Dual Effect of Cyclic GMP on Renal Brush Border Na-H Antiporter (43323)

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Abstract. **The Na-H antiporter of renal-brush border membranes is inhibited by cyclic AMP and stimulated by protein kinase C. The proximal tubule contains guanylate cyclase and is capable of cyclic GMP production. The effect of cGMP on renal Na-H antiporter activity was analyzed in phosphorylated brush border membranes by ^{22}Na uptake in the presence or absence of 1 m*M* amiloride. 8-Bromo cyclic GMP (1 μM) increased the amiloride-sensitive ^{22}Na uptake in control from 1.26 ± 0.13 to 1.54 ± 0.12 nmol/mg/protein/10 sec, $P < 0.01$, without altering the amiloride-insensitive component. In the absence of exogenous ATP, cGMP also stimulated the amiloride-sensitive ^{22}Na uptake, which can be explained by the presence of endogenous ATP in concentrations of up to 50 μM in the membranes. In ATP-depleted membrane vesicles, however, cGMP inhibited the amiloride-sensitive ^{22}Na uptake. These data indicate that cGMP acts on the Na-H antiporter by at least two different mechanisms, one of which is ATP dependent. It is likely that cGMP-dependent protein kinase mediates the stimulatory effects seen in the presence of ATP, and the inhibition seen in ATP-depleted membranes results from cGMP direct action on the Na-H antiporter.** [P.S.E.B.M. 1991, Vol 198]

The brush border Na-H antiporter is the main system responsible for acidification in the proximal tubule. This system is regulated by intracellular pH, acid base status, and hormones. In addition, this system is inhibited by cyclic AMP through its kinase and stimulated by protein kinase C (1, 2). The proximal tubule is capable of generating cyclic guanosine monophosphate (cyclic GMP; 3, 4) in response to angiotensin II and other stimuli (5, 6), but the effect of cyclic GMP on renal Na-H antiporter is unknown. In intestinal brush border membranes, both cyclic GMP and cyclic AMP (7) inhibit the Na-H antiporter. In the kidney, the effect of cyclic GMP on this transport system is unknown, but recent studies have shown that cyclic GMP directly inhibits a cation channel in the inner medullary collecting duct (8). The purpose of this study was to investigate the effect of cyclic GMP on the renal Na-H antiporter.

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Materials and Methods

Membrane Preparation. Purified renal brush border membrane vesicles (BBMV) were prepared from cortical homogenates of New Zealand White male rabbits using differential and ionic precipitation techniques as described previously (9), except that Ca^{2+} was omitted in the solution. Calcium-free membranes were used in most of the experiments because calcium decreases the activity of the Na-H antiporter. Calcium-free membranes were also used to exclude the possibility that the effect of cyclic GMP was not mediated through calcium-dependent protein kinases. Purified ATP-depleted renal BBMV were prepared by modification of the Mg^{2+} aggregation technique described by Aronson (10). In this technique, 0.05 m*M* EDTA and 0.05 m*M* EGTA were added to the solution. In these membrane vesicles, ATP depletion was accomplished by incubation at 25°C for 15 min (11). With this procedure, ATP was reduced to very minimal concentrations. In both preparations, BBMV were enriched 13- and 11-fold in alkaline phosphatase and γ-glutamyl transferase, respectively. Na-K-ATPase was not enriched, suggesting a lack of basolateral membrane contamination.

ATP Assay. Endogenous ATP in the membrane vesicles (intravesicular and membrane bound) was measured using an ATP luciferase assay. Intravesicular and membrane-bound ATP were extracted as described

by Rood *et al.* (11). Membrane vesicles were first treated with 0.1% saponin at 4°C for 10 min and then spun for 10 min at 100,000*g*. The resulting supernatant, which contained intravesicular ATP, was saved for ATP assay. The pellet, which was resuspended in buffer containing 200 mm of mannitol, 40 m*M* Tris (pH 9.20), 5 m*M* Mg gluconate, and 2 m*M* of EGTA, was boiled for 15 min and spun at 100,000*g* for 5 min. The latter supernatant, which contained the membrane-bound ATP, was also used to measure ATP. ATP was measured by a bioluminescent assay kit from the Sigma Chemical Co. Results are expressed for total ATP, which represents intravesicular plus membrane-bound ATP.

***In-Vitro* Phosphorylation and Electrophoresis.** BBMV were phosphorylated (1) by incubating at 30°C for 5 min in 1:4 hypotonic solution containing 10 m*M* $MgCl_2$, 10 m*M* KF, 5 m*M* 2-(*N*-morpholino)ethane sulfonic acid-Tris buffer (pH 6.80), and 50 μM adenosine triphosphate. 8-Bromo cyclic GMP (1 μM) and/or H-8, a protein kinase inhibitor, was added whenever necessary. Protein phosphorylation by ATP and cyclic GMP was assessed by gel electrophoresis by using modification of the method of Laemmli (10). *In vitro* phosphorylation of membrane vesicles was carried out as described above, with the addition of 5–10 μCi [^{32}P] ATP. The reaction was stopped by the addition of 1:2 solution containing 20% glycerol, 9% sodium dodecyl sulfate, 10% 2-mercaptoethanol, and 125 m*M* Tris. Samples were heated at 95°C for 4 min and then cooled on ice. Seventy to 120 μg of membrane protein were loaded on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis discontinuous gel and ran at 25 mA/gel for the stacking gel and 35 mA/gel for the separating gel. The gels were stained with Coomassie blue, dried, and autoradiographed.

Na-H Antiporter Activity and H^+ Permeability. ^{22}Na uptake was measured by the rapid filtration technique in both phosphorylated and unphosphorylated BBMV, as described by Cohn *et al.* (12), in the presence of pH gradient and in the absence and presence of 1 m*M* amiloride. Proton permeability was assessed by dissipation of acridine orange fluorescence, as described previously (9). In brief, membrane vesicles loaded with 150 m*M* *N*-methylglutamine gluconate at pH 6.0 with and without ATP and with and without cyclic GMP were added to cuvette containing 150 m*M* *N*-methylglutamine gluconate, 10 m*M* Hepes Tris (pH 7.4), and 6 μM acridine orange, and the fluorescence was monitored in a Perkin-Elmer spectrofluorometer with an excitation wavelength of 493 and emission at 530 m*M*. The acid pH inside the vesicle caused rapid quenching of acridine orange fluorescence. The rate of dissipation of acridine orange reflects the rate of H^+ dissipation from the inside to the outside of the vesicle and it has been used as an index of H^+ permeability. The methods correlated well with the measurements of H^+ permeability, taking into account the buffer capacity of the vesicles (13).

All chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO) except acridine orange, which was obtained from Eastman Kodak (Rochester, NY). Sodium-22 was purchased from the Amersham Corp. (Arlington Hts, IL). Statistical significance was calculated by a paired *t* test. Data are expressed as mean ± SE.

Results

Figure 1 shows a sodium dodecyl sulfate-polyacrylamide gel electrophoresis autoradiogram of brush border membranes phosphorylated with [^{32}P]ATP alone and with ATP plus 1 μM 8-bromo cyclic GMP. It is clear that cyclic GMP phosphorylated several brush border membrane proteins. Figure 2 shows ^{22}Na uptake in the presence and absence of 10^{-3} *M* amiloride in Ca^{2+}-free brush border membranes loaded with 50 μM ATP (interior pH 6.40) and suspended in buffer (pH 7.40). Amiloride decreased ^{22}Na uptake by 73%, indicating the presence of Na-H antiporter. Cyclic GMP (10^{-6} *M*) increased ^{22}Na uptake significantly from 1.73 ± 0.15 to 1.98 ± 0.13 nmol/mg protein/10 sec, $P < 0.01$. The increase in ^{22}Na uptake by cyclic GMP was

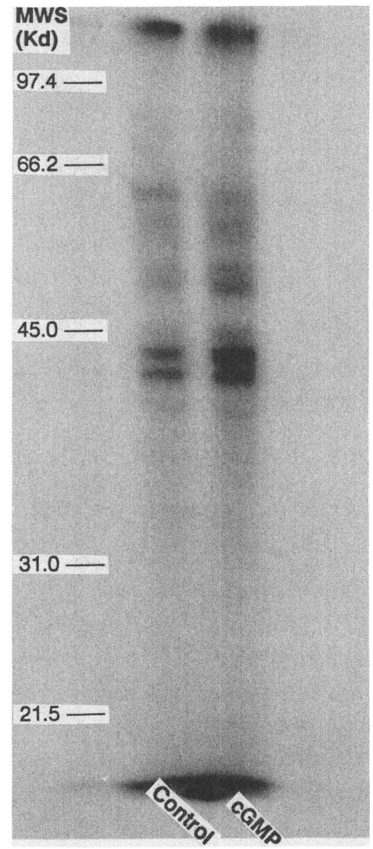

Figure 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis autoradiogram of brush border membranes phosphorylated with (left lane) [^{32}P]ATP and [^{32}P]ATP plus 10^{-6} *M* cyclic GMP.

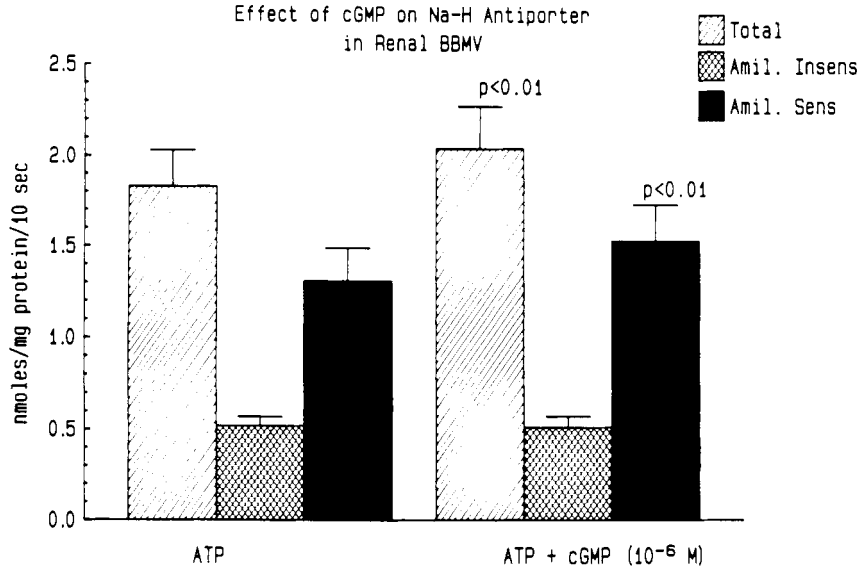

Figure 2. Effect of 10^{-6} *M* cGMP on Na-H antiporter of phosphorylated renal BBMV in the presence and absence of 10^{-3} *M* amiloride (n = 15).

totally accounted for by an increase in amiloride-sensitive ^{22}Na uptake (1.26 ± 0.13 vs 1.54 ± 0.12 nmol/mg protein/10 sec), while the amiloride-insensitive component was not affected (0.47 ± 0.04 vs 0.45 ± 0.04 nmol/mg protein/10 sec). The 25% increase in amiloride-sensitive ^{22}Na uptake is similar in magnitude to the increase in Na-H antiporter observed with stimulation of the antiporter by protein kinase C (2). Equilibrium values of ^{22}Na uptake at 60 min, however, were not different between control and cyclic GMP (10^{-6} *M*)-treated vesicles (0.48 ± 0.04 vs 0.34 ± 0.06 nmol/mg protein/60 min, $n = 4$, NS). In membranes prepared with calcium, the activity of the Na-H antiporter was lower, and this is in agreement with results of other investigators (13). Under these conditions, cyclic GMP also resulted in 25% stimulation of ^{22}Na uptake from 0.74 ± 0.10 to 0.91 ± 0.13 nmol/mg protein/10 sec, $P < 0.01$. To determine whether the effect of cyclic GMP was dependent on the presence of addition of ATP to the membranes, we examined the effect of GMP on the Na-H antiporter in the absence of exogenous ATP. Under these conditions, cyclic GMP again stimulated the total and amiloride-sensitive ^{22}Na uptake without affecting the amiloride-insensitive component (Fig. 3). The increase in the amiloride-sensitive component from 1.31 ± 0.18 to 1.53 ± 0.20 nmol/mg protein/10 sec, $P < 0.01$, was identical to that observed in the presence of exogenous ATP.

To ascertain that the stimulatory effect of cGMP on the Na-H antiporter was not secondary to permeability changes of the membrane, we measured H^+ permeability, as assessed by acridine orange fluorescence dissipation in the absence of Na^+ (14). Experiments were performed in the presence and absence of ATP. Table I shows that H^+ permeability was not altered by cyclic GMP in the presence of ATP and, hence, that the increase in Na-H antiporter activity cannot be explained by alterations in H^+ permeability. In the absence of ATP, H^+ permeability was also not different between control and cyclic GMP (10^{-6} *M*)-treated vesicles (control, 0.14 ± 0.02, vs cyclic GMP, 0.16 ± 0.03 arbitrary fluorescence units/mg protein/min, NS, $n = 4$).

The finding that cyclic GMP stimulated Na-H antiporter activity in the absence of exogenous ATP could be explained either by an effect independent of activation of cyclic GMP-dependent protein kinase (16) or alternatively by the presence of endogenous ATP in the membranes in an amount sufficient to effect phosphorylation of cyclic GMP-dependent protein kinase. We measured ATP in membranes used for experiments in Figure 3 and found that these membranes contained 50 μmol/mg protein of ATP. Since these membranes have an average volume of 1 μl/mg of protein, the concentration of ATP is on the average 50 μ*M*, an amount sufficient to phosphorylate cyclic GMP-dependent protein kinase, which has a K_m for ATP of 30 μ*M* (15). The results in Figure 3, therefore, could be explained by an effect of cyclic GMP through an ATP-dependent mechanism, i.e., through phosphorylation of cyclic GMP-dependent protein kinase utilizing endogenous ATP. To further explore the role of cyclic GMP-dependent protein kinase, we examined the putative inhibitor of this protein, H-8, on cyclic GMP stimulation of the Na-H antiporter. Membranes were phosphorylated with ATP, and preliminary experiments indicated that H-8 did not affect ^{22}Na uptake. The effect of cyclic GMP on Na-H antiporter activity

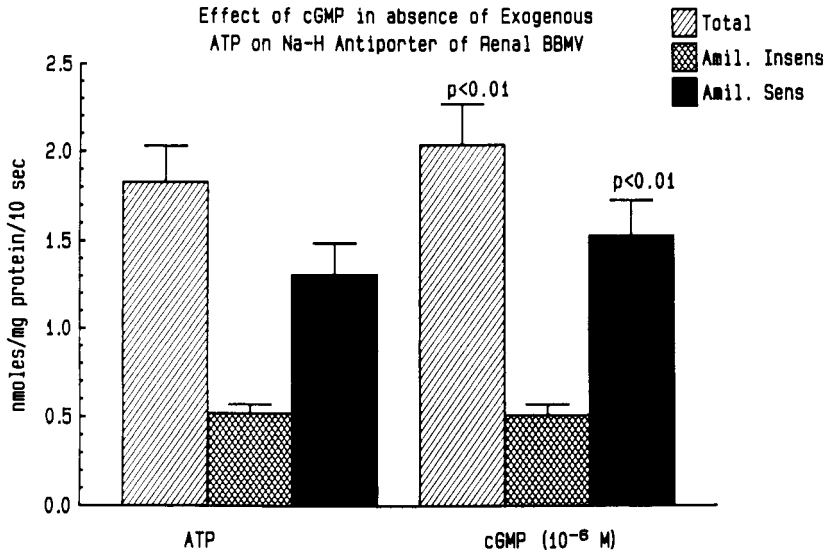

Figure 3. Effect of 10^{-6} *M* cGMP on Na-H antiporter of unphosphorylated renal BBMV in the presence and absence of 10^{-3} *M* amiloride (n = 10).

Table I. H^+ Permeability in Renal BBMV Measured by Acridine Orange Fluorescence Dissipation[a]

	FU/mg protein/min
ATP	0.34 ± 0.05
ATP + cGMP	0.37 ± 0.06
cGMP	0.37 ± 0.06

[a] n = 4–6 experiments. FU, arbitrary fluorescence units.

was measured in paired experiments in which the effects of cGMP were assessed in the presence and absence of H-8 (1–5 μM). Cyclic GMP stimulated the Na-H antiporter by 32% as compared with controls. H-8 did not inhibit the stimulatory effect of cyclic GMP on the Na-H antiporter (cyclic GMP, 1.14 ± 0.16 nmol/mg protein/10 sec; cyclic GMP + H-8, 1.21 ± 0.04 nmol/mg protein/10 sec, NS, n = 5). We did not use higher concentrations of H-8, because at higher concentrations H-8 inhibits protein kinases A and C (17).

Since cyclic GMP has been shown to have an effect on Na^+ transport independent of its kinase (13), we examined the effect of 10^{-6} *M* cyclic GMP on the Na-H antiporter activity of brush border membranes depleted of ATP. The depletion of ATP was verified by measuring the ATP content of these vesicles and we showed that these membranes contained less than 1 μM ATP (range, 0.37–1 μM ATP). Figure 4 shows the effect of 10^{-6} *M* cyclic GMP on ^{22}Na uptake in ATP-depleted membranes. In contrast to the stimulatory effect in the presence of ATP, cyclic GMP consistently inhibited Na-H antiporter activity in ATP-depleted membranes. Again, the inhibitory effect of cyclic GMP on these membranes was accounted for by inhibition of the amiloride-sensitive component of the Na-H antiporter.

Discussion

The present study was aimed at studying the effect of cyclic GMP on the renal brush border Na-H antiporter. This system is responsible for 85% of the HCO_3 reabsorption in the proximal tubule (1). The proximal tubule contains guanylate cyclase, which is activated by several hormones, including angiotensin II and vitamin D, to form cyclic GMP (5, 6). These hormones have been shown to increase HCO_3 reabsorption in the proximal tubule (18–20). The present studies showing an effect of cyclic GMP, in the presence of ATP to stimulate Na-H antiporter, provide an explanation, at least in part, for the effect of these hormones on the stimulation of HCO_3 reabsorption in the proximal tubule. It is reasonable to postulate that this effect of cyclic GMP is mediated through cyclic GMP protein kinase.

Although cyclic GMP-dependent protein kinase was not measured in the present studies, previous studies have suggested that the presence of cyclic GMP-dependent protein kinase parallels the presence of guanylate cyclase activity (17); hence, it is reasonable to postulate the presence of cyclic GMP-dependent protein kinase on brush border membranes that are rich in guanylate cyclase.

The failure of H-8 to prevent the effect of cGMP does not rule out an effect of cyclic GMP, through its kinase, since the concentration used may not have been sufficiently high to achieve inhibition of cyclic GMP-dependent protein kinase. As discussed above, higher concentrations were not used because cyclic AMP-dependent protein kinase and protein kinase C are inhibited by high concentrations of H-8. Cyclic GMP stimulated the Na-H antiporter by 25%, an effect that is of the same magnitude as that described for protein

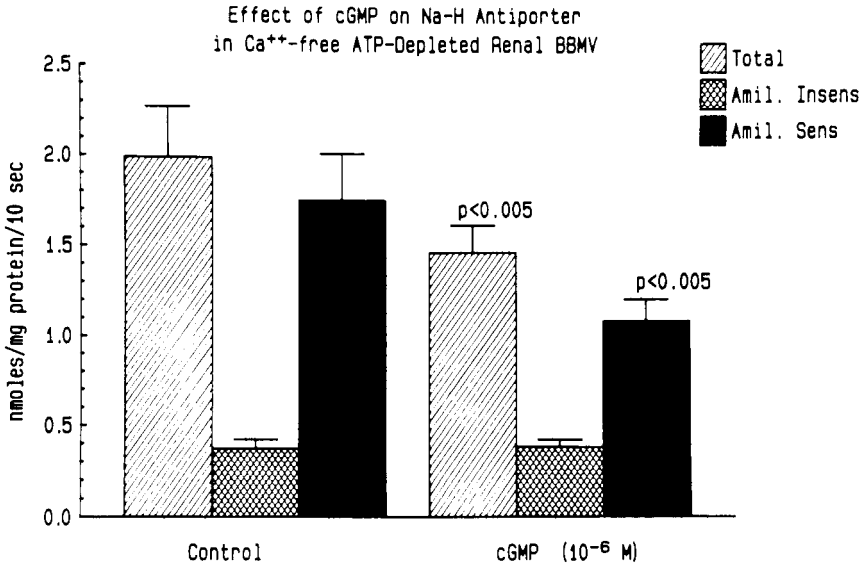

Figure 4. Effect of 10^{-6} *M* cGMP on Na-H antiporter of ATP-depleted renal BBMV in the presence and absence of 10^{-3} *M* amiloride ($n = 6$).

kinase C on the renal Na-H antiporter. It must be emphasized, however, that the effect *in vivo* may be of higher magnitude because certain hormones, e.g., angiotensin II, may increase HCO_3 reabsorption by decreasing cyclic AMP and increasing cyclic GMP and protein kinase C, all of which will result in the enhancement of HCO_3 reabsorption (18, 19). The effect of cyclic GMP on the activation of the Na-H antiporter was not mediated by change in proton permeability or changes in the volume to the vesicles (see Results).

In addition to the stimulatory effect of cyclic GMP on the Na-H antiporter in the presence of ATP, we observed an inhibitory effect of cyclic GMP on the Na-H antiporter in the absence of ATP; therefore, this effect is not mediated by cyclic GMP-dependent protein kinase. These results are in agreement with previous studies showing an effect of cyclic GMP on Na^+ channels independent of kinase (8).

The results of present studies, thus, clearly demonstrate a dual effect of cyclic GMP on the Na-H antiporter activity; a stimulatory effect, mediated apparently through cyclic GMP-dependent protein kinase, and an inhibitory effect, not dependent on kinase activity. Despite intense investigation of the regulation of the Na-H antiporter, only one previous study has examined the effect of cyclic GMP on the opossum kidney cell line and found that high concentrations of cyclic GMP (10^{-4} *M*) inhibited the Na-H antiporter (21). The results of the present study demonstrate a dual effect of physiologic concentration of cyclic GMP on the renal Na-H antiporter. Since under the physiologic condition, ATP is available in large quantities in the proximal tubule, we postulate that *in vivo* cyclic GMP stimulates the renal Na-H antiporter.

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