Myosin regulation of NKCC1: effects on cAMP-mediated Cl\(^{-}\) secretion in intestinal epithelia

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Hecht, Gail, and Athanasia Koutsouris. Myosin regulation of NKCC1: effects on cAMP-mediated Cl\(^{-}\) secretion in intestinal epithelia. Am. J. Physiol. 277 (Cell Physiol. 46): C441–C447, 1999.—The basally located actin cytoskeleton has been demonstrated previously to regulate Cl\(^{-}\) secretion from intestinal epithelia via its effects on the Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter (NKCC1). In nontransporting epithelia, inhibition of myosin light chain kinase (MLCK) prevents cell-shrinkage-induced activation of NKCC1. The aim of this study was to investigate the role of myosin in the regulation of secretagogue-stimulated Cl\(^{-}\) secretion in intestinal epithelia. The human intestinal epithelial cell line T84 was used for these studies. Prevention of myosin light chain phosphorylation with the MLCK inhibitor ML-9 or ML-7 and inhibition of myosin ATPase with butanedione monoxime (BDM) attenuated cAMP but not Ca\(^{2+}\)-mediated Cl\(^{-}\) secretion. Both ML-9 and BDM diminished cAMP activation of NKCC1. Neither apical Cl\(^{-}\) channel activity, basolateral K\(^{+}\) channel activity, nor Na\(^{+}\)-K\(^{+}\)-ATPase were affected by these agents. Cytoskeletal disruption of actin filaments to which MLCK is localized (27) and thus activates MLCK enzyme. In fact, activation of MLCK would serve to contract the cell, thus altering cell shape in response to changes in cell volume. Such a link between cell volume-induced activation of MLCK and NKCC1 would explain the orchestration of cellular responses to volume changes.

The regulatory mechanisms governing NKCC1 activity in transporting epithelia appear, however, to be different. Matthews et al. (23) have shown that, in intestinal epithelial T84 cells, NKCC1 activation by hypotonicity, but not hypertonicity, is attenuated by stabilization of actin microfilaments with phalloidin. In contrast, the actin destabilizer cytochalasin D diminished NKCC1 activation in response to hypertonicity but not hypotonicity. These findings suggest that hypotonicity-induced NKCC1 activity requires an intact cytoskeleton but not microfilament rearrangement. In fact, this study demonstrated that hypotonic, but not hypertonic, states induced actin rearrangements similar to that seen in response to cAMP-mediated secretagogues such as forskolin. It should be noted, however, that, although both hypertonicity and hypotonicity activated NKCC1 in T84 cells, active Cl\(^{-}\) secretion did not occur, highlighting the fact that NKCC1 serves at least a dual role for the cell, including regulation of cell volume and active secretion of Cl\(^{-}\).

Although a clear role of the actin cytoskeleton in cAMP-mediated Cl\(^{-}\) secretion has been demonstrated, the exact nature of involvement remains undefined. Similarly, the participation of MLCK in cell-shrinkage-elicited NKCC1 activation in endothelial and Ehrlich ascites cells has been shown, but the mechanism is not fully understood. Although inhibition of MLCK prevents both myosin light chain phosphorylation and NKCC1 activation, cotransporter phosphorylation is

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not disturbed (15). It has been suggested that cel-
volume-induced cytoskeleton reorganization is the sig-
nal that activates NKCC1 in this particular model.

The involvement of myosin in the regulation of Cl-
secretion has not been examined. In general, the move-
ment of actin microfilaments is dependent on interac-
tions with myosin. Specifically, actin associates with
only the phosphorylated form of myosin light chain.
This interaction then results in the hydrolysis of ATP
by actin-activated myosin ATPase and filament move-
ment (1). The aim of this study was to determine
whether myosin is involved in regulating Cl− secretion
from intestinal epithelial cells. For these studies, the
well-characterized intestinal epithelial cell line T84
was used. The action of myosin was interrupted in two
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was used. The action of myosin was interrupted in two
ways. First, the phosphorylation of myosin light chain
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was prevented by MLCK inhibitors ML-9 and ML-7.

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METHODS

T84 cell culture. T84 cells, a generous gift from Dr. Kim
Barrett (University of California, San Diego, CA), were
grown as previously described (21) in a 1:1 (vol/vol) mixture of
Dulbecco-Vogt modified Eagle’s medium and Ham’s F-12 plus
6% newborn calf serum. For electrophysiological studies, cells
were grown to confluence on 0.33-cm² collagen-coated perme-
able supports (Transwell; Costar, Cambridge, MA). For radio-
isootope studies, 0.6-cm² collagen-coated permeable supports
were used.

Storage and use of BDM. BDM (Sigma Chemical, St. Louis,
MO) was stored in powder form at room temperature in the
dark. On the day of the experiment, a fresh 0.5 M stock was
made in water by vigorously vortexing. Just before experiment-
ation, a 10 mM dilution was made in tissue culture medium.
BDM has been shown to remain stable at 37°C in tissue
culture medium for at least 60 min (6).

Measurement of short-circuit current. Short-circuit current
(Isc) was determined using a simplified technique for measur-
ing electrical parameters of cultured monolayers as published
by Madara et al. (20). A voltage clamp (Bioengineering
Department, University of Iowa, Iowa City, IA) was inter-
faced with a pair of calomel electrodes immersed in saturated
KCl with a pair of Ag-AgCl electrodes immersed in Ringer
solution. Agar bridges connected the electrodes and the
medium surrounding the cultured monolayers. The tips of the
bridges were cleaned with 70% ethanol and rinsed in sterile
PBS before contact with the tissue culture medium. Under
voltage-clamped conditions, Isc was measured in response to
either carbachol (10–6 M) or forskolin (10–6 M) in the presence
or absence of ML-9, ML-7, or BDM.

CAMP extraction. Cells were grown to confluence on 24-well
plates. Medium was removed and replaced with PBS contain-
ing forskolin alone or forskolin plus BDM for the appropriate
time periods at room temperature. PBS was removed and 200
µl of ice-cold 65% (vol/vol) ethanol were added. The super-
natant was collected on ice, centrifuged at 2,000 g for 15 min at
4°C, transferred into new tubes, and dried in a vacuum oven.
CAMP concentrations were assessed utilizing the nonacetyla-
tion assay protocol for the CAMP enzyme immunoassay
system (Amersham, Arlington Heights, IL).

Fluorescent staining of actin. Fluorescent staining of F-
actin was performed using rhodamine-labeled phalloidin
(Molecular Probes, Eugene, OR). Monolayers were rinsed
in PBS, fixed for 10 min at room temperature in 3.7% formalde-
hyde, and permeabilized for 5 min in ice-cold acetone. They
were stained at room temperature in the dark for 30 min with
rhodamine phalloidin, rinsed, and mounted in Slow Fade
(Molecular Probes). Stained monolayers were examined and
photographed using epifluorescence microscopy. Photographs
were scanned by using DeskScan II and the images were
compiled in PowerPoint.

RESULTS

Inhibition of myosin light chain phosphorylation or
myosin II ATPase attenuates cAMP-mediated Isc. Confluent
T84 monolayers grown on collagen-coated perme-
able supports were stimulated by adding either the
Ca2+–mediated secretagogue carbachol (10–4 M) or the
CAMP-mediated secretagogue forskolin (10–4 M) to the
basal reservoir. The Isc response was measured every
minute in the presence or absence of either ML-9 or
BDM. The peak responses, 2 min for carbachol and 10
min for forskolin, are shown in Fig. 1. Neither ML-9 nor
BDM attenuated the Isc response to carbachol. In fact,
BDM significantly increased the secretory response. In
contrast, both ML-9 and BDM significantly diminished
the forskolin-induced Isc. This differential response is
reminiscent of findings observed with phalloidin stabi-
lization of F-actin (25).

125I and 86Rb efflux studies. T84 cell monolayers were
grown to confluence on 0.6-cm² collagen-coated permeable supports
(Transwell; Costar, Cambridge, MA). Monolayers were loaded
with 2 µCi of either 125I or 86Rb by incubating for 3 h.
Monolayers were then rapidly washed four times with HEPES-
phosphate-buffered Ringer solution (HPBR) composed of (in
mM) 135 NaCl, 5 KCl, 3.33 NaH2PO4, 0.83 Na2HPO4, 1 CaCl2,
1 MgCl2, 5 HEPES, and 10 glucose. A “sample/replace”
technique (28) was used to determine the rate constants of
125I and 86Rb efflux. These studies estimate the secretagogue-
mediated activation of Cl− and K+ channels, respectively
(28). Four baseline samples were obtained before addition of
secretagogue. Samples were collected every 2 min following
activation with secretagogue. Residual intracellular radio-
activity was determined by extracting cells with 1 ml of 0.1 N
NaOH and by counting samples in a scintillation counter. The
efflux rate constant was calculated as [ln(R2) – ln(R1)]/(t2 –
t1), where R2 is percent of radioactivity remaining in the
monolayer at time t2 (28).

86Rb uptake. The secretagogue-stimulated uptake of 86Rb
was used to assess NKCC1 activity. T84 monolayers grown
on permeable supports were treated or not with forskolin in
the presence and absence of bumetanide (20 µM for 20 min)
and/or BDM and ML-9. HPBR containing 2 µCi/ml of 86Rb
was then added to the basolateral reservoir of monolayers,
ant was allowed to proceed over 3 min. Uptake was
halted by placing the monolayers in ice-cold buffer composed of
100 mM MgCl2 and 10 mM Tris·HCl, pH 7.5. Radioactivity
was extracted from monolayers with 0.1 N NaOH and counted
in a scintillation counter. Protein concentration was deter-
ned by the Bradford assay. Na+–K+–2Cl− cotransporter
activity was defined as the bumetanide-sensitive portion of
86Rb uptake.

Statistical analysis. Data are presented as means ± SE
and were analyzed using Student’s t-test. Significance was
defined as P ≤ 0.05.
To ensure that BDM did not decrease forskolin-elicited $I_{sc}$ by interfering with cAMP production, the concentration of cAMP was measured in monolayers treated with forskolin alone and forskolin plus BDM (Fig. 2). There was no significant difference in the increase in cAMP in response to forskolin alone and forskolin plus BDM after either 1 or 5 min. Interestingly, by 8 min, the increase in cAMP was more than twice that in response to forskolin plus BDM compared with forskolin alone. Despite this increase in cAMP, forskolin-stimulated $I_{sc}$ is attenuated. BDM-induced increase in cAMP may account for the synergistic $I_{sc}$ response to carbachol seen in Fig. 1.

Dose-response studies with both ML-9 and BDM are shown in Fig. 3, A and B, respectively. The maximum concentration of ML-9 tested was 40 µM, because specificity is lost with higher concentrations. The concentrations used for the remainder of the experiments were 40 µM ML-9 and 10 mM BDM. To confirm the specificity of these agents for myosin-based activities, a more selective inhibitor of MLCK, ML-7, was also tested. ML-7 decreased forskolin-stimulated $I_{sc}$ in a fashion similar to that of ML-9 and BDM (98 ± 4, 77 ± 8, and 71 ± 6 µA/cm² for forskolin alone, forskolin + 5 µM ML-7, and forskolin + 10 µM ML-7, respectively; n = 4), indicating that myosin-driven processes are
involved in the regulation of NCCK1 activity induced by cAMP.

Studies were then performed to determine whether inhibition of myosin-based cytoskeletal activity was effective in reversing established forskolin-induced $I_{sc}$. At the peak of the $I_{sc}$ response (~10 min after exposure to forskolin) either BDM or ML-9 was added to the monolayers, and $I_{sc}$ was followed for 30 min. Neither BDM nor ML-9 was able to attenuate forskolin-induced $I_{sc}$ once steady-state currents had been established (83 ± 2.6, 83 ± 4.5, and 91 ± 2.0 μA/cm² for forskolin alone, forskolin + BDM, and forskolin + ML-9, respectively; n = 4 in each group; not significant). This finding was not unexpected, because at this point cytoskeletal remodeling would have already occurred.

Neither apical Cl⁻ nor basolateral K⁺ channel activity is blocked by BDM. The majority of $I_{sc}$ from stimulated T84 cell monolayers is attributable to apical Cl⁻ secretion (8). The coordinated activities of several transporters are required for vectorial Cl⁻ secretion to ensue. To determine whether myosin-altering agents interfere with apical Cl⁻ channel or basal K⁺ channel activity, apical $^{125}$I and basolateral $^{86}$Rb efflux studies were performed. This approach has been validated in T84 cell monolayers (28) and is routinely used as a method to examine the activities of these specific channels (22, 23). Figure 4 shows that neither Cl⁻ channel activity (A) in response to forskolin nor basolateral K⁺ channel activity (B) is altered by BDM.

Effect of ML-9 and BDM on Na⁺-K⁺-2Cl⁻ cotransport activity. NKCC1 activity can be assessed by measuring the bumetanide-sensitive, basolateral uptake of $^{86}$Rb. Both ML-9 and BDM (Fig. 5) significantly decreased forskolin-stimulated, bumetanide-sensitive uptake of $^{86}$Rb, whereas the bumetanide-insensitive component, representing primarily Na⁺-K⁺-ATPase activity, remained unchanged. The decrease in forskolin-stimulated $I_{sc}$ by bumetanide was identical to that seen with inhibition of myosin ATPase by BDM (Fig. 6).

An intact actin cytoskeleton is required for myosin-perturbing agents to inhibit cAMP-induced Cl⁻ secretion. If the effects of the agents used herein are truly acting by interference with actomyosin interactions, one would predict that disruption of the actin cytoskeleton would prevent their ability to diminish cAMP-stimulated Cl⁻ secretion. To test this prediction, monolayers were incubated with cytochalasin D (20 μM) for 10 min before stimulation with forskolin in the presence or absence of ML-9 or BDM. Cytochalasin completely prevented the effects of ML-9 on forskolin-elicted $I_{sc}$ and nearly ablated the inhibitory impact of BDM (Fig. 7). These findings suggest that an intact actin cytoskeleton is required for ML-9 and BDM to interfere with cAMP-driven Cl⁻ secretion.

cAMP-induced rearrangement of actin microfilaments is blocked by both ML-9 and BDM. Because actin microfilament rearrangement is required for cAMP-elicted Cl⁻ secretion and microfilament movement is myosin based, we hypothesized that myosin-perturbing agents attenuate Cl⁻ secretion by preventing cytoskeletal reorganization. To investigate this possibility, the effect of ML-9 and BDM on forskolin-stimulated actin filament rearrangement was assessed by fluorescence microscopy. Figure 8 demonstrates the previously described pattern of basal stress fibers in control and forskolin-treated T84 monolayers. In control monolayers (Fig. 8A), actin filaments are seen spanning the basal pole of cells in a homogeneous manner. After treatment with 1 μM forskolin, basal actin filaments are redistributed to the periphery of cells as thick ropelike strands. The central area of cells is rather devoid of stress fibers (Fig. 8B). Figure 8, C and D, demonstrates that both ML-9 and BDM prevent the forskolin-induced rearrangement of basal actin filaments.

**DISCUSSION**

The activity of the Na⁺-K⁺-2Cl⁻ cotransporter is crucial for maintaining cellular homeostasis. This transporter is key in regulating the cell volume of numerous
cell types. In transporting epithelia, NKCC1 is important for regulating intracellular salt concentrations in the face of dramatic changes. In view of the critical functions that NKCC1 serves, it should not be surprising to find that the regulation of the activity of this transporter is complex and perhaps variable between cell types. Several general mechanisms of regulation have been identified and include direct phosphorylation of NKCC1 (18, 19), alterations in \([\text{Cl}^-]_i\) (10, 13), and cytoskeletal-dependent mechanisms (13, 22, 23, 29). Understanding the role of the latter presents a challenge, yet elegant studies published previously have provided key insights. Matthews et al. (22) were the first to show that stabilization of the actin cytoskeleton of intestinal epithelial cells attenuated cAMP-driven Cl\(^-\) secretion by inhibiting NKCC1 activity (22). Whether NKCC1 activation occurred via direct effects of cAMP or in response to diminished \([\text{Cl}^-]_i\), was not known. By comparing cAMP-induced activation of NKCC1 in cells expressing, or not, the apical Cl\(^-\) channel cystic fibrosis transmembrane conductance regulator, these investigators concluded that NKCC1 can be activated by pathways independent of apical Cl\(^-\) efflux, although mechanisms dependent on and independent from \([\text{Cl}^-]_i\), may work in tandem (24). It was determined, however, that cytoskeletal remodeling was required for ion uptake by NKCC1.

The effect of hypertonic challenge on active transporting epithelia may, however, be different. Using the intestinal epithelial T84 cell model, Matthews et al. (23) have shown that hypertonicity-induced activation of NKCC1 was not altered by the actin-stabilizing agent phalloidin. This suggests that cytoskeletal movement was not affected by MLCK inhibition. These data led the investigators to conclude that cell volume status, volume-regulating transporters such as NKCC1, and cytoskeletal contraction are closely linked.

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Fig. 5. ML-9 and BDM significantly diminish cAMP-stimulated NKCC1 activity. Bumetanide-sensitive uptake of \(^{86}\text{Rb}\) was used to assess activity of NKCC1. Both inhibition of myosin light chain phosphorylation with ML-9 and inhibition of actin-activated myosin ATPase with BDM attenuated activity of NKCC1 in response to forskolin (n = 6 each group; P = 0.003 for forskolin vs. forskolin + ML-9; P = 0.006 for forskolin vs. forskolin + BDM). In contrast, there was no significant change in bumetanide-insensitive component of \(^{86}\text{Rb}\) uptake, which primarily represents \(\text{Na}^+\text{-K}^+\)-ATPase activity.

Fig. 6. BDM and the well-characterized NKCC1 inhibitor bumetanide have similar effects on cAMP-stimulated \(I_{\text{sc}}\). Pretreatment of monolayers with either bumetanide or BDM decreased \(I_{\text{sc}}\) in response to forskolin by \(\sim 50\%\). This corresponds closely to percentage decrease in NKCC1 activity by these agents as shown in Fig. 5.

Fig. 7. Cytochalasin D prevents attenuation of cAMP-stimulated \(I_{\text{sc}}\) by myosin-perturbing agents. T84 monolayers were treated with cytochalasin D before stimulation with forskolin in absence or presence of either ML-9 or BDM. Cytochalasin completely ablated ML-9 attenuation of \(I_{\text{sc}}\) (n = 4; P = 0.34 for forskolin vs. forskolin + ML-9 + cytochalasin D) and partially reversed the effect of BDM.
be uncoupled from the apical membrane transport events, namely Cl\textsuperscript{2\textprimessmall} efflux.

Because the cytoskeleton appears to play such a key role in one of the major physiological functions provided by intestinal epithelia, namely vectorial ion transport, we wanted to investigate whether the previously described cAMP-induced, actin-mediated activation of NKCC1 was myosin based. Actin microfilament movement in nonmuscle cells, such as epithelia, is regulated in the manner as has been described for smooth muscle cells (5). Actin interacts only with the phosphorylated form of myosin light chain. This protein is phosphorylated by the specific enzyme MLCK. The interaction of actin with phosphorylated myosin light chain activates myosin ATPase. It is the energy from this reaction that leads to microfilament movement. In the present study, we interrupted this process in two ways. First, MLCK activity was blocked with an enzyme inhibitor, ML-9 or ML-7, thus preventing myosin light chain phosphorylation. Second, the terminal step in regulating actin microfilament movement was prevented by inhibiting myosin ATPase activity with BDM. Interruption at either of these steps resulted in the same outcome: attenuation of NKCC1 activity in response to cAMP and decreased apical Cl\textsuperscript{2\textprimessmall} efflux. These findings confirm that active contraction of actin microfilaments, through interactions with myosin, is required for NKCC1 activation and Cl\textsuperscript{2\textprimessmall} secretion stimulated by cAMP.

The signal by which MLCK is activated remains unidentified. Increased [Ca\textsuperscript{2\textprimessmall}] is the sole identified stimulator of MLCK. Shrinkage-induced activation of MLCK in endothelial cells, however, appears to not result from increased [Ca\textsuperscript{2\textprimessmall}], (15). In addition, increased [Ca\textsuperscript{2\textprimessmall}], could not be demonstrated to correlate with myosin light chain phosphorylation in hypertonicity-challenged mesangial cells (27), suggesting that a novel mechanism may be responsible. Previous studies have demonstrated that [Ca\textsuperscript{2\textprimessmall}], and the level of myosin light chain phosphorylation do not always correlate (2). However, a GTP-dependent mechanism that enhances myosin light chain phosphorylation and muscle contraction at a fixed concentration of Ca\textsuperscript{2\textprimessmall} has been identified (26). This has been termed "GTP-induced increase in Ca\textsuperscript{2\textprimessmall} sensitivity." The small GTPase Rho is responsible (12) for this event. In fact, Rho has been found to regulate myosin light chain phosphorylation by two separate pathways. First, activated Rho kinase can phosphorylate the myosin-binding subunit of myosin phosphatase, thereby inactivating myosin phosphatase (14). Second, Rho kinase itself has been shown to phosphorylate myosin light chain at the same site as MLCK and subsequently activate myosin ATPase (3). In many contractile models, both pathways have been demonstrated to be required to increase myosin light chain phosphorylation (17). Interestingly, the binding of halides to heterotrimeric G proteins has been shown to alter GTPase activity (11). Whether activated Rho is involved in the regulation of NKCC1 and ultimately vectorial ion transport is not known. It is intriguing to speculate, in view of the role of myosin light chain phosphorylation in NKCC1 activation yet in the absence of increased [Ca\textsuperscript{2\textprimessmall}], that Rho GTPases may control the cytoskeletal regulation of NKCC1.

Fig. 8. ML-9 and BDM prevent forskolin-induced rearrangement of actin microfilaments. Cell monolayers were pretreated or not with ML-9 or BDM and then exposed to forskolin for 10 min. Actin microfilaments were stained as described in METHODS. A: In control monolayers, basally located actin microfilaments are seen spanning the entire cell. B: After a 10-min exposure to forskolin, microfilaments have rearranged into thickened ropelike strands primarily situated at cell periphery. Both ML-9 (C) and BDM (D) prevent rearrangement of actin microfilaments, which is reminiscent of the effects of the actin microfilament stabilizer, phalloidin.
In summary, this study demonstrates that phosphorylation of myosin light chain and subsequent contraction of actin-myosin bundles are crucial to the cAMP activation of NKCC1 and subsequent apical Cl− efflux. These data serve to highlight the importance of this regulatory pathway in transporting epithelia, much as has been shown previously for shrinkage-induced NKCC1 activation in endothelial cells. Additional studies will allow the deciphering of the signals involved in stimulating the cytoskeletal changes that so closely govern these crucial cellular events.

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