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Identification of a regulated Na/K/Cl cotransport system in a distal nephron cell line

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Lack of an adequate cell model has limited investigation of Na/K/Cl cotransporter regulation in the kidney. Using A6 cells, an amphibian distal renal cell line, we observed that 63% of rubidium uptake in confluent A6 monolayers was ouabain-insensitive. Ouabain-insensitive rubidium uptake was inhibited in a dose-dependent fashion by furosemide (IC_{50} 6.6 μM) or bumetanide (IC_{50} 1.7 μM). Kinetic studies confirmed that furosemide-sensitive rubidium uptake had features consistent with cotransporter activity in other cell lines. Futhermore, specific binding of [^{3}H]bumetanide occurred with a capacity of 8.6 pmol/mg protein and a K_d of 1.6 μM bumetanide. Finally, furosemide-sensitive rubidium uptake was rapidly regulated by a calcium inophore, the phorbol ester PDBu, forskolin, and adenosine. These data demonstrate an Na/K/Cl cotransport system in the A6 cell which will serve as a useful model for studying cotransporter regulation by endogenous signaling pathways.

Introduction

The Na/K/Cl cotransporter mediates electroneutral reabsorption of sodium, potassium, and chloride and is involved in cell volume regulation and vectorial ion transport in secretory and absorptive epithelia (reviewed in Refs. 1–4). In the kidney, regional expression allows the cotransporter to perform the specific functions of dilution of luminal filtrate at the thick ascending limb of Henle and establishment of renal medullary ion gradients [2–4]. In spite of its important role in kidney function, the biochemical features and physiology of the Na/K/Cl cotransporter remain poorly understood. Although Na/K/Cl cotransport and its regulation have been studied in epithelial models such as shark rectal gland [5] and flounder intestine [6,7], these models may have limited applicability to cotransport regulation in the kidney. Lack of an adequate renal cell model has hampered investigation of renal Na/K/Cl cotransporter regulation.

The Na/K/Cl cotransporter has been previously described in two renal epithelial cell lines, one derived from porcine (LLC-PK_1) [8,9] and one from canine (MDCK) [9–11] kidney. The LLC-PK_1 cell exhibits morphologic and transport features of a proximal renal cell [8,12,13], but expression of vasopressin receptors [13] and lack of endogenous PTH receptors [12] make the LLC-PK_1 cell a less specific model of renal epithelium. The MDCK cell displays distal nephron characteristics [10,14] but only expresses a small amount of Na/K/Cl cotransport activity [9,11,14,15]. Neither the LLC-PK_1 nor the MDCK cell cotransporter is known to be regulated by endogenous receptors or cell signaling processes. To gain insight into Na/K/Cl cotransport regulation in the kidney, we sought a cell culture model with the following features: (1) morphological and physiological characteristics of a polarized renal epithelium, (2) related transport processes which are well characterized and (3) endogenous membrane receptors and signaling processes which may be functionally coupled to Na/K/Cl cotransport.

The A6 cell line offers a near-ideal model for the study of Na/K/Cl cotransport. Derived from the kidney of the African frog *Xenopus laevis*, the A6 cell forms a tight epithelium which is polarized [16–18] and maintains transepithelial transport of sodium [16]. Importantly, the A6 cell displays long term regulation of Na^+/K^+-ATPase by aldosterone [18–20] and expresses membrane ion channels for sodium [17,21], potassium [22], and chloride [23]. These features will allow study of Na/K/Cl cotransport in the context of a traditional distal nephron epithelial cell. In this study, we observe ion transport with features characteristic of Na/K/Cl cotransporter activity in the A6 cell line. In addition,

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we demonstrate high levels of saturable [^{3}H]bumetanide binding in these cells. Finally, we provide evidence for regulation of cotransporter activity by cell signaling processes. These observations demonstrate the utility of the A6 cell as a model for further study of regulation of Na/K/Cl cotransport activity by endogenous or transfected membrane receptors.

Materials and Methods

Cell Culture

A6 cells were obtained from American Tissue Culture Collection (Rockville, MD) and maintained in Coon's Hams F-12 (3 parts) and Leibovitz (7 parts) medium equilibrated to a sodium concentration of 104 mM (pH 7.4) and supplemented with 5% fetal calf serum. All culture reagents were obtained from Gibco (Grand Island, NY). The cells were incubated at 27°C in humidified atmosphere containing 5% CO_2 and 95% air and grown to confluent monolayers either on porous polycarbonate membranes or on plastic cell-culture dishes (Costar, Cambridge, MA) as indicated. In these culture conditions, A6 cells exhibit tight junctions and apical brush borders as confirmed by electron microscopy studies.

Rubidium Uptake Studies

Confluent monolayers were equilibrated in transport buffer for 20 min (Earle's solution: Na^+ 143 mM, K^+ 5.4 mM, Mg^{2+} 0.8 mM, Ca^{2+} 1.8 mM, Cl^- 125 mM, Hepes 15 mM, PO_4^{3-} 0.2 mM, pH 7.4) then treated with agonist or vehicle for the specified time. Uptake buffer was (27°C) phosphate-free Earle's solution that contained ^{86}Rb (approx. 4000 cpm/nmol K^+), 10 mM ouabain, in the presence or absence of 500 μM furosemide. Under these conditions, rubidium uptake was linear for 6 min (data not shown). After 4 min, the monolayers were washed three times with sodium-free (choline-substituted) Earle's at 0°C and solubilized in scintillation fluid for counting. Na/K/Cl cotransporter activity was determined by subtracting furosemide-insensitive from total uptake values.

Bumetanide-binding studies

[^{3}H]Bumetanide was synthesized and purified as previously described [28] and the specific activity of the final product was 17 Ci/mmol. Preliminary studies with confluent monolayers of A6 cells in plastic 6-well culture plates indicated that specific binding achieved equilibrium within 40 min. The wells were washed twice with 3 ml Earle's solution and then incubated for 40 min at 27°C in Earle's solution with increasing concentrations of [^{3}H]bumetanide in the presence or absence of an excess of unlabelled bumetanide (40 μM). During the incubation with labelled bumetanide, the culture plates were kept on a platform rocker in a humidified incubator with 5% CO_2. The labelled bumetanide solutions were then aspirated and each well was washed three times with 2 ml of ice-cold 1 mM $CaCl_2$, 20 mM, Hepes. The cells were solubilized by a 15-min incubation in 2 ml 0.1 M NaOH, 1 mg/ml sodium saponin and binding determined by liquid scintillation counting. Samples were performed in duplicate and factored by protein concentration.

Results

Previous studies have validated that rubidium uptake can be used to measure potassium transport by the cell processes of either Na^+/K^+-ATPase or Na/K/Cl cotransporter [3,14,24,25]. To fully characterize rubidium uptake in A6 cells, initial transport studies were performed on cells grown on semipermeable membranes to allow equal access to rubidium to all cell surfaces. When both sides of the membrane were allowed to participate in transport (Fig. 1), addition of 10 mM ouabain decreased rubidium uptake by 14%. In contrast, addition 500 μM furosemide, a potent inhibitor of Na/K/Cl cotransporter activity [2,4,6], decreased rubidium uptake by 93%. To determine if the apex of the cell contributed to furosemide-sensitive rubidium uptake, transport by the basolateral surface was also measured. Fig. 1 shows that the basolateral membrane alone contributed less than half of the total furosemide-sensitive rubidium uptake. Therefore, though the transport process was not exclusively polarized, more than 50% of total furosemide-sensitive rubidium uptake was accounted for by the apical cell surface. These results suggest that A6 cells exhibit significant levels of Na/K/Cl cotransport activity and

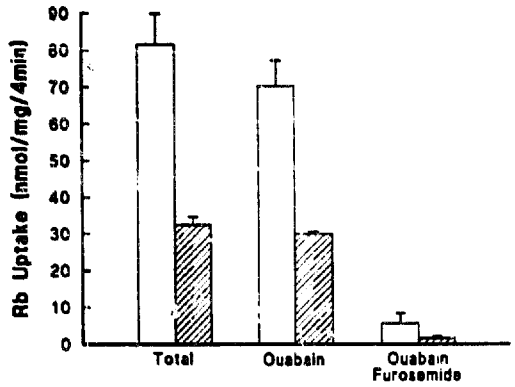

Fig. 1. Rubidium uptake by A6 cell monolayers on semipermeable membranes. Rubidium uptake by both sides (open bars) or by the basal side (hatched bars) of the monolayer was measured after treatment with 10 mM ouabain in the presence or absence of 500 μM furosemide. Values are means ± S.E. (n = 4–10).

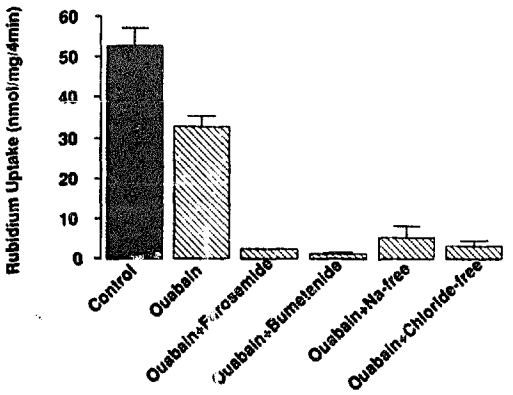

Fig. 2. Characterization of rubidium uptake in confluent A6 cell monolayers. Rubidium uptake was measured after treatment with 10 mM ouabain, 500 μM furosemide, 500 μM bumetanide, choline-substituted or methylsulfate-substituted Earle's solution. Values represent means ± S.E. (n = 5).

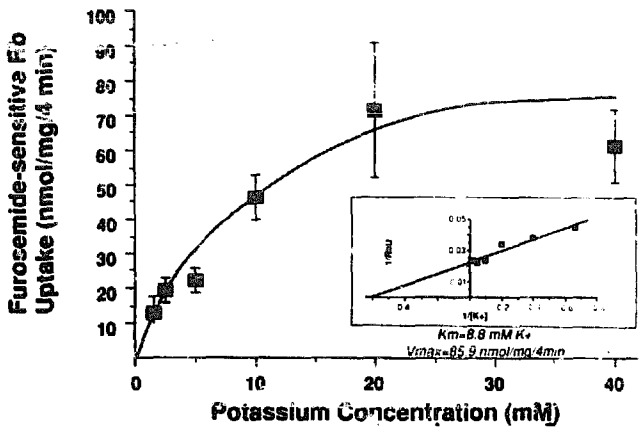

Fig. 3. The relationship between extracellular potassium and furosemide-sensitive rubidium uptake in confluent A6 cell monolayers. Uptake of rubidium by intact A6 monolayers was measured in the presence of 10 mM ouabain with or without 500 μM furosemide. Increasing concentrations of extracellular potassium in the uptake buffer were used and furosemide-sensitive values were derived. Values represent means ± S.E. (all studies in duplicate; n = 3). Inset shows Lineweaver-Burk conversion of the same data.

that this transport process is present on the apical cell surface.

To emphasize cotransport activity by the apical membrane, the following studies were performed on A6 cells grown in plastic wells in monolayer. Fig. 2 shows that in confluent A6 monolayers, ouabain inhibited rubidium uptake by 37%. The further addition of either furosemide (500 μM) or bumetanide (500 μM) caused 96–98% inhibition of the ouabain-insensitive component of rubidium uptake. We further demonstrated that either furosemide or bumetanide decreased rubidium uptake in dose-dependent fashion, with IC_{50} values of 6.6 μM and 1.7 μM, respectively. In addition, Fig. 2 also shows that removal of extracellular sodium (by choline substitution) or chloride (by methylsulfate substitution) caused inhibition of rubidium uptake similar to that produced by furosemide or bumetanide. These results support the premise that apical furosemide-sensitive rubidium uptake in A6 cells has inhibitor specificities and ion dependence characteristic of Na/K/Cl cotransport.

As shown in Fig. 3, furosemide-sensitive uptake was a process that was saturable with increasing concentrations of extracellular potassium. The inset demonstrates that under these conditions the transport process followed Michaelis-Menten kinetics. Lineweaver-Burk analysis shows that furosemide-sensitive rubidium uptake, used as a measure of Na/K/Cl cotransport, displayed an apparent K_m of 8.8 mM K^+ and V_{max} of 85.9 nmol/mg protein per 4 min. These results demonstrate that rubidium uptake in A6 cells follows classic transport kinetics, and furthermore that the kinetic features of this transport process are similar to those reported for Na/K/Cl cotransporter in other renal cell lines [8,10,14].

To more fully characterize Na/K/Cl cotransporter expression, we performed binding studies with [^{3}H]bumetanide ligand. As shown in Fig. 4, we observed specific binding with a total binding capacity of approx. 7.4 pmol/mg protein and K_d of 1.6 μM bumetanide. Using the V_{max} determined from Fig. 3, the estimated turnover of the cotransporter in A6 cells is 42 s^{-1} at 27°C. Thus, A6 cells in these conditions express high levels of the Na/K/Cl cotransporter.

After demonstrating functional and pharmacological features characteristic of the Na/K/Cl cotransporter in A6 cells, we considered possible regulatory mechanisms for cotransporter activity. We measured rubidium uptakes in A6 cells after exposure to forskolin, ionomycin, and the active phorbol ester phorbol 2,3-dibutyrate (PDBu). As seen in Fig. 5, after 10 min of treatment with PDBu, an activator of protein kinase C, there was 49% inhibition of furosemide-sensitive rubidium uptake. Protein kinase C was involved in this

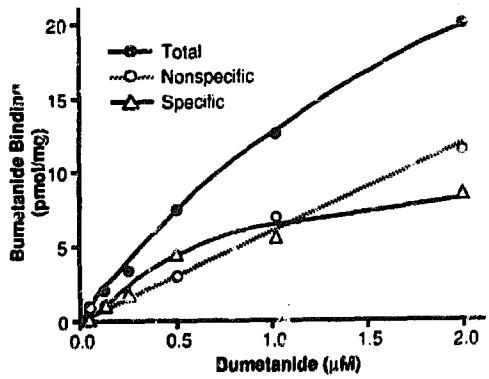

Fig. 4. [^{3}H]Bumetanide binding to A6 cell monolayer. Total (●), nonspecific (○), and specific (△) binding of [^{3}H]bumetanide to A6 cell monolayers is shown for a single representative study. Binding capacity was 8.6 ± 4.2 pmol/mg protein and K_d was 1.6 ± 0.2 μM from three different binding experiments performed in duplicate.

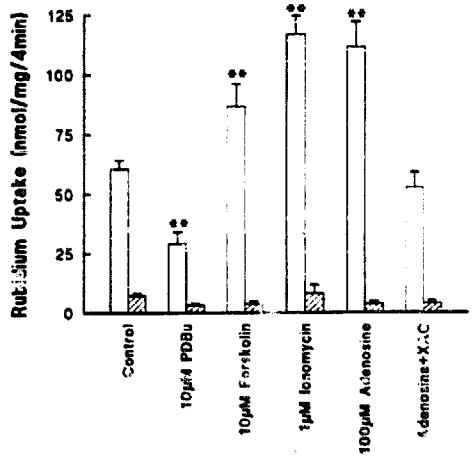

Fig. 5. Regulation of furosemide-sensitive rubidium uptake by cell signaling processes. A6 monolayers were incubated with phorbol 2,3-dibutyrate (10 μM PDBu), forskolin (10 μM), ionomycin (1 μM), adenosine (100 μM), or adenosine + 8-(4-[*N*-2(aminoethyl)-carbamoylmethoxy]phenyl)-1,3-dipropylxanthine (XAC, 1 μM) for 10 min prior to measuring the uptake of rubidium. Uptake solutions contained ouabain (10 mM, open bars) with or without furosemide (500 μM, hatched bars). Values represent means + S.E. (**, $P < 0.005$; $n = 6$–10).

inhibition since inactivation of protein kinase C by overnight treatment with 16 μM PDBu decreased the short-term response to active phorbol ester (12% inhibition; P = NS; $n = 5$) and since the inactive phorbol ester 4α-phorbol did not alter furosemide-sensitive uptake ($n = 4$). Short-term treatment with either the calcium ionophore ionomycin or forskolin, an activator of adenylyl cyclase, significantly stimulated Na/K/Cl cotransport. Finally, application of adenosine (100 μM) also rapidly stimulated cotransport activity, and the response was blocked by an adenosine receptor antagonist 8-(4-[*N*(2-aminoethyl)-carbamoylmethoxy]phenyl)-1,3-dipropylxanthine (XAC, 1 μM). These results suggest that Na/K/Cl cotransporter activity is regulated by intrinsic intracellular signaling mechanisms

Discussion

These studies establish that the A6 cell, an amphibian distal nephron cell line, displays Na/K/Cl cotransport. Specifically, cellular uptake of rubidium is sensitive to established cotransport inhibitors and is dependent on the presence of extracellular sodium and chloride. Further characterization of the transport of rubidium shows that it has kinetic features similar to LLC-PK$_1$ and MDCK cells, other renal cell lines known to express the cotransporter [8,10,14]. Intact A6 cells bind [^{3}H]bumetanide in a saturable manner with a capacity of 8.6 pmol/mg protein and K_d of 1.6 μM. The affinity of the [^{3}H]bumetanide analogue corresponds well with the IC_{50} for bumetanide and rubidium uptake, supporting the notion that the binding site is associated with Na/K/Cl cotransport in A6 cells. Notably the affinity of the binding site in A6 cells is lower than that for either bovine kidney [6], winter flounder intestine [6], avian red cells [27], and canine kidney [28], but is comparable to that observed in Ehrlich ascites cells [29] and shark rectal gland [3,5]. The physiological and pharmacological findings are extended by the observation that cell-signaling mechanisms rapidly regulate Na/K/Cl activity in the A6 cell.

The A6 cell originates from kidney tissue and faithfully expresses transport and morphological features of distal nephron cells. First, the A6 cell differentiates in culture to a polarized, high-resistance epithelium [16,18–20,23,30]. Conditions such as aldosterone treatment and growth on semipermeable supports favor basolateral distribution of Na/K ATPase [18,20,29]. Second, the A6 cell has apical sodium channels which are markedly sensitive to amiloride and contribute to transepithelial sodium flux [17,21]. Third, the A6 cell displays a variety of other cation [22] and anion [23] channels, some of which are similar to ion channels present in principal cells of the cortical collecting tubule [1]. As with polarity characteristics, expression of ion channels depends on cell-culture conditions [23]. In the current studies the A6 cells are arguably less differentiated. For example, confluent cells on plastic culture-plates display significant inhibition of rubidium uptake by ouabain, suggesting that this population of A6 cells does not have polarized Na^+/K^+-ATPase. However, the cells in this study express certain notable features: first, ouabain-sensitive rubidium uptake is present, consistent with endogenous Na^+/K^+-ATPase activity; second, native purinergic receptors for adenosine, described previously [16,31], are expressed in the cells used in this study and are coupled to adenylyl cyclase (data not shown); third, these A6 cells in patch-clamp experiments display macroscopic currents which are due to the presence of chloride channels (A. Mangel and G. Fitz, unpublished observations). Preliminary studies show that individual whole-cell currents have either linear (approx. 50% of cells tested) or outwardly rectified (approx. 45% of cells) current-voltage relationships, consistent with at least two types of chloride channels described in A6 cells [23]. Fourth, electron microscopy studies indicate that the cells exhibit brush borders and tight junctions, basic morphology of a polarized epithelium. Thus, in spite of the expected heterogeneity within the cell line, the A6 cell has transport and morphological characteristics of distal nephron cells, well-characterized related transport processes, and endogenous receptor expression, which provide a suitable context to study transport regulation.

These studies also establish that the Na/K/Cl cotransporter of A6 cells undergoes modification of activity as a consequence of signal transduction systems. Elevations of intracellular calcium and activation of

cyclic-AMP-dependent protein kinase stimulate the A6 Na/K/Cl cotransporter. Interestingly, activation of protein kinase C inhibits cotransport activity, suggesting that the intracellular signals following receptor-mediated activation of phospholipase C, namely cytosolic calcium and protein kinase C, may have opposing effects on Na/K/Cl cotransport activity. Protein kinase C has similar actions in both HT-29 enterocytes [32] and endothelial cells [24–26]. An array of cell types respond to second-messenger systems, but the same signaling event in different tissues can cause divergent transport responses. For example, Na/K/Cl cotransport is inhibited in endothelial cells [25] but stimulated in avian red cells [26,33] by cAMP and presumably cAMP-dependent protein kinase. Several biochemical or physiological features could explain this response diversity, such as differences in related cell-transport processes, phosphorylation of intermediate substrate proteins [15], or multiple isoforms of the Na/K/Cl cotransporter. For instance, in the A6 cell the response to cytosolic calcium or cAMP could be due to a change in chloride channels [1,4,34,35]. Alternatively, biochemical studies suggest that the Na/K/Cl cotransporter in A6 cells is structurally unique and is modified by activation of cAMP-dependent protein kinase. Using a bumetanide analogue photolabel, [4-^{3}H]benzoyl-5-sulfamoyl-3-(thenyloxy)benzoic acid, previously shown to label either a 150-kDa protein in duck red blood cells [33] or a 120-kDa protein in flounder intestine [7], a 125-kDa protein is identified in A6 cells (J. Middleton, unpublished observations). Furthermore, binding of the photolabel is markedly enhanced after treatment of cells with forskolin, similar to results from avian red cells [33], raising the possibility that the cAMP signal alters the substrate for bumetanide binding. Finally, extracellular adenosine augments Na/K/Cl cotransport activity in A6 cells, an effect that is blocked by a sufficient concentration of receptor antagonist to block both A_1 and A_2 receptors [36]. Though full characterization of the specific adenosine receptor and signaling-mechanism pathway will be necessary, this adenosine response may contribute to that previously reported for intact A6 monolayers [16]. These results confirm that the Na/K/Cl cotransporter in A6 cells shares at least some regulatory mechanisms and biochemical features of the cotransporter in other tissues.

The current studies advance the A6 cell as an alternative cell model to study Na/K/Cl cotransport regulation. The A6 cell in culture retains biochemical and transport features which will provide insight into cotransport regulation not only in a range of cell types but specifically in distal renal cells. Further work can elucidate the precise relationship between established membrane receptors, signaling pathways, ion channels, and Na/K/Cl cotransport.

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