Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2008;22:487-496

Accepted: October 17, 2008

Vasopressin Regulates the Phosphorylation State of AMP-activated Protein Kinase (AMPK) in MDCK-C7 Cells

Charity Nofziger[1], Kameljit Kalsi[2], T. Aaron West[1], Deborah Baines[2] and Bonnie L. Blazer-Yost[1]

[1]Department of Biology, Indiana University Purdue University at Indianapolis, Indianapolis, [2]Ion Channels and Cell Signalling Research Centre, Division of Basic Medical Sciences, St. George's, University of London, London

Key Words
PKA • CFTR • Principal cell • Okadaic acid • ENaC • LKB1 • cAMP

Abstract
AMP-activated protein kinase (AMPK) is a regulatory kinase coupling cellular metabolism with ion transport. Madin-Darby Canine Kidney-Clone 7 (MDCK-C7) cells possess characteristics of the renal principal cell type, express the cystic fibrosis transmembrane regulator and the epithelial Na^+ channel, and display NPPB and amiloride-sensitive transepithelial transport when stimulated with [Arg^8]-vasopressin. [Arg^8]-vasopressin binding to its receptor on the basolateral membrane of MDCK-C7 results in cAMP production, activation of cAMP-dependent protein kinase A (PKA), and increases in Cl^- and Na^+ transport. Ussing-style electrophysiology showed that the PKA inhibitor, H89, blocked Cl^- and Na^+ transport. Unexpectedly, [Arg^8]-vasopressin stimulation resulted in the dephosphorylation of $pAMPK^{thr172}$. H89 did not prevent this, suggesting that the dephosphorylation is independent of PKA. 24 hour, but not 15 minute, incubation with the AMPK activator, AICAR, also blocked [Arg^8]-vasopressin-stimulated currents. Contrary to previous studies, immunoblotting revealed that AICAR did not increase abundance of the active, phosphorylated form of AMPK ($pAMPK^{thr172}$); although, AICAR treatment significantly blocked [Arg^8]-vasopressin -stimulated cAMP production. [Arg^8]-vasopressin still caused $pAMPK^{thr172}$ dephosphorylation in the presence of AICAR, suggesting that this effect is also independent of cAMP. In summary, these data suggest [Arg^8]-vasopressin regulates AMPK phosphorylation and that AICAR inhibits ion transport independently of AMPK in MDCK-C7 cells.

Introduction

[Arg^8]-vasopressin binding to the V2 receptor on the basolateral membrane of principal cells results in production of cAMP and concomitant activation of cAMP-dependent protein kinase A (PKA). This enzyme plays multiple roles in the cell, one of which is the regulation of ion transport processes, including Cl^- and Na^+ transport via the cystic fibrosis transmembrane conductance regulator (CFTR) and epithelial Na^+ channel (ENaC),

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1015-8987/08/0226-0487$24.50/0

Accessible online at:
www.karger.com/cpb

Bonnie L. Blazer-Yost
Indiana University Purdue University at Indianapolis
Department of Biology
723 West Michigan Street, Indianapolis (USA)
Tel. +1 317 278-1145, Fax +1317 274-2846, E-Mail bblazer@iupui.edu

respectively [1, 2]. The activities of the aforementioned ion channels in various epithelial cell models (human colonic adenocarcinoma T84 cells, mouse principal kidney cortical collecting duct clone 4 cells, and human lung adenocarcinoma H441 cells) have also been shown to be negatively regulated by AMP-activated protein kinase (AMPK) [3-8]. Moreover, AMPK has been reported to regulate transporters other than CFTR and ENaC, including the $Na^+/K^+/2Cl^-$ co-transporter (NKCC) and the Na^+/K^+ ATPase [8, 9]. AMPK has therefore been described as a cellular energy-ion transport coupling kinase [10].

AMPK is a serine/threonine (ser/thr) protein kinase composed of a catalytic (α) and regulatory subunits (β & γ), each of which are expressed as multiple isoforms [11]. The activity of this enzyme can be regulated by changes in cellular adenosine monophosphate/adenosine triphosphate (AMP/ATP) ratios during periods of cellular stress. The binding of AMP to the catalytic subunit of AMPK causes an allosteric change allowing the enzyme to be more easily phosphorylated and activated by upstream AMPK kinases (AMPKKs). Known AMPKKs include LKB1, the calcium/calmodulin-dependent protein kinase kinases α and β (CaMMKα & CaMMKβ) and transforming growth factor-β-activated kinase (TAK1) [11]. Each AMPKK can phosphorylate the thr^{172} position of the AMPKα subunit. Thr^{172} has been the most intensively studied phosphorylation site on AMPK and is considered to be important in determining the degree of enzyme activation.

The Madin-Darby Canine Kidney-Clone 7 (MDCK-C7) cell line is a continuous cell line derived from dog principal cells. These epithelial cells, in contrast to MDCK-C11 cells, have retained multiple characteristics of the renal principal cell type, including high transepithelial resistances (≥ 1000 Ω•cm^2) and Na^+-retaining responses to aldosterone, insulin-like growth factor-1, and [Arg^8]-vasopressin [12-14]. Moreover, MDCK-C7 cells express CFTR, as do *in vivo* kidney cells [15-19]. For these reasons, this principal cell model was chosen to test the hypothesis that AMPK regulates CFTR and ENaC-mediated ion transport stimulated by [Arg^8]-vasopressin. We report for the first time, that [Arg^8]-vasopressin inhibits AMPK activity. In addition, the AMPK activator, 5-Aminoimidazole-4-carboxyamide ribonucleoside (AICAR) inhibits [Arg^8]-vasopressin stimulated Cl^- and Na^+ flux. However, this effect of AICAR is independent of AMPK activation and is, at least in part, the result of an inhibition of cAMP production in response to [Arg^8]-vasopressin. These data highlight the complex interaction between cAMP and AMPK signaling pathways and show that AICAR can elicit AMPK-independent effects on ion transport processes in MDCK-C7 cells.

Materials and Methods

Materials

Madine Darby Canine Kidney, clone-7 (MDCK-C7) cells were a kind gift from Prof. Hans Oberliethner (Meunster, Germany). H89 and AICAR were purchased from Biomol (Plymouth Meeting, PA) and prepared in DMSO. [Arg^8]-vasopressin was purchased as a solution and okadaic acid (OA) was prepared in DMSO. Both were from Sigma Aldrich (St. Louis, MO). Amiloride, also from Sigma Aldrich, was prepared in double-distilled water. Nystatin was prepared in DMSO and was from Sigma Aldrich. Primary antibodies (raised in rabbit) directed against αAMPK, phospho-α$AMPK^{thr172}$, ACC, and phospho-ACC^{ser79} were from Cell Signaling (Danvers, MA). The antibodies used for αAMPK and phospho-α$AMPK^{thr172}$ detection recognize both α1 and α2 isoforms of AMPK. The antibodies used for ACC and phospho-ACC^{ser79} detection recognize both αACC and βACC. The anti-LKB1 antibody was from Abcam (Cambridge, MA). Mouse anti-β-actin antibody was purchased from Novus Biologicals (Littleton, CO). Goat-anti rabbit-HRP and goat anti-mouse-HRP secondary antibodies were purchased from Upstate (Lake Placid, NY). The Direct Cyclic AMP Enzyme Immunoassay Kit was from Assay Designs, Inc. (Ann Arbor, MI).

Cell culture

MDCK-C7 cells were cultured at 37°C in a humified incubator gassed with 5% CO_2. Culture media consisted of DMEM/F12 base media (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (ICN Biochemicals Inc., Irvine, CA), 1 mM glutamax®, 25 U/mL penicillin, 25 mg/mL streptomycin (Invitrogen), and 12 mg/L ciprofloxacin (Mediatech Inc., Herndon, VA). Cells were maintained in plastic culture flasks. For electrophysiological and biochemical analyses, the cells were subcultured onto permeable supports (Costar® Transwells, Corning, Acton, MA) for at least 14 days. Fresh media was supplied to all cell cultures three times per week.

Electrophysiology

Ussing-style electrophysiology was used to investigate net transepithelial ion flux in the form of short-circuit current (SCC). Cells grown on 0.4 μM Costar® Transwell filters were assembled into Ussing chambers; the potential difference was clamped to zero and the resulting SCC measured. Transepithelial resistance was monitored throughout the entire duration of each electrophysiological experiment by applying a 2mV pulse every 200 seconds. Only cells that maintained a resistance ≥ 1000 Ω•cm^2 were included in statistical analyses. For electrophysiological experiments, [Arg^8]-vasopressin (100mU/mL) was added to the serosal bathing media; H89 (20μM) or vehicle (DMSO) was added bilaterally; amiloride (10μM) was

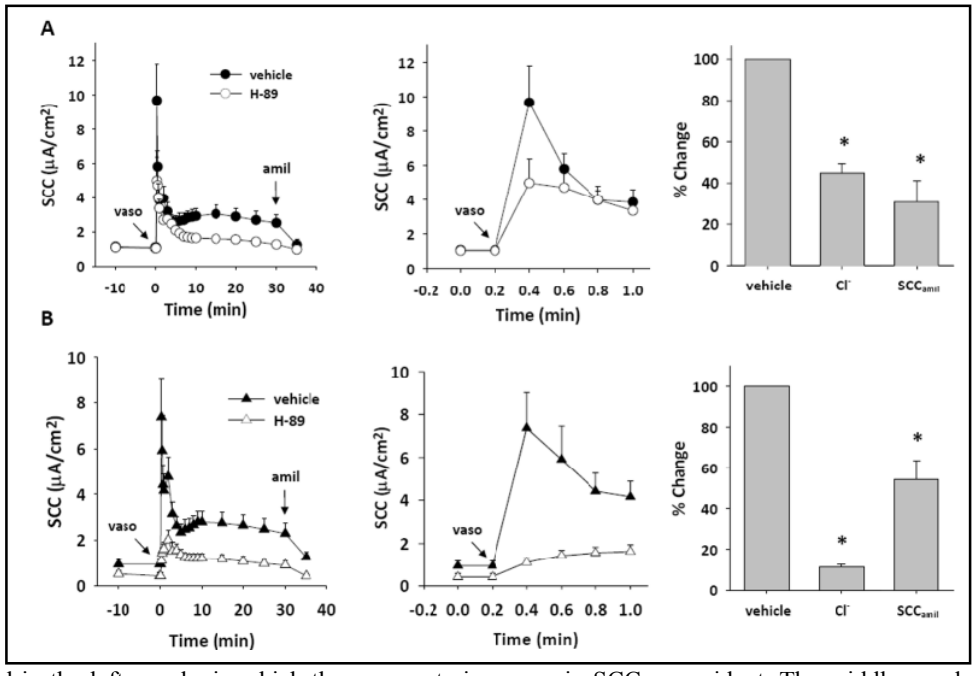

Fig. 1. PKA is required for [Arg^8]-vasopressin -stimulated increases in SCC. Panel A: Polarized MDCK-C7 cells were assembled into Ussing chambers and allowed to stabilize. 10 min prior to stimulation with [Arg^8]-vasopressin (100mU/mL), vehicle (DMSO) or H89 (20 μM) was added to the apical and serosal bathing compartments. Panel B: While in culture, polarized MDCK-C7 cells were treated as those in Panel A, except for 24 hrs. After assembly into Ussing chambers, vehicle or H89 was re-added to the apical and serosal bathing compartments. All cells were stimulated with [Arg^8]-vasopressin (100 mU/mL) at t = 0 min on the time scale. Amiloride (10 μM) was added 30 min. later. The entire duration of the electrophysiology experiments are depicted in the left panels, in which three separate increases in SCC are evident. The middle panels show an expanded timescale emphasizing the effect of H89 on the first increase (Cl^- secretion) in SCC elicited by the addition of [Arg^8]-vasopressin. In the right panels, the magnitude of Cl^- secretion and Na^+ reabsorption (SCC_{amil}) was calculated as $SCC_{t=0.4min}$ - $SCC_{t=0min}$ and $SCC_{t=35min}$ - $SCC_{t=30min}$, respectively. The magnitudes in H89-treated cells were normalized to those in matched vehicle-treated cells. H89 did not cause any significant changes in transepithelial resistance. Symbols and bars represent the mean ± SEM for 7 individual experiments where *$p \leq 0.001$ by a one-way ANOVA followed by Tukey's post-hoc test. Vaso, [Arg^8]-vasopressin; amil, amiloride.

added to the apical bathing media.

For experiments involving nystatin, physiological Cl^- Ringers solution (in mM; 140 NaCl, 5 KCl, 0.36 K_2HPO_4, 0.44 KH_2PO_4, 1.3 $CaCl_2$, 0.5 $MgCl_2$, 4.2 $NaHCO_3$, 10 HEPES, 5 D-glucose, pH 7.2 with Tris-base) or low Cl^- Ringers solution (in mM; 2.5 NaCl, 133.3 sodium gluconate, 5 potassium gluconate, 0.36 K_2HPO_4, 0.44 KH_2PO_4, 5.7 $CaCl_2$, 0.5 $MgCl_2$, 4.2 $NaHCO_3$, 10 HEPES, 5 D-glucose, pH 7.2 with Tris-base) were used to bath cultures during SCC experiments. The final Cl^- concentrations were 149mM and 14.9mM, respectively.

Immunoblotting

MDCK-C7 cells grown on permeable supports were washed twice with cold Hank's Balanced Salt Solution (Mediatech Inc., Herndon, VA). Cell lysis buffer (4% SDS, 10% glycerol in 0.5 M Tris, pH 6.8) was added directly to epithelial cultures and cells were scraped with a rubber policeman. 9 μL protease inhibitor cocktail (Sigma) per mL cell lysis buffer was added to prevent protein degradation. Protein concentrations in samples were determined with the DC protein assay (Biorad, Hercules, CA). 20 μg of protein were separated by SDS-PAGE on 7.5% acrylamide gels and transferred onto PVDF blotting membrane. Membranes were blocked for 1 hr at room temperature in 5% BSA diluted in wash buffer (0.01% Tween-20 in TBS, pH 7.6). All primary antibodies were diluted in 5% BSA diluted in wash buffer. Membranes were incubated with primary antibody overnight at 4°C, and then incubated in 3 changes of wash buffer for 5 min. each. This was followed by 1 hr incubation in secondary antibody, diluted with 5% milk in wash buffer, at room temperature. Three, 5 min. incubations in wash buffer followed. Protein bands were visualized with West Dura Enhanced Chemiluminescent Reagent and developed either on ClearBlue film (Pierce, Rockford, IL) or with a Molecular Imager ChemiDoc XRS System (Biorad). In order to compare relative amounts of $pACC^{ser79}$ and $pAMPK^{thr172}$, as well as ACC and AMPK, PVDF blotting membranes were cut lengthwise after transfer at ~ 75 kDa and 50 kDa. This yielded three pieces of membrane for immunoblotting - one for $pACC^{ser79}$ or ACC, one for $pAMPK^{thr172}$ or AMPK, and one for β-actin (loading control).

Nucleotide Extraction and High Performance Liquid Chromatography

Polarized MDCK-C7 cells were washed twice with ice-cold HBSS on ice. Cells were scraped in 500 μL 0.4 M cold perchloric acid on ice and spun for 3 min. at 9825 x *g*. A fixed volume of supernatant was removed and neutralized with 3 M K_3PO_4 and extracts were analyzed with high performance liquid chromatography (HPLC) according to the reverse-phase procedures described previously [20, 21]. The equipment used was the Hewlett-Packard 1100 series linked to a diode array detector. The perchlorate precipitate was re-suspended in 500 μL 0.5 M NaOH and the protein content was determined using a Bradford assay.

cAMP Assay

Polarized MDCK-C7 cells were treated serosally and apically with DMSO or AICAR (1mM) for 24 hrs followed by stimulation with or without [Arg^8]-vasopressin (100mU/mL) for 10 seconds. Each culture was washed twice with 37°C Hank's Balanced Salt Solution and incubated for 10 min. with 1% Triton X-100 in 0.1M HCl at 37°C. Lysates were centrifuged for 1 min. at 14,243 x *g* to remove cellular debris. Protein concentrations and cAMP concentrations per sample were determined with the RC/DC Protein Assay and the Direct Cyclic AMP Enzyme Immunoassay Kit, respectively. Final cAMP concentrations were calculated as pmol cAMP/mg protein.

Statistics

Differences between two or more groups in a given experiment were analyzed by a one-way ANOVA followed by a Tukey's post-hoc test using SPSS 14.0 statistical software. Student's t-test was used to analyze experiments containing only two groups. Differences were considered significant where $p \leq 0.05$. Symbols in line and bar graphs represent means ± SEM. Line and bar graphs were generated using Sigma Plot 2000 graphing software.

Results

Effect of [Arg^8]-vasopressin on ion transport

Stimulation with [Arg^8]-vasopressin induces a triphasic increase in transepithelial ion transport, measured as SCC, in MDCK-C7 principal cells. This electrophysiological response has been previously characterized by ourselves as transient Cl^- secretion via CFTR, followed by a putative calcium-activated K^+ flux, and finally reabsorptive Na^+ movement via ENaC [13] (Fig. 1A and B). The increase in Cl^- secretion and Na^+ reabsorption elicited by [Arg^8]-vasopressin stimulation were significantly blocked by short-term (10 min) and long-term (24 hr) treatment with H89, a PKA inhibitor (Fig. 1A and B respectively), supporting a role for the PKA signaling pathway in mediating these ion transport events.

Effect of [Arg^8]-vasopressin on AMPK activity

Activation of AMPK has been shown to negatively regulate CFTR and ENaC in multiple epithelial cell models [3-8]. Therefore, we postulated that changes in [Arg^8]-vasopressin -stimulated SCC may involve AMPK as well as PKA. To verify the contribution of each kinase, control cells and those treated with H89 were analyzed for changes in the active, phosphorylated form of AMPK (pAMPK^{thr172}). Acetyl co-A carboxylase (ACC) is a downstream target of AMPK. Accordingly, we used the phosphorylation of ACC at ser^{79} to confirm activation of AMPK. Fig. 2 shows that no changes in pAMPK^{thr172} or pACC^{ser79} were detected in the presence of short-term (10min) or long-term (24hrs) H89 (compare lanes 1, 3, & 5 or 2, 4, & 6). Unexpectedly, stimulation with [Arg^8]-vasopressin resulted in a dramatic reduction of pAMPK^{thr172}, which was translated to pACC^{ser79}. These changes were independent of any changes in the total protein pools of AMPK and ACC (compare lanes 1 & 2). In addition, the dephosphorylation of AMPK and ACC by [Arg^8]-vasopressin was not affected by H89 (compare lanes 3 & 5 or 4 & 6). Taken together these data indicate that [Arg^8]-vasopressin reduces AMPK activity but this effect is not mediated via PKA or cAMP.

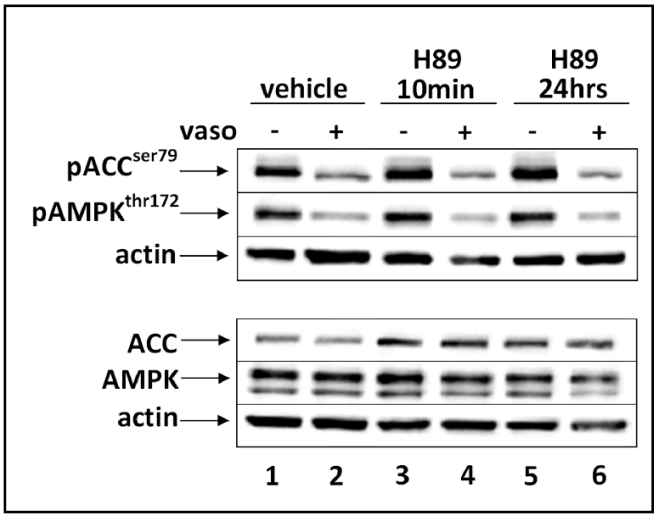

Fig. 2. [Arg^8]-vasopressin dephosphorylates AMPK independently of PKA. Polarized MDCK-C7 cells were treated with either vehicle (DMSO) or H89 (20 μM) bilaterally for 10 min or 24 hrs and stimulated with [Arg^8]-vasopressin (100 mU/mL) for ~20 seconds prior to lysis. Cells were solubilized and proteins were separated by SDS-PAGE and transferred to PVDF in preparation for immunoblotting. PVDFs were cut into thirds and probed for pACC^{ser79} and pAMPK^{thr172} (upper panels) or ACC and AMPK (lower panels). β-actin served as a loading control. Bands corresponding to the predicted molecular weights of ACC & pACC^{ser79} (180 kDa), AMPK & pAMPK^{thr172} (62 kDa) and actin (42 kDa) were detected. This is a representative immunoblot of three individual experiments. The numbers 1-6 refer to the lane number.

Effect of AICAR on [Arg^8]-vasopressin -stimulated ion transport

To further investigate the role of AMPK on [Arg^8]-vasopressin stimulated ion transport we used AICAR, an AMP analogue known to result in the activation of AMPK. Fig. 3A shows that short-term (15 min) incubation with

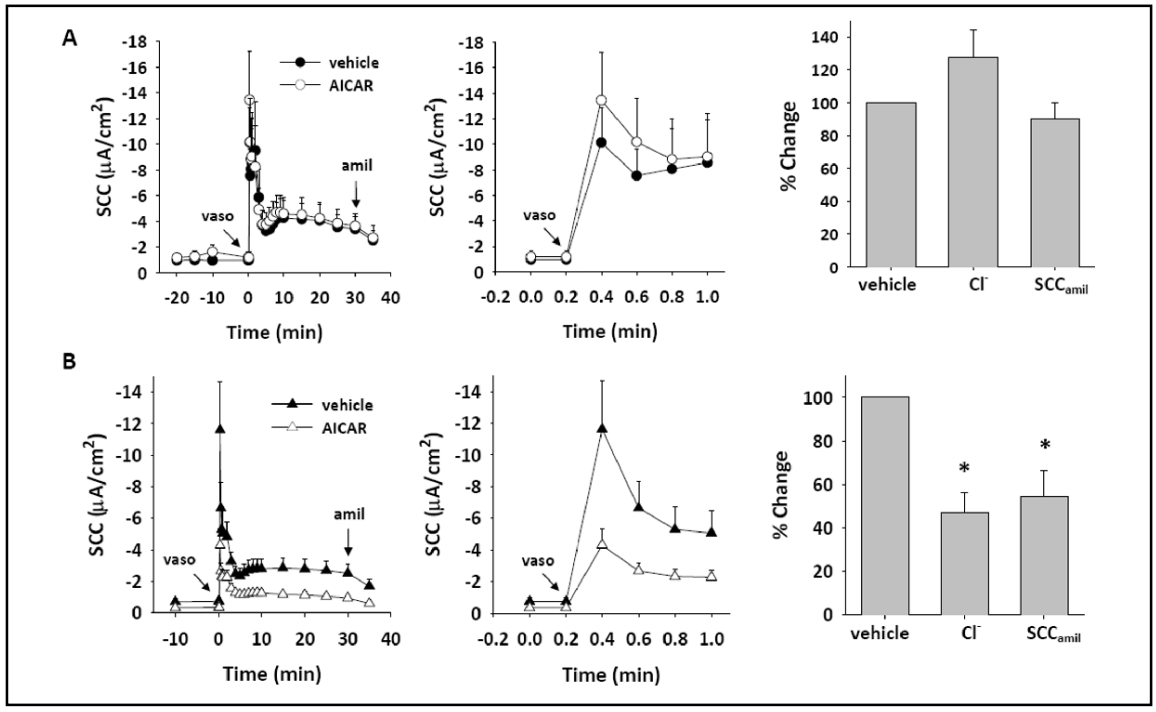

Fig. 3. AICAR inhibits [Arg^8]-vasopressin -stimulated ion transport. Panel A: Polarized MDCK-C7 cells were assembled into Ussing chambers and allowed to stabilize. 15 min prior to stimulation with [Arg^8]-vasopressin (100mU/mL), vehicle (DMSO) or AICAR (1 mM) was added to the apical and serosal bathing compartments. Panel B: While in culture, polarized MDCK-C7 cells were treated as those in Panel A, except for 24 hrs. After assembly into Ussing chambers, vehicle or AICAR was re-added to the apical and serosal bathing compartments. All cells were stimulated with [Arg^8]-vasopressin (100 mU/mL) at t = 0 min on the time scale. Amiloride (10 μM) was added 30 min. later. The entire duration of the electrophysiology experiments are depicted in the left panels. The right panels show an expanded timescale emphasizing the effect of AICAR on Cl^- secretion elicited by the addition of [Arg^8]-vasopressin. In the right panels, the magnitude of Cl^- secretion and amount of SCC_{amil} was calculated as $SCC_{t=0.4min}$ - $SCC_{t=0min}$ and $SCC_{t=35min}$ - $SCC_{t=30min}$, respectively. The magnitudes in AICAR-treated cells were normalized to that in matched vehicle-treated cells. AICAR did not cause any significant changes in transepithelial resistances. Symbols and bars represent the mean ± SEM for 14 individual experiments where *$p \leq 0.02$ by a one-way ANOVA followed by Tukey's post-hoc test. Vaso, [Arg^8]-vasopressin; amil, amiloride.

AICAR was without effect on ion transport induced by [Arg^8]-vasopressin. Conversely, long-term (24 hrs) challenge with AICAR significantly blocked [Arg^8]-vasopressin induced Cl^- secretion (by ~47.1 ± 9.0%) and Na^+ reabsorption (by ~54.3 ± 12.2%) (Fig. 3B). We also permeabilized the basolateral membrane with nystatin, thereby eliminating any ion conductances that alter Cl^- secretion, and imposed a Cl^- gradient across the epithelium. AICAR (24 hrs) significantly inhibited apical Cl^- secretion under permeabilized conditions, suggesting that the compound is affecting the activity of Cl^- channels located in the apical membrane (Fig. 4).

Effect of AICAR on AMPK activity

To verify the involvement of AMPK in the observations described in the previous section, cells treated both short- and long-term with AICAR were analyzed for $pAMPK^{thr172}$ and $pACC^{ser79}$ abundances. It is shown in Fig. 5 that AICAR did not result in any substantial changes in the abundance of $pAMPK^{thr172}$ at either incubation period (compare lanes 1 & 3, 2 & 4, 5 & 7, or 6 & 8). Moreover, [Arg^8]-vasopressin caused a similar decrease in $pAMPK^{thr172}$ and $pACC^{ser79}$ levels in the presence of AICAR as previously observed in the absence of AICAR (refer to Fig. 2). These data indicate that AICAR decreases Cl^- secretion and Na^+ reabsorption independently of AMPK activation.

Effect of AICAR on cAMP

[Arg^8]-vasopressin elevates cAMP and activates PKA to increase CFTR and ENaC activity. We therefore investigated the effect of AICAR on total cellular cAMP

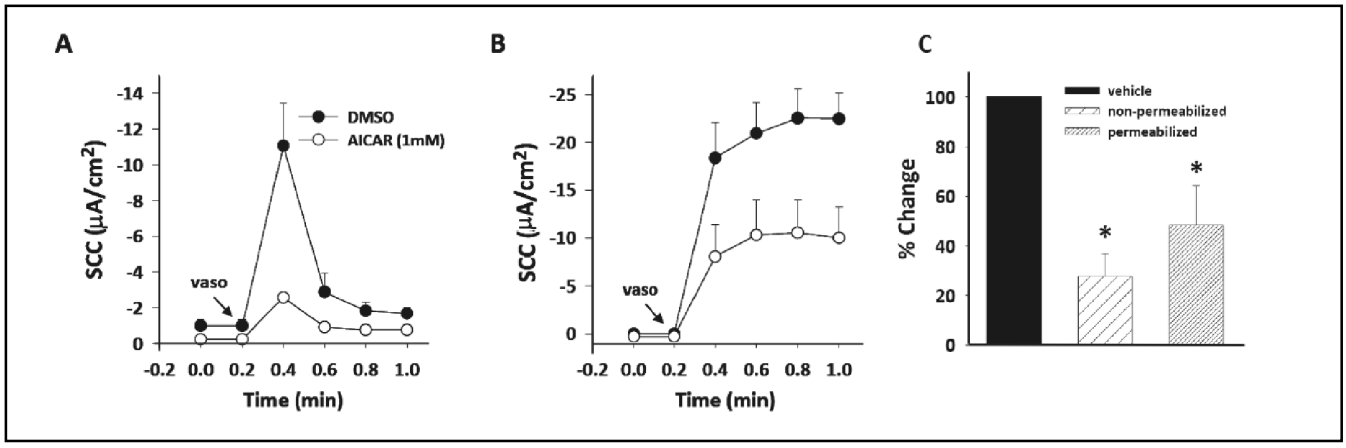

Fig. 4. Basolateral membrane permeabilization does not rescue AICAR-induced inhibition of Cl^- secretion. While in culture, polarized MDCK-C7 cells were treated serosally with DMSO (vehicle, black circles) or AICAR (1mM, white circles) for 24 hrs. Cells were subsequently assembled into Ussing chambers. Cultures in panel A were bathed in symmetrical Ringers solution ($[Cl^-]$ = 150 mM). Cultures in panel B were bathed in asymmetrical Ringers solution ($[Cl^-]_{apical}$ = 15 mM, $[Cl^-]_{serosal}$ = 150 mM) and treated with nystatin (280 U/mL) 30 min. prior to hormone stimulation. All cultures were treated apically with amiloride 10 min. prior to stimulation with $[Arg^8]$-vasopressin (at time = 0 min) to block Na^+ reabsorption via ENaC. Panel C: The magnitude of $[Arg^8]$-vasopressin-stimulated Cl^- transport in AICAR-treated cultures was normalized to that of the respective (permeabilized or non-permeabilized) vehicle-treated cultures and expressed as a percent. AICAR did not cause any significant changes in transepithelial resistance. Bar graphs represent the mean ± SEM of 3 experiments, where *$p \leq 0.05$ compared to the vehicle-treated as determined by a one-way ANOVA followed by Tukey's post-hoc test. The magnitude of Cl^- secretion from non-permeabilized cultures treated with AICAR was not statistically different from that in permeabilized cultures. Vaso = $[Arg^8]$-vasopressin.

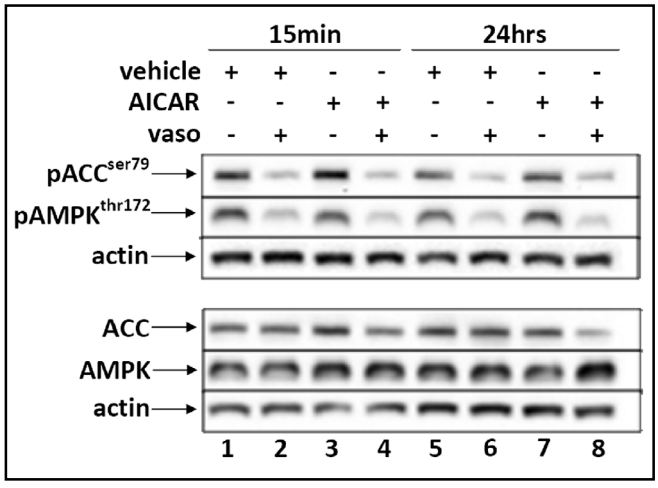

Fig. 5. AICAR does not regulate AMPK phosphorylation in MDCK-C7 cells. Polarized MDCK-C7 cells were treated either with vehicle (DMSO) or AICAR (1mM) bilaterally for 15 minutes or 24 hrs and stimulated with $[Arg^8]$-vasopressin (100mU/mL) for ~20 seconds prior to lysis. Cells were solubilized and proteins were separated by SDS-PAGE and transferred to PVDF in preparation for immunoblotting. PVDFs were cut into thirds and probed for $pACC^{ser79}$ and $pAMPK^{thr172}$ (upper panels) or ACC and AMPK (lower panels). β-actin served as a loading control. Bands corresponding to the predicted molecular weights of ACC & $pACC^{ser79}$ (180 kDa), AMPK & $pAMPK^{thr172}$ (62 kDa) and actin (42 kDa) were detected. This is a representative immunoblot of three individual experiments. The numbers 1-8 refer to the lane number.

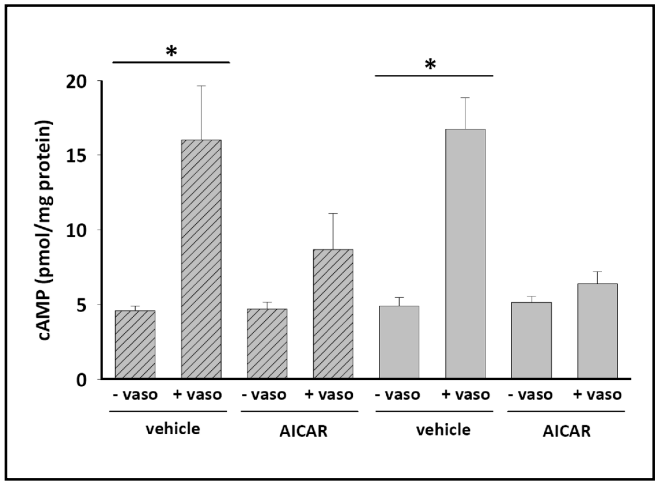

Fig. 6. AICAR inhibits cAMP production. Polarized MDCK-C7 cells were treated with vehicle (DMSO) or AICAR (1 mM) bilaterally for 15 min (hashed bars) or 24 hrs (solid bars), stimulated with or without $[Arg^8]$-vasopressin (100 mU/mL) for ~20 sec and assayed for cAMP content. Bars represent the means ± SEM for 6 individual experiments. *$p \leq 0.05$ by a one-way ANOVA followed by Tukey's post-hoc test.

levels. Surprisingly, both short- and long-term challenge with AICAR inhibited cAMP production following $[Arg^8]$-vasopressin stimulation (Fig. 6). These results are only partially consistent with the ion transport results described

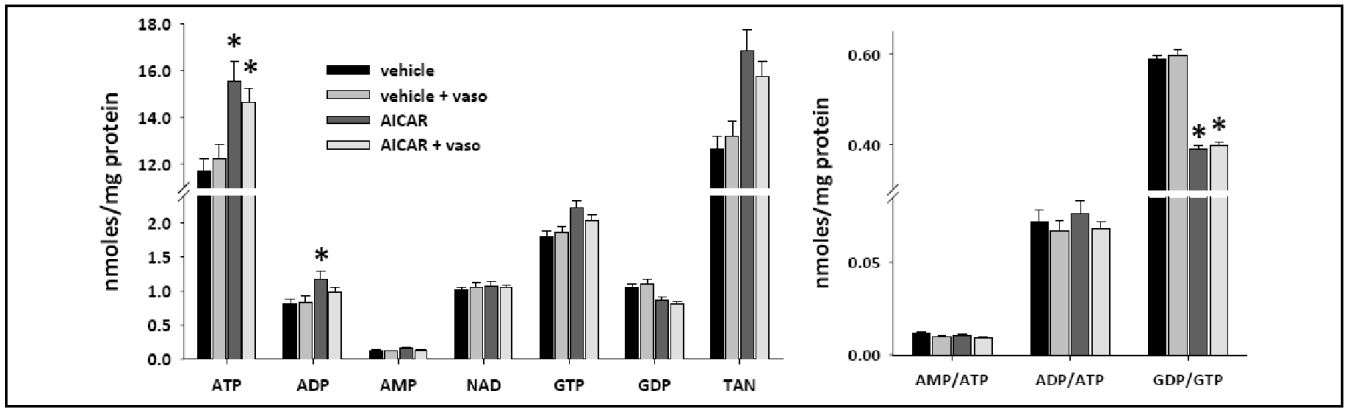

Fig. 7. Longterm AICAR treatment lowers GDP/GTP but does not affect total adenine nucleotide levels. Polarized MDCK-C7 cells were treated bilaterally for 24 hrs with vehicle (DMSO) or AICAR (1 mM) and stimulated with or without [Arg^8]-vasopressin (100 mU/mL) for ~20 sec. Cells were prepared for nucleotide extraction and HPLC. Nucleotide levels were normalized to the amount of protein in each sample. Bars represent the means ± SEM for 3 individual experiments containing 3 replicates each. *p ≤ 0.05 by a one-way ANOVA followed by Tukey's post-hoc test. ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; NAD, nicotinamide dinucleotide; GTP, guanosine triphosphate; GDP, guanosine diphosphate; TAN, total adenine nucleotide.

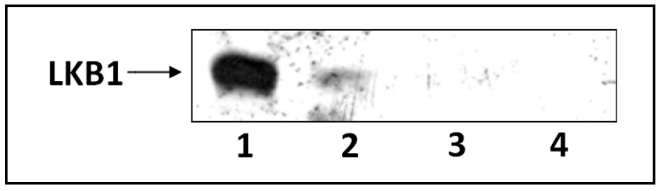

Fig. 8. LKB1 is not expressed in three separate principal cell lines. Polarized H441 (lane 1), A6 (lane 2), mpkCCD$_{cl4}$ (lane 3), and MDCK-C7 (lane 4) cells were solubilized. Equal amounts of protein were separated by SDS-PAGE and transferred to PVDF in preparation for immunoblotting. The predicted molecular weight of LKB1 is 48 kDa, however, the antibody used to identify the protein is advertised to detect a 55 kDa band. A prominent band of ~56 kDa was detected in H441 cells. A faint band was detected in A6 cells, and no band was detected in either the mpkCCD$_{cl4}$ or MDCK-C7 cells. This image is representative of one experiment.

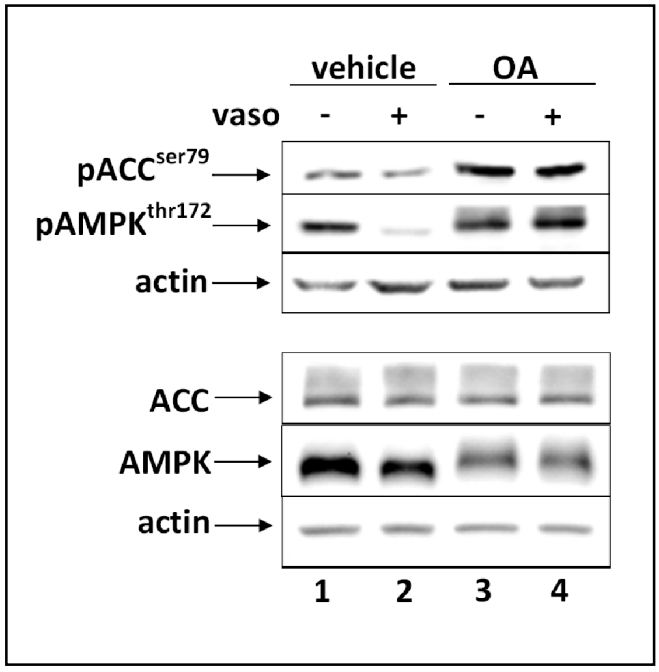

Fig. 9. Okadaic acid prevents AMPK and ACC dephosphorylation. Polarized MDCK-C7 cells were treated with vehicle (DMSO) or okadaic acid (1 μM) bilaterally for 30 minutes and stimulated with or without [Arg^8]-vasopressin (100 mU/mL) for ~20 seconds prior to lysis. Cells were solubilized and proteins were separated by SDS-PAGE and transferred to PVDF in preparation for immunoblotting. PVDFs were cut into thirds and probed for pACC^{ser79} and pAMPK^{thr172} (upper panels) or ACC and AMPK (lower panels). β-actin served as a loading control. Bands corresponding to the predicted molecular weights of ACC & pACC^{ser79} (180 kDa), AMPK & pAMPK^{thr172} (62 kDa) and actin (42 kDa) were detected. This is a representative immunoblot of three individual experiments. The numbers 1-4 refer to the lane number.

in Fig. 3, where only long-term AICAR treatment, but not short-term, significantly blocked ion transport.

Effect of AICAR and [Arg^8]-vasopressin on cellular nucleotides

The cellular nucleotide pool has been demonstrated to change in cells treated with AICAR. We investigated whether such changes may underlie the effects elicited by AICAR. Long-term AICAR treatment caused a significant increase in total adenine nucleotide concentrations, which was not altered after [Arg^8]-vasopressin stimulation (Fig. 7). This can be attributed to an increase in both AMP and ATP; however, no changes in the AMP/ATP ratio were detected. AICAR signifi-

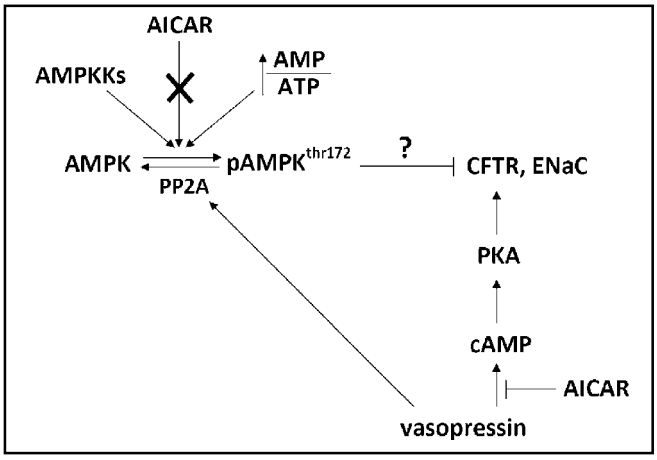

Fig. 10. Schematic representation of [Arg^8]-vasopressin and AICAR regulation of AMPK, ENaC and CFTR in MDCK-C7 cells. [Arg^8]-vasopressin stimulates CFTR and ENaC activity by increasing the production of cAMP, which in turn activates PKA. H89 inhibits PKA activity. In the current studies, AICAR inhibits the production of cAMP. AMPK can be activated by increases in AMP/ATP, followed by phosphorylation by AMPKKs. Conversely, AMPK can be de-activated by dephosphorylation via PP2A. In the current studies, AMPK is not activated by AICAR. [Arg^8]-vasopressin dephosphorylates AMPK, probably by PP2A activation. It is still unknown what role AMPK plays in regulation of CFTR and ENaC activity in MDCK-C7 cells.

cantly decreased GDP concentrations and increased GTP concentrations, resulting in a reduced GDP/GTP ratio. In addition, [Arg^8]-vasopressin, did not alter any nucleotide concentrations. Thus, the [Arg^8]-vasopressin -induced dephosphorylation of AMPK was not via a decrease in the AMP/ATP ratio, which would result in an allosteric change in AMPK, making this less able to be phosphorylated by AMPKKs.

MDCK-C7 cells do not express LKB1

It was unexpected that AICAR did not increase pAMPKthr172 or cellular nucleotide levels. AICAR-induced activation of AMPK is thought to require the upstream kinase, LKB1. Therefore, we hypothesized that this protein may not be expressed in MDCK-C7 cells. Fig. 8 shows that no LKB1 could be detected in the MDCK-C7 cells. Moreover, the *Xenopus laevis* A6 and mpkCCD$_{cl4}$ (mouse principal kidney cortical collecting duct clone 4) cell lines, both of which model the principal cell type, showed little to no LKB1 expression. The protein was however, detected in H441 cells, as previously shown [7].

Effect of okadaic acid on [Arg^8]-vasopressin induced dephosphorylation of AMPK

As [Arg^8]-vasopressin had no effect on cellular nucleotide pools, we turned our attention to the role of the ser/thr phosphatases in dephosphorylation of AMPK. There are 2 families-phosphatase 1 (PP1) and phosphatase 2 (PP2)-the latter being composed of 3 sub-families, 2A, 2B, and 2C. Okadaic acid (OA) is a well-known phosphatase inhibitor, and can be used at concentrations of up to 1 μM to inhibit PP2A independently from PP1s, PP2B, or PP2C [22]. As expected, acute (30 min.) incubation with OA resulted in substantial increases in both pAMPK^{thr172} and pACC^{ser79} abundances (Fig. 9). This verifies that PP2A mediates, at least in part, the dephosphorylation of pAMPK^{thr172} to AMPK. Moreover, OA prevented [Arg^8]-vasopressin -induced dephosphorylation of AMPK indicating that [Arg^8]-vasopressin could dephosphorylate AMPK via activation of PP2A.

Discussion

It is well established that [Arg^8]-vasopressin orchestrates its effects on Cl^- and Na^+ transport (through CFTR and ENaC, respectively) via elevation of cAMP and activation of PKA. H89 blocks PKA activity by competing with the ATP-binding site of the kinase [23] and, consistent with previous studies, we showed that inhibition of PKA activity inhibited [Arg^8]-vasopressin-induced rises in Cl^- and Na^+ transport in MDCK-C7 cells. Interestingly, we also found that [Arg^8]-vasopressin caused a dramatic decrease in pAMPK^{thr179} levels; an effect that is independent of PKA since the dephosphorylation was evident in the presence of H89. Since phosphorylation of AMPK in epithelial cells has been shown to decrease Na^+ and Cl^- transport [3-8] these data would support a coordinated response to [Arg^8]-vasopressin whereby there is downregulation of an inhibitory pathway (AMPK) together with an upregulation of a stimulatory pathway (PKA).

The role of AMPK in ion transport regulation was further studied using AICAR-a pharmacological activator of AMPK. SCC studies showed that 15 minute treatment with AICAR did not significantly alter [Arg^8]-vasopressin-stimulated ion transport. While this was unexpected, as others have shown decreases in Cl^- currents within 2 hrs of AICAR treatment [4], a 24 hr AICAR treatment did inhibit [Arg^8]-vasopressin-stimulated Cl^- and Na^+ flux. AICAR uptake into the cell occurs via nucleoside transporters, and the compound is then phosphorylated

into ZMP by adenosine kinase [24]. ZMP activates AMPK independently of changes in the AMP/ATP ratio.

Previously published literature showed that AICAR inhibits forskolin-stimulated Cl^- currents via activation of AMPK [4, 5]. Unexpectedly, we could not detect substantial changes in AMPK activation (as defined by changes in $pAMPK^{thr179}$) after short- or long-term AICAR challenge. These data suggest that in MDCK-C7 cells, AICAR inhibits ion transport via an AMPK-independent mechanism. While this is the first report of such a mechanism in epithelial cells, AICAR-mediated, AMPK-independent apoptosis has been described in Jurkat cells [25]. Furthermore, the biological effects of AICAR in Jurkat cells did not rely on the conversion of the activator into ZMP. In addition, inhibition of adenosine kinase prevented the activation of AMPK in human polarized retinal pigment epithelial cells [26]. Therefore, low adenosine kinase activity in MDCK-C7 cells may account for AICAR being unable to activate AMPK. We found that AICAR caused a significant increase in both ATP and ADP, which is in direct contrast to a recent study performed in H441 cells where AICAR significantly decreased ATP and ADP [8]. AICAR can be further metabolized into ATP, ADP, and AMP in the cell as it is an intermediate of the *de novo* purine biosynthetic pathway [27]. Thus, our data would support AICAR conversion to ZMP in MDCK-C7 cells. Why then AICAR does not activate AMPK in MDCK-C7 cells is not known; however, AICAR activation of AMPK is thought to require the upstream kinase, LKB1. We could not detect LKB1 in the MDCK-C7 cells nor two other principal cell models, the A6 and $mpkCCD_{cl4}$ cell lines. The antibody used against LKB1 is reported to cross-react with human, mouse, and rat. Therefore, the antibody may not recognize dog LKB1. This is, however, unlikely, in that no LKB1 was detected in the mouse $mpkCCD_{cl4}$ (an alternative principal cell line) cells. Thus, it is possible that the absence of LKB1 and/or other upstream kinases prevents AMPK activation by AICAR in the MDCK-C7 cells.

As $[Arg^8]$-vasopressin elevates cAMP to stimulate ion transport, we speculated that 24 hr AICAR challenge may alter cellular cAMP levels. Surprisingly, both short- and long-term AICAR treatment inhibited cAMP production after $[Arg^8]$-vasopressin stimulation. There was a small rise in cAMP in response to $[Arg^8]$-vasopressin with 15 min AICAR treatment. Even though the slight increase in cAMP by $[Arg^8]$-vasopressin was not significantly different from control-treated cells, there may still have been enough cAMP to activate ion transport. Walker et al showed that 48 hour treatment with AICAR lowered forskolin-stimulated Cl^- currents in mouse colon and decreased apical CFTR expression in T-84 cells-effects that were shown without demonstrating activation of AMPK [6]. Such an effect of AICAR (to lower apical CFTR expression) is consistent with the permeabilization studies described in Fig. 4. Therefore, our observation of decreased Cl^- current with 24 hr AICAR challenge may be a combined effect of lowered cAMP production and CFTR expression.

Earlier published reports state that PP2A mediates the dephosphorylation of AMPK at thr^{172} [22] and that PP2A is activated by cAMP-elevating agents [28, 29]. We confirmed that PP2A dephosphorylates AMPK in MDCK-C7 cells using the phosphatase inhibitor, OA. Moreover, $[Arg^8]$-vasopressin-induced dephosphorylation of AMPK is reversed by OA, indicating that this effect is mediated by PP2A. However, the vasopressin stimulated dephosphorylation of AMPK is independent of cAMP as demonstrated by the results in Figure 6 showing vasopressin-induced dephosphorylation in the presence of AICAR which inhibits cAMP production. Thus, we speculate that $[Arg^8]$-vasopressin may activate PP2A by a mechanism that does not rely on increases in cAMP.

In conclusion, $[Arg^8]$-vasopressin stimulates Cl^- and Na^+ transport in MDCK-C7 cells via elevation of cAMP and activation of PKA, and potentially, as shown here, by inhibition of AMPK. $[Arg^8]$-vasopressin resulted in a dramatic down-regluation of $pAMPK^{thr179}$, potentially via activation of PP2A. AICAR decreased $[Arg^8]$-vasopressin induced Cl^- and Na^+ transport by preventing the elevation of cAMP without activation of AMPK. A schematic representation of these observations is shown in Fig. 10. This is the first description in epithelial cells that $[Arg^8]$-vasopressin is a potent regulator of AMPK phosphorylation and that AICAR can modify ion transport independently of AMPK activation. These data show that the signaling pathways regulating ion channel function in epithelial cells are undoubtedly complex and vary considerably between cell types. More work will be required to elucidate the interactions between these pathways. In the interim, in the light of these data, we suggest that biological effects attributed to AICAR (and thus AMPK) must be analyzed with caution.

Acknowledgements

The authors would like to sincerely thank Dr. Christie Thomas (University of Iowa) for preparing the H441 cell lysate used for Fig. 8.

References

1 Snyder PM, Olson DR, Kabra R, Zhou R, Steines JC: cAMP and serum and glucocorticoid-inducible kinase (SGK) regulate the epithelial Na^+ channel through convergent phosphorylation of nedd4-2. J Biol Chem 2004;279:45753-45758.

2 Tabcharani JA, Chang XB, Riordan JR, Hanrahan JW: Phosphorylation-regulated Cl^- channel in CHO cells stably expressing the cystic fibrosis gene. Nature 1991;352:628-631.

3 Carattino MD, Edinger RS, Grieser HJ, Wise R, Neumann D, Schlattner U, Johnson JP, Kleyman TR, Hallows KR: Epithelial sodium channel inhibition by AMP-activated protein kinase in oocytes and polarized renal epithelial cells. J Biol Chem 2005;280:17608-17616.

4 Hallows KR, Kobinger GP, Wilson JM, Witters LA, Foskett JK: Physiological modulation of CFTR activity by AMP-activated protein kinase in polarized T84 cells. Am J Physiol Cell Physiol 2003;284:C1297-1308.

5 Hallows KR, Raghuram V, Kemp BE, Witters LA, Foskett JK: Inhibition of cystic fibrosis transmembrane conductance regulator by novel interaction with the metabolic sensor AMP-activated protein kinase. J Clin Invest 2000;105:1711-1721.

6 Walker J, Jijon HB, Churchill T, Kulka M, Madsen KL: Activation of AMP-activated protein kinase reduces cAMP-mediated epithelial chloride secretion. Am J Physiol Gastrointest Liver Physiol 2003;285:G850-860.

7 Woollhead AM, Scott JW, Hardie DG, Baines DL: Phenformin and 5-aminoimidazole-4-carboxamide-1-beta-d-ribofuranoside (AICAR) activation of AMP-activated protein kinase inhibits transepithelial Na^+ transport across H441 lung cells. J Physiol 2005;566:781-792.

8 Woollhead AM, Sivagnanasundaram J, Kalsi KK, Pucovsky V, Pellatt LJ, Scott JW, Mustard KJ, Hardie DG, Baines DL: Pharmacological activators of AMP-activated protein kinase have different effects on Na^+ transport processes across human lung epithelial cells. Brit J Pharm 2007;151:1204-1215.

9 Fraser SA, Gimenez I, Cook N, Jennings I, Katerelos M, Katsis F, Levidiotis V, Kemp BE, Power DA: Regulation of the renal-specific Na^+-K^+-$2Cl^-$ co-transporter NKCC2 by AMP-activated protein kinase (AMPK). Biochem J 2007;405:85-93.

10 Hardie DG, Hawley SA, Scott JW: AMP-activated protein kinase-development of the energy sensor concept. J Physiol 2006;574:7-15.

11 Hardie DG: AMP-activated protein kinase as a drug target. Annu Rev Pharmacol Toxicol 2007;47:185-210.

12 Blazer-Yost BL, Record RD, Oberleithner H: Characterization of hormone-stimulated Na^+ transport in a high-resistance clone of the MDCK cell line. Pflugers Arch 1996;432:685-691.

13 Lahr TF, Record RD, Hoover DK, Hughes CL, Blazer-Yost BL: Characterization of the ion transport responses to ADH in the MDCK-C7 cell line. Pflugers Arch 2000;439:610-617.

14 Wunsch S, Gekle M, Kersting U, Schuricht B, Oberleithner H: Phenotypically and karyotypically distinct Madin-Darby canine kidney cell clones respond differently to alkaline stress. J Cell Physiol 1995;164:164-171.

15 Crawford I, Maloney PC, Zeitlin PL, Guggino WB, Hyde SC, Turley H, Gatter KC, Harris A, Higgins CF: Immunocytochemical localization of the cystic fibrosis gene product CFTR. Proc. Natl Acad Sci USA 1991;88:9262-9266.

16 Larbig M, Jansen S, Dorsch M, Bernhard W, Bellmann B, Dorin JR, Porteous DJ, Von Der Hardt H, Steinmetz I, Hedrich HJ, Tuemmler B, Tschernig T: Residual CFTR expression varies with age in CFTR(tm1Hgu) cystic fibrosis mice: Impact on morphology and physiology. Pathobiol 2002;70:89-97.

17 Morales MM, Carroll TP, Morita T, Schwiebert EM, Devuyst O, Wilson PD, Lopes AG, Stanton BA, Dietz HC, Cutting GR, Guggino WB: Both the wild type and a functional isoform of CFTR are expressed in kidney. Am. J. Physiol Renal Physiol 1996;270:F1038-1048.

18 Stanton BA: Cystic fibrosis transmembrane conductance regulator (CFTR) and renal function. Wiener Klinische Wochenschrift 1997;109:457-464.

19 Todd-Turla KM, Rusvai E, Naray-Fejes-Toth A, Fejes-Toth G: CFTR expression in cortical collecting duct cells. Am J. Physiol Renal Physiol 1996;270:F237-244.

20 Kalsi KK, Smolenski RT, Pritchard RD, Khaghani A, Seymour AM, Yacoub MH: Energetics and function of the failing human heart with dilated or hypertrophic cardiomyopathy. Eur J Clin Invest 1999;29:469-477.

21 Smolenski RT, Lachno DR, Ledingham SJ, Yacoub MH: Determination of sixteen nucleotides, nucleosides and bases using high-performance liquid chromatography and its application to the study of purine metabolism in hearts for transplantation. J Chromatog 1990;527:414-420.

22 Millward TA, Zolnierowicz S, Hemmings BA: Regulation of protein kinase cascades by protein phosphatase 2A. Trends Biochem Sci 1999;24:186-191.

23 Chijiwa T, Mishima A, Hagiwara M, Sano M, Hayashi K, Inoue T, Naito K, Toshioka T, Hidaka H: Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, n-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. J Biol Chem 1990;265:5267-5272.

24 Henin N, Vincent MF, Van den Berghe G: Stimulation of rat liver AMP-activated protein kinase by AMP analogues. Biochim Biophys Acta 1996;1290:197-203.

25 Lopez JM, Santidrian AF, Campas C, Gil J: 5-aminoimidazole-4-carboxamide riboside induces apoptosis in jurkat cells, but the AMP-activated protein kinase is not involved. Biochem J 2003;370:1027-1032.

26 Qin S, De Vries GW: Alpha2 but not alpha1 AMP-activated protein kinase mediates oxidative stress-induced inhibition of retinal pigment epithelium cell phagocytosis of photoreceptor outer segments. J Biol Chem 2008;283:6744-6751.

27 Vincent MF, Marangos PJ, Gruber HE, Van den Berghe G: Inhibition by AICA riboside of gluconeogenesis in isolated rat hepatocytes. Diabetes 1991;40:1259-1266.

28 Feschenko MS, Stevenson E, Nairn AC, Sweadner KJ: A novel cAMP-stimulated pathway in protein phosphatase 2A activation. J Pharmacol Exp Ther 2002;302:111-118.

29 Hurley RL, Barre LK, Wood SD, Anderson KA, Kemp BE, Means AR, Witters LA: Regulation of AMP-activated protein kinase by multisite phosphorylation in response to agents that elevate cellular camp. J Biol Chem 2006;281:36662-36672.