

# Hormonal regulation of the epithelial Na<sup>+</sup> channel: From amphibians to mammals

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## Abstract

High-resistance epithelia derived from amphibian sources such as frog skin, toad urinary bladder, and the A6 *Xenopus laevis* kidney cell line have been widely used to elucidate the underlying mechanisms involved in the regulation of vectorial ion transport. More recently, the isolation of high-resistance mammalian cell lines has provided model systems in which to study differences and similarities between the regulation of ion transporter function in amphibian and mammalian renal epithelia. In the present study, we have compared the natriuretic (Na<sup>+</sup> retaining) responses to aldosterone, insulin, and vasotocin/vasopressin in the A6 and mpkCCD<sub>cl4</sub> (mouse principal cells of the kidney cortical collecting duct) cell lines. The functional responses of the epithelial Na<sup>+</sup> channel (ENaC) to hormonal stimulation were remarkably similar in both the amphibian and mammalian lines. In addition, insulin- and aldosterone-stimulated, reabsorptive Na<sup>+</sup> transport in both cell lines requires the presence of functional PI3-kinase.

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**Keywords:** Aldosterone; Insulin; Vasopressin; Vasotocin; A6; mpkCCD<sub>cl4</sub>; ENaC; Kidney

## 1. Introduction

All terrestrial vertebrates face a constant challenge in maintaining proper osmolarity of body fluids. This challenge is met physiologically by regulating the balance between the intake and loss of salt and water. Regulation of the multimeric epithelial Na<sup>+</sup> channel (ENaC) is a key factor in maintenance of salt and water homeostasis. Under-scoring the importance of this channel is the finding that three hormones, aldosterone, insulin, and arginine vasopressin (AVP), found in most mammals or arginine vasotocin (AVT), found in other vertebrates, directly control the activity of ENaC. In mammals, this channel plays a role in the regulation of salt and fluid balance in several organs, notably the lung, intestine, sweat duct, and kidney. It is in the latter organ where whole body homeostasis is maintained, predominately via hormonal control of reabsorptive Na<sup>+</sup> movement through ENaC. Amphibians also express

highly regulated salt and water transporters, including ENaC, in order to maintain a balance with the external environment. In amphibians, homeostatic control of Na<sup>+</sup> transport takes place across the epithelial cells of the skin and urinary bladders as well as via the kidney.

For humans, renal regulation of Na<sup>+</sup> is key to maintaining normal blood pressure. The high incidence of salt-sensitive hypertension in Western societies attests to problems with the efficiency of the system when faced with salt excess. The importance of studies focusing on the elucidation of pathways involved in ENaC regulation has been substantiated by the finding of rare monogenetic human diseases that are the result of mutated ENaC subunits. Naturally occurring gain-of-function mutations result in serious hypertension in toddlers while loss-of-function mutations result in hypotension and salt wasting in the neonatal period (Chang et al., 1996; Shimkets et al., 1994, respectively).

Since the pioneering studies of Hans Ussing and his colleagues (Koefoed-Johnsen and Ussing, 1958; Ussing and Zerahn, 1951), amphibian tissues have served as model systems for the study of ion transport in polarized,

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high-resistance epithelia. For many years, frog skin, toad bladders, and the A6 cell line derived from the *Xenopus laevis* kidney were the models used to investigate the functional characteristics of transport proteins responsible for vectorial ion movement, including ENaC. These model systems were also used to characterize the unique biochemical characteristics distinguishing the apical from the basolateral membranes, as well as the nature of the tight junctions, which maintain selective differences between the two membranes.

Isolated amphibian epithelia have proven to be remarkably applicable models of the transport characteristics of the principal cells of the mammalian distal nephron in that they respond to physiologically relevant concentrations of all hormones that normally control ENaC activity (Crabbe, 1961; Handler, 1983; Herrera, 1965; Leaf and Dempsey, 1960; Rafferty, 1969; Singer et al., 1969). The complexity of the mammalian kidney makes correlative functional and biochemical experiments difficult to perform, thus much of our knowledge regarding hormonal control of Na<sup>+</sup> transport was first discovered in isolated amphibian epithelia and subsequently confirmed in mammalian tissues. After confirmation of each component of regulation, investigators often returned to the amphibian tissues to explore additional aspects of the complexities of multiple hormonal regulation of this single transporter.

More recently, continuous lines representing the mammalian renal principal cell type have been derived. These model systems express the characteristics defining the principal cell type in vivo, including a high-resistance phenotype and natriferic (Na<sup>+</sup> retaining) responses to aldosterone, insulin, and AVP. We have examined hormonal responses in multiple mammalian lines: Madin–Darby canine kidney (MDCK)—C7 subclone, M1 mouse cortical collecting duct line, and mouse principal cells of the kidney cortical collecting duct (mpkCCD<sub>cl4</sub>) (Blazer-Yost et al., 1996; Lahr et al., 2000; Nofziger et al., 2005a,b; Wagner et al., 2005). Of these, the mpkCCD<sub>cl4</sub> line exhibits the most robust responses. From a renal perspective, we are now in a position to directly compare the amphibian principal cell models with their mammalian counterparts. In the current studies, we compare and contrast hormonal responses in the A6 amphibian cell line with those of the mpkCCD<sub>cl4</sub> line.

## 2. Methods and materials

### 2.1. Materials

Stock hormone solutions were prepared 1000-fold concentrated as follows: bovine insulin (Sigma Chemical, St. Louis, MO) in 10 mM HCl, and aldosterone (Acros Organics purchased through Fisher Chemical, Chicago, IL) in methanol. Arginine vasopressin (Sigma Chemical) stock solution was purchased as an aqueous solution. Arginine vasotocin (Sigma Chemical) was purchased as a salt and prepared in serum-free media. LY294002 (2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one) and 5-nitro-2-(3-phenylpropyl amino)-benzoic acid (NPPB) (Biomol, Plymouth Meeting, PA) were prepared in DMSO. Amiloride (Sigma Chemical) stock was prepared in distilled water.

### 2.2. Cell culture

mpkCCD<sub>cl4</sub> cells were grown at either 33 or 37 °C in a humidified incubator gassed with 5% CO<sub>2</sub>. Mammalian culture media were Dulbecco's modified Eagle's medium (DMEM); Ham's F12 base media (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (ICN Biochemicals, Irvine, CA), 1 mM Glutamax (Invitrogen), 25 U/mL penicillin, 25 mg/mL streptomycin, and 12 mg/L ciprofloxacin (Voigt Global Distribution; Kansas City, MO).

The A6 cell line was grown at 27 °C in a humidified incubator gassed with 4.5% CO<sub>2</sub>. Culture media consisted of 7 parts Coon's F12 High Zinc and 3 parts Leibovitz's L15 (Irvine Scientific; Santa Ana, CA), supplemented with 10% calf serum (ICN Biochemicals), 25 mM NaHCO<sub>3</sub>, 25 U/mL penicillin, 25 mg/mL streptomycin, 1 mM Glutamax, and 12 mg/L ciprofloxacin.

Cell lines were maintained in plastic culture flasks with thrice weekly feeding. For electrophysiological experiments, cells were subcultured onto 24-mm Transwell inserts (CoStar, Fisher Chemical) in 6-well plates for at least 14 days.

### 2.3. Electrophysiology

Ion flux across the cellular monolayers was determined by the electrophysiological method of short-circuit current (Koefoed-Johnsen and Ussing, 1958). During electrophysiological experiments, monolayers were mounted in a modified water-jacketed Ussing chamber and connected to a voltage clamp apparatus via electrodes. Cells were bathed in serum-free media maintained at 37 °C (mammalian) or 27 °C (amphibian) and circulated by a 5% CO<sub>2</sub>–95% O<sub>2</sub> gas lift. The spontaneous transepithelial potential difference was clamped to zero and the short-circuit current ( $I_{SC}$ ) was monitored as a measure of net ion transport. Transepithelial resistance was measured throughout each experiment by applying 2 mV pulses across the monolayer every 200 s and measuring the resultant current deflection. Only monolayers with a transepithelial resistance >1000 Ω cm<sup>2</sup> were used in statistical analyses. Monolayers were allowed to reach a stable, relatively unchanging baseline prior to the addition of effectors. Insulin (100 nM), AVP/AVT (4.4 × 10<sup>-8</sup> M or various concentrations for dose-response studies) or aldosterone (2.7 μM) were added to the serosal bathing media only. The time of hormone addition is defined as time zero in all experiments. In phosphoinositide signaling pathway experiments, LY294002 (50 μM) was added bilaterally. The addition of amiloride (10 μM) to the apical bathing media at the end of each experiment verified the amount of  $I_{SC}$  due to net Na<sup>+</sup> flux via ENaC. In some experiments, NPPB (100 μM) was added bilaterally following amiloride addition in order to determine the amount of  $I_{SC}$  due to Cl<sup>-</sup> secretion.

Statistical comparisons were performed using either an unpaired Student's *t* test, where  $p \leq 0.05$  between groups was considered statistically significant, or a one-way analysis of variance (ANOVA) followed by a Tukey HSD post hoc test, where  $p \leq 0.01$  between groups was considered statistically significant.

## 3. Results

Aldosterone, a steroid hormone, is the final effector of the renin–angiotensin–mineralocorticoid axis and exerts its action on epithelial cells expressing the mineralocorticoid receptor. Findings from previous studies of the dose–response relationship of aldosterone in these cell lines (Bens et al., 1999; Lyoussi and Crabbe, 1996) provided a maximal concentration used to compare the natriferic responses in the A6 amphibian kidney cell line to those observed in the mpkCCD<sub>cl4</sub> cell line (Fig. 1).

The effect of insulin on transepithelial Na<sup>+</sup> transport has been previously demonstrated in amphibian model systems (Herrera, 1965; Record et al., 1996) as well as in whole

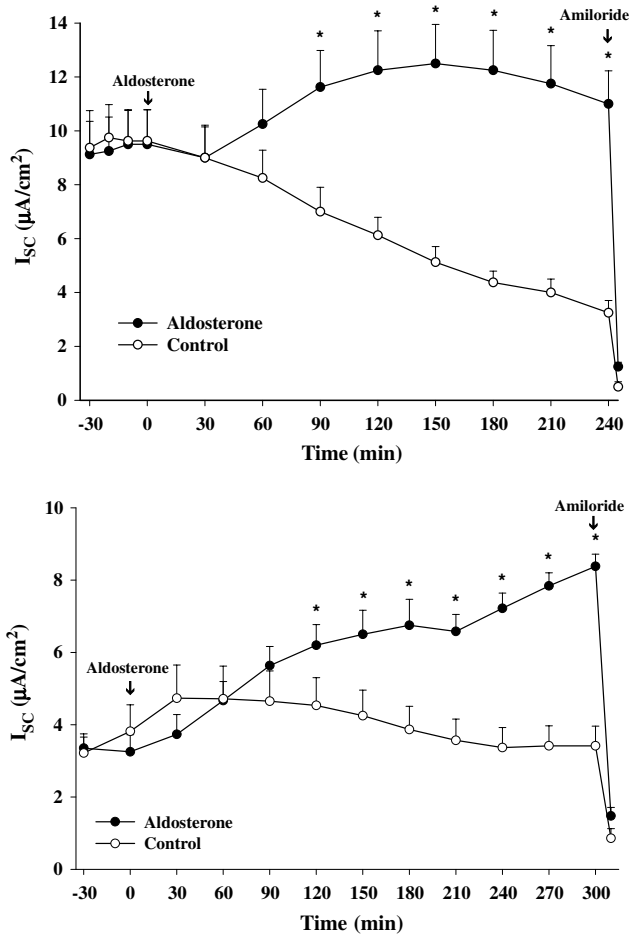


Fig. 1. Aldosterone-induced  $Na^+$   $I_{SC}$  in renal principal cells. In each series of experiments, a maximal dose of aldosterone ( $2.7 \mu M$ ) was added at  $time = 0 \text{ min}$  to the serosal bathing media of monolayers formed by either the mpkCCD<sub>cl4</sub> (upper panel) or A6 cell line (lower panel). Amiloride ( $10 \mu M$ ) was added apically at the end of each experiment. Symbols represent the mean of  $n$  experiments  $\pm$  SEM. As determined by Student's  $t$  test, aldosterone caused a significant increase in reabsorptive  $Na^+$  flux beginning at 90 and 120 min post-hormone addition in mpkCCD<sub>cl4</sub> ( $n = 8$ ) and A6 ( $n = 6$ ) cells, respectively.  $*p \leq 0.05$ .

animals (DeFronzo, 1981) and in the mpkCCD<sub>cl4</sub> cell line (Nofziger et al., 2005b). The goal of the present study was to compare natriuretic responses of the A6 and mpkCCD<sub>cl4</sub> lines to a maximal dose of insulin. The insulin-stimulated increase in  $I_{SC}$  observed in each cell line is shown in Fig. 2.

We have previously shown that, in the A6 cell line, basal-, insulin-, and aldosterone-stimulated transport are mediated via the phosphoinositide (PI) signaling pathway (Blazer-Yost et al., 1999; Record et al., 1998). Because these studies have not been previously extended to mammalian principal cell lines, we used the mpkCCD<sub>cl4</sub> cells to explore the effect of inhibiting phosphoinositide 3-kinase (PI3-kinase), an enzyme activated early in the PI pathway. Fig. 3 shows the responses of the mpkCCD<sub>cl4</sub> cells to both insulin and aldosterone after preincubation (30 min) with LY294002 (50  $\mu M$ )—a reversible inhibitor of PI3-kinase (Cheatham et al., 1994). As with the A6 cells, the PI3-kinase inhibitor

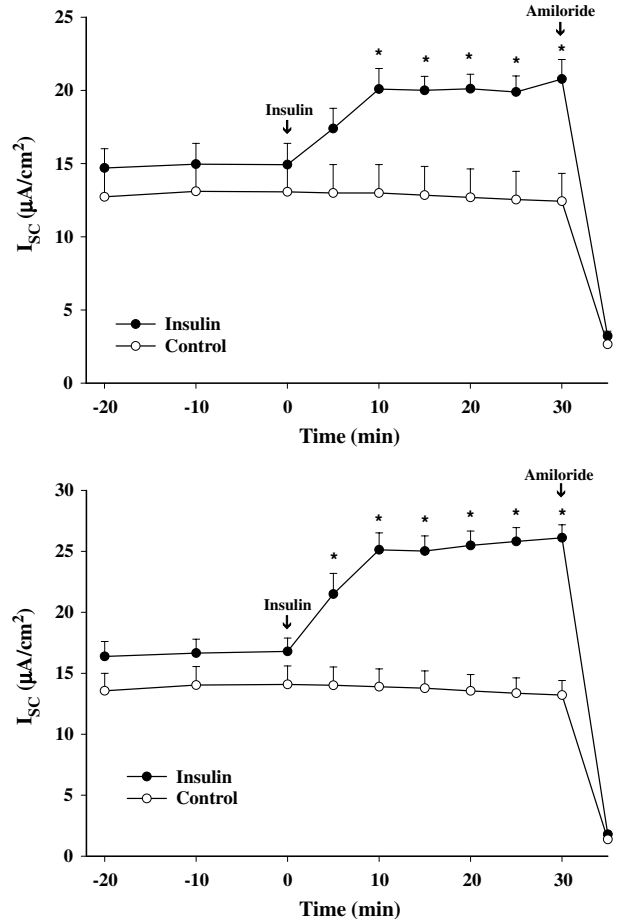


Fig. 2. Insulin-induced  $Na^+$   $I_{SC}$  in renal principal epithelial cells. Insulin ( $100 \text{ nM}$ ) was added to the serosal bathing media of monolayers formed by either the mpkCCD<sub>cl4</sub> (upper panel) or A6 cell line (lower panel) at  $time = 0 \text{ min}$ . Amiloride ( $10 \mu M$ ) was added to the apical bathing media 30 min following insulin stimulation. Symbols represent the mean of  $n$  experiments  $\pm$  SEM. As determined by Student's  $t$  test, insulin induced a significant increase in reabsorptive  $Na^+$  flux beginning at 10 and 5 min post-hormone addition in mpkCCD<sub>cl4</sub> ( $n = 6$ ) and A6 ( $n = 7$ ) cells, respectively.  $*p \leq 0.05$ .

did not adversely affect cellular viability as indicated by the maintenance of a high transepithelial resistance throughout the duration of the experiments (data not shown), but did significantly inhibit hormonal stimulation of  $Na^+$  flux via ENaC in the mpkCCD<sub>cl4</sub> cells.

AVP in most mammals, or AVT in other vertebrates, are peptide hormones which cause the insertion of water channels (aquaporins) into the apical membrane of epithelial cells via activation of the adenylate cyclase/cAMP signaling pathway. Interestingly, this hormone also causes the insertion of ENaC into the apical membrane thereby resulting in a concomitant increase in transepithelial  $Na^+$  flux (Blazer-Yost et al., 2001; Els and Helman, 1989). AVP (in mpkCCD<sub>cl4</sub> cells) and AVT (in A6 cells) dose-dependent stimulation of  $I_{SC}$  is shown in Fig. 4. AVP (Fig. 4A, upper panel) elicits a bi-phasic ion transport response in the mpkCCD<sub>cl4</sub> cells. Specifically, there is a rapid and transient increase in  $I_{SC}$ , followed by a slower, sustained rise in transepithelial flux. In contrast, AVT stimulated a relatively slow

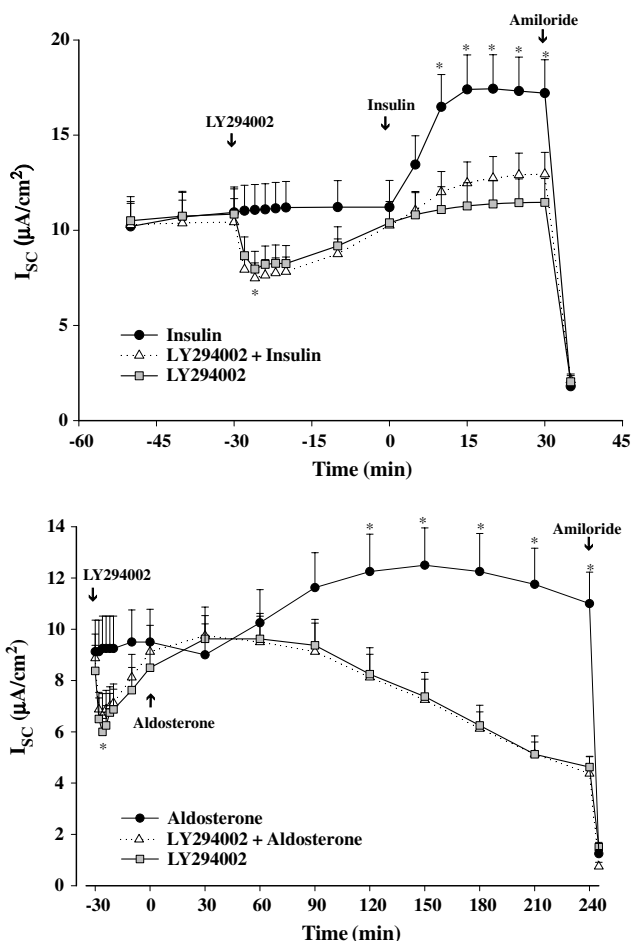


Fig. 3. Insulin- and aldosterone-stimulated ENaC activity in the mpkCCD<sub>c14</sub> cell line is dependent on PI3-kinase. LY294002 (50  $\mu$ M) was added bilaterally at time = -30 min. Insulin (100 nM) (upper panel) or aldosterone (2.7  $\mu$ M) (lower panel) was added serosally at time = 0 min. Amiloride (10  $\mu$ M) was added to the apical bathing media at time = 30 min. Symbols represent the mean of  $n$  experiments  $\pm$  SEM. LY294002 pretreatment prevented both aldosterone ( $n = 8$ ) and insulin ( $n = 15$ )-stimulated increases in  $I_{sc}$ . \*Statistical significance compared to cells treated only with LY294002 ( $p \leq 0.05$ ; Student's  $t$  test).

rise in  $I_{sc}$  (Fig. 4A, lower panel). The dose–response curves for the stimulation of net ion transport ( $\Delta I_{sc}$ ) elicited by AVP or AVT are shown in Fig. 4B.

To determine which portion of the ion transport responses elicited by either AVP or AVT was ENaC-mediated, mpkCCD<sub>c14</sub> and A6 cells were pretreated with amiloride and subsequently stimulated with the appropriate hormone (Fig. 5). The initial, transient increase in  $I_{sc}$  induced by AVP in the mpkCCD<sub>c14</sub> cells is not mediated by ENaC (Fig. 5A, upper panel). This transient, amiloride-insensitive increase within the first minute after the addition of AVP is analogous to a CFTR-mediated anion secretory transport that we previously characterized in the MDCK-C7 cell line (Lahr et al., 2000). Thus in the mammalian cells, anion secretion is temporally separated from Na<sup>+</sup> absorption.

In the amphibian cell line, the two ion transport events are not as clearly separated. Fig. 5B shows that in the A6 line, AVT induces a sustained amiloride-insensitive rise in

$I_{sc}$  that is maintained over the 30 min incubation period. The addition of NPPB, a chloride channel blocker, inhibited the remaining, AVT-stimulated  $I_{sc}$  in the A6 cell line suggesting that this is due to an anion secretory event. To determine whether the magnitude of the amiloride-insensitive component changed or remained constant over varying AVT concentrations, we calculated the percentage of amiloride-sensitive and -insensitive  $I_{sc}$  over the dose range (Fig. 6). Only the  $4.4 \times 10^{-11}$  M AVT dose induced a significantly smaller percentage of amiloride-insensitive current. In the mpkCCD<sub>c14</sub> cell line, the relative percentage of amiloride-sensitive compared to insensitive  $I_{sc}$  did not change over the entire dose range (data not shown).

#### 4. Discussion

ENaC has been conserved through evolution. In *Caenorhabditis elegans*, ENaC-related family members termed degenerins have been shown to be mechano-sensitive proteins (Huang and Chalfie, 1994). In vertebrates, the three ENaC subunits form a Na<sup>+</sup>-specific transporter. Each of the three subunits expressed in the amphibian-derived A6 cell line exhibit ~60% sequence identity with the rat homologs (Puoti et al., 1995). What is truly remarkable, however, is the conservation of function in vertebrates. At a single channel level, the kinetics of the amphibian and mammalian channels are virtually indistinguishable and their ion selectivity and amiloride sensitivity are strikingly similar (Puoti et al., 1995). Our previous studies comparing insulin dose–response relationships between the A6 and mpkCCD<sub>c14</sub> lines (Nofziger et al., 2005b) as well as the studies in this manuscript show that the hormone responses are also conserved.

When comparing the natriuretic effect of aldosterone, both the mammalian and amphibian cells show very similar responses. In both lines, the aldosterone-stimulated increase in amiloride-sensitive ion flux is manifested after a 90- to 120-min delay (Fig. 1), consistent with the known protein synthesis-dependent mechanism of action of this steroid hormone on ENaC-mediated transepithelial Na<sup>+</sup> flux.

Recently, we completed dose–response curves for insulin stimulation of ENaC in the A6 cell line, the M1 cell line, and the mpkCCD<sub>c14</sub> line. Interestingly, the amphibian A6 cell line exhibited a dose–response relationship that was virtually identical to that of the M1 and mpkCCD<sub>c14</sub> lines and showed a half maximal stimulatory concentration within the rodent circulating insulin levels (Nofziger et al., 2005b).

From a survival standpoint, it is logical that a multifunctional hormone like insulin results in Na<sup>+</sup> retention at the same time that it positively affects glucose uptake into muscle and fat cells. In humans, interest in insulin-stimulated Na<sup>+</sup> reabsorption has risen in recent years because of the increasing incidence of hyperinsulinemic states such as Metabolic Syndrome. Metabolic Syndrome is characterized by insulin resistance leading to hyperinsulinemia which is usually accompanied by obesity, hypertension, and

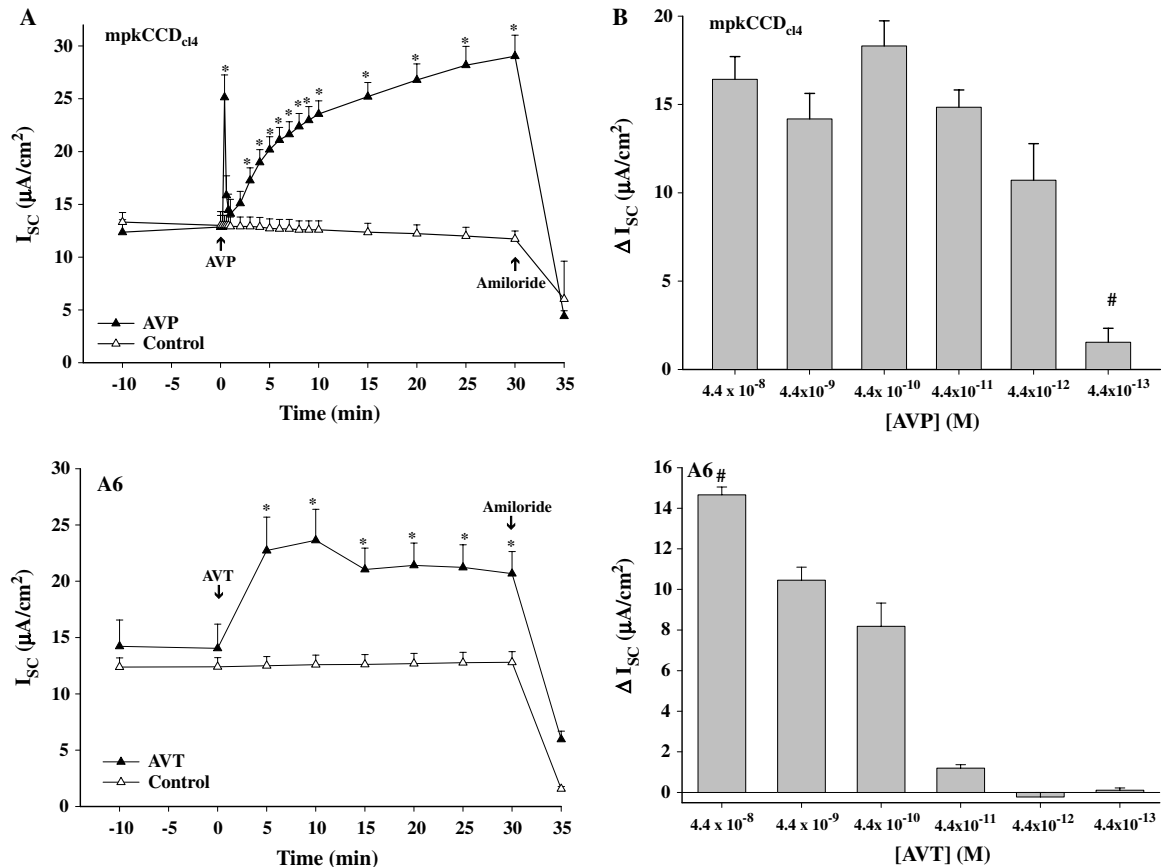


Fig. 4. Dose–response curves for AVP- and AVT-stimulated changes in net ion flux. (A) Representative ion transport responses induced by maximal concentrations of AVP ( $4.4 \times 10^{-8}$  M) in mpkCCD<sub>cl4</sub> cells (upper panel) and AVT ( $4.4 \times 10^{-8}$  M) in A6 cells (lower panel). Either AVP or AVT was added to the serosal bathing media at *time* = 0 min. Amiloride was added apically 30 min after hormonal stimulation. Symbols represent the mean of *n* experiments  $\pm$  SEM. *n* = 5 for mpkCCD<sub>cl4</sub> experimental and control cells. In the A6 experiments, *n* = 19 for experimental and *n* = 10 for control cells. \*Statistically significant increase over the respective control cells as determined by Student's *t* test ( $p \leq 0.05$ ). (B) Dose–response relationships for AVP in mpkCCD<sub>cl4</sub> cells (upper panel) and AVT in A6 cells (lower panel). The magnitude ( $\Delta I_{SC}$ ) of  $I_{SC}$  stimulated by varying concentrations of AVP in mpkCCD<sub>cl4</sub> cells (upper panel) and AVT in A6 cells (lower panel) is depicted as bar graphs representing the mean of *n* experiments  $\pm$  SEM. The data are shown as maximal responses at *time* = 30 min in the mpkCCD<sub>cl4</sub> ( $\Delta I_{SC} = I_{SC \ t=30 \text{ min}} - I_{SC \ t=0 \text{ min}}$ ) and *time* 7 = min in the A6 cells ( $\Delta I_{SC} = I_{SC \ t=7 \text{ min}} - I_{SC \ t=0 \text{ min}}$ ). For mpkCCD<sub>cl4</sub> cells, *n* for each AVP concentration is between 7 and 23, and for A6 cells, *n* for each AVT concentration is 4. # Statistical significance from other concentrations as determined by a one-way ANOVA ( $p \leq 0.01$ ).

hypertriglyceridemia. A variety of studies have indicated that there is a correlation between hyperinsulinemia and hypertension that appears to be independent of age, gender or degree of obesity (Reaven, 1999). It is our contention that the hypertension often seen in Metabolic Syndrome may be linked to the hyperinsulinemic state.

In the A6 cell line, both insulin- and aldosterone-stimulated transport require the activity of PI3-kinase. The effects of PI3-kinase inhibition on basal- and hormone-stimulated transport in the amphibian A6 cell line have been shown previously (Blazer-Yost et al., 1999; Record et al., 1998). In the A6 cell line, LY294002 completely inhibited basal transport and this inhibition was not reversed as long as the inhibitor was present. Interestingly, in the mammalian cells (Fig. 3), pretreatment with LY294002 partially inhibits basal current and this inhibition reverses with time allowing the basal transport to return to control levels within 30 min. The reason for the difference in degree and duration of inhibition of the basal vectorial transport

between amphibian and mammalian cells is unknown. The mammalian cells appear to have compensatory mechanisms, which can overcome the basal inhibitory effects of the LY294002 compound. Whether these represent recruitment or activation of channels via a PI3-kinase-independent mechanism is not known. Regardless of the mechanism of the compensatory effect on basal transport, in the mammalian cells, LY294002 completely inhibits a subsequent response to either insulin or aldosterone.

We have recently shown that in response to insulin stimulation, ENaC is translocated from a diffuse intracellular pool into the apical plasma membrane, but that the channel may follow an indirect pathway to the apical face of the cell (Blazer-Yost et al., 2003, 2004). Apical membrane targeting is dependent on the activity of PI3-kinase and can be blocked by LY294002 (Record et al., 1998). Within 1 min after insulin stimulation, ENaC is co-localized with PI3-kinase, predominately along the lateral surfaces of the epithelial cells. Blocking PI3-kinase activation with LY294002

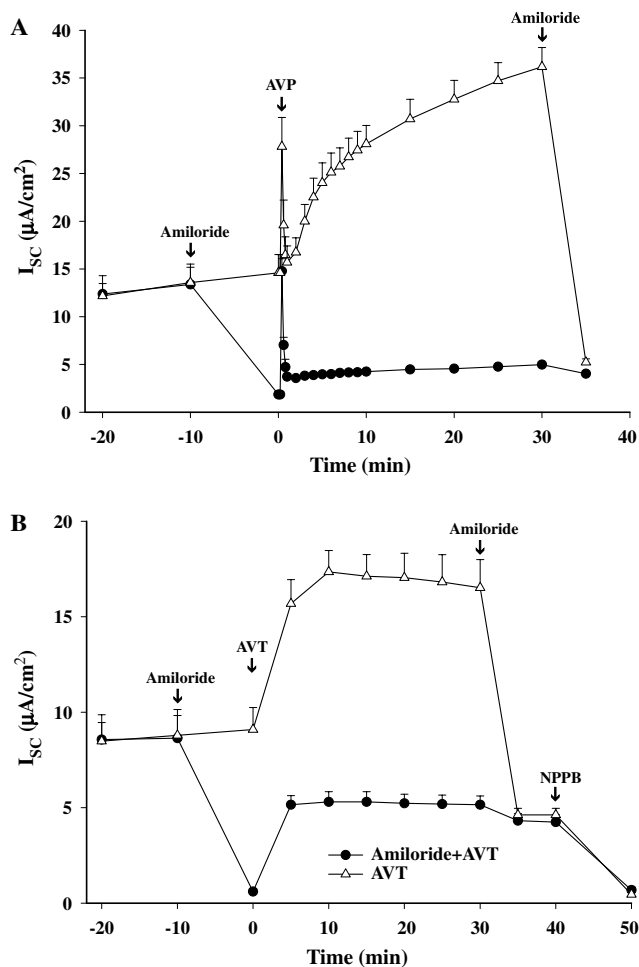


Fig. 5. The responses to AVP and AVT are composed of amiloride-sensitive and -insensitive ion transport components. (A) mpkCCD<sub>cl4</sub> or (B) A6 cells were pretreated apically with or without amiloride (10  $\mu$ M) for 10 min and subsequently stimulated with AVP ( $4.4 \times 10^{-8}$  M) or AVT ( $4.4 \times 10^{-8}$  M). Amiloride was added again 30 min following hormonal stimulation. NPPB (100  $\mu$ M) was added to A6 cells 5 min following the last amiloride addition.

does not prevent the insulin-stimulated co-localization of ENaC and PI3-kinase; rather it blocks the translocation of the complex to the lateral membrane (Blazer-Yost et al., 2003). Although the interaction of the two proteins is necessary for ENaC insertion into the apical membrane, the co-localization is no longer present once ENaC has reached the apical membrane. It is unlikely that the recruitment of ENaC to the area of the lateral membrane is indicative of ENaC insertion into this membrane because transmembrane, multi-subunit channel proteins are unable to cross tight junctions. We have recently used live cell confocal imaging to demonstrate that one of the conduits for the transmembrane signal transduction is the production of phosphatidylinositol 3,4,5-tris-phosphate (PIP3). Lipids of the cytoplasmic leaflet have been shown to cross the junctional complex (Dragsten et al., 1981; van Meer and Simons, 1986). PIP3 is produced exclusively at the cell membrane and is found in A6 cells only after stimulation of PI3-kinase (Record et al., 1998). Therefore, this transiently

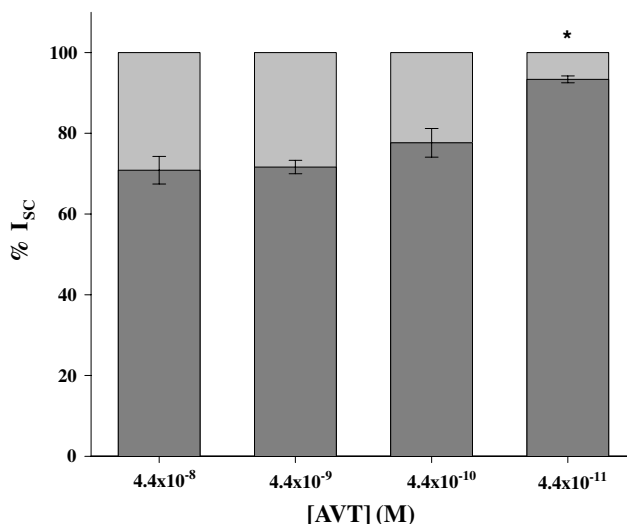


Fig. 6. Percentage of amiloride-sensitive and -insensitive ion flux stimulated by varying concentrations of AVT. The percentages of amiloride-sensitive (dark gray) and amiloride-insensitive (light gray)  $I_{sc}$  from A6 cells stimulated with varying concentrations of AVT are shown as bar graphs representing the means of  $n$  experiments  $\pm$  SEM.  $n = 4$  for each AVT concentration. \*Statistical significance between all groups as determined by a one-way ANOVA ( $p \leq 0.01$ ).

synthesized lipid is an ideal candidate for transmitting a signal from the basolateral surface of the membrane to the apical membrane without diffusion through the cellular cytoplasm.

Given the clinical importance of regulation of ENaC, it will be of interest to determine whether this novel, membrane delimited, signaling pathway described above is also present in mammalian cells. The first step toward this goal is the current demonstration that in the mammalian cell line, insulin- and aldosterone-stimulated  $Na^+$  transport require the presence of PI3-kinase (Fig. 3).

In response to AVT/AVP stimulation, both the amphibian and mammalian cell lines exhibit increases in net ion transport. Interestingly, these responses appear to be due to the activation of at least two transporters, ENaC and CFTR. The dose–response curves for the peptide hormone responses show the combined ion transport response (Fig. 4). In mammals and lower vertebrates, the circulating concentrations of the appropriate hormones are in the low picomolar ranges (Kloas and Hanke, 1990; Konno et al., 2005; Liedtke and Friedman, 2003; Wood et al., 2001). Of significant importance is the finding that the sub-maximal responses to AVP/AVT observed in the present study correspond with those of physiologically relevant circulating concentrations found in both amphibians and rodents, thus substantiating these cell culture models.

In the A6 cell line, a sustained amiloride-insensitive transport response was uncovered after pretreatment with amiloride. Addition of NPPB after amiloride treatment suggests that  $Cl^-$  secretion is the primary factor in the amiloride-insensitive transport component (Fig. 5). These findings are in agreement with previous studies showing  $Cl^-$  secretion in the A6 cells in response to the addition of

permeable cAMP analogs (Yanase and Handler, 1986). The pathway stimulated in response to either AVT or AVP has been shown to involve increases in cAMP and the subsequent activation of PKA which can, in turn, stimulate transport through both ENaC and CFTR.

In contrast, in response to AVP, the mpkCCD<sub>cl4</sub> cell line exhibits a clear bi-phasic response. The initial, transient phase is amiloride insensitive (Fig. 5). We have characterized a similar response to AVP in a subclone of the Madin–Darby canine kidney (MDCK-C7) cell line as anion secretion mediated through activation of CFTR (Blazer-Yost et al., 1996; Lahr et al., 2000).

The notable difference between the amphibian and mammalian cells is the duration of the amiloride-insensitive response. In the A6 cells, this component is sustained throughout the AVT-stimulated response while in the mpkCCD<sub>cl4</sub> cells, the analogous amiloride-insensitive transport has a very rapid onset and is only transiently expressed. The mechanisms underlying the observed differences are unknown. There could be intracellular differences in driving forces between the cell types, differential expression of the pertinent transport proteins, a difference in the amount or type of intracellular regulators, or a combination of these or other possibilities.

Despite the disparities observed with regard to the anion secretory phase elicited by AVT/AVP, the natriferic responses of the two lines resemble one another with respect to both magnitude and time-course. These data suggest that, as with the previous two hormones, Na<sup>+</sup> reabsorption in response to AVT/AVP is conserved from amphibians to mammals.

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