Regulation of the Renal Na-HCO₃ Cotransporter: IV. Mechanisms of the Stimulatory Effect of Angiotensin II¹,²

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ABSTRACT
Angiotensin II stimulates proximal tubule acidification by activating both the Na-H antiporter and the Na-HCO₃ cotransporter. The mechanism whereby angiotensin II stimulates the Na-HCO₃ cotransporter was investigated in renal cortical basolateral membrane vesicles of the rabbit by measuring ²²Na uptake in the presence of HCO₃ and gluconate. Na-HCO₃ cotransporter activity (expressed in nanomoles per milligram of protein per 3 s) was taken as the difference in ²²Na uptake in the presence of HCO₃ and gluconate. Angiotensin II stimulated Na-HCO₃ cotransporter activity significantly (control, 1.5 ± 0.4; angiotensin II, 3.3 ± 0.6, P < 0.05), and this stimulation was prevented by the angiotensin II receptor antagonist DuP 753. Angiotensin II has been shown to stimulate both pertussis toxin-sensitive G₁ protein and pertussis toxin-insensitive G₉ protein. In the presence of pertussis toxin, angiotensin II (10⁻¹¹ M) failed to stimulate the Na-HCO₃ cotransporter, suggesting a role of G₁ protein in mediating this effect. In the presence of a polyclonal antibody against G₁ protein, angiotensin II failed to stimulate the Na-HCO₃ cotransporter (control, 1.6 ± 0.4; angiotensin II, 3.9 ± 0.9; angiotensin II + G₁, 1.2 ± 0.7). Angiotensin II stimulated inositol trisphosphate release, and this effect could be blocked by the phospholipase C inhibitor U73122, suggesting a role of phospholipase C or A₂ in this effect of angiotensin II. In the presence of the protein kinase C inhibitor calphostin C (50 nM), angiotensin II also failed to stimulate the Na-HCO₃ cotransporter. These results demonstrate that angiotensin II stimulates the renal Na-HCO₃ cotransporter by interacting with a specific angiotensin II receptor and that this stimulation is mediated by the activation of G₁ and G₉ proteins.

Key Words: Na-HCO₃ cotransporter, angiotensin II, G protein, phospholipase C

Angiotensin II (All) plays an important role in proximal HCO₃ reabsorption. Microperfusion studies have shown that All strongly stimulates HCO₃ reabsorption by specific All receptors in the proximal tubule (1). Additional studies have shown that All stimulation caused an increase in both brush border Na-H antiporter and basolateral Na-HCO₃ cotransporter activities (2-4). The mechanism whereby All affects the Na-H antiporter has been studied in detail, and it has been suggested that All stimulates this antiporter by several mechanisms including the inhibition of the adenylate cyclase/cAMP system and the activation of phospholipase C (PLC) and A₂ (PLA₂) (5-9). All has been shown to stimulate pertussis toxin (PTX)-sensitive G₁ and PTX-insensitive G₉ protein (9,10). We have recently shown that G proteins play an important role in the regulation of the Na-HCO₃ cotransporter, with Gα stimulating this transport system (11). In this study, we sought to define the mechanisms whereby All stimulates the Na-HCO₃ cotransporter.

METHODS
Membrane Vesicle Preparation
Purified renal basolateral membrane vesicles (BLMV) were prepared from cortical homogenates of New Zealand White rabbits by differential and gradient centrifugation by ionic precipitation techniques, as previously described (12). The BLMV were enriched 10- to 13-fold in Na-K-ATPase activity with minimal cross-contamination of brush border membranes, as assessed by alkaline phosphatase and γ-glutamyl transferase activities.

Isolation of Rabbit Proximal Tubule Cells
The separation of the proximal tubules was done by the method of Chung et al. (13), as previously described (14,15). In brief, New Zealand White rabbits (5 to 6 lb) were anesthetized with pentobarbital (50 mg/kg). The aorta was cannulated, and the kidneys were perfused in situ with sterile serum-free medium (Dulbecco modified Eagle medium [DMEM]/F12) containing particulate iron oxide. The kidneys were dissected free from the abdominal cavity, the capsule was removed, and the cortex was dissected free from medullary tissue and homogenized in a Polytron tissue homogenizer (Brinkmann Instruments, Inc., Westbury, NY). The homogenate was serially filtered through three nylon mesh screens having pore sizes of 250, 74, and 44 μm. The material that traversed the former screen contained both tubules and glomeruli, the latter being removed with a sterile magnet. The resultant tubule suspension was used for the measurement of inositol trisphosphate (IP₃) and arachidonic acid release.
Measurement of Na-HCO₃ Cotransporter Activity

²²Na uptake was measured at 3 s by the rapid filtration technique as previously described (12). BLMV were preincubated for 1 h at room temperature in a solution containing 200 mM sucrose and 50 mM N-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES) (pH 7.50) with Tris and 1 mM magnesium gluconate. The suspension was spun at 30,000 rpm with a Beckmann Ti 50.2 rotor (Beckmann Instruments, Inc., Palo Alto, CA) for 30 min at 4°C, and the resulting pellet was resuspended in the same solution. The reaction was started by the addition of 100 to 150 μg of membrane protein to uptake medium containing 40 mM sodium gluconate, 1 mM magnesium gluconate, and 50 mM HEPES (pH 7.50) with KOH, as well as 1 μCi of ²²[NaCl in the presence of 25 mM KHCO₃ or potassium gluconate. After 3 s of incubation at room temperature, the reaction was stopped by the addition of 4 mL of ice-cold stop solution containing 200 mM sucrose and 50 mM HEPES (pH 7.50) with Tris and subsequent pouring onto a 0.45-μm-pore-size prewetted Millipore filter (Millipore, Bedford, MA). Filters were washed three times more, and radioactivity was measured by scintillation spectrometry. HCO₃-dependent ²²Na uptake was taken as the difference in ²²Na uptake in the presence or absence of an inwardly directed HCO₃ gradient (HCO₃ was replaced by gluconate).

Measurement of IP₃ Release

IP₃ released by isolated proximal tubule cells was measured as described (16) with some modifications. In brief, cells were labeled with [³H]myo-inositol in DMEM/F12 with 0.5% fetal calf serum for 48 h at 37°C. After the cells were washed three times with DMEM/F12 with 0.5% fetal calf serum, the cells were incubated either with vehicle, 10⁻¹¹ M All, or 10⁻¹⁰ M All and 10⁻⁵ M U73122 for 30 min at 37°C. The reaction was stopped by the addition of 1 mL of ice-cold methanol. IP₃ was extracted with CHCl₃:MeOH:H₂O in a ratio of 2:1:1. The upper aqueous phase was applied to Dowex formate columns (100 to 200 mesh), after which the columns were washed thoroughly with 30 mL of solution containing 0.3 M NH₄ formate and 0.1 M formic acid. The IP₃ fraction was eluted with 10 mL of 2 M NH₄ formate and 0.1 M formic acid. The eluate contained the 1,4,5, 1,3,4 and 2,4,5 isomers of IP₃ and the 1,3,4,5 isomer of IP₃. The radioactivity of the eluate was subsequently measured by scintillation spectrometry.

Measurement of Arachidonic Acid Release

Arachidonic acid released by isolated rabbit proximal tubule cells was measured as described (16). Cells were labeled with 0.30 μCi/mL [³H]arachidonic acid in DMEM containing 0.5% fetal calf serum for 4 h at 37°C. After the cells were washed with DMEM with fetal calf serum, they were incubated with 10⁻¹¹ M All or with 10⁻¹⁰ M All and 10⁻⁶ M mepacrine for 30 min at 37°C. One milliliter of the media was then aspirated, and radioactivity was measured by scintillation spectrometry.

Materials

²²NaCl was purchased from Amersham (Arlington Heights, IL), and [³H]myo-inositol and [³H]arachidonic acid were from NEN-DuPont (Boston, MA). DuP 753 was a gift from the DuPont Merck Pharmaceutical Company (Wilming- ton, DE). U73122 was obtained from Research Biochemicals International (Natick, MA). Calphostin C was from Calbio- chem (San Diego, CA), anti-G₁ was from Upstate Biotechnol- ogy Incorporated (Lake Placid, NY), DMEM/F12 was from Atlanta Biologicals (Norcross, GA), and fetal calf serum was from GIBCO BRL (Grand Island, NY). PTX, anti-rabbit immu- noglobulin (IgG), and all other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

Statistical Analysis

Experiments were done in triplicate and results are presented as mean ± SE. Statistical significance was analyzed by the use of the paired t test and analysis of variance as appropriate.

RESULTS

Effect of All on the Na-HCO₃ Cotransporter and the Role of G₁ Protein

Other investigators have shown that All has a dual effect on proximal tubular transport, with physiologic concentration, stimulating Na transport, whereas pharmacologic concentrations inhibit it (17–19). In this study, we examined the effect of a physiologic concentration of All (10⁻¹¹ M) on the Na-HCO₃ cotransporter activity. Figure 1 shows the effect of 10⁻¹¹ M All on the ²²Na uptake by BLMV in the presence of HCO₃ or gluconate. All significantly increased ²²Na uptake in the presence of HCO₃, from 5.91 ± 0.45 to 6.66 ± 0.52 nmol/mg of protein per 3 s (P < 0.05), but not in the presence of gluconate, 4.31 ± 0.41 versus 3.74 ± 0.44 nmol/mg of protein per 3 s (not significant). Thus, Na-HCO₃ cotransporter activity, the difference in ²²Na uptake in the presence of HCO₃ and gluconate, was significantly increased by All from 1.60 ± 0.31 to 2.92 ± 0.52 nmol/mg of protein per 3 s, (P < 0.01). This is in agreement with results reported by other investigators (3,4). Figure 2 shows that the effect of All to stimulate the Na-HCO₃ cotransporter activity in BLMV was totally prevented by the All receptor antagonist DuP 753 from 3.42 ± 0.54 to 1.27 ± 0.33 nmol/mg of protein per 3 s (P < 0.05).

Because All has been shown to stimulate PTX-sensitive G₁ protein (5–8,20–22) and we have previously shown that purified G₁ protein stimulated the Na-HCO₃ cotransporter (11), we investigated the role of this protein in mediating the effect of All on the Na-HCO₃ cotransporter. For this purpose, we examined the effect of All in the presence and in the absence of either PTX or a polyclonal antibody against G₁ protein. Figure 3 shows that in the presence of PTX, the effect of All to stimulate the Na-HCO₃ cotransporter was totally prevented (in nanomoles per milligram of protein per 3 s: control, 1.05 ± 0.32; All 1.77 ± 0.22; All + PTX, −0.24 ± 0.59; P < 0.02 for control versus All and P < 0.05 for All versus All + PTX). Figure 4 shows that the effect of All to stimulate the Na-HCO₃ cotransporter was totally prevented by a polyclonal antibody against Ga₅, but not by IgG (in nanomoles per milligram of protein per 3 s: control, 1.59 ± 0.69; All + IgG, 3.88 ± 0.85; All + anti-Ga₅,
All and the Renal Na-HCO₃ Cotransporter

Figure 1. Effect of All on ²²Na uptake by BLMV in the presence of HCO₃ (open bar) or gluconate (speckled bar). HCO₃-dependent ²²Na uptake (solid bar), the difference in ²²Na uptake in the presence of HCO₃ and gluconate, was taken as the activity of the Na-HCO₃ cotransporter. BLMV were incubated either with vehicle (control) or with 10⁻¹¹ M All for 15 min. Each datum point represents the mean and SE of experiments done in triplicates from 11 membrane preparations.

Figure 2. Effect of DuP753 on the stimulatory effect of All on the activity of the Na-HCO₃ cotransporter in BLMV treated with vehicle (open bar), 10⁻¹¹ M All (solid bar), or 10⁻¹¹ M All + 10⁻⁵ M DuP753 (diagonal bar). N = 6 in each group.

Figure 3. Effect of PTX on the stimulatory effect of All on Na-HCO₃ cotransporter activity. HCO₃-dependent ²²Na uptake in control BLMV (open bar), All-treated BLMV (solid bar), and All + PTX (100 ng)-treated BLMV (speckled bar). N = 5 in each group.

To examine the possible role of adenylate cyclase inhibition on the effect of All on the Na-HCO₃ cotransporter, we studied the effect of dideoxyadenosine (DDA), 10⁻⁴ M, an adenylate cyclase inhibitor (23). Figure 5 shows that the addition of DDA to BLMV was associated with a stimulation of the Na-HCO₃ cotransporter activity (from 1.96 ± 0.37 to 0.37 to 5.35 ± 0.77 nmol/mg of protein per 3 s; P < 0.05), suggesting that the inhibition of adenylate cyclase in BLMV increases the activity of the Na-HCO₃ cotransporter. In the presence of DDA, the effect of All to stimulate the cotransporter was similar to that of All alone (All, 4.73 ± 0.74 versus All + DDA, 4.20 ± 0.77 nmol/mg of protein per 3 s; not significant).

Role of PLC and Protein Kinase C on the Effect of All on the Na-HCO₃ Cotransporter

Because All stimulates PLC in other cells (9,10,24-26), thereby activating protein kinase C, we decided to
investigate the possible role of this pathway on the effect of AII on the Na-HCO₃ cotransporter. AII (10⁻¹¹ M), stimulated IP₃ release by 62% as compared with control. This effect was prevented by U73122 (10⁻⁵ M), a PLC/A2 inhibitor. The functional effect of the PLC inhibitor U73122 to prevent the effect of AII to stimulate the Na-HCO₃ cotransporter was investigated, but the results were inconclusive because U73122 (10⁻⁵ M) caused inhibition of the Na-HCO₃ cotransporter (data not shown). The ability of U73122 to inhibit both PLC and PLA₂ may have accounted for the inconsistent effect on the activity of the cotransporter.

Figure 6 shows the effect of the protein kinase C inhibitor calphostin C on stimulation of the Na-HCO₃ cotransporter activity. At 50 nM calphostin C, which is the IC₅₀ for the inhibition of protein kinase C, this highly specific inhibitor completely prevented the stimulatory effect of AII on the Na-HCO₃ cotransporter activity (in nanomoles per milligram of protein per 3 s: control, 1.33 ± 0.34; AII, 4.91 ± 1.29; AII + calphostin C, 1.33 ± 0.36; P < 0.05 for both control versus AII and AII versus AII + calphostin C). Of interest is the fact that in the presence of calphostin C, the activity of the Na-HCO₃ cotransporter was returned to baseline control levels.

Role of PLA₂ on the Effect of AII on the Cotransporter

AII has been shown to stimulate PLA₂ and to release arachidonic acid in brush border membranes, in Madin-Darby canine kidney cells, and in cultured mesangial cells (8,16,26). In this study, we investigated the effect of AII, 10⁻¹¹ M, on the release of arachidonic acid by basolateral membranes. All increased the release of arachidonic acid by 42% above control levels (AII, 142.25 ± 13.34), and this effect was prevented by mepacrine, 10⁻⁶ M (AII + mepacrine: 82.60 ± 15.81). We have recently shown that arachidonic acid (10⁻⁶ M) directly inhibits the Na-HCO₃ cotransporter (1.22 ± 0.35 versus 0.18 ± 0.59 nmol/mg of protein per 3 s; \( P < 0.05 \)), and thus, the effect of AII to stimulate PLA₂ and PLC could have a dual effect on the Na-HCO₃ cotransporter, with PLA₂ stimulation inhibiting the cotransporter and PLC activation enhancing the activity of the cotransporter.

We examined the effect of 10⁻⁶ M mepacrine on the activity of the Na-HCO₃ cotransporter in the absence of AII, and we observed that this inhibitor had a variable effect on the activity of the cotransporter. In the majority of experiments, it did not alter the activity of the cotransporter, whereas in some, it appeared to inhibit the activity (data not shown). It was therefore difficult to ascertain the effect of mepacrine in modulating the effect of AII on the cotransporter.

DISCUSSION

These studies were aimed at identifying the mechanisms responsible for the stimulation of the Na-HCO₃ cotransporter by AII. Our results confirm the results of a previous study that a physiologic concentration of AII stimulated the Na-HCO₃ cotransporter in basolateral membranes of the rabbit (4). These findings are also in agreement with other studies showing an effect of AII to cause a parallel stimulation of the brush border Na-H antipporter and of the basolateral Na-HCO₃ cotransporter (3). This effect of AII to stimulate the Na-HCO₃ cotransporter is prevented by DuP 753, suggesting that it is mediated by the AT₁ receptor (27).

Several signaling pathways have been suggested to explain the effect of AII in other tissues and in the kidney, particularly in the proximal tubules (5-8,20,21,24,25,28-31). These pathways include the inhibition of adenylate cyclase by a G, coupled mechanism and cAMP-independent pathways including an increase in cytosolic Ca²⁺ and the activation of protein kinase C, PLA₂, and prostaglandin metabolites. The stimulation of these different pathways by AII has been shown to be different among different species and tissues and also to be concentration dependent. The basolateral membrane of the kidney has been shown to have a high-affinity receptor density for AII with Kₐ (dissociation constant) in the order of 2 nM (31,32). The mechanism whereby AII alters transport is clearly concentration dependent in that physiologic concentrations of AII, 10⁻¹¹ to 10⁻⁶ M, stimulate most transport systems whereas pharmacologic concentrations seem to inhibit Na transport (17-19).

We have chosen to study only the relevant physiologic concentration of AII on the cotransporter, and this concentration of AII had been clearly shown to decrease cAMP production by cultured rabbit proximal tubule cells through a G₁-dependent mechanism.
Figure 5. Effect of DDA on the activity of the Na-HCO₃ cotransporter. BLMV were treated with vehicle (open bar), 10⁻¹¹ M AII (solid bar), 10⁻⁴ M DDA (speckled bar), or AII + DDA (diagonal bar). N = 4 in each group.

Figure 6. Effect of calphostin C on the stimulatory effect of AII on the Na-HCO₃ cotransporter activity in control BLMV (open bar), BLMV treated with 10⁻¹¹ M AII (solid bar), and BLMV treated with 10⁻¹¹ M AII and 50 nM calphostin C (speckled bar). N = 7 in each group. For experiments with calphostin C, the compound was protected from light.

We have previously shown that the cAMP/protein kinase A system inhibits the Na-HCO₃ cotransporter (33) and that PTX also inhibited the cotransporter through a G₁-dependent mechanism (11). Thus, the observed effect of AII to stimulate the Na-HCO₃ cotransporter could, at least in part, be explained by a decrease in cAMP with a consequent removal of the inhibitory effect of protein kinase A on the cotransporter. To address this issue, we examined the effect of DDA alone on the activity of the Na-HCO₃ cotransporter and showed that this compound resulted in significant stimulation of the cotransporter. These results are compatible with the hypothesis that the inhibition of adenyl cyclase increases the activity of the cotransporter. In the presence of DDA, AII stimulates the Na-HCO₃ cotransporter to the same level as that observed in the absence of DDA. Similar results have been reported for the effect of AII to stimulate the Na-H antiporter (6). These results are compatible with the hypothesis that part of the effect of AII to stimulate the cotransporter is mediated by the inhibition of adenyl cyclase. We recognize, however, that the lack of an additive effect of DDA and AII may be because the stimulation of the cotransporter caused by the inhibition of cAMP is potent enough so as to prevent the stimulation of the cotransporter by AII through other pathways.

Taken together, the above experiments suggest a cAMP-dependent mechanism for the stimulation of the Na-HCO₃ cotransporter by AII. These results do not however, exclude the possibility that other signal transduction pathways are also involved in modulating the effect of AII on the cotransporter.

AII has been shown to stimulate PLA₂ in rabbit cultured proximal tubules (30) and brush border membranes (8). This study confirms that a physiologic concentration of AII causes the release of arachidonic acid from isolated proximal tubule cells prelabeled with [³H]arachidonic acid and that this effect was inhibited by the PLA₂ inhibitor mepacrine. Arachidonic acid has been suggested to stimulate the brush border Na-H antiporter, and this has been proposed to be one of the mechanism whereby PLA₂ activation stimulates this transporter (8). Recent studies from our laboratory have shown that parathyroid hormone also releases arachidonic acid and inhibits the cotransporter (34). In addition, arachidonic acid by itself inhibits the cotransporter and an inhibitor of arachi-
donic acid metabolism, eicosatetraynoic acid, prevented the effect of parathyroid hormone. These findings therefore strongly suggest that the activation of the PLA2 pathway cannot account for the stimulatory effect of All on the cotransporter because this pathway causes inhibition rather than stimulation of the cotransporter. Although mepacrine inhibited arachidonate release, its effect on the cotransporter activity could not be completely investigated because it tended to inhibit the cotransporter in the absence of All.

Studies in vascular smooth muscle have suggested that the effect of All to stimulate Na-H exchange is, at least in this tissue, mediated by protein kinase C activation (9). We have previously shown that protein kinase C activation stimulates the Na-HCO3 cotransporter in basolateral membranes (33), and preliminary studies in our laboratory showed the same effect in primary cultures of the proximal tubules (35). Studies in primary cultures of proximal tubules by other investigators have failed to show IP3 release by concentrations of All > 10^{-8} M (30), but lower concentrations of All were not studied, and we found that 10^{-11} M All clearly stimulated IP3 release and that this effect could be blocked by the PLC inhibitor U73122. These results suggest that All stimulates protein kinase C in proximal tubule cells. The effect of the PLC inhibitor on the Na-HCO3 cotransporter again could not be studied because the compound either has no specific effect to inhibit the cotransporter or alternatively blocks both PLC and PLA2, resulting in opposing effects on the activity of the cotransporter. Further evidence that protein kinase C was involved in the stimulation of the Na-HCO3 cotransporter by All is supported by the finding that the protein kinase C inhibitor calphostin C prevented the effect of All on the cotransporter. Calphostin C has been shown to be a potent and more specific inhibitor of protein kinase C, and at the concentration used in this study, it does not inhibit protein kinase A or other kinases (36). In summary, the results of this study demonstrate that All stimulates the Na-HCO3 cotransporter by two distinct pathways, a cAMP-dependent mechanism and a protein kinase C-dependent mechanism.

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