A Small Molecule Pyrazolo[3,4-d]Pyrimidinone Inhibitor of Zipper-Interacting Protein Kinase Suppresses Calcium Sensitization of Vascular Smooth Muscle

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ABSTRACT

A novel inhibitor of zipper-interacting protein kinase (ZIPK) was used to examine the involvement of ZIPK in the regulation of smooth muscle contraction. Pretreatment of de-endothelialized rat caudal arterial smooth muscle strips with the pyrazolo[3,4-d]pyrimidinone inhibitor 2-((1-(3-chlorophenyl)-4-oxo-4,5-dihydro-1H-pyrazolo [3,4-d]-pyrimidin-6-yl)thio)propanamide (HS38) decreased the velocity of contraction (time to reach half-maximal force) induced by the ph osphatase inhibitor calyculin A in the presence of Ca^{2+} without affecting maximal force development. This effect was reversed following washout of HS38 and correlated with a reduction in the rate of phosphorylation of myosin 20-kDa regulatory light chains (LC20) but not of protein kinase C-potentiated inhibitory protein for myosin phosphatase of 17 kDa (CPI-17), prostate apoptosis response-4, or myosin phosphatase-targeting subunit 1 (MYPT1), all of which have been implicated in the regulation of vascular contractility. A structural analog of HS38, with inhibitory activity toward proviral integrations of Moloney (PIM) virus 3 kinase but not ZIPK, had no effect on calyculin A-induced contraction or protein phosphorylations. We conclude that a pool of constitutively active ZIPK is involved in regulation of vascular smooth muscle contraction through direct phosphorylation of LC20 upon inhibition of myosin light chain phosphatase activity. HS38 also significantly attenuated both phasic and tonic contractile responses elicited by phenylephrine, angiotensin II, endothelin-1, U46619, and K+–induced membrane depolarization in the presence of Ca^{2+}, which correlated with inhibition of phosphorylation of LC20, MYPT1, and CPI-17. These effects of HS38 suggest that ZIPK also lies downstream from G protein–coupled receptors that signal through both Ga_{12/13} and Ga_{q/11}.

Introduction

The coordinated regulation of tone via protein kinases is a key functional property of vascular smooth muscle (VSM), and it is not surprising that a variety of signal transduction mechanisms can regulate VSM tone (Hirano, 2007; Kim et al., 2008; Wynne et al., 2009). Of central importance to VSM tone generation is the relationship between cytosolic Ca^{2+} concentrations ([Ca^{2+}]_i) and force generation. Greater force can be obtained in the absence of a change in [Ca^{2+}], through a Ca^{2+} sensitization process. Since blood flow is proportional to the fourth power of the vessel radius (Sutera and Skalak, 1993), fine control of VSM tone is essential for maintenance of normal cardiovascular function. This is achieved primarily via signaling pathways that lead to Ca^{2+} sensitization of the contractile response. Furthermore, alterations in the sensitivity of VSM cells to [Ca^{2+}], have been hypothesized to underlie many cardiovascular diseases. For example, increased VSM contractility contributes to the severe arterial narrowing observed in vasospasm, chronic hypertension, and the decreased dilatory responses of arteries in obesity, diabetes, and aging (Katsumata et al., 1997; Uehata et al., 1997; Masumoto et al., 2002; Georgescu et al., 2011; Takeya et al., 2014).

ABBREVIATIONS: Ang II, angiotensin II; CLa, calycin A; CPI-17, protein kinase C-potentiated inhibitory protein for myosin phosphatase of 17 kDa; DAPK, death-associated protein kinase; ET-1, endothelin-1; GEF, guanine nucleotide exchange factor; GPCR, G protein-coupled receptor; HS38, 2-((1-(3-chlorophenyl)-4-oxo-4,5-dihydro-1H-pyrazolo [3,4-d]-pyrimidin-6-yl)thio)propanamide; HS43, 1-(3-chlorophenyl)-6-((2-hydroxyethyl)thio)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one; LC20, myosin 20-kDa regulatory light chain; LZ, leucine zipper; MLCP, myosin light chain phosphatase; MYPT1, myosin phosphatase-targeting subunit 1; Par-4, prostate apoptosis response-4; PE, phenylephrine; PIM, proviral integrations of Moloney virus; PKC, protein kinase C; ROCK, Rho-associated coiled-coil-containing protein kinase; VSM, vascular smooth muscle; ZIPK, zipper-interacting protein kinase.
The biochemical systems that govern Ca\textsuperscript{2+} sensitization in VSM are complex and multifactorial; however, zipper-interacting protein kinase (ZIPK) has emerged as one of the most important. ZIPK has been linked to the regulation of diverse cellular processes, including cell motility, smooth muscle contraction, and programmed cell death (reviewed in Haystead, 2005; Ihara and MacDonald, 2007; Shiloh et al., 2014; Usui et al., 2014). ZIPK possesses an N-terminal kinase domain, central autoinhibitory region, nuclear localization sequence, and C-terminal leucine zipper (LZ) motif. ZIPK is a member of a larger family of protein kinases known as the death-associated protein kinases (DAPKs), and is also known as DAPK3. The kinase domain is most closely related to other DAPKs but also shares significant sequence and structural conservation with calmodulin-dependent kinase family members such as myosin light chain kinase (MLCK) (Ihara et al., 2007; Moffat et al., 2011; Shiloh et al., 2014). In contrast to MLCK and the other DAPKs, ZIPK does not have a calmodulin-binding domain, and its activity is regulated independently of Ca\textsuperscript{2+}.

ZIPK appears to be a key regulator of VSM contractility, and the kinase is implicated in diphosphorylation of the myosin 20-kDa regulatory light chain (LC\textsubscript{20}) subunits of myosin II at Ser\textsuperscript{19} and Thr\textsuperscript{18} (Murata-Hori et al., 1999; Niro and Ikebe, 2001; Borman et al., 2002; Moffat et al., 2011; Carlson et al., 2013); phosphorylation of the myosin phosphate-targeting subunit 1 (MYPT1) of myosin light chain phosphatase (MLCP) at the inhibitory sites, Thr\textsuperscript{697} and Thr\textsuperscript{855} (Endo et al., 2004; MacDonald et al., 2001a); and phosphorylation of the protein kinase C (PKC)–potentiated inhibitory protein for myosin phosphatase of 17 kDa (CPI-17) at Thr\textsuperscript{38} (MacDonald et al., 2001b). Ultimately, these targets are thought to be the primary mediators of ZIPK effects on VSM contraction; however, several novel ZIPK regulators have been revealed in recent years. Notably, the prostate apoptosis response-4 (Par-4) protein is suggested to facilitate VSM contraction by acting as a cytoskeletal scaffold for ZIPK (Vetterkind and Morgan, 2009; Vetterkind et al., 2010). While the upstream activating pathways for ZIPK have yet to be resolved, evidence suggests that the kinase can be activated in VSM in response to external stimuli (MacDonald et al., 2001a; Graves et al., 2005; Hagerty et al., 2007), and ZIPK can be phosphorylated by Rho-associated coiled-coil-containing protein kinase (ROCK) (Hagerty et al., 2007).

Pharmacological agents can provide powerful insight into signaling mechanisms if used appropriately. Four independent groups have recently reported the development of novel ZIPK inhibitory compounds: 1) Velentza et al. (2003) reported the first small molecule DAPK inhibitor, an alkyllated 3-amino-6-phenylpyridazine; 2) Okamoto et al. (2009, 2010) used structure-based, virtual screening to identify DAPK1 and ZIPK inhibitors with a 2-phenyl-4-(3-pyridinylmethylene)-5-(4H)-oxazolone core; 3) Huber et al. (2012) identified inhibitory oxo-β-carboline compounds that do not rely on canonical ATP competition; and 4) Carlson et al. (2013) used a chemoproteomic fluorescence linked enzyme chemoproteomic strategy screening to identify an argl-thiopyrazolo[3,4-d]pyrimidinone—i.e., 2-[1-(3-chlorophenyl)-4-oxo-4,5-dihydro-1H-pyrazolo [3,4-d]-pyrimidin-6-yl]thio)propanamide (HS38)—that competitively inhibits ZIPK. HS38 displays similar or greater potency for ZIPK and has fewer off-target liabilities than other published ZIPK inhibitors (Carlson et al., 2013). The availability of a cell-permeable, potent, and selective inhibitor provides a novel opportunity to examine aspects of ZIPK signaling in VSM that were previously not attainable, and we have used HS38 to study the mechanistic role of ZIPK in Ca\textsuperscript{2+} sensitization of VSM.

### Materials and Methods

**Materials.** HS38 and 1-(3-chlorophenyl)-6-(2-hydroxyethyl)thio)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (HS43) were synthesized in the Haystead Laboratory at Duke University as previously described (Carlson et al., 2013). Only trace impurities were identified (<1% by liquid chromatography coupled to mass spectrometry). Calyculin A (CLA), angiotensin II (AngII), endothelin-1 (ET-1), and U46619 were purchased from EMD Millipore (Billerica, MA) and phenylephrine (PE) was obtained from Sigma Chemical Co. (St. Louis, MO). The Supersignal West Fermo Chemiluminescence Kit was purchased from GE Healthcare (Piscataway, NJ). Antibodies specific for CPI-17 and Par-4 were purchased from EMD Millipore. Phospho-specific antibodies for MYPT1 phosphorylated at Thr\textsuperscript{697} or Thr\textsuperscript{855} were purchased from EMD Millipore. Phos-Tag-acrylamide was purchased from NARD Chemicals, Inc. (Kobe City, Japan). Purified ROCK2 was purchased from SignalChem (Richmond, British Columbia, Canada). LC\textsubscript{20} (Hathaway and Haebeler, 1983) and MLCK (Nghi et al., 1994) proteins were purified from chicken gizzard as previously described. All other chemicals were reagent grade and were obtained from Sigma Chemical Co. or VWR (Mississauga, Ontario, Canada).

**In Vitro Kinase Assays.** ROCK2 and MLCK activities were assayed at 25°C with LC\textsubscript{20} protein substrate under standard conditions (25 mM HEPES, pH 7.4, 1 mM Mg\textsubscript{Cl\texttwo}, 0.2 mM ATP, and 2 µCi of [γ\textsuperscript{32P}]ATP). Reactions were initiated by the addition of ATP and terminated by spotting the mixture onto phosphocellulose P81 paper. After extensive washing with 20 mM H\textsubscript{3}PO\textsubscript{4}, 32P incorporation was determined by scintillation counting.

**Tissue Preparation and Force Measurements.** Caudal arteries were removed from male Sprague-Dawley rats (~300 g) that had been anesthetized and euthanized according to protocols approved by the University of Calgary Animal Care and Use Committee. The arteries were cleaned of excess adventitia, denuded of endothelium, and cut into helical strips (1.5 mm × 6 mm). Muscle strips were mounted on a Grass isometric force transducer (FT03C) and force was recorded as previously described (Moffat et al., 2011). Intact tissues were treated with CLA (0.5 µM) in HEPES-buffered Tyrode’s solution containing 137 mM NaCl, 2.7 mM KCl, 1 mM Mg\textsubscript{Cl\texttwo}, 1.8 mM Ca\textsubscript{Cl\texttwo}, 5.6 mM glucose, and 10 mM HEPES, pH 7.4. Isolated smooth muscle strips, preincubated with HS38 or vehicle (dimethylsulfoxide), were also stimulated with the following contractile agonists in HEPES-buffered Tyrode’s solution: the thromboxane A\textsubscript{2} mimetic U46619, ET-1, AngII, PE, or KCl (87 mM KCl). After extensive washing with 20 mM H\textsubscript{3}PO\textsubscript{4}, 32P incorporation was determined by scintillation counting.

**Analysis of LC\textsubscript{20} MYPT1, Par-4, and CPI-17 Phosphorylation.** Protein was extracted from each lyophilized tissue in 1 ml of SDS-gel sample buffer with constant shaking for 16 hours at 4°C. Tissue proteins were resolved by Phos-Tag SDS-PAGE and western blotting for the measurement of LC\textsubscript{20}, Par-4 and CPI-17 phosphorylation (Takeya et al., 2008; Moffat et al., 2011; Ihara et al., 2015), and by SDS-PAGE and western blotting with phosphospecific antibodies for MYPT1 phosphorylation (Grassie et al., 2012; Mills et al., 2015). For CPI-17, 12.5% acrylamide and 30 µM Phos-Tag reagent were used, and proteins were transferred to polyvinylidene difluoride (Roche Life Science, Quebec, Canada) overnight at 25 V and 4°C in 10 mM CAPS, pH 11, 10% methanol. For LC\textsubscript{20}, 12.5% acrylamide and 50 µM Phos-Tag reagent were used, and proteins were transferred to
polyvinylidene difluoride overnight at 27 V and 4°C in 25 mM Tris-HCl, pH 7.5, 192 mM glycine, 10% (v/v) methanol. Following transfer, proteins were fixed on the membrane by incubation for 20 minutes with 0.5% glutaraldehyde in phosphate-buffered saline and non-specific binding sites were blocked with 5% (w/v) nonfat dry milk (for LC20) or with 2% enhanced chemiluminescence blocking reagent (for CPI-17) in Tris-buffered saline/Tween 20 for 1 hour at room temperature. Membranes were incubated overnight with anti-LC20 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:500 dilution in 1% (w/v) nonfat dry milk. Membranes were then incubated for 1 hour with

Fig. 1. The effects of HS38 on the activities of Rho-associated coiled coil-containing kinase and MLCK, and the rate of force development and maximal tension elicited by the phosphatase inhibitor CLa. (A and B) Effects of increasing concentrations of HS38 on the activities of ROCK2 and MLCK, respectively. (C and D) Representative contractile responses of de-endothelialized rat caudal arterial smooth muscle strips to CLa (0.5 μM) in the presence of vehicle (dimethylsulfoxide) or HS38 (50 μM), respectively. (E) The time required to reach half-maximal contraction (t1/2) after application of CLa in the presence of increasing concentrations of HS38 was calculated and normalized to the value in the absence of HS38. #, Significantly different from the value in the absence of HS38 (analysis of variance, Dunnett’s post hoc test, P < 0.05). (F) The maximal contractile force developed during CLa exposure (0.5 μM for 60 minutes) at different concentrations of HS38 was expressed as a percentage of the initial reference contraction to 87 mM KCl. Values in (E and F) indicate the mean ± S.E.M. (n = 4–7).
horseradish peroxidase–conjugated secondary antibody (Chemicon, Temecula, CA) at 1:10,000 dilution or for 2 hours with anti-CPI-17 (EMD Millipore) at 1:5000 dilution, and then for 1 hour with biotin-labeled secondary antibody (EMD Millipore) at 1:10,000 dilution, followed by streptavidin-horseradish peroxidase (Pierce Chemical Co., Rockford, IL) at 1:5000 dilution for 30 minutes. For Par-4, 7.5% acrylamide and 60 μM Phos-Tag reagent were used, proteins were transferred to 0.2-μm nitrocellulose membrane overnight at 28 V and 4°C in 25 mM Tris-HCl, pH 7.5, 192 mM glycine, 10% (v/v) methanol, blocked with 5% skim milk in Tris-buffered saline/Tween 20 for 1 hour at room temperature, and incubated with anti-Par-4 (Abcam, Toronto, Ontario, Canada) at 1:5000 dilution for 2 hours at room temperature, followed by anti-rabbit IgG (EMD Millipore) at 1:5000 dilution for 1 hour at room temperature. For analysis of MYPT1 phosphorylation, tissue homogenates were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with anti-[phospho-Thr855]-MYPT1 (EMD Millipore) at 1:3000 dilution, and [phospho-Thr697]-MYPT1 (EMD Millipore) at 1:3500 dilution or anti-β-actin (Abcam; Toronto, Canada). Monoclonal anti-Par-4 antibody was used as a loading control with antibodies from Cytoskeleton Inc. (Denver, CO; 1:5000 dilution). All western blots were visualized with West Femto enhanced chemiluminescence reagent using a LAS4000 Imaging Station (GE Healthcare), ensuring that the representative signal occurred in the linear range. Quantification was performed by densitometry with ImageQuant TL software (GE Healthcare). LC20 phosphorylation stoichiometry was calculated as follows: mol signal occurred in the linear range. Quantification was performed by densitometry with ImageQuant TL software (GE Healthcare). 

Data Analysis. Values are presented as the mean ± S.E.M., with n indicating the number of animals (tissue experiments). Data were analyzed by Student's t test (two-tailed). For comparison of multiple groups, significance was determined by two-way analysis of variance with Dunnett's post hoc test. Differences were considered to be statistically significant when P < 0.05.

Results

The pyrazolo[3,4-d]pyrimidinone derivative HS38 was previously characterized as a potent inhibitor of ZIPK (Carlson et al., 2013). HS38 acts as a competitive inhibitor with respect to ATP and was reported to be most potent toward ZIPK (Kd = 280 nM), DAPK1 (Kd = 300 nM), and proviral integrations of Moloney virus 3 (PIM3) (Kd = 810 nM), 10-fold less potent toward IRAK4 and PIM1, 100-fold less potent against PIM2 and MLCK, and inactive against ROCK2. Indeed, more detailed in vitro kinetic assessments of the inhibitory potential of HS38 toward ROCK2 and MLCK indicated that it has no significant effect on these contractile kinases at concentrations up to 100 μM (Fig. 1, A and B). We employed isolated rat caudal arterial smooth muscle strips as a model VSM tissue and assessed the concentration and time dependence of HS38 effects on contractile responses. Muscle strips were incubated in Ca2+-containing HEPES buffer with HS38 or vehicle prior to addition of the type 1 and 2A phosphatase inhibitor CLa, which by inhibiting endogenous MLCP activity unmasks basal Ca2+-independent LC20 kinase activities to induce a slow, sustained contraction (Wilson et al., 2005b; Sutherland and MacDonald et al., 2013). HS38 acts as a competitive inhibitor with respect to ATP and was reported to be most potent toward ZIPK (Carlson et al., 2013). HS38 acts as a competitive inhibitor with respect to ATP and was reported to be most potent toward ZIPK (Carlson et al., 2013).

**TABLE 1**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 94)</th>
<th>HS38 (n = 28)</th>
</tr>
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<tbody>
<tr>
<td>Latency (s)</td>
<td>361.0 ± 15.1</td>
<td>654.7 ± 60.6</td>
</tr>
<tr>
<td>t1/2 from stimulation (s)</td>
<td>1082.9 ± 37.9</td>
<td>1792.0 ± 110.8</td>
</tr>
<tr>
<td>% KC3 contraction</td>
<td>702.0 ± 25.6</td>
<td>1170.1 ± 64.0</td>
</tr>
<tr>
<td>1/2 from contraction (s)</td>
<td>158.3 ± 5.1</td>
<td>161.7 ± 8.4</td>
</tr>
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*Denotes CLa (0.5 μM) alone.
*Denotes CLa + HS38 (50 μM).

Fig. 2. Reversibility of the inhibitory effect of HS38 on CLa-induced contraction. De-endothelialized rat caudal arterial smooth muscle strips in HEPES-buffered Tyrode's solution were preincubated for 30 minutes (A) or 3 hours (B) with vehicle (dimethylsulfoxide) or HS38 (100 μM). HS38 was retained or washed out for 30 minutes prior to addition of CLa (0.5 μM) for 3 hours to elicit a sustained contractile response. The time required to reach half-maximal force (t1/2) was calculated and normalized to the vehicle control, which had never seen HS38. Values indicate the mean ± S.E.M. (n = 3) with the control value set at 100%. *, Significantly different from the force evoked in the absence of HS38 (vehicle control); #, significantly different from the force evoked in the presence of HS38; nsd, no significant difference from vehicle control. Statistical significance assessed by analysis of variance with Dunnett's post hoc test, P < 0.05.
Walsh, 2012). HS38 appeared to attenuate the velocity of the contractile responses, whereas the steady-state level of force induced by CLa was unaffected by ZIPK inhibition (Fig. 1, C and D), a result that was previously demonstrated (Carlson et al., 2013). As shown in Table 1, the mean steady-state force induced by CLa was ~160% of that induced by KCl (87 mM) and was unaffected by pretreatment with HS38. We did observe variability in the steady-state force levels induced by CLa relative to KCl from tissue to tissue, as indicated by the S.D. values in parentheses in Table 1. This variability can be attributed to variations in the dimensions of the muscle strips. The latency period, i.e., the time from addition of CLa to the onset of contraction, and the half-time from the initiation of contraction to the plateau of the contractile response, in addition to the $t_{1/2}$ values (the half-time from addition of CLa to the plateau of the contractile response) were all significantly increased in the presence of HS38 (Table 1). Significant increases in $t_{1/2}$ were observed at HS38 concentrations $\geq 10 \mu$M, and maximal effects (~2-fold increase in $t_{1/2}$) were observed at HS38 concentrations $\geq 50 \mu$M (Fig. 1E). On the other hand, increasing HS38 concentration did not affect the maximal developed force (Fig. 1F).

We addressed the possibility that HS38 may influence [Ca$^{2+}$], by examining Fluo-4-loaded, de-endothelialized rat caudal artery strips. As shown in Supplemental Fig. 1, HS38 had no effect on the depolarization-induced Ca$^{2+}$ transient.

Finally, the effects of HS38 on the contractile response to CLa were found to be reversible. Treatment of de-endothelialized rat caudal arterial smooth muscle strips with HS38 (50 $\mu$M for 30 minutes or 3 hour), followed by washout, restored $t_{1/2}$ values of CLa-induced contraction to that observed with no prior exposure to HS38 (Fig. 2, A and B).

The effect of HS38 on LC20 phosphorylation was assessed to determine if inhibition of LC20 phosphorylation underlies the HS38-induced reduction of the rate of CLa-induced contraction. HS38 suppressed CLa-induced LC20 phosphorylation in

![Fig. 3. The effect of HS38 on CLa-induced LC20 phosphorylation. LC20 phosphorylation was analyzed by Phos-Tag SDS-PAGE with detection of unphosphorylated (LC20-0P), monophosphorylated (LC20-1P), diphosphorylated (LC20-2P), and triphosphorylated (LC20-3P) forms of LC20 by western blotting with anti-LC20. (A) The effect of increasing concentrations of HS38 on LC20 phosphorylation induced by CLa treatment (0.5 $\mu$M for 60 minutes). A representative western blot is shown above cumulative quantitative data. Phosphorylation stoichiometry (mol Pi/mol LC20) was calculated as described under Materials and Methods. (B and C) Representative western blots and cumulative quantitative data showing the time courses of LC20 phosphorylation in response to CLa (0.5 $\mu$M) in the presence of vehicle (dimethylsulfoxide) (B) or HS38 (100 $\mu$M) (C). The various LC20 bands were quantified by scanning densitometry and expressed as a percentage of the total LC20 signal. (D) LC20 phosphorylation stoichiometry is expressed as mol P/mol LC20. #, Significantly different from the corresponding value in the absence of HS38 (analysis of variance with Dunnett’s post hoc test, $P < 0.05$). Values in (A) and (B–D) indicate the mean ± S.E.M. [n = 4–7 in (A) and n = 4 in (B–D)].]
a concentration-dependent manner, with significant inhibition observed at HS38 concentrations ≥25 μM (Fig. 3A). The inhibitory effect of HS38 on LC20 phosphorylation was confirmed by analysis of the time course of CLa-induced phosphorylation of LC20 in the absence and presence of HS38 (100 μM) (Fig. 3, B–D). The fact that LC20 phosphorylation was not abolished by HS38 is consistent with the unmasking of the activities of other kinases, such as integrin-linked kinase (Wilson et al., 2005b), upon CLa treatment.

ZIPK was originally isolated from smooth muscle as a MLCP-associated kinase (MacDonald et al., 2001a), and studies have demonstrated its ability to phosphorylate MYPT1 (the regulatory, myosin-targeting subunit of the phosphatase) in vitro and in situ (MacDonald et al., 2001a; Endo et al., 2004; Moffat et al., 2011). A basal level of phosphorylation was observed at both Thr inhibitory sites, Thr697 (Fig. 4A) and Thr855 (Fig. 4D). Exposure of smooth muscle strips to HS38 reduced the basal phosphorylation observed under resting conditions (time zero) at both sites (Fig. 4, B and E), thus there is likely some basal ZIPK activity (or other kinase activity influenced by ZIPK) that contributes to MYPT1 phosphorylation under rest/basal conditions. It is noticeable that the electrophoretic mobility of phospho-MYPT1-immunoreactive bands decreased with time of exposure to CLa, suggesting that MYPT1 is hyperphosphorylated at multiple sites upon phosphatase inhibition. After normalizing the MYPT1 phosphorylation immunosignals against α-actin to account for loading variability, we were also able to report the change in phosphorylation signal relative to the initial basal/resting level. The application of HS38 resulted in an unexpected accumulation of MYPT1 phosphorylation at both Thr697 (Fig. 4C) and Thr855 (Fig. 4F) when compared with CLa stimulation alone. We have previously observed increased MYPT1 phosphorylation upon blockade of ZIPK signaling in rat caudal artery (Moffat et al., 2011); this observation was made during loading of recombinant, kinase-dead ZIPK protein into Triton-skinned muscle strips.

**Fig. 4.** The effect of HS38 on CLa-induced phosphorylation of MYPT1 at Thr697 and Thr855. The time courses of MYPT1 phosphorylation at Thr697 (A–C) and Thr855 (D–E) were analyzed by SDS-PAGE and western blotting with phosphospecific antibodies following stimulation of caudal arterial smooth muscle strips with CLa (0.5 μM) in the presence of vehicle (dimethylsulfoxide) (A and D) or HS38 (100 μM) (B and E). Cumulative quantitative data showing the time course of MYPT1 phosphorylation (fold increase relative to levels at time zero) for Thr697 (C) and Thr855 (F) in the absence and presence of HS38 (100 μM) are provided. α-Actin was used as the loading control. Data are representative of four independent experiments. *, Statistical significance assessed with the Student’s t test (two-tailed), P < 0.05.
with MLCP inhibition by microcystin-LR. We have interpreted this phenomenon to be a result of coincident unmasking of kinase activities with MLCP inhibitors and suppression of ZIPK activity. The resulting changes associated with the contractile architecture may allow the phosphorylation sites of MYPT1 to become more accessible, as theorized by Vetterkind et al. (2010) and Vetterkind and Morgan (2009).

The in vitro phosphorylation of CPI-17 by ZIPK has been reported previously (MacDonald et al., 2001b). Treatment of caudal arterial smooth muscle strips with CLa resulted in slow stoichiometric phosphorylation of CPI-17 at several sites, as shown by Phos-Tag SDS-PAGE (Fig. 5A). In this case, HS38 had no effect on CLa-induced CPI-17 phosphorylation stoichiometry (Fig. 5B).

The Par-4 (36 kDa) protein is expressed in differentiated VSM cells (Vetterkind and Morgan, 2009; Vetterkind et al., 2010) and is suggested to facilitate contraction by acting as a cytoskeletal scaffold for ZIPK. Therefore, we investigated the effects of HS38 on Par-4 phosphorylation in response to CLa stimulation of caudal arterial smooth muscle and observed that CLa treatment induced the phosphorylation of Par-4 at multiple sites (Fig. 6A). Eight distinct phosphorylated species were detected by Phos-Tag SDS-PAGE; however, HS38 (100 μM) had no effect on the rate or maximal stoichiometry of Par-4 phosphorylation (Fig. 6, A–C).

Although HS38 has been described as a potent and selective ZIPK inhibitor, it does retain some off-target activity toward members of the PIM kinase family (Carlson et al., 2013), which have roles in cell growth, proliferation, apoptosis, and regulation of signal transduction cascades (Alvarado et al., 2012). Currently, there are no reports of PIM kinases contributing to smooth muscle contractility; however, we felt it prudent to assess whether any of the observed effects of HS38 on VSM contractility could be attributed to inhibition of PIM kinases. In this regard, a structural ortholog (HS43) was developed from the HS38 backbone (Carlson et al., 2013). This compound retains selective potency toward PIM3 with minimal activity toward ZIPK. The HS43 compound was applied to de-endothelialized rat caudal arterial strips, and the contractile responses to CLa were monitored (Fig. 7A). No effect was observed for HS43 on the velocity of contraction (Fig. 7B) or maximal force (Fig. 7C). Finally, CLa-induced phosphorylations of LC20 (Fig. 7D), Par-4 (Fig. 7, E and F), and MYPT1 (Fig. 7, G and H) were also unaffected by inhibition of PIM3 kinase activity with HS43.
The development of a potent and selective ZIPK inhibitor provides the opportunity to examine upstream receptor-signaling modules that are linked to ZIPK-dependent contractile responses. Isolated rat caudal arterial smooth muscle strips were again employed since receptor-mediated contractile responses have been extensively explored in this VSM tissue (Weber et al., 1999; Mita et al., 2002; Wilson et al., 2005a,b; Grassie et al., 2012). The typical contractile response of this tissue to agonist stimulation can be divided into an initial, rapid phasic response and a sustained, steady-state tonic response. The effects of HS38 pretreatment on the contractile responses to a range of agonists were investigated (Fig. 8A). PE (1 μM), ET-1 (0.1 μM), and high-[K+] extracellular solution (87 mM KCl) induced robust contractile responses; other stimuli [the thromboxane A2 mimetic U46619 (1 μM) and AngII (0.1 μM)] elicited contractile responses of reduced magnitude. Both the force developed in the early phasic response (i.e., peak tension at 2–5 minutes) and the sustained tonic response (i.e., sustained force at 15 minutes) to all agonists were significantly inhibited by pretreatment with HS38 (Fig. 8, B and C).

Agonist-induced contractions were associated with LC20 phosphorylation exclusively at a single site (Fig. 9A), previously identified as Ser19, as expected (e.g., Sutherland and Walsh, 2012). LC20 phosphorylation was significantly increased at the peak of the contractile response to PE, U46619, KCl, and ET-1 (Fig. 9, A and B). All of these agonist-induced increases in LC20 phosphorylation were abolished or significantly reduced by preincubation of the tissue with HS38 (Fig. 9, A and B). In addition, the data showed no change in LC20 phosphorylation...
following stimulation with AngII; however, a significant decrease was observed during AngII treatment in the presence of HS38. The effect of HS38 on agonist-induced phosphorylation of MYPT1 at Thr697 and Thr855 was also investigated. PE-induced Thr697 phosphorylation was reduced by $\sim50\%$ by HS38. Thr855 phosphorylation induced by PE, KCl and ET-1 was also suppressed by $\sim50\%$ by HS38 (Fig. 9, C and D). Finally, agonist-induced phosphorylation of CPI-17 was assessed in the absence or presence of HS38 (Fig. 9, E and F). Stimulation with all agonists (i.e., PE, U46619, KCl, AngII, and ET-1) enhanced CPI-17 phosphorylation relative to control, and HS38 pre-treatment significantly suppressed the increase in CPI-17 phosphorylation induced by PE, AngII, and ET-1. In contrast, HS38 treatment had no influence on CPI-17 phosphorylation induced with U46619 or KCl.

**Discussion**

The inhibitory potential of the HS38 molecule against ZIPK was first defined in 2013, and some basic assessments of its effects on smooth muscle cells and tissues were completed to support its potential utility as a lead scaffold for future development and application to cardiovascular systems (Carlson et al., 2013). In this regard, HS38 could alter ex vivo contractile force.
development of various smooth muscles isolated from rabbit, rat, and mouse. HS38 could attenuate LC20 phosphorylation in human coronary and aorta VSM cells in response to stimulation with serum and sphingosine-1-phosphate, respectively. In addition, HS38 suppressed Ca\textsuperscript{2+}-independent force production and LC20 phosphorylation in permeabilized rabbit ileum treated with the MLCP inhibitor microcystin-LR. The lag time to the onset of force was increased, and the rate of force development was decreased. Additional pilot studies with rat caudal artery revealed the ability of HS38 to attenuate LC20 phosphorylation and contractile force development. The initial findings supported a novel hypothesis that the ability of ZIPK to regulate the Ca\textsuperscript{2+}-sensitization of smooth muscle contraction is conserved across species and smooth muscle tissues. Collectively, these results provided conclusive support for the role of ZIPK in the Ca\textsuperscript{2+}-sensitizing pathways that regulate VSM contractile tone.

Intriguing differences were observed for the ZIPK-dependent contractile responses of caudal arterial strips, depending on the mode of activation. Treatment with CLa to inhibit type 1 and 2A phosphatases (including MLCP, a type 1 phosphatase), and thereby unmask endogenous kinase activities, yielded robust LC20 mono- and diphosphorylation that was sensitive to ZIPK inhibition by HS38; however, the suppression of ZIPK activity did not relieve MLCP inhibition, i.e., significant amounts of CLa-induced MYPT1 phosphorylation at Thr697 and Thr855 were still observed in the presence of HS38. In contrast, agonist-induced stimulation of G protein-coupled receptor (GPCRs) and activation of downstream Ca\textsuperscript{2+} sensitization pathways elicited notable effects on phosphorylation of MYPT1 and CPI-17, as well as LC20. Based on these distinct responses, we speculate that two functional pools of ZIPK exist in this VSM tissue: 1) a constitutively active ZIPK associated with the myosin II compartment, and 2) an inactive ZIPK pool that is intimately associated with MLCP and its regulatory apparatus (e.g., Par-4, CPI-17, and the myosin phosphatase-Rho interacting protein, M-RIP). The first pool of ZIPK would be available to

Fig. 9. The effect of HS38 on phosphorylation of LC20, MYPT1, and CPI-17 evoked by various agonists. De-endothelialized rat caudal arterial smooth muscle strips were treated with the indicated agonists in the absence or presence of HS38 (100 \mu M): phenylephrine (PE) 1 \mu M; thromboxane A\textsubscript{2} mimetic (U46619, 1 \mu M); high-[K\textsuperscript{+}] extracellular solution (87 mM KCl); AngII (0.1 \mu M); and ET-1 (0.1 \mu M). Tissues were quick frozen for western blot analysis at the peak of contraction (PE and KCl: 1 minute; U46619, AngII and ET-1: 5 minutes). (A) LC20 phosphorylation was analyzed by Phos-Tag SDS-PAGE and western blotting with anti-LC20. (B) Immunoreactive bands from (A) were quantified by scanning densitometry and phosphorylation stoichiometry (mol P/mol LC20) was calculated as described under Materials and Methods. (C) The phosphorylation of MYPT1 at Thr697 and Thr855 was analyzed by western blotting with phosphospecific antibodies using α-actin as a loading control. Cumulative quantitative data are presented in (D) as the % change in Thr697 or Thr855 phosphorylation with treatment of HS38 relative to agonist in the presence of vehicle control. (E) CPI-17 phosphorylation was analyzed by Phos-Tag SDS-PAGE and western blotting with anti-CPI-17. (F) CPI-17 bands were quantified by scanning densitometry and phosphorylation stoichiometry (mol P/mol CPI-17) was calculated as described for LC20 under Materials and Methods. VSM strips treated with CLa (0.5 \mu M for 60 minutes) were used as positive controls in (A), (C), and (E). Values indicate the mean ± S.E.M. (n = 3). #, Significantly different from phosphorylation levels under resting conditions (tissue incubated in HEPES-buffered Tyrode’s solution); * significantly different from agonist stimulation in the absence of HS38 using the Student’s t test (two-tailed), P < 0.05.
ZIPK and Vascular Smooth Muscle Contraction

Fig. 10. Proposed signaling mechanisms for the regulation of VSM contraction by ZIPK. The coupling of agonist-activated GPCRs to phospholipase C$\beta$ (PLC$\beta$) via the heterotrimeric G protein $G_{\alpha1}$ results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to inositol 1,4,5-trisphosphate (IP$_3$), which releases Ca$^{2+}$ from the sarcoplasmic reticulum (SR) via IP$_3$ receptors (Ca$^{2+}$ channels) in the SR membrane. This results in activation of Ca$^{2+}$/calmodulin (CaM)-dependent MLCK, which phosphorylates the 20-kDa regulatory light chains of myosin II at Ser19. Agonist stimulation of GPCRs also results in the activation of ROCK via G$_{12/13}$, a Rho-guanine nucleotide exchange factor (RhoGEF) and the small GTPase RhoA. Activated ROCK leads to inhibition of MLCP activity, increased LC$_{20}$ phosphorylation, and smooth muscle contraction. VSM contraction can be evoked independently of Ca$^{2+}$ by direct phosphorylation of MYPT1 at Thr697 and Thr855, or indirectly via phosphorylation of CPI-17 at Thr38. Two discrete pools of ZIPK play distinct roles in Ca$^{2+}$ sensitization of VSM contraction: 1) a pool of constitutively active ZIPK (indicated in green), whose activity is unmasked by inhibition of MLCP activity, for example, by CLa and phosphorylates LC$_{20}$ at Thr18 and Ser19, and 2) a pool of ZIPK (indicated in blue) is activated by phosphorylation by ROCK, leading to phosphorylation of MYPT1 and CPI-17 and decreased MLCP activity. Other kinases, including integrin-linked kinase (ILK), can also phosphorylate LC$_{20}$ at Thr18 and Ser19, as well as CPI-17 at Thr38 and MYPT1 at Thr697 and Thr855.
pharmacomechanical coupling of agonists and contractile force development involves changes in [Ca\(^{2+}\)], as well as activation of Ca\(^{2+}\)-sensitizing signal transduction pathways (Wetttschureck and Offermanns, 2005; Wynne et al., 2009). The former may involve Gr\(_{q11}\)-mediated PLC\(\beta\) activation with downstream diacylglycerol and inositol 1,4,5-trisphosphate effects on internal Ca\(^{2+}\) stores. The latter may involve Gr\(_{q11}\) or Gr\(_{12/13}\)-mediated activation of PKC and/or RhoA/ROCK. Given the fact that these agonist/receptor pairs activate similar downstream signaling pathways in VSM, it is clear why inhibition of ZIPK activity by HS38 attenuated the contractile responses to all these agonists.

We addressed the possibility that HS38 influenced [Ca\(^{2+}\)]\(_{c}\) (Supplemental Fig. 1) since Ca\(^{2+}\)-dependent MLCK activity is absolutely required for both the phasic and tonic components of the contractile responses to agonists and depolarization. During the sustained contractile response, when [Ca\(^{2+}\)]\(_{c}\) has declined, the MLCK activity is low relative to its activation during the phasic response, and without inhibition of MLCP activity will be insufficient to maintain LC\(_{20}\) phosphorylation and force (Mita and Walsh, 1997; Mita et al., 2002). Given that HS38 had no effect on depolarization-induced Ca\(^{2+}\) signaling or MLCK activity but inhibited both contraction and MYPT1 phosphorylation (which has been implicated in the mechanism of Ca\(^{2+}\) sensitization), it is reasonable to conclude that ZIPK is involved in Ca\(^{2+}\) sensitization but not Ca\(^{2+}\)-induced contraction induced by membrane depolarization. Inhibition of agonist-induced contraction by HS38 also involves Ca\(^{2+}\) desensitization; therefore, we cannot rule out an additional inhibitory effect on Ca\(^{2+}\) signaling.

There is good evidence indicating that ZIPK is regulated by phosphorylation at multiple sites by ROCK1 (Hagerty et al., 2007) and DAPK1 (Shani et al., 2004). The involvement of RhoA/ROCK in Ca\(^{2+}\) sensitization of VSM contraction has been unequivocally demonstrated. In this regard, ROCK can be activated in a variety of smooth muscle types in response to a large number of different contractile agonists (Puetz et al., 2009). The activation of RhoA/ROCK by Gr\(_{q11}\) is mediated by guanine nucleotide exchange factors (GEFs)—including the GEFs p115-RhoGEF and PDZ-RhoGEF, and leukemia-associated Rho guanine—and can be stimulated by administration of AngII, ET-1, or U46619 (Amin et al., 2013; Puetz et al., 2002). Given that this agonist/receptor pairs activate similar downstream signaling pathways in VSM, it is clear why inhibition of ZIPK activity by HS38 attenuated the contractile responses to all these agonists.

Alternatively, it is possible that ZIPK is primarily integrated with Gr\(_{q11}\)-PLC\(\beta\) and PKC signaling to regulate Ca\(^{2+}\) sensitization. ZIPK can phosphorylate CPI-17 at the phosphatase-inhibitory Thr38 site in vitro (MacDonald et al., 2001b). Therefore, it is possible that PKC may activate ZIPK or vice versa. A recent report by Xu et al. (2010) revealed that PKC/CPI-17 and ZIPK might participate to regulate the Ca\(^{2+}\) sensitivity of mesenteric arterial constriction after hemorrhagic shock. These authors suggested an intermediary role for ZIPK, whereby it acts as a bridge between PKC at the plasma membrane and MLCP at the contractile apparatus. Therefore, additional in vivo evaluation of ZIPK, especially in relation to the RhoA/ROCK and PKC pathways, will be important to further our understanding of signal transduction crosstalk of Ca\(^{2+}\) sensitizing pathways, and perhaps also lead to better treatments for cardiovascular diseases.

References


ZIPK and Vascular Smooth Muscle Contraction


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