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Myosin light chain phosphorylation-dependent modulation of volume-regulated anion channels in macrovascular endothelium

Bernd Nilius[a,*], Jean Prenen[a], Michael P. Walsh[b], Iris Carton[a], Mathieu Bollen[c], Guy Droogmans[a], Jan Eggermont[a]

[a] *Laboratorium voor Fysiologie, Campus Gasthuisberg, Herestraat 49, KU Leuven, B-3000 Leuven, Belgium*
[b] *Smooth Muscle Research Group and Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Calgary, 3330 Hospital Drive N.W., Calgary, Alb. T2N 4N1, Canada*
[c] *Laboratorium voor Biochemie, Campus Gasthuisberg, KU Leuven, B-3000 Leuven, Belgium*

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Abstract The Rho/Rho-associated kinase (ROK) pathway has been shown to modulate volume-regulated anion channels (VRAC) in cultured calf pulmonary artery endothelial (CPAE) cells. Since Rho/ROK can increase myosin light chain phosphorylation, we have now studied the effects of inhibitors of myosin light chain kinase (MLCK) or myosin light chain phosphatase (MLCP) on VRAC in CPAE. Application of ML-9, an MLCK inhibitor, inhibited VRAC, both when applied extracellularly or when dialyzed into the cell. A similar inhibitory effect was obtained by dialyzing the cells with AV25, a specific MLCK inhibitory peptide. Conversely, NIPP1$^{191-210}$, an MLCP inhibitory peptide, potentiated the activation of VRAC by a 25% hypotonic stimulus. These data indicate that activation of VRAC is modulated by MLC phosphorylation.

Key words: Chloride channel; Endothelium; Cytoskeleton; Myosin light chain kinase; Myosin light chain phosphatase

1. Introduction

Outwardly rectifying Cl^- channels regulated by changes in cell volume (volume-regulated anion channels, VRAC) have been described in many mammalian and non-mammalian cell types (for reviews see [1–3]). These channels are involved in several important physiological processes such as cell volume regulation, transport of osmolytes, regulation of the membrane potential and they may be required for progression through the cell cycle [4]. In endothelial cells, VRAC can also be activated by shear stress [5] suggesting an additional role for VRAC in endothelial mechanosensitivity.

In spite of the well documented biophysical description of $I_{Cl,swell}$ (i.e. the Cl^- current through VRAC) in a wide panel of cellular systems, it is still not understood which processes control the gating of this channel. Several groups have provided evidence that VRAC activation requires one or more protein (de)phosphorylation steps, but the identity of the kinases (protein tyrosine kinases, PKC) or phosphatases as well as their targets remain unknown and/or controversial [6–10]. We have proposed a pathway specifically for vascular endothelial cells whereby cell swelling reduces intracellular ionic strength which triggers one or more tyrosine phosphorylation steps that directly or indirectly activate VRAC [11,12]. In addition, a role for GTP-binding proteins in the activation of VRAC has been proposed since intracellular application of GTPγS transiently activates VRAC in several cell types [7,13–15]. Pretreatment with the *Clostridium botulinum* C3 exoenzyme which inactivates Rho GTPases by ADP-ribosylation, reduced the cell swelling-induced I^- efflux in intestinal epithelial cells and $I_{Cl,swell}$ in vascular endothelial cells [16,17]. Furthermore, Y-27632, a selective inhibitor of the Rho-associated kinase ROK (also known as ROCK or Rho kinase; one of the downstream targets for Rho) also inhibited VRAC activity [17]. Thus, these data point to the Rho/ROK pathway as a critical part in the signal transduction cascade regulating VRAC.

Rho GTPase controls stress fiber formation via at least two different downstream effectors, Dia1 and ROK [18,19]. Dia1 induces profilin-mediated actin polymerization [19], whereas ROK promotes myosin light chain (MLC) phosphorylation by phosphorylating the myosin binding subunit of MLC phosphatase thereby inhibiting the phosphatase activity [20,21]. In non-muscle cells, MLC phosphorylation is a prerequisite for actomyosin interaction and hence for stress fiber assembly [22]. In addition, it is well accepted that cytoskeletal components, including myosin, are key players in mechanosensing properties of endothelial cells [23,24].

Since on the one hand VRAC can be inhibited by inactivating Rho or ROK and on the other hand the Rho/ROK pathway is a known activator of MLC phosphorylation, we investigated whether inhibitors of MLC phosphorylation and dephosphorylation could affect the activation of VRAC.

2. Materials and methods

2.1. Cell culture

Cultured calf pulmonary artery endothelial cells (CPAE, purchased from American Type Culture Collection, ATCC, # CCL 209) were grown in Dulbecco's modified Eagle's medium containing 10% human serum, 2 mM L-glutamine, 2 U/ml penicillin and 2 mg/ml streptomycin.

2.2. Solutions

The standard extracellular solution was a Krebs solution containing (in mM): 150 NaCl, 6 KCl, 1 $MgCl_2$, 1.5 $CaCl_2$, 10 glucose, 10 HEPES, pH 7.4 with NaOH. The osmolality of this solution, as measured with a vapor pressure osmometer (Wescor 5500, Schlag, Gladbach, Germany), was 320 ± 5 mOsm. At the beginning of the patch-clamp recordings, the Krebs solution was replaced by an isotonic Cs^+ solution containing (in mM): 105 NaCl, 6 CsCl, 1 $MgCl_2$, 1.5 $CaCl_2$, 10 glucose, 90 mannitol, 10 HEPES, pH 7.4 with NaOH.

*Corresponding author. Fax: (32)-16-34 5991.
E-mail: bernd.nilius@med.kuleuven.ac.be

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The 12.5% (280 ± 5 mOsm) and 25% (240 ± 5 mOsm) hypotonic solutions (HTS) were obtained by omitting respectively 45 mM and 90 mM mannitol from this solution. In all experiments, a pipette solution was used containing (in mM) 40 CsCl, 100 Cs-aspartate, 1 $MgCl_2$, 1.93 $CaCl_2$, 5 EGTA, 4 Na_2ATP, 10 HEPES, pH 7.2 with CsOH (290 mOsm). The concentration of free Ca^{2+} in this solution was buffered at 100 nM, which is below the threshold for activation of Ca^{2+} activated Cl^- currents but sufficient for activation of VRAC by cell swelling in CPAE cells. Only VRAC is activated under these conditions. An accompanying background current through non-selective cation channels is negligible (current density at +100 mV less than 5 pA/pF).

2.3. Inhibitors

The MLC kinase inhibitor, 1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride (ML-9) [25] was purchased from Sigma. More specific inhibition of myosin light chain kinase (MLCK) was obtained with the AV25 peptide [26] which was included in the pipette solution at a concentration of 1 mM. The sequence of AV25 is AKKLAKDRMKKYMARRKLQKAGHAV (2.93 kDa). As a control peptide, which does not affect MLCK activity, we used a peptide derived from calponin (residues 145–163, $p^{145-163}$) which is structurally similar to AV25. The control peptide (2.38 kDa, sequence AEKQQRRFQPEKLREGRNI) was also used at a concentration of 1 mM in the pipette. Both peptides were synthesized in the Peptide Synthesis Core Facility at the University of Calgary, Alb., Canada. The inhibitory peptide for myosin light chain phosphatase (MLCP), $NIPP1^{191-210}$ (2.47 kDa, sequence RPKRKRKNSRVTFSEDDEII), and the mutated control peptide $NIPP1^{191-210}$ (V201A, F203A) (2.37 kDa, sequence RPKRKRKNSRATASEDDEII) were synthesized at the KU Leuven [27].

2.4. Electrophysiological recordings

Currents were monitored with an EPC-9 (Heka Electronics, Lambrecht, Pfalz, Germany) patch-clamp amplifier. Patch electrodes had DC resistances between 2 and 6 MΩ. An Ag-AgCl wire was used as reference electrode. In Cl^- substitution experiments, a 3 M KCl-agar bridge was used and membrane voltages were corrected for liquid junction potentials if necessary. Whole-cell membrane currents were measured using ruptured patches. Currents were sampled at 1 or 2 ms intervals and filtered at 500 or 200 Hz, respectively. Pipettes with a resistance below 2 MΩ were used to ensure effective diffusion of the pipette solution into the cytosol. Between 50% and 70% of the series resistance was electronically compensated to minimize voltage errors. The following voltage protocol was applied every 15 s from a holding potential of −20 mV: a step to −80 mV for 0.6 s, followed by a step to −100 mV for 0.2 s and a 2.6 s linear voltage ramp to +100 mV. The time course of the whole-cell current at −100 and +100 mV was reconstructed from the currents recorded at these potentials during successive voltage ramps. Current-voltage relations were obtained from the currents measured during the linear voltage ramps. The effects of the tested inhibitors were determined when $I_{Cl,\,swell}$ had reached a stationary value.

2.5. Statistics

Experiments were performed at room temperature (22–25°C). Pooled data are given as the mean ± S.E.M. from n cells. Significance was tested using Student's paired or unpaired t-tests. Differences were considered significant at the level of $P < 0.05$.

3. Results

3.1. Inhibition of VRAC by MLCK blockers

To investigate the role of MLC phosphorylation in VRAC activation we used ML-9 which is a well documented inhibitor of smooth muscle MLCK [25] and which is also capable of blocking agonist-induced MLC phosphorylation in endothelial cells [28]. In a first approach, we activated $I_{Cl,swell}$ with 25% HTS after which ML-9 (50 μM) was administered to the bath solution. As shown in Fig. 1A,B, ML-9 exerted a rather slow and voltage-independent inhibition of the pre-activated $I_{Cl,swell}$. 50 μM ML-9 reduced the current density of $I_{Cl,swell}$ activated by 25% HTS at +100 mV to 61 ± 7% ($n = 7$, Fig. 1C). The inhibitory effect of ML-9 on $I_{Cl,swell}$ was more pro-

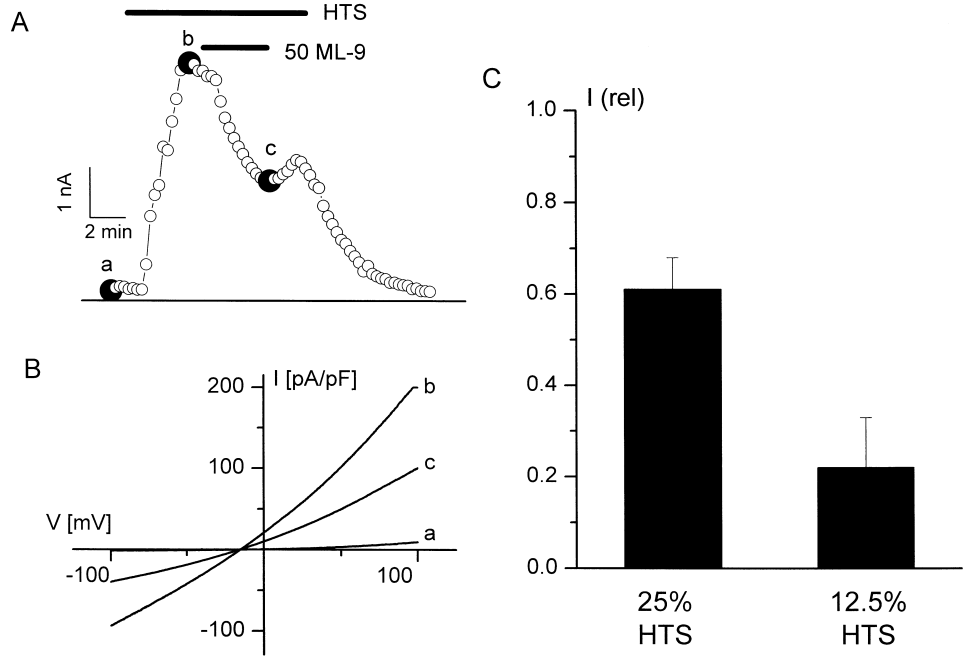

Fig. 1. Block of VRAC by extracellularly applied ML-9. A: Time course of membrane current at +100 mV. $I_{Cl,swell}$ was activated by 25% HTS and 50 μM ML-9 was applied to the bath solution after $I_{Cl,swell}$ had reached a constant level. Note the slow inhibition of the current by ML-9. In the absence of ML-9 the current remains elevated during the HTS exposure (see also Fig. 2A). B: *IV* curves measured at the times indicated in (A). Currents reversed at −17 mV which is close to the theoretical E_{Cl} of −25 mV. No shift in reversal potential was observed after application of ML-9. Inhibition of $I_{Cl,swell}$ is identical at all voltages. C: Pooled current amplitudes at +100 mV in the presence of 50 μM ML-9 and normalized to the maximal current during HTS exposure, either 25% ($n = 7$) or 12.5% ($n = 6$). Note the stronger inhibition at the weaker hypotonic stimulus.

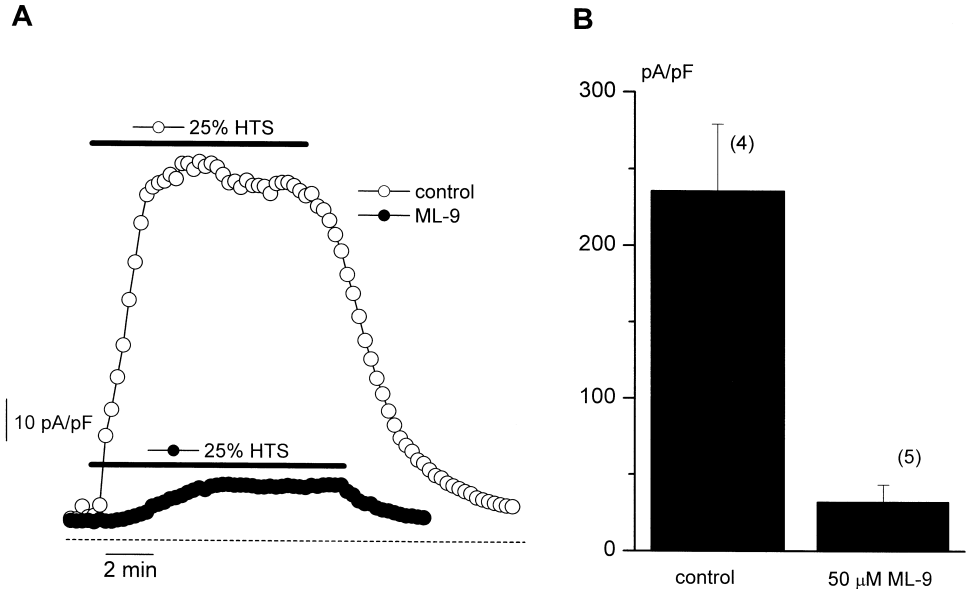

Fig. 2. Block of VRAC by ML-9 included in the patch pipette. A: Time course of membrane current at +100 mV in a control cell (open circles) and a cell dialyzed with 50 μM ML-9 via the patch pipette (filled circles). Cells were challenged by 25% HTS as indicated by the solid bars. B: Pooled data comparing the current densities of $I_{Cl,swell}$ activated by 25% HTS at +100 mV in control cells and in cells dialyzed with 50 μM ML-9 via the pipette.

nounced when a weaker hypotonic challenge (12.5% HTS) was applied in which case the current amplitude was reduced to 22 ± 11% of the control ($n = 6$, Fig. 1C).

In a second series of experiments we included ML-9 in the patch pipette (50 μM). This significantly delayed the activation of VRAC during a 25% HTS stimulus (Fig. 2A). The time to half-maximal activation of $I_{Cl,swell}$ increased from 95 ± 16 s ($n = 11$) under control conditions (same batch of cells) to 288 ± 42 s ($n = 5$) in the presence of 50 μM ML-9 in the pipette. In addition, the maximal current density decreased at all potentials (not shown). At +100 mV, ML-9 induced a decrease of $I_{Cl,swell}$ from 235 ± 43 ($n = 4$) to 32 ± 11 pA/pF ($n = 5$, Fig. 2C).

To further substantiate the role of MLCK in activating VRAC and to verify that the ML-9 effect was due to MLCK inhibition (as opposed to a direct inhibition of VRAC), we used a synthetic peptide (AV25) based on the autoinhibitory domain of MLCK that has been shown to block MLCK activity [26]. Activation of MLCK occurs via a Ca^{2+}/calmodulin-induced conformational change that removes the autoinhibitory domain from the active site, thereby allowing access of its substrate myosin. The AV25 peptide mimics the autoinhibitory domain by binding to the active site and blocking access to myosin. As shown in Fig. 3, inclusion of AV25 (1 mM) in the pipette strongly reduced VRAC activation by a 25% HTS stimulus. At +100 mV,

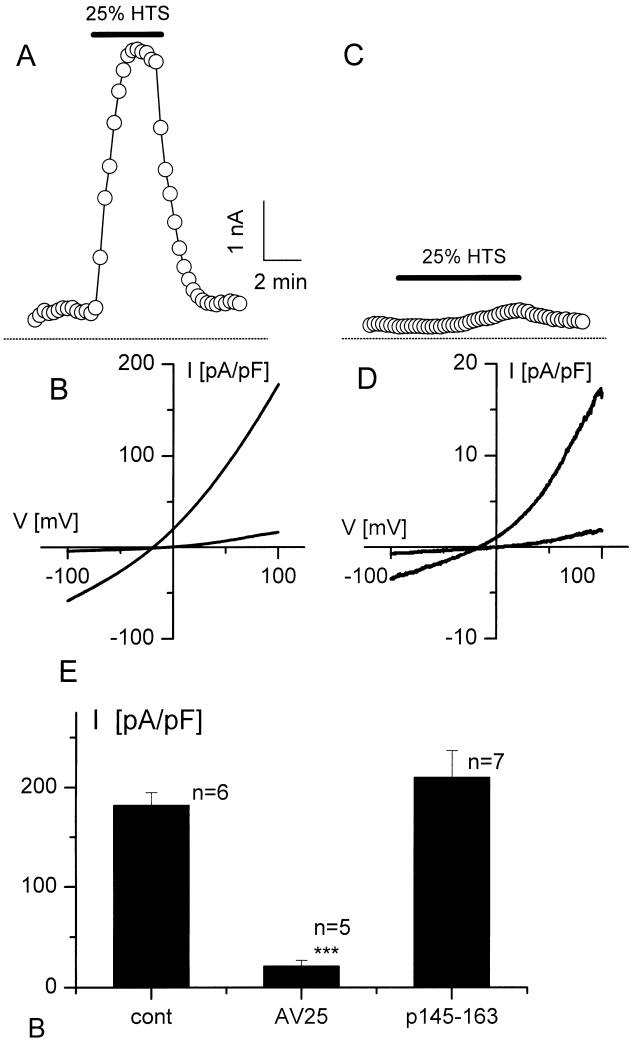

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Fig. 3. Block of VRAC by the specific MLCK inhibitory peptide AV25. A: $I_{Cl,swell}$ at +100 mV activated with 25% HTS in a control cell (left) and in a cell dialyzed with 1 mM AV25 via the pipette (right). Note the reduction in current amplitude and the slower activation. B: Current-voltage relationships measured at the times indicated in (A) for the control cell (left) and the cell dialyzed with the AV25 peptide (right). C: Pooled data from several cells comparing the IV curves at +100 mV under control conditions, in the presence of the AV25 peptide and in the presence of $p^{145-163}$, the calponin-derived control peptide.

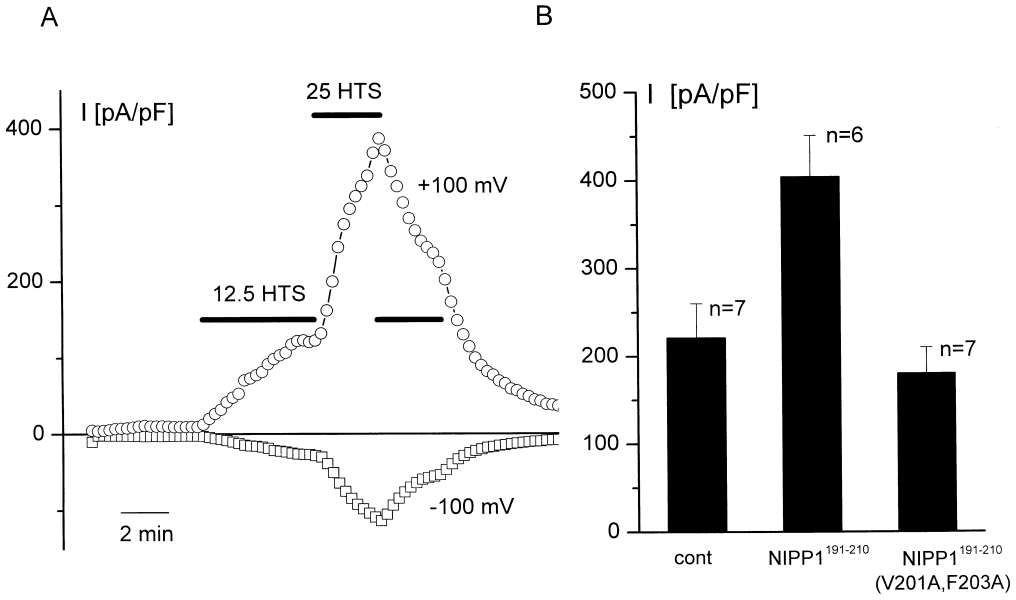

Fig. 4. Potentiation of VRAC by an inhibitory peptide of MLCP. A: Time course of membrane current in CPAE cells at +100 and −100 mV in the presence of 1 mM NIPP1$^{191-210}$ peptide in the pipette during a 12.5 and 25% hypotonic stimulus. Note the extremely high current density at 25% HTS. B: Pooled data for CPAE cells, either control or treated with 1 mM of the active NIPP1$^{191-210}$ or the inactive NIPP1$^{191-210}$ (V201A, F203A) peptide (mean ± S.E.M. of current densities at +100 mV for 25% HTS).

$I_{Cl,swell}$ reached 182 ± 13 pA/pF ($n = 6$) in control cells, but was reduced to 21 ± 6 pA/pF ($n = 5$) in AV25-treated cells. A control peptide (p$^{145-163}$) which does not affect MLCK activity, did not significantly affect $I_{Cl,swell}$ (210 ± 22 pA/pF at +100 mV; $n = 7$) in comparison to non-treated cells. In addition to the inhibition of the current amplitude, AV25 also delayed $I_{Cl,swell}$ activation similar to our observations with ML-9 (Fig. 2). The time to half-maximal activation at +100 mV was increased to 261 ± 29 s ($n = 5$) in AV25-treated cells compared to 95 ± 16 s ($n = 11$) in control cells.

3.2. Potentiation of VRAC by a MLCP inhibitory peptide

If ML-9 and the AV25 peptide inhibit VRAC by blocking MLCK activity, then it should also be possible to activate or potentiate VRAC by blocking MLC dephosphorylation. Smooth muscle MLCP is a type 1 serine/threonine protein phosphatase (PP1) and the functional holoenzyme is composed of three subunits: the δ-isoform of the catalytic subunit PP1$_C$, the myosin-targeting subunit MYPT1 and a MYPT1-associated protein of 20 kDa of unknown function [29]. Synthetic peptides containing a RVXF-motif alleviate the interaction between the regulatory subunit and the catalytic subunit PP1$_C$ and thereby destroy specific properties of the holoenzyme [30]. To functionally interfere with the MLCP activity in CPAE cells, we used the synthetic peptide, NIPP1$^{191-210}$, which includes the RVXF sequence (residues 200–203) of the nuclear regulator NIPP1 and which has been shown to act as a competitor of various regulators of PP1$_C$ [27].

When administered via the patch pipette, NIPP1$^{191-210}$ (1 mM) had no effect on the basal current under isotonic conditions. However, it significantly potentiated $I_{Cl,swell}$ evoked by 25% HTS (Fig. 4): 404 ± 47 pA/pF ($n = 6$) versus 221 ± 39 pA/pF ($n = 7$) in control cells (both at +100 mV). The current potentiated by NIPP1$^{191-210}$ had a similar reversal potential (between −25 and −15 mV) as $I_{Cl,swell}$ in control cells. In contrast, the control peptide NIPP1$^{191-210}$ (V201A, F203A) which does not have a functional RVXF-motif, had no effect on $I_{Cl,swell}$ when applied at 1 mM via the patch pipette: 180 ± 30 pA/pF ($n = 7$) at +100 mV. The NIPP1$^{191-210}$ peptide did not significantly increase $I_{Cl,swell}$ when cells were stimulated with a weaker hypotonic stimulus (12.5% HTS): 154 ± 21 pA/pF ($n = 5$) at +100 mV in the presence of 1 mM versus 127 ± 32 pA/pF ($n = 7$) at +100 mV in control cells.

4. Discussion

In this study we demonstrate that VRAC in macrovascular endothelial cells is inhibited by blockers of MLCK and potentiated by inhibition of MLCP, thereby identifying a functional link between MLC phosphorylation and VRAC activation. An important question with respect to the underlying mechanism is whether MLC phosphorylation directly participates in the VRAC activation cascade or, alternatively, whether MLC phosphorylation affects VRAC activity indirectly by setting the extent of cell swelling during a hypotonic stimulus. We have recently shown that activation of $I_{Cl,swell}$ does not correlate with changes in cell volume and that a decrease in intracellular ionic strength is the primary trigger for channel activation [12]. This lack of correlation effectively excludes cell volume as the link between MLC phosphorylation and VRAC activity. We therefore conclude that MLC phosphorylation is a critical parameter for one or more steps in the VRAC activation cascade. This prompts the question whether a mere increase in MLC phosphorylation is sufficient to trigger VRAC activation. Several observations suggest a negative answer to this question. First, thrombin which has been shown to increase MLC phosphorylation via inhibition of MLCP in endothelial cells [31] and also to activate endothelial MLCK [32], does not activate VRAC under isotonic conditions, although it can potentiate VRAC once it is pre-activated by a hypotonic stimulus [33]. Second, the

$NIPP1^{191-210}$ peptide, which acts as a competitor for the RVXF-mediated interaction of various regulators with $PP1_C$, had no effect on membrane current under isotonic conditions. MLCK is activated in a Ca^{2+}/calmodulin-dependent way [32]. Although there is no direct correlation between $[Ca^{2+}]_i$ and VRAC [15], we have shown that a permissive concentration of Ca^{2+} is required to activate VRAC yet there is no correlation between cytosolic Ca^{2+} and VRAC activation [34]. It appears that MLC phosphorylation is required, but not sufficient to activate VRAC in CPAE cells. A possible explanation for such a permissive role could be that actomyosin interaction which in non-muscle cells depends on MLC phosphorylation [18], creates a scaffold for proteins involved in the VRAC activation cascade (for a discussion of the role of the cytoskeleton in cell signaling, see [35]). Such a molecular organization would then allow an efficient transmission of the primary trigger (reduction in ionic strength) to VRAC.

A positive correlation between on the one hand MLCK activity and/or degree of MLC phosphorylation and on the other hand transporter activity has also been reported for the $Na^+/K^+/2\ Cl^-$ cotransporter in endothelial cells [36], and for the Na^+/H^+ antiporter in rat primary astrocytes [37]. How MLCK activity or MLC phosphorylation determine $Na^+/K^+/2\ Cl^-$ or cotransporter Na^+/H^+ antiporter activity remains to be established, but a MLC phosphorylation-dependent organization of the activation cascade could be the common denominator.

Finally, our data are also compatible with a model whereby the previously described effects of the Rho/ROK pathway on VRAC activity [17] are at least in part mediated via changes in MLC phosphorylation.

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