J. Membrane Biol. 109, 65–72 (1989)

The Journal of
Membrane Biology

Activation of K^+ Channels in Renal Medullary Vesicles by cAMP-Dependent Protein Kinase

W. Brian Reeves, Glenn A. McDonald*, Pramod Mehta*, and Thomas E. Andreoli
Division of Nephrology, Department of Internal Medicine, University of Arkansas College of Medicine, Little Rock, Arkansas

Summary. ADH, acting through cAMP, increases the potassium conductance of apical membranes of mouse medullary thick ascending limbs of Henle. The present studies tested whether exposure of renal medullary apical membranes in vitro to the catalytic subunit of cAMP-dependent protein kinase resulted in an increase in potassium conductance. Apical membrane vesicles prepared from rabbit outer renal medulla demonstrated bumetanide- and chloride-sensitive $^{22}Na^+$ uptake and barium-sensitive, voltage-dependent $^{86}Rb^+$ influx. When vesicles were loaded with purified catalytic subunit of cAMP-dependent protein kinase (150 mU/ml), 1 mM ATP, and 50 mM KCl, the barium-sensitive $^{86}Rb^+$ influx increased from 361 ± 138 to 528 ± 120 pM/mg prot · 30 sec ($P < 0.01$). This increase was inhibited completely when heat-stable protein kinase inhibitor (1 μg/ml) was also present in the vesicle solutions. The stimulation of $^{86}Rb^+$ uptake by protein kinase required ATP rather than ADP. It also required opening of the vesicles by hypotonic shock, presumably to allow the kinase free access to the cytoplasmic face of the membranes. We conclude that cAMP-dependent protein kinase-mediated phosphorylation of apical membranes from the renal medulla increases the potassium conductance of these membranes. This mechanism may account for the ADH-mediated increase in potassium conductance in the mouse mTALH.

Key Words ADH · cAMP-dependent protein kinase · mTALH K^+ channels

Introduction

K^+ is the predominant, and possibly the sole, conductive species crossing apical membranes of mouse medullary thick ascending limbs of Henle (mTALH) [13]. This paper provides evidence that the antidiuretic hormone (ADH)-mediated increase in apical K^+ conductance of microperfused mTALH segments [15] may be referable to ATP-dependent phosphorylation of apical membranes by cAMP-dependent protein kinase.

In isolated mouse mTALH segments, ADH, operating via the adenylate cyclase cascade [14], increases the rate of net Cl^- absorption [14], the spontaneous transepithelial voltage [14], and G_c (mS cm^{-2}), the Ba^{2+}-sensitive transcellular electrical conductance [13, 23, 27]. There is general agreement that this increase in G_c includes an increase in g^b_{Cl} (mS cm^{-2}), the Cl^- conductance of basolateral membranes [23, 27], and that concomitantly, V_{bl} (mV), the basolateral membrane voltage, is depolarized [13, 27].

At least two classes of explanations have been set forth to account for this ADH-dependent increase in g^b_{Cl}. Schlatter and Greger [27] have argued that ADH produces a primary increase in g^b_{Cl}. Alternatively, we have found that luminal furosemide abolishes the ADH-dependent increases in G_c and in g^b_{Cl} [23]. Accordingly, we have proposed that ADH increases intracellular Cl^- activity by increasing the functional number of apical $Na^+:K^+:2Cl^-$ cotransport units, and that this rise in intracellular Cl^- activity produces a secondary increase in g^b_{Cl} [23]. Furthermore, in the presence of luminal furosemide, dibutyryl-cyclic adenosine monophosphate (db-cAMP) increases the rate of net K^+ secretion in isolated mouse mTALH segments [15]. Accordingly, we have proposed that, in mouse mTALH segments, ADH exerts an admittance effect on apical plasma membranes, that is, to increase the functional number of both $Na^+:K^+:2Cl^-$ cotransport units and K^+ channels [15, 22, 23].

The present experiments were designed to evaluate the mechanism for this hormone-mediated increase in apical K^+ conductance. Specifically, we tested the effects of ATP and the catalytic subunit of cAMP-dependent protein kinase on Ba^{2+}-sensitive, bumetanide- and ouabain-insensitive $^{86}Rb^+$ influx into renal medullary vesicles loaded with KCl.

The results indicate that loading the vesicles with ATP and with the catalytic subunit of cAMP-

* *Present address:* Division of Nephrology, University of Texas Medical School, Houston, Texas.

dependent protein kinase produces a specific stimulation of conductive Ba^{2+}-sensitive, bumetanide- and ouabain-insensitive $^{86}Rb^+$ uptake into such vesicles. Accordingly, we conclude that ATP-dependent phosphorylation of apical plasma membranes of mTALH segments by cAMP-dependent protein kinase may activate K^+ channels already present in those membranes. A preliminary report of these findings has been presented elsewhere [25].

Materials and Methods

In prior studies, we prepared membrane vesicles from rat outer medulla using a modification [24] of a method described by Jorgensen [16]. Kinne et al. [17] recently described a method for making renal medullary vesicles from rabbit outer medulla in which there was enrichment of alkaline phosphatase activity and de-enrichment of (Na^+ + K^+)-ATPase activity. Thus in the present experiments, we adopted the method of Kinne et al. [17] to prepare membrane vesicles from rabbit outer medulla.

The outer strip of medulla was dissected, minced to a fine paste, suspended in 20 volumes of 250 mM sucrose, 10 mM Tris, 16 mM HEPES (pH 7.4) and homogenized for 11 strokes in a Teflon-glass homogenizer attached to a rotary drill turning at 800 rpm. The resulting homogenate was centrifuged at 2300 rpm for 10 min (Sorval SS-34 rotor). The pellet was discarded and the supernatant fluid was centrifuged at 11,700 rpm for 20 min. The supernatant was retained and the fluffy white upper layer of the pellet was gently removed and resuspended in the homogenizing buffer and spun again at 11,700 rpm for 20 min. This step was repeated and the three supernatants were combined and centrifuged at 20,000 rpm for 40 min. The resulting pellet was then suspended in approximately 1000 μl of buffer solution by drawing the vesicles back and forth through a 27-gauge needle.

Procedure for Loading Vesicles

Vesicles were loaded with the appropriate salts, enzymes, and nucleotides by hypotonic shock. 300 μl of vesicles suspended in 250 mM sucrose were diluted into 6 ml of a solution containing 10 mM KCl, 5 mM KF, 1 mM $MgCl_2$, 1 μM $CaCl_2$, 10 mM Tris, 16 mM HEPES, 0.5 mM ouabain and 1 mM sodium ATP (pH 7.4). Vesicles so loaded served as the control vesicles for all experiments. The catalytic subunit (*see below*) of cAMP-dependent protein kinase (150 mU/ml) and/or an inhibitor (*see below*) of cAMP-dependent protein kinase (1 μg/ml) were added when appropriate. Likewise, in some experiments (*see* Fig. 4), ADP was substituted for ATP. The suspension was incubated on ice for 15 min before centrifugation at 48,000 × *g* for 40 min.

The pellets were resuspended in a solution containing (in mM): 40 KCl, 10 KF, 1 $MgCl_2$, 0.001 $CaCl_2$, 0.5 ouabain, 10 Tris, 16 HEPES, 200 sucrose (no ATP or protein kinase) and centrifuged at 48,000 × *g* for 40 min. This step was repeated once. The final pellet was resuspended to a volume of 300 μl.

Procedure for Measuring $^{86}Rb^+$ Uptake

The conditions for $^{86}Rb^+$ uptake were similar to those described by Burnham et al. [3]. Specifically, we assayed Ba^{2+}-sensitive $^{86}Rb^+$ uptake, which was ouabain- and bumetanide-insensitive in vesicles loaded, as described above, with 1 mM ATP. 100 μl of vesicles were eluted through a Dowex D50W ion exchange column (previously equilibrated with Tris and poured in a Pasteur pipette) with 300 μl of 300 mM sucrose, 20 mM Tris, 16 mM HEPES in order to remove the potassium (or other cation) from the extravesicular solution. Isotope uptake was initiated by adding 150 μl of the Dowex-treated vesicle suspension to 50 μl of isotope solution, resulting in final concentrations of (in mM): 0.25 $^{86}RbCl$ (10^6 cpm/50 μl), 250 sucrose, 0.5 ouabain, 0.1 bumetanide, 10 Tris, 16 HEPES, ± 2 $BaCl_2$. At specified intervals, 50 μl aliquots of the reaction mixture were transferred to a cold Dowex column and the vesicles were eluted with 1 ml of a cold 250 mM sucrose solution into counting vials. All assays were carried out in duplicate or triplicate. The activity of the eluate was measured on a liquid scintillation counter.

$^{22}Na^+$ Uptake

$^{22}Na^+$ uptake was measured as described previously [17, 24]. Specifically, $^{22}Na^+$ uptake was determined in triplicate by a rapid filtration technique. Vesicles containing 100 mM sucrose, 1 mM $MgSO_4$, 20 mM triethanolamine (pH 7.4) were added to a $^{22}Na^+$ containing solution so that the final composition of the reaction mixture was 100 mM sucrose, 55 mM KCl or K gluconate, 0.5 mM $^{22}NaCl$, 1 mM $MgSO_4$, 20 mM triethanolamine, 1 mM amiloride (pH 7.4). Transport was terminated after 5 sec by the addition of 1.5 ml of a cold stop solution containing 150 mM KNO_3, 20 mM triethanolamine. The diluted sample was immediately filtered through a 0.45 μm filter and washed with four additional aliquots of cold stop. Activity of the $^{22}Na^+$ retained on the filter was determined by liquid scintillation photometry.

Assays

Protein was measured by the method of Lowry et al. [21]. The plasma membrane vesicles were assayed for alkaline phosphatase and (Na^+ + K^+)-ATPase activity. Alkaline phosphatase was determined from the rate of *p*-nitrophenol production using *p*-nitrophenol phosphate as a substrate (Sigma Technical Bulletin No. 104). The (Na^+ + K^+)-ATPase activity was determined spectrophotometrically using a regenerating coupled enzyme assay [5].

Materials

The catalytic subunit of cAMP-dependent protein kinase (PK-CSU), generously provided by Dr. E.J. Weinman, was prepared from rabbit skeletal muscle using the procedure of Beavo et al. [2]. The protein kinase inhibitor (also provided by Dr. E.J. Weinman) was prepared from rabbit skeletal muscle according to the procedure of Whitehouse and Walsh [31]. $^{22}NaCl$ and $^{86}RbCl$ were obtained from New England Nuclear. All other chemicals were of the highest grade available.

Results

The vesicles were prepared by a slight modification of the procedure of Kinne et al. [17]. The enrichment in alkaline phosphatase of vesicles over the starting homogenate was 4.8 ± 0.6 (SEM; $n = 4$; $P <$

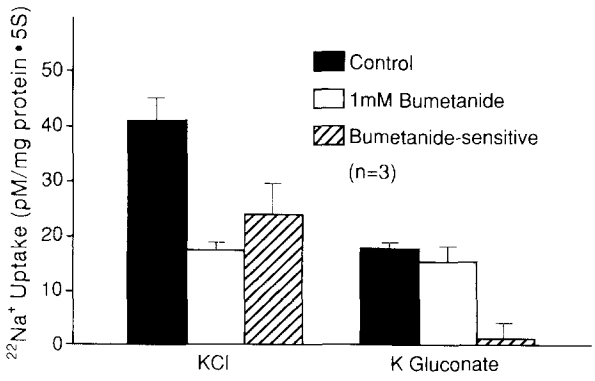

Fig. 1. $^{22}Na^+$ uptake into medullary vesicles was determined as described in Materials and Methods in the presence and absence of 1 mM bumetanide. The inwardly directed salt gradient was either KCl or K gluconate. The results are expressed as mean values ± SEM

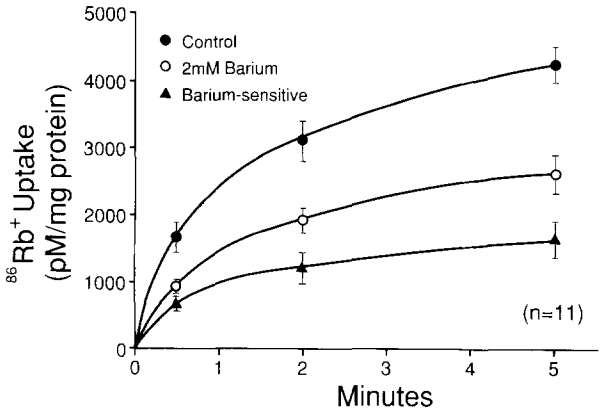

Fig. 2. The time course of $^{86}Rb^+$ into medullary vesicles. $^{86}Rb^+$ uptake driven by an outwardly directed KCl gradient was measured as described in Materials and Methods at each of the indicated times. Paired measurements of $^{86}Rb^+$ uptake at each of the indicated times were made with and without 2 mM Ba^{2+} in aqueous media. The results are expressed as mean values ± SEM

0.01), which is similar to that reported by Kinne et al. [17]. In our experiments, we did not observe de-enrichment of $(Na^+ + K^+)$-ATPase activity in renal medullary vesicles. Instead, the $(Na^+ + K^+)$-ATPase activity of the medullary vesicles was indistinguishable from the activity in the starting homogenate, that is, enriched 1.4 ± 0.3 times ($n = 4$; P = NS).

$^{22}Na^+$ Uptake Studies

Since alkaline phosphatase activity is not a specific marker for apical membranes, we also examined the vesicles for a functional marker of apical membrane activity. Specifically, as in prior studies with rat renal medullary vesicles [24], we evaluated bumetanide-sensitive $^{22}Na^+$ uptake driven by an inwardly directed KCl gradient (*see* Materials and Methods). In rat renal medullary vesicles, such bumetanide-sensitive $^{22}Na^+$ uptake depends on the simultaneous presence of both K^+ and Cl^- [24]. Likewise, the results presented in Fig. 1 show clearly that, in the rabbit renal medullary vesicles used in the present experiments, 10^{-3} M bumetanide inhibited 58% of $^{22}Na^+$ uptake driven by an inwardly directed KCl gradient. Moreover, this bumetanide-sensitive $^{22}Na^+$ uptake was eliminated completely when gluconate replaced Cl^-. These results are closely comparable to those of Kinne et al. [17] in rabbit medullary vesicles, to those obtained by us in rat medullary vesicles [24], and to the results of other workers in medullary vesicles from various species [6, 19].

These results indicate that the rabbit medullary vesicles used in the present studies contained, at a minimum, apical mTALH membranes. However, the data from $(Na^+ + K^+)$-ATPase enrichment listed above also show that the vesicles had about the same activity for this basolateral enzyme marker as the starting homogenate. Thus we consider that the vesicles used in the present experiments contained primarily apical mTALH membranes, but there also was present an admixture of basolateral membranes. The latter observation is consistent with our earlier findings with rat medullary vesicles [24], as well as with the observations of others on medullary vesicles from different mammalian species [6, 16, 17, 19].

$^{86}Rb^+$ Uptake

Apical membranes of TALH cells contain barium-sensitive K^+ channels as the principal conductive element [9, 10, 13]. To evaluate the presence of such a K^+ conductance in these vesicles, we studied the time course of Ba^{2+}-sensitive, ouabain- and bumetanide-insensitive $^{86}Rb^+$ uptake into vesicles loaded with 50 mM KCl, 1 mM ATP (*see* Materials and Methods). The results presented in Fig. 2 show that approximately 40% of the total $^{86}Rb^+$ uptake was inhibited by 2 mM $BaCl_2$. This relatively prolonged uptake phase is consistent with the results of Burnham et al. [3] and is typical of conductive K^+ transport when measured under these conditions [8].

The nature of the $^{86}Rb^+$ uptake pathway was examined further by testing the ionic specificity of Ba^{2+}-sensitive $^{86}Rb^+$ uptake. Table 1 presents the results of experiments in which we compared $^{86}Rb^+$ uptake at 30 sec into vesicles loaded with 1 mM ATP and with either KCl, NaCl or potassium isethionate. When Cl^- was replaced by isethionate, both the to-

Table 1. Ionic requirements for $^{86}Rb^+$ uptake into medullary membrane vesicles

Intravesicular salt	$^{86}Rb^+$ uptake (pM/mg protein · 30 sec)		
	0 Ba^{2+}	2 mM Ba^{2+}	Ba^{2+}-sensitive uptake
KCl	1659 ± 119	934 ± 50	725 ± 77
K isethionate	2756 ± 190	1833 ± 207	923 ± 69[a]
NaCl	410 ± 35	261 ± 27	148 ± 10[a]
	(n = 11)		

Vesicles were loaded as described in Materials and Methods with either 50 mM KCl, 50 mM K isethionate, or 50 mM NaCl. The barium-sensitive component is the mean paired difference between values with and without 2 mM Ba^{2+}. The results are expressed as mean values ± SEM.
[a] ($P < 0.001$ *vs.* KCl).

tal $^{86}Rb^+$ uptake and the Ba^{2+}-sensitive $^{86}Rb^+$ uptake increased. Thus $^{86}Rb^+$ uptake did not depend on the simultaneous presence of K^+ and Cl^-. The results shown in Table 1 also indicate that, when the vesicles were loaded with NaCl rather than with KCl, both the $^{86}Rb^+$ uptake and Ba^{2+}-sensitive $^{86}Rb^+$ uptake were strikingly reduced. These results indicate that the $^{86}Rb^+$ uptake pathway was specific for K^+ with respect to Na^+.

It was pertinent to evaluate the possibility that the pathway for $^{86}Rb^+$ influx was conductive. To test this view, we measured relative intravesicular voltage with the voltage sensitive dye DiS-C_3-(5) [32]. Figure 3 shows tracings from a representative experiment of the relative fluorescence intensities when identical aliquots of vesicles containing 100 mM potassium were diluted into solutions containing 2 μM DiS-C_3-(5) and either 100 mM K^+ or zero K^+, and either 2 mM Ba^{2+} or zero Ba^{2+}. The data indicate that Ba^{2+} had little effect on the relative intravesicular voltage, as estimated by fluorescence intensity, when the potassium concentrations inside and outside the vesicles were equal. However, when the vesicles were diluted into a zero K^+ medium, the change in fluorescence intensity indicated that the vesicle interior hyperpolarized, and that this hyperpolarization was reversed by barium. Finally, the addition of valinomycin resulted in hyperpolarization of these vesicles even in the presence of barium. Thus when taken together, the data in Table 1 and Fig. 3 indicate that, in these vesicles, Ba^{2+}-sensitive, ouabain- and bumetanide-insensitive $^{86}Rb^+$ influx driven by an outwardly directed K^+ gradient occurred via a conductive pathway that was highly selective for K^+ with respect to Na^+, and did not require Cl^-.

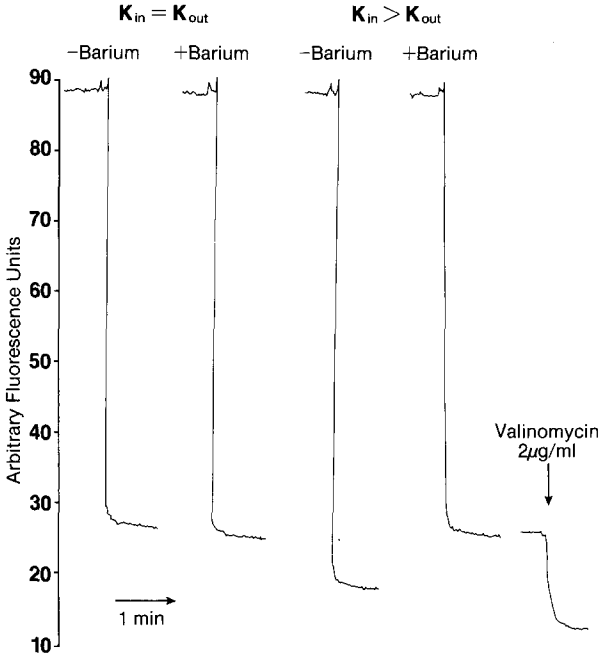

Fig. 3. Intravesicular voltage determined by DiS-C_3-(5) fluorescence [32]. Vesicles containing 100 mM K gluconate were diluted into a solution of either 100 mM K gluconate ($K_{in} = K_{out}$) or 100 mM Na gluconate ($K_{in} > K_{out}$), with and without 2 mM $BaCl_2$ and containing 2 μM DiS-C_3-(5)

Effects of the Catalytic Subunit of cAMP-Dependent Protein Kinase

In earlier studies, we found that dibutyryl-cAMP augmented the rate of net K^+ secretion in isolated mouse mTALH segments exposed to 10^{-4} M luminal furosemide [15]. Moreover, in cultured chick kidney cells, ADH and forskolin activate apical K^+ channels [11]. Thus it can be argued that ADH, operating via cAMP, increases the functional number of K^+ channels in mTALH segments [13, 15]. Accordingly, in the present experiments, we evaluated the possibility that the Ba^{2+}-sensitive component of $^{86}Rb^+$ uptake might be modulated by cAMP-dependent protein kinase.

Specifically, as in all experiments, the renal medullary vesicles were loaded by hypotonic shock with 1.0 mM Mg^{2+} and with 1 mM ATP as described in Materials and Methods. Identical aliquots of vesicles were also loaded with 150 mU/ml of purified catalytic subunit of cAMP-dependent protein kinase (PK-CSU) and, in some instances, with 1 μg/ml of purified protein kinase inhibitor (PKI). This experimental protocol permitted paired comparisons among identical aliquots of vesicles loaded with three different groups of agents, that is: vesicles loaded only with 1 mM ATP, vesicles loaded

Table 2. Effects of ATP and the catalytic subunit of cAMP-dependent protein kinase on $^{86}Rb^+$ uptake by medullary membrane vesicles loaded with potassium chloride

Condition	$^{86}Rb^+$ uptake (pM/mg protein · 30 sec)			Δ Ba^{2+}-sensitive uptake
	0 Ba^{2+}	2 mM Ba^{2+}	Ba^{2+}-sensitive uptake	
ATP	1154 ± 344	793 ± 212	361 ± 138	
				Δ = 167 ± 47 ($P < 0.01$)
ATP, PK-CSU	1307 ± 403	778 ± 241	528 ± 170	
				Δ = 209 ± 86 ($P < 0.05$)
ATP, PK-CSU, PKI	1068 ± 299	748 ± 209 ($n = 4$)	319 ± 90	

Vesicles were loaded by hypotonic shock, as described in Materials and Methods and in Results, with either 1 mM ATP, ATP and the catalytic subunit of cAMP-dependent protein kinase (PK-CSU) or ATP, PK and heat-stable protein kinase inhibitor (PKI). The intravesicular salt was KCl. The results are expressed as mean values ± SEM.

with 1 mM ATP plus the catalytic subunit of cAMP-dependent protein kinase, and vesicles containing 1 mM ATP, PK-CSU, and purified protein kinase inhibitor. In all instances, the vesicles were also loaded with 10 mM KF to inhibit protein phosphatases [1], and with 1 mM $MgCl_2$ and 0.001 mM $CaCl_2$ (*see* Materials and Methods). Finally, as indicated in Materials and Methods, the external solutions uniformly contained 0.5 mM ouabain and 0.1 mM bumetanide.

Table 2 shows the effects of the catalytic subunit of cAMP-dependent protein kinase and of the protein kinase inhibitor on the two components of $^{86}Rb^+$ uptake into vesicles containing 50 mM K^+. Loading the vesicles with PK-CSU resulted in a 46% increase in the Ba^{2+}-sensitive component of $^{86}Rb^+$ uptake, that is, from 361 ± 138 pM/mg protein · 30 sec to 528 ± 170 pM/mg protein · 30 sec ($P < 0.01$). However, PK-CSU had no effect on the Ba^{2+}-insensitive component of $^{86}Rb^+$ uptake. The results presented in Table 2 also indicate clearly that this enhancement of Ba^{2+}-sensitive $^{86}Rb^+$ uptake by PK-CSU could be blocked entirely by PKI and that the latter agent had no statistically discernible effect on the Ba^{2+}-insensitive component of $^{86}Rb^+$ uptake.

Figure 4 illustrates the results of paired experiments in which we tested nucleotide specificity for the enhancement of Ba^{2+}-sensitive $^{86}Rb^+$ uptake by PK-CSU. The vesicles were loaded under three different sets of conditions: 1 mM ATP; 1 mM ATP plus PK-CSU; or 1 mM ADP plus PK-CSU. The results presented in Fig. 4 show clearly, in agreement with the results in Table 2, that PK-CSU produced about a 35% increase in Ba^{2+}-sensitive $^{86}Rb^+$ uptake in vesicles loaded with 1 mM ATP. However, PK-CSU did not augment Ba^{2+}-sensitive $^{86}Rb^+$ influx in vesicles containing 1 mM ADP instead of 1 mM ATP.

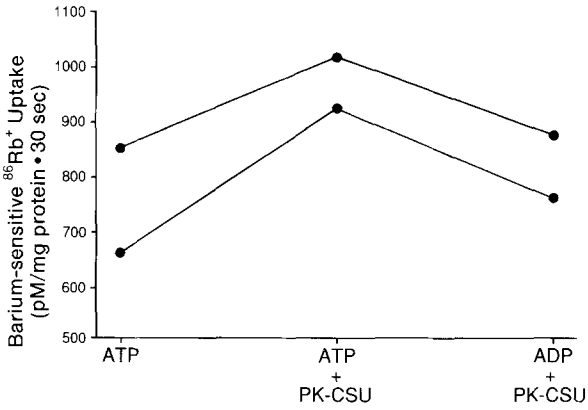

Fig. 4. Effects of ATP, ADP and PK-CSU on stimulation of $^{86}Rb^+$ uptake driven by an outwardly directed KCl gradient. Identical aliquots of vesicles were loaded as described in Materials and Methods with either 1 mM ATP, 1 mM ATP plus PK-CSU or 1 mM ADP plus PK-CSU. The lines connect values for individual experiments

As noted earlier, the vesicles used in these experiments are primarily apical membranes with an admixture of basolateral membranes. Thus we wished to exclude the possibility that the increase in $^{86}Rb^+$ uptake in vesicles loaded with ATP and PK-CSU (Table 2) occurred because these agents affected Cl^- conductance, or activated a KCl symport

Table 3. Effects of ATP and the catalytic subunit of cAMP-dependent protein kinase on $^{86}Rb^+$ uptake by medullary vesicles loaded with potassium isethionate

Condition	$^{86}Rb^+$ uptake (pM/mg protein · 30 sec)			ΔBa^{2+}-sensitive uptake
	0 Ba^{2+}	2 mM Ba^{2+}	Ba^{2+}-sensitive uptake	
ATP	2026 ± 276	1052 ± 98	974 ± 188	
				Δ = 198 ± 62 ($P < 0.01$)
ATP, PK-CSU	2223 ± 208	1051 ± 66	1172 ± 149	
				Δ = 157 ± 27 ($P < 0.01$)
ATP, PK-CSU, PKI	2051 ± 233	1036 ± 88 (n = 7)	1015 ± 151	

The experiments were carried out as described in Table 2, except that the vesicles contained potassium isethionate rather than KCl. The results are expressed as mean values ± SEM.

Table 4. Effect of vesicle-loading procedure on the stimulation of $^{86}Rb^+$ uptake by PK-CSU

	Ba^{2+}-sensitive $^{86}Rb^+$ uptake (pM/mg protein · 30 sec) Loading condition	
	Isotonic	Hypotonic
ATP	586 ± 50	441 ± 54
ATP + PK-CSU	628 ± 41	669 ± 52
Δ	42 ± 23 (P = NS)	228 ± 23 ($P < 0.005$)
	[n = 3]	

Identical aliquots of vesicles were exposed to ATP and the catalytic subunit of cAMP-dependent protein kinase using either a hypotonic solution (*see* Materials and Methods) or an isotonic solution (230 mM sucrose added to the hypotonic solutions). The intravesicular salt was KCl. All subsequent steps and the transport assay were identical, as described in the text. The results are expressed as mean values ± SEM.

process, in basolateral vesicles present in the preparation.

Accordingly, we evaluated the effects of ATP, PK-CSU and PKI on $^{86}Rb^+$ uptake into vesicles containing potassium isethionate rather than KCl. The results of these experiments, presented in Table 3, show clearly that, with vesicles containing potassium isethionate, the catalytic subunit of cAMP-dependent protein kinase augmented specifically the Ba^{2+}-sensitive component of $^{86}Rb^+$ uptake and that this enhancement was blocked by PKI. Moreover, an unpaired comparison of the results presented in Tables 2 and 3 indicates that the magnitude of the increase in $^{86}Rb^+$ uptake produced by PK-CSU was nearly the same when vesicles contained either KCl (Table 2, 167 ± 47 pM/mg protein · 30 sec) or potassium isethionate (Table 3, 198 ± 62 pM/mg protein · 30 sec).

Effect of Vesicle Loading Procedure

Hypotonic shock provides a way of opening and resealing vesicles, so that moieties present in aqueous media can be incorporated into the intravesicular space [30]. The catalytic subunit of cAMP-dependent protein kinase operates on the cytoplasmic face of membranes [7, 26, 30]. Thus we compared the effects of PK-CSU on $^{86}Rb^+$ uptake in vesicles where the vesicle-loading procedure was carried out either under hypotonic conditions, as described in Materials and Methods, or when the vesicle-loading solution also contained sufficient sucrose (230 mM) to make the loading solution isotonic to the vesicular interior.

The results of these experiments are shown in Table 4. When the vesicle-loading solutions were hypotonic, PK-CSU enhanced $^{86}Rb^+$ uptake by 228 ± 23 pM/mg proteins · 30 sec, a result in close quantitative agreement with the results presented in Tables 2 and 3. The results in Table 4 also show that, when the vesicle-loading solutions were isotonic, PK-CSU had no significant effect on $^{86}Rb^+$ uptake. These results are consistent with the view that the vesicles were impermeable to ATP and/or the catalytic subunit of cAMP-dependent protein kinase, and that opening of vesicles was required for incorporation of these agents. Thus the site, or sites, affected by PK-CSU were inside oriented in these

vesicles, that is, it is probable that the vesicles were right-side out.

Discussion

At least three lines of evidence indicate that ADH, operating through the adenylate cyclase cascade, may increase the K^+ conductance of apical membranes in certain regions of the nephron. First, in isolated mouse mTALH segments, db-cAMP increases the rate of net K^+ secretion in cells that are uniformly hyperpolarized to about −70 mV by application of 10^{-4} M luminal furosemide [15]. Second, in cultured chick kidney cells, Guggino et al. [11] have found that ADH or forskolin increases the fractional open time of Ca^{2+}-activated, Ba^{2+}-sensitive K^+ channels in apical membranes. Finally, Klaerke et al. [18] have reported that the activity of K^+ channels purified from renal medullary vesicles and incorporated into liposomes is apparently increased by phosphorylation with cAMP-dependent protein kinase. Moreover, cAMP-dependent phosphorylation also increases K^+ conductance in a variety of other tissues [4, 20]. Thus in the experiments reported in this paper, we evaluated the effects of ATP and the catalytic subunit of cAMP-dependent protein kinase on K^+ conductance in renal medullary vesicles. Based on the present results, the following conclusions are pertinent.

First, Ba^{2+}-sensitive, ouabain- and bumetanide-insensitive $^{86}Rb^+$ uptake into the renal medullary vesicles increased when K isethionate replaced KCl as the intravesicular salt, and was virtually abolished when NaCl replaced KCl as the intravesicular salt (Table 1). Moreover, the results with the voltage-sensitive dye DiS-C_3-(5) indicate that an outwardly directed K^+ gradient hyperpolarized the vesicle interior, that Ba^{2+} reversed this hyperpolarization and that, under these circumstances, valinomycin again hyperpolarized the vesicle interior (Fig. 3). We conclude from these data that, in these renal medullary vesicles, Ba^{2+}-sensitive $^{86}Rb^+$ uptake at 30 sec provides a reasonable index to conductive K^+ transport.

As indicated in Results, the renal medullary vesicles were primarily from apical membranes (Fig. 1). Thus we infer that Ba^{2+}-sensitive $^{86}Rb^+$ uptake occurred primarily in apical membranes. However, as noted in Results, the renal medullary membranes also contained an admixture of basolateral membranes. And basolateral membranes in intact mTALH segments have a K^+ conductance [29]. Accordingly, it is possible that a portion of the Ba^{2+}-sensitive $^{86}Rb^+$ uptake observed in the present experiments may also involve basolateral membrane vesicles.

Second, the results presented in Table 2 indicate clearly that, in the presence of 1 mM Mg^{2+} and 1 μM Ca^{2+}, conductive Ba^{2+}-sensitive $^{86}Rb^+$ influx driven by a KCl gradient was enhanced by nearly 50% by ATP and the catalytic subunit of cAMP-dependent protein kinase. However, the latter two agents did not affect Ba^{2+}-sensitive $^{86}Rb^+$ uptake when the vesicles were not exposed to hypotonic shock (Table 4). Since cAMP-dependent protein kinase operates on the cytoplasmic face of membranes [7, 26, 30], we conclude from the results in Tables 2–4 and Fig. 4 that the renal medullary vesicles were chiefly right-side out, and that phosphorylation of the cytoplasmic surface of these vesicles by ATP and the catalytic subunit of cAMP-dependent protein kinase increased the conductance of K^+ channels present in these vesicles. These K^+ channels may represent primarily apical membrane K^+ channels. However, cAMP-dependent events enhance basolateral K^+ conductance in toad urinary bladder [4]. Likewise, the present data do not exclude the possibility that ATP and the catalytic subunit of cAMP-dependent protein kinase also affected K^+ conductance in basolateral membrane vesicles.

The present experiments provide no information about whether such phosphorylation involved K^+ channels directly, or sites vicinal to K^+ channels. But taken together, the results presented in this paper are consistent with the view that the mechanism by which ADH increases the functional number of K^+ channels in apical membranes of the mouse mTALH [13, 23], and possibly other renal epithelia [11], involves activation of K^+ channels already present in apical membranes by phosphorylation of apical membrane sites via cAMP-dependent protein kinase. This type of mechanism would therefore differ fundamentally from the actions of ADH in enhancing the osmotic water permeability of amphibian epithelia and mammalian collecting ducts by promoting the insertion of water-permeable membrane units into apical membranes [12, 28].

We are grateful to Ms. Clementine M. Whitman for her customarily excellent secretarial assistance. Supported by NIH grant (7 R01 DK25540). W.B. Reeves is a Squibb-American Heart Association Clinician-Scientist (85-442).

References

1. Ballou, L.M., Fischer, E.H. 1986. Phosphoprotein phosphatases. *In:* The Enzymes. Vol. 17, pp. 312–361. P.O. Boyer and E.G. Krebs, editors. Academic, Orlando

2. Beavo, J.A., Bechtel, P.J., Krebs, E.G. 1974. Preparations of homogenous cyclic AMP-dependent protein kinase(s) and its subunits from rabbit skeletal muscle. *Methods Enzymol.* **38:**299–308
3. Burnham, C., Braw, R., Karlish, S.J.D. 1986. A Ca-dependent K channel in "luminal" membranes from renal outer medulla. *J. Membrane Biol.* **93:**177–186
4. Driessche, W. van, Erlij, D. 1988. Activation of K^+ conductance in basolateral membrane of toad urinary bladder by oxytocin and cAMP. *Am. J. Physiol.* **254:**C816–C821
5. Dubinsky, W.P., Monti, L.B. 1986. Resolution of apical from basolateral membranes of shark rectal gland. *Am. J. Physiol.* **251:**C721–C726
6. Eveloff, J., Kinne, R. 1983. Sodium-chloride transport in the medullary thick ascending limb of Henle's loop: Evidence for a sodium-chloride cotransport system in plasma membrane vesicles. *J. Membrane Biol.* **72:**173–181
7. Ewald, D.A., Williams, A., Levitan, I.B. 1985. Modulation of single Ca^{2+}-dependent K^+ channel activity by protein phosphorylation. *Nature (London)* **315:**503–506
8. Garty, H., Rudy, B., Karlish, S.J.D. 1983. A simple and sensitive procedure for measuring isotope fluxes through ion-specific channels in heterogenous populations of membrane vesicles. *J. Biol. Chem.* **258:**13094–13099
9. Greger, R., Schlatter, E. 1983. Properties of the luminal membrane of the cortical thick ascending limb of Henle's loop of rabbit kidney. *Pfluegers Arch.* **396:**315–324
10. Guggino, S.E., Guggino, W.B., Green, N., Sacktor, B. 1987. Ca^{2+}-activated K^+ channels in cultured medullary thick ascending limb cells. *Am. J. Physiol.* **252:**C121–C127
11. Guggino, S.E., Suarez-Isla, B.A., Guggino, W.B., Sacktor, B. 1985. Forskolin and antidiuretic hormone stimulate a Ca^{2+}-activated K^+ channel in cultured kidney cells. *Am. J. Physiol.* **249:**F448–F455
12. Handler, J.S. 1988. Antidiuretic hormone moves membranes. *Am. J. Physiol.* **255:**F375–F382
13. Hebert, S.C., Andreoli, T.E. 1984. Effects of antidiuretic hormone on cellular conductive pathways in mouse medullary thick ascending limbs of Henle: II. Determinants of the ADH-mediated increases in transepithelial voltage and in net Cl^- absorption. *J. Membrane Biol.* **80:**221–233
14. Hebert, S.C., Culpepper, R.M., Andreoli, T.E. 1981. NaCl transport in mouse medullary thick ascending limbs: I. Functional nephron heterogeneity and ADH-stimulated NaCl cotransport. *Am. J. Physiol.* **241:**F412–F431
15. Hebert, S.C., Friedman, P.A., Andreoli, T.E. 1984. Effects of antidiuretic hormone on cellular conductive pathways in mouse medullary thick ascending limbs of Henle: I. ADH increases transcellular conductance pathways. *J. Membrane Biol.* **80:**210–219
16. Jorgensen, P.L. 1974. Purification and characterization of (Na^+, K^+)-ATPase: III. Purification from the outer medulla of mammalian kidney after selective removal of membrane components by sodium dodecylsulphase. *Biochim. Biophys. Acta* **356:**36–52
17. Kinne, R., Kinne-Saffran, E., Scholermann, B., Schutz, H. 1986. The anion specificity of the sodium-potassium-chloride cotransporter in rabbit kidney outer medulla: Studies on medullary plasma membranes. *Pfluegers Arch.* **407(Suppl 2):**S168–S173
18. Klaerke, D.A., Petersen, J., Jorgensen, P.L. 1987. Purification of Ca^{2+}-activated K^+ channel protein in calmodulin affinity columns after detergent solubilization of luminal membranes from outer renal medulla. *FEBS Lett.* **216:**211–216
19. Koenig, B., Cicapito, S., Kinne, R. 1983. Chloride transport in the thick ascending limb of Henle's loop: Potassium independence and stoichiometry of the NaCl cotransport system in plasma membrane vesicles. *Pfluegers Arch.* **399:**173–179
20. Levitan, I.B. 1985. Phosphorylation of ion channels. *J. Membrane Biol.* **87:**177–190
21. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193:**265–275
22. Molony, D.A., Reeves, W.B., Andreoli, T.E. 1987. Some transport characteristics of mammalian renal diluting segments. *Miner. Electrolyte Metab.* **13:**442–450
23. Molony, D.A., Reeves, W.B., Hebert, S.C., Andreoli, T.E. 1987. ADH increases apical Na^+, K^+, $2Cl^-$ entry in mouse medullary thick ascending limbs of Henle. *Am. J. Physiol.* **252:**F177–F187
24. Reeves, W.B., Dudley, M.A., Mehta, P., Andreoli, T.E. 1988. Diluting power of isolated thick ascending limbs of Henle: II. Osmotic gradients modulate bumetanide-sensitive $^{22}Na^+$ influx in medullary vesicles. *Am. J. Physiol. (in press)*
25. Reeves, W.B., Mehta, P., Andreoli, T.E. 1988. ATP and cAMP-dependent protein kinase increase K^+ conductance in renal medullary membrane vesicles. *Clin. Res.* **36:**525A
26. Roth, J., Grunfeld, C. 1985. Mechanism of action of peptide hormones and catecholamines. *In:* Textbook of Endocrinology. (7th Ed.) J.D. Wilson and D.W. Foster, editors. pp. 76–122. W.B. Saunders, Philadelphia
27. Schlatter, E., Greger, R. 1985. cAMP increases the basolateral Cl^- conductance in the isolated perfused medullary thick ascending limb of Henle's loop of the mouse. *Pfluegers Arch.* **405:**367–376
28. Strange, K.S., Willingham, M.C., Handler, J.S., Harris, H.W., Jr. 1988. Apical membrane endocytosis via coated pits is stimulated by removal of antidiuretic hormone from isolated, perfused rabbit cortical collecting tubule. *J. Membrane Biol.* **103:**17–28
29. Teulon, J., Paulais, M., Bouthier, M. 1987. A Ca^{2+}-activated cation-selective channel in the basolateral membrane of the cortical thick ascending limb of Henle's loop of the mouse. *Biochim. Biophys. Acta* **905:**125–132
30. Weinman, E.J., Shenolikar, S., Kahn, A.M. 1987. cAMP associated inhibition of the Na^+-H^+ exchanger in rabbit kidney brush border membranes. *Am. J. Physiol.* **252:**F19–F25
31. Whitehouse, S., Walsh, D.A. 1987. Inhibitor protein of the cAMP-dependent protein kinase: Characteristics and purification. *Methods Enzymol.* **99:**80–93
32. Wright, E.M. 1984. Electrophysiology of plasma membrane vesicles. *Am. J. Physiol.* **246:**F363–F372

Received 1 December 1988; revised 30 January 1989