activity (16). Furthermore, the related AV25 peptide inhibits LC20 phosphorylation by ZIPK in vitro with an IC50 value of 0.6 ± 0.05 μM (30). In this study, kinetic analysis (Supplemental Fig. S1, A and B; supplemental material for this article is available online at the American Journal of Physiology Gastrointestinal and Liver Physiology website) indicated that AV25, like SM-1, exhibited competitive inhibition kinetics with respect to LC20 substrate. A secondary plot of apparent K_m as a function of [AV25] produced a K_i value of 1.8 ± 0.4 μM (Supplemental Fig. S1B). This K_i value was 1.8-fold lower than that obtained for SM-1 (3.4 μM) (16). AV25, even at very high concentrations, had no effect on ILK activity; furthermore, AV25 demonstrated weak inhibition of ROK, with an apparent IC50 value >75 μM (data not shown). Additional experiments were carried out to confirm that AV25 interacts with full-length ZIPK. AV25-agarose removed full-length, myc-tagged ZIPK from HEK293 cell lysates (Supplemental Fig. S1C). Overall, these results indicate that AV25, like SM-1, is an effective in vitro inhibitor of ZIPK with absence of inhibitory potential against other Ca2+-sensitizing kinases (i.e., ROK and ILK) and that AV25 interacts with ZIPK.

**In situ effects of AV25 on Ca2+-independent, microcystin-induced contraction of rat ileal smooth muscle.** We examined the effect of AV25 on Ca2+-independent, microcystin-induced contraction. Application of microcystin, an inhibitor of protein phosphatases type-1 and -2A (PP1 and PP2A, respectively), unmasks basal Ca2+-independent protein kinase activity (29) and causes the development of sustained force in Triton-skinned ileal smooth muscle strips under Ca2+-free conditions (Fig. 1A). Treatment with AV25 potentiated the Ca2+-independent, microcystin-induced contraction (Fig. 1B). We previously reported that SM-1 exhibits nonspecific binding to calmodulin (16); however, AV25-induced potentiation of microcystin-induced force was unaffected following the removal of calmodulin by application of the calmodulin antagonist, trifluoperazine (31), at the plateau of Ca2+-induced contraction (Fig. 1, C and D).

Intestinal smooth muscle is associated with robust MLCP activity (8). Increases in microcystin concentration to levels...
greater than 10 μM do not elicit further increases in MLCP inactivation and force development (16). We observed similar levels of AV25 potentiation over a range of microcystin concentrations, 1–30 μM (Fig. 2), with maximal potentiation observed at 1 μM microcystin. The potentiation of contractile force induced by AV25 was significantly reduced at higher microcystin concentrations. This implies that the mechanism of AV25 potentiation involves Ca2+-independent LC20 kinase activity (i.e., direct phosphorylation of LC20) with coincident inhibition of MLCP activity. The Ca2+-independent LC20 kinase activity revealed by AV25 treatment is likely to be tightly associated with the contractile filaments because AV25-induced potentiation was retained following Triton skinning of the smooth muscle.

**Effects of AV25 on the phosphorylation of LC20, MYPT1, and CPI-17 during microcystin-induced contraction of ileal smooth muscle.** To further assess the mechanism underlying AV25 potentiation, we examined whether the AV25-induced potentiation in Ca2+-free conditions was associated with changes in the phosphorylation of LC20, MYPT1, and/or CPI-17. The application of 1 μM microcystin stimulated a weak contraction in Ca2+-free (G10) solution (Fig. 3A), and this small microcystin-induced contraction was dramatically increased with AV25 treatment. Indeed, under these conditions the potentiation by AV25 peptide was the most profound. Smooth muscle tissues were frozen 45 min after application of microcystin in the absence or presence of AV25 and subjected to Western blot analysis. LC20 monophosphorylation was significantly increased in the presence of AV25 (32.2 ± 1.6% vs. 23.1 ± 3.0%). LC20 diphosphorylation was also higher after microcystin administration in the presence of AV25 (4.6 ± 0.5% vs. 1.6 ± 0.3%; Fig. 3B). In addition, pretreatment with AV25 was associated with increased [Thr-697]-MYPT1 phosphorylation (Fig. 3C), suggesting that the AV25-induced potentiation was mediated at least in part by inhibition of MLCP through phosphorylation of MYPT1. However, [Thr-38]-CPI-17 phosphorylation was not affected by AV25 treatment (Fig. 3D).

**Effects of protein kinase inhibitors on AV25-induced potentiation of Ca2+-independent, microcystin-induced contraction of ileal smooth muscle.** The AV25-induced potentiation was abolished by pretreatment with the broad-specificity kinase inhibitor, staurosporine (Fig. 4), indicating that the potentiating effect of AV25 is protein kinase dependent. In our next series of experiments, therefore, we attempted to identify the protein kinase(s) responsible for the AV25-induced potentiation of Ca2+-independent, microcystin-induced contraction. ROK can induce Ca2+ sensitization in smooth muscle (12); however, pretreatment with Y27632, a selective inhibitor of ROK, had no effect on AV25 potentiation (Fig. 5A). We have recently linked PKC and MAPK pathways to Ca2+-independent, microcystin-induced contraction in rat ileal smooth muscle (18). AV25 potentiation was enhanced by pretreatment with broad-specificity PKC inhibitor, GF109203x (Fig. 5B); MEK inhibitor, PD98059 (Fig. 5C); and p38MAPK inhibitor, SB203580 (Fig. 5D). As summarized in Fig. 5E, the force observed 25 min after the application of microcystin was increased twofold in control (addition of AV25 in the absence of protein kinase inhibitors), sevenfold with GF109203x, 14-fold with PD98059, and ninefold with SB203580. The apparent increases in AV25-induced potentiation appear to result from inhibition of PKC and MAPK contributions to microcystin-induced contraction in the control experiments (i.e., absence of AV25 peptide). Thus it appears that the ROK, PKC, and MAPK pathways were not responsible for AV25-induced potentiation of Ca2+-independent, microcystin-induced contractile force in ileum.

**In situ effects of AV25 on Ca2+-independent, ZIPK-induced contraction of rat ileal smooth muscle.** We also examined the ability of AV25 to block smooth muscle contraction induced under Ca2+-free conditions by the addition of exogenous ZIPK. Application of constitutively active ZIPK(1–320) (5 μM) elicited Ca2+-independent contraction in Triton-skinned ileal smooth muscle strips (Fig. 6A). Pretreatment with AV25 failed to inhibit ZIPK-induced contraction, whereas SM-1 was previously shown to inhibit this Ca2+-independent, ZIPK-induced contraction (16). Surprisingly, the application of AV25 resulted in significant potentiation of the sustained tension in this tissue (Fig. 6, B and C). The force (39.8 ± 4.4%, n = 6) obtained 30 min after application of ZIPK in the presence of AV25 was significantly greater than the force (27.5 ± 3.9%, n = 6) observed in the absence of the peptide. Furthermore, the potentiation of ZIPK-induced contraction by AV25 was accompanied by increased LC20 phosphorylation (Fig. 6D). The amount of diphosphorylated LC20 was significantly increased from 22.4 ± 2.0% to 38.9 ± 3.9% of total LC20 (Fig. 6E). A corresponding decrease in the amount of unphosphorylated LC20 was also observed. The following LC20 phosphorylation stoichiometries were calculated: 0.65 ± 0.03 mol Pi/mol LC20 (exogenous ZIPK addition) and 0.98 ± 0.06 mol Pi/mol LC20 (exogenous ZIPK and AV25 addition).

**Effects of SM-1 on AV25-induced potentiation of Ca2+-independent, microcystin-induced contraction of ileal smooth muscle.** To examine whether ZIPK itself was involved in the AV25-dependent potentiation of ileal smooth muscle contraction, we carried out experiments using the SM-1 peptide. SM-1 has been previously used in permeabilized ileal smooth muscle as an inhibitor of ZIPK (16). Interestingly, pretreatment with SM-1 (50 μM) abolished AV25-induced potentiation (Fig. 7) although SM-1 alone did not have any effect on Ca2+-inde-
pendent, microcystin-induced contraction. These results suggest that, even though AV25 was an in vitro inhibitor of ZIPK, activation of the ZIPK pathway is apparent following application of AV25 to ileal smooth muscle.

Effects of KD-ZIPK(1–320) on AV25-induced potentiation of Ca\(^{2+}\)-independent, microcystin-induced contraction of ileal smooth muscle.

To further examine the paradoxical role of ZIPK in the response to AV25, a dominant-negative form of ZIPK [i.e., KD-ZIPK(1–320)] was added to Triton-skinned ileal smooth muscle strips to test whether the AV25-dependent potentiation of microcystin-induced contraction could be blocked. A significant amount of myc-tagged, KD-ZIPK(1–320) was retained in the skinned strips. After incubation, densitometric analysis of Western blots indicated that KD-ZIPK(1–320) levels rose to 2.7-\(\pm\)0.2-fold greater than endogenous ZIPK levels (Fig. 8A). Furthermore, we examined whether endogenous levels of ZIPK or ILK were affected by treatment of Triton-skinned ileal strips with AV25 or KD-ZIPK(1–320). Endogenous ZIPK levels were unaltered by either treatment, and endogenous ILK levels were unaffected by AV25 application. However, application of KD-ZIPK(1–320) did result in a 55% reduction in the amount of endogenous ILK retained in the permeabilized ileal strip. When we examined the microcystin-induced contractile responses of KD-ZIPK(1–320)-treated ileal strips, the AV25-induced potentiation was abolished (Fig. 8B), suggesting that the potentiating effect of AV25 is dependent on signaling through ZIPK. It is possible that the pool of exogenous KD-ZIPK(1–320) acts as a molecular sponge to reduce AV25 availability in the muscle strip. This postulate was also supported by our earlier finding that AV25 could augment Ca\(^{2+}\)-independent contraction induced by application of exogenous, constitutively active ZIPK(1–320).

DISCUSSION

Smooth muscle is clearly defined as a distinct muscle group, separate from cardiac and skeletal muscle (28). The contraction of smooth muscle is regulated by autonomic neural innervation as well as hormones, autocrine/paracrine agents, and other local chemical signals. All smooth muscle beds rely on Ca\(^{2+}\)-dependent activation of MLCK and LC\(_{20}\) phosphorylation to initiate crossbridge cycling between actin and myosin fila-
ments (7, 12, 24). Nonetheless, different smooth muscle beds exhibit striking heterogeneity in the functional regulation of this contractile process. Smooth muscle can be broadly classified into slowly contracting tonic (vascular) and relatively fast-contracting phasic (visceral) types (25). In response to depolarization, tonic smooth muscles exhibit slow, sustained contractions, whereas phasic smooth muscles generate more rapid, transient contractions (25). Both the contractile machinery (1, 19) and the transduction of signals (13) that regulate the contractile process are believed to underlie the different contractile properties of tonic and phasic smooth muscles.

The AV25 peptide was originally developed as an inhibitor of MLCK and has been used in smooth muscle physiology to explore both Ca\(^{2+}\)/calmodulin-dependent activation of MLCK and Ca\(^{2+}\)-independent, microcystin-induced contraction by AV25. Force was measured in the absence and presence of microcystin with (hatched bars) or without (open bars) AV25. The amount of AV25-dependent potentiation is also displayed (solid bars). Error bars indicate means \(\pm\) SE \((n = 5)\). *Significantly different (Student’s t-test, \(P < 0.05\)); n.s., not significantly different. Forces observed at pCa 4.5 and pCa 9.0 were designated as 100% and 0%, respectively.

**Fig. 4.** Effect of staurosporine on AV25-induced potentiation of microcystin-induced contraction of rat ileal smooth muscle. A: broad-spectrum kinase inhibitor staurosporine (3 \(\mu\)M) blocked both microcystin-induced contraction and AV25 potentiation of contraction of \(\beta\)-escin-permeabilized ileal smooth muscle. Cumulative results \((B)\) are presented for the effects of staurosporine on the potentiation of Ca\(^{2+}\)-independent, microcystin-induced contraction by AV25. Force was measured in the absence and presence of staurosporine 25 and 45 min after application of 1 \(\mu\)M microcystin with (hatched bars) or without (open bars) AV25. The amount of AV25-dependent potentiation is also displayed (solid bars). Error bars indicate means \(\pm\) SE \((n = 5)\). *Significantly different (Student’s t-test, \(P < 0.05\)); n.s., not significantly different. Forces observed at pCa 4.5 and pCa 9.0 were designated as 100% and 0%, respectively.

**Fig. 5.** Effect of Rho-associated kinase (ROK), PKC, and MAPK inhibitors on AV25-induced potentiation of microcystin-induced contraction of rat ileal smooth muscle. The AV25 potentiation of microcystin-induced contraction of \(\beta\)-escin-permeabilized ileal smooth muscle was unaffected by addition of the ROK inhibitor Y27632 (10 \(\mu\)M, \(A\)); AV25-induced potentiation was increased by addition of the PKC inhibitor GF109203x (100 nM, \(B\)), the MEK inhibitor PD98059 (10 \(\mu\)M, \(C\)), or the p38 MAPK inhibitor SB203580 (10 \(\mu\)M, \(D\)). Cumulative results \((E)\) are presented for the effects of kinase inhibitors on the potentiation of microcystin-induced contraction by AV25. Force was measured in the presence of the indicated inhibitors 25 min after application of 1 \(\mu\)M microcystin without (open bars) or with (hatched bars) AV25. The amount of AV25-dependent potentiation is also displayed (solid bars). Error bars indicate means \(\pm\) SE \((n = 5)\). *Significantly different from the force generated in the absence of AV25 (Student’s t-test, \(P < 0.05\)). #Significantly different from the AV25-dependent potentiation observed in the absence of kinase inhibitors (Student’s t-test, \(P < 0.05\)). Forces observed at pCa 4.5 and pCa 9.0 were designated as 100% and 0%, respectively.
These modifications did not significantly alter the in vitro inhibitory potency of AV25 toward smooth muscle MLCK (29) or ZIPK (Supplemental Fig. S1A, Ref. 30), disparate results were obtained when ileal smooth muscle contraction was monitored in the presence of the two peptides. SM-1 blocked Ca^{2+}-independent contraction of Triton-skinned rat ileal smooth muscle strips induced by application of either exogenous or recombinant ZIPK or microcystin. The application of AV25 peptide caused potentiation of contraction in rat ileal (this study) and colonic (data not presented) smooth muscles, and, interestingly, this AV25-induced potentiation was not observed in rat caudal artery (29, 30).

We have identified increased LC_{20} mono- and diphosphorylation as well as enhanced [Thr-697]-MYPT1 phosphorylation, although in vitro enzymatic studies have defined AV25 to be an inhibitor of ZIPK (Supplemental Fig. S1A and Ref. 30), experiments presented in this study illustrate that the mechanism of this contractile potentiation in situ was most likely through a paradoxical activation of ZIPK, leading to increased LC_{20} mono- and diphosphorylation as well as enhanced [Thr-697]-MYPT1 phosphorylation but not [Thr-38]-CPI-17 phosphorylation.

The SM-1 and AV25 peptides have been used extensively in smooth muscle under in situ conditions to inhibit MLCK. The peptides are modeled on the pseudosubstrate, autoinhibitory region of chicken gizzard MLCK (29). AV25 is three amino acids longer than SM-1 at the COOH terminus, corresponding to residues 783–807 of chicken gizzard MLCK, with three substitutions: Trp-800 replaced by Leu to attenuate interactions to residues 783–807 of chicken gizzard MLCK, with three substitutions; Trp-800 replaced by Leu to attenuate interactions, with Ser-787 and Thr-803 replaced by Ala to avoid peptide phosphorylation. Although these modifications did not significantly alter the in vitro inhibitory potency of AV25 toward smooth muscle MLCK (29) or ZIPK (Supplemental Fig. S1A), disparate results were obtained when ileal smooth muscle contraction was monitored in the presence of the two peptides. SM-1 blocked Ca^{2+}-independent contraction of Triton-skinned rat ileal smooth muscle strips isolated from rat ileum were treated with ZIPK (5 μM) in Ca^{2+}-free solution (G10). B: magnitude of ZIPK(1–320)-induced steady-state contraction was increased following the addition of AV25. C: cumulative results are representative of 6 independent experiments. Error bars indicate means ± SE. *Significantly different (Student’s t-test, P < 0.05); n.s., not significantly different. D: Triton-skinned muscle strips were quick frozen following treatment with G10, ZIPK(1–320), or ZIPK(1–320) plus AV25 (100 nM). Phosphorylated and unphosphorylated LC_{20} were separated by urea/glycerol gel electrophoresis and detected by Western blotting with anti-LC_{20}. E: LC_{20} blots were quantified by scanning densitometry. Different exposure times were used for the quantification to ensure that signals lie within the linear range of the relationship between protein amount and signal intensity in each case. The data are expressed as percentages of total LC_{20} for unphosphorylated (P0-LC_{20}; open bars), monophosphorylated (P1-LC_{20}; hatched bars), and diphosphorylated (P2-LC_{20}; solid bars) bands. Significant differences are indicated (P < 0.05) between control (G10 + ZIPK) and AV25-treated muscle. Phosphorylation stoichiometries are presented in parentheses above the data bars.

![Fig. 6. AV25 potentiates Ca^{2+}-independent, zipper-interacting protein kinase (ZIPK)-induced contraction and LC_{20} phosphorylation in Triton-skinned rat ileal smooth muscle.](https://example.com/fig6)

**A** Triton-skinned, longitudinal smooth muscle strips isolated from rat ileum were treated with ZIPK (5 μM) in Ca^{2+}-free solution (G10). **B**: magnitude of ZIPK(1–320)-induced steady-state contraction was increased following the addition of AV25. **C**: cumulative results are representative of 6 independent experiments. Error bars indicate means ± SE. *Significantly different (Student’s t-test, P < 0.05); n.s., not significantly different. **D**: Triton-skinned muscle strips were quick frozen following treatment with G10, ZIPK(1–320), or ZIPK(1–320) plus AV25 (100 μM). Phosphorylated and unphosphorylated LC_{20} were separated by urea/glycerol gel electrophoresis and detected by Western blotting with anti-LC_{20}. E: LC_{20} blots were quantified by scanning densitometry. Different exposure times were used for the quantification to ensure that signals lie within the linear range of the relationship between protein amount and signal intensity in each case. The data are expressed as percentages of total LC_{20} for unphosphorylated (P0-LC_{20}; open bars), monophosphorylated (P1-LC_{20}; hatched bars), and diphosphorylated (P2-LC_{20}; solid bars) bands. Significant differences are indicated (P < 0.05) between control (G10 + ZIPK) and AV25-treated muscle. Phosphorylation stoichiometries are presented in parentheses above the data bars.

**Fig. 7. Effect of SM-1 on AV25-induced potentiation of Ca^{2+}-independent contraction. AV25 potentiation of Ca^{2+}-independent contraction of β-escin-permeabilized rat ileal smooth muscle strips induced by microcystin was blocked by pretreatment of the tissue with 50 μM SM-1. Forces observed at pCa 4.5 and pCa 9.0 were designated as 100% and 0%, respectively. A representative trace of n = 5 independent experiments is shown.****
potentiation. ERK and p38MAPK pathways do not contribute to Ca^{2+}-independent, microcystin-induced contraction in vascular tissues (30), so it was reasonable for us to predict that one of these protein kinase modules was involved in the AV25-induced contractile potentiation in rat ileum. Unexpectedly, neither of these protein kinases was involved, and therefore, ILK and/or ZIPK itself are the most likely contributors to the AV25-induced potentiation.

ZIPK and ILK levels are higher in phasic (ileum and colon) compared with tonic smooth muscle (aorta, caudal, and femoral arteries), determined with the use of Western blotting analysis (data not shown). Different protein levels of the kinases involved in regulation of vascular smooth muscle contractility may also contribute to the unique contractile properties of the two smooth muscle types. Higher ZIPK and ILK levels may suggest a more critical role for these kinases in phasic (ileum and colon) smooth muscle regulation. Furthermore, the levels of ZIPK were unchanged in ileum following Triton X-100 skinning, suggesting that the majority, if not all, of this protein is tightly bound to the contractile apparatus.

Unfortunately, specific inhibitors for ZIPK or ILK are not available. However, the use of dominant-negative proteins to elucidate protein function has proved to be successful in the past. This method has provided insights into the molecular mechanisms of action of a number of signaling protein families, including hormone and growth factor receptors, protein kinases, and protein phosphatases. Therefore, to address the issue of the involvement of ILK or ZIPK in the AV25-dependent potentiation of microcystin-induced contraction, we developed a KD ZIPK variant. When this dominant-negative ZIPK protein was added to Triton-skinned ileal smooth muscle, the potentiation of microcystin-induced contraction by AV25 was blocked. Although earlier experiments were completed in β-escin-permeabilized tissue, it was considered necessary to utilize Triton X-100-skinned tissues to obtain efficient ZIPK replacement. However, as shown in Figs. 7 and 8, the contractile responses elicited by addition of microcystin were unchanged regardless of β-escin or Triton X-100 treatment, respectively. These results suggest that the signaling architecture of the tissue was not altered by either β-escin permeabilization or Triton-skinning to affect AV25 potentiation.

Pretreatment of rat ileum with SM-1 abolished AV25-induced potentiation. Because SM-1 was previously demonstrated to attenuate ZIPK signaling in ileal smooth muscle (16) and has no inhibitory effect on ILK (30), this result further supports ZIPK dependency of the potentiating actions of AV25 peptide. In addition, previous reports have demonstrated that phosphoinositide 3-kinase (PI3K) can regulate ILK-dependent Ca^{2+} sensitization in smooth muscle (14). We examined whether inhibition of PI3K (LY294002, 10 μM) had any effect on microcystin-induced contraction and AV25-dependent potentiation. Our findings (Supplemental Fig. S2) rule out any involvement of the PI3K/ILK nexus in the contractile responses to AV25. We conclude, therefore, that ZIPK provides the dominant pathway for AV25-induced contraction of intestinal smooth muscle.

It is feasible that the observed AV25 effects are mediated through an unknown inhibitor of ZIPK. Blocking the inhibition of ZIPK would increase its activity and lead to the observed contractile effects. This is most likely not the case because treatment with the KD-ZIPK(1–320) suggests a direct mechanism. Therefore, we speculate that AV25 is binding directly to endogenous ZIPK and initiating a conformational change that increases the activity of ZIPK. This phenomenon would most likely be mediated through a mechanism in which the autoinhibitory interaction of the pseudosubstrate region with the catalytic domain of ZIPK is attenuated. The differences in AV25 effects on ZIPK seen in vitro compared with in situ could be explained by structural perturbations induced upon binding of ZIPK to the MYPT1 subunit of MLCP. The association of ZIPK with MYPT1 has been previously reported (5, 20); however, the specific amino acid residues of MYPT1 required for ZIPK binding remain to be identified. One could predict that an interaction between ZIPK and MYPT1 could alter the actions of AV25. For example, it is possible that residues of ZIPK required for AV25 binding are inaccessible upon association with MYPT1, and therefore, the interaction of the peptide with ZIPK may be directed to alternative sites. Closer examination of the region of ZIPK known to act in an autoinhibitory manner (9, 11) reveals a number of putative pseudosubstrate motifs (Supplemental Fig. S3A). Our mutational examination of these putative pseudosubstrate sequences has identified some variability in their contribution to ZIPK.
activity. For example, replacement of basic residues with Ala in four putative pseudosubstrate sequences leads to inhibition of ZIPK (Supplemental Fig. S3B). This result suggests that these pseudosubstrate motifs are not all inhibitory in nature, and slight modifications to the ZIPK structure induced by AV25 binding could augment kinase activity. Future experiments will explore the putative mechanism of AV25-induced activation of ZIPK. For example, pretreatment of ileal smooth muscle with antibodies raised against the hypothesized AV25 binding site of ZIPK would be predicted to prevent AV25-induced activation of ZIPK, confirming both the binding site of AV25 within ZIPK as well as its mechanism of action.

ZIPK has been shown to phosphorylate both MYPT1 (3, 20) and LC20 (3, 22) in vitro; however, the validation of ZIPK binding site of ZIPK would be predicted to prevent AV25-induced activation of myosin phosphatase-associated kinase induces Ca2+ sensitization via myosin phosphatase inhibition. J Biol Chem 277: 23441–23446, 2002.


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