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Angiotensin II Directly Increases Rabbit Renal Brush-Border Membrane Sodium Transport: Presence of Local Signal Transduction System

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Summary. In the present study, we have examined the direct actions of angiotensin II (AII) in rabbit renal brush border membrane (BBM) where binding sites for AII exist. Addition of AII (10^{-11}–10^{-7} M) was found to stimulate $^{22}Na^-$ uptake by the isolated BBM vesicles directly. AII did not affect the Na^+-dependent BBM glucose uptake, and the effect of AII on BBM $^{22}Na^+$ uptake was inhibited by amiloride, suggesting the involvement of Na^+/H^+ exchange mechanism. BBM proton permeability as assessed by acridine orange quenching was not affected by AII, indicating the direct effect of AII on Na^+/H^+ antiport system.

In search of the signal transduction mechanism, it was found that AII activated BBM phospholipase A_2 (PLA) and that BBM contains a 42-kDa guanine nucleotide-binding regulatory protein (G-protein) that underwent pertussis toxin (PTX)-catalyzed ADP-ribosylation. Addition of GTP potentitated, while GDP-βS or PTX abolished, the effects of AII on BBM PLA and $^{22}Na^+$ uptake, suggesting the involvement of G-protein in AII's actions. On the other hand, inhibition of PLA by mepacrine prevented AII's effect on BBM $^{22}Na^+$ uptake, and activation of PLA by mellitin or addition of arachidonic acid similarly enhanced BBM $^{22}Na^+$ uptake, suggesting the role of PLA activation in mediating AII's effect on BBM $^{22}Na^+$ uptake.

In summary, results of the present study show a direct stimulatory effect of AII on BBM Na^+/H^+ antiport system, and suggest the presence of a local signal transduction system involving G-protein mediated PLA activation.

Key Words angiotensin II · renal brush border membrane · Na^+/H^+ exchange · phospholipase A_2 · GTP-binding protein

Introduction

Angiotensin II (AII) is a powerful effector agent in the regulation of extracellular volume and exerts an important influence on renal sodium (Na^+) excretion. AII modulates renal Na^+ excretion through stimulation of aldosterone secretion and through its direct action on the kidney. In the kidney, besides its effect on renal hemodynamics, AII also affects tubular Na^+ transport directly [12]. Substantial evidence from receptor binding [4, 5, 7, 22] and transport studies [6, 11, 13, 18–20, 30, 32] has indicated that proximal tubule serves as the target site for AII's action. Both in vivo micropuncture [11, 13, 18–20] and in vitro tubule perfusion studies [6, 32] showed that peritubular application of AII induced a dose-dependent bimodal effect on proximal tubule Na^+ transport. At lower concentrations (10^{-12}–10^{-10} M), AII enhanced proximal tubule Na^+ transport, whereas at higher concentration (10^{-9}–10^{-6} M), AII suppressed proximal tubule Na^+ transport. The cellular mechanism underlying such bimodal effect of AII remains unclear. Traditionally, AII is considered to affect proximal tubule transport through binding to its receptor on the peritubular basolateral membrane (BLM), which triggers intracellular events and alters Na^+ transport across the luminal brush border membrane (BBM). Thus, it has been proposed that AII at lower concentrations may suppress BLM adenylate cyclase and stimulate proximal tubule Na^+ transport by removing the inhibitory effect of cAMP [12, 20, 38]. On the other hand, AII at higher concentrations may inhibit proximal tubule Na^+ transport through an increase in intracellular calcium levels [6, 12, 24, 35].

In contrast to this peritubular approach, AII binding sites were recently found to be distributed uniformly on both luminal BBM and peritubular BLM in rat proximal tubules [4, 5, 7]. The function of AII binding sites on luminal BBM is not known. Although these binding sites may simply serve as the site for AII absorption and degradation [25], it is also possible that these binding sites may function as hormone receptors and provide another route for AII's action in renal proximal tubule. Indeed, a luminal effect of AII to increase proximal tubule Na^+

transport has been suggested in previous reports [13, 19, 20]. To further substantiate such a luminal action of AII in renal proximal tubule, we have tested the direct effect of AII on BBM Na^+ transport in the present study. The results show that AII directly stimulates BBM Na^+ transport through Na^+/H^+ antiport system and suggest the role of GTP-binding protein (G-protein)-mediated activation of BBM phospholipase A_2 (PLA).

Materials and Methods

Animals

New Zealand White male rabbits, weighing 1.5–2.0 kg, were used in these studies. The animals were maintained on an *ad lib* diet of standard rabbit chow with free access to tap water for drinking.

BBM Vesicle Preparation

Purified BBM vesicles were prepared from rabbit renal cortex by the conventional magnesium-precipitation method [3]. Purification of BBM preparation, as assessed by the enrichment of BBM enzyme marker, alkaline phosphatase [17], was 11.3 ± 0.9 (n = 12) fold higher than the initial cortex homogenate. Other non-BBM enzyme markers including Na,K-ATPase of BLM [27], cytochrome C oxidase of mitochondria [36] and glucose-6-phosphatase of endoplasmic reticulum [14] were 0.86 ± 0.01, 0.30 ± 0.05 and 0.66 ± 0.1 (n = 12) fold lower than the cortex homogenate, respectively.

Measurements of BBM Na^+ and Glucose Uptake

BBM vesicles were prepared and suspended in a medium comprised of 300 mM mannitol, 10 mM $MgSO_4$ and 10 mM Tris, 16 mM HEPES, pH 7.5. Transport experiments were performed at 24°C by a Millipore rapid-filtration procedure [1], and the radioactivity of the filter membrane was counted in a liquid scintillation counter (LS5801, Beckman). $^{22}Na^+$ uptake was measured in the presence of an outward proton-gradient induced by preincubation of vesicles for 2 hr at 24°C in a pre-equilibration medium comprised of 272 mM mannitol, 10 mM $MgSO_4$, 9 mM Tris, 14 mM HEPES and 30 mM MES, pH 6.0. Uptake was initiated by incubation of vesicles with Na^+ uptake medium comprised of 1 mM $^{22}NaCl$ (2.5 μCi/ml), 286 mM mannitol, 2 mM $MgSO_4$, 13 mM Tris, 15 mM HEPES, 6 mM MES, pH 7.5, and was stopped by an ice-cold (4°C) isosmotic solution. The amiloride-sensitive $^{22}Na^+$ uptake was calculated as the difference between the uptakes with and without amiloride (1 mM) in the uptake solution. For ^{14}C-glucose uptake, BBM vesicles suspended in the medium containing 300 mM mannitol, 10 mM $MgSO_4$ and 10 mM Tris, 16 mM HEPES, pH 7.5, were incubated with the uptake medium comprised of 100 mM mannitol, 20 mM HEPES, 100 mM NaCl or KCl and 0.1 mM ^{14}C-glucose (2.5 μCi/ml), pH 7.5 and was stopped by an ice-cold (4°C) isosmotic solution. The Na^+-dependent ^{14}C-glucose uptake was calculated as the difference between uptakes in the presence and absence of Na^+ in the uptake medium. Uptakes were calculated from the accumulated $^{22}Na^+$ or ^{14}C-glucose and expressed in moles per milligram protein per unit time. A correction for $^{22}Na^+$ or ^{14}C-glucose binding to the filters was made by subtracting the radioactivity measured at zero time. All measurements were carried out in triplicate with freshly prepared membranes. Protein concentration was assayed using Coomassie Brilliant blue G250 with bovine serum albumin as the reference protein [33].

Measurement of BBM Proton Permeability

BBM proton permeability was determined with acridine orange (AO) using the base-pulse technique [28]. BBM vesicles (100 μg) equilibrated in 10 μl pre-equilibration medium (pH 6.0) were added to a cuvette containing 980 μl of the same medium to which 10 μM AO had been added. After 3–5 min of equilibration, 10 μl of 1 M Tris-base was added to the cuvette and the recovery of fluorescence followed for a further 5 min. In control experiments vesicles were pre-equilibrated with 5 mM KCl, and following the base pulse, 2 μl of nigericin (1 mg/ml) were added to the cuvette during the recovery phase. Fluorescence was monitored on a Perkin-Elmer LS-5 fluorometer (Norwalk, CT) at 490 nm excitation and 540 nm emission with slit width set at 5 nm. Acidification of the vesicle interior with respect to medium pH results in the intravesicular trapping of AO in its protonated form and is associated with quenching of the fluorescence signal. Recovery of AO fluorescence indicates dissipation of the pH gradient and was used as a measure of proton permeability.

BBM ^{125}I-AII Binding Assay

The binding assay was performed with BBM vesicles (80 μg protein/tube) suspended in pre-equilibration medium and incubated in the Na^+ uptake medium (final vol 200 μl) containing also ^{125}I-AII (15 fmol or 0.05 μCi/tube) at 24°C for varying times and then washed by an ice-cold (4°C) isosmotic solution. Bound radioactivity was determined by Millipore rapid-filtration procedure similar to that for uptake studies. Nonspecific binding was measured as the amount of ^{125}I-AII bound in the presence of excess (1 nmol/tube) unlabeled AII. The difference between total and nonspecific bindings was calculated as the specific binding of AII.

Pertussis Toxin and ADP-Ribosylation of BBM Proteins

G-protein substrates of pertussis toxin (PTX) were assayed using PTX-catalyzed incorporation of ^{32}P-ADP-ribose from ^{32}P-NAD. PTX dissolved in pre-equilibration medium was activated prior to use by 30 min incubation at 24°C with 10 mM dithiothreitol. For ADP-ribosylation, BBM vesicles (4 mg protein/tube) were incubated with or without activated PTX (500 ng/ml) in the pre-equilibration medium (500 μl) containing also 2.5 mM ATP, 2 mM GTP, 10 mM thymidine, 10 mM isoniazid, and 10 μM ^{32}P-NAD (4 Ci/mmol). The reaction was carried out at 24°C for 2 hr and stopped by dilution with 1 ml of ice-cold isosmotic solution. The mixture was centrifuged at 40,000 × g for 10 min, and the pellets were washed twice with stop solution. The final membrane pellets were resuspended in 500 μl of a solution containing 62.5 mM Tris-Cl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol and 5% β-mercaptoethanol and loaded into the wells of a 7.5% SDS-polyacrylamide gel for electrophoresis. The gels were stained for

protein by utilizing Coomassie blue and autoradiographs were taken by exposure of Kodak X-Omat film to the dried gels at −70°C for 4 days. Same amount of protein was loaded on each lane of the gels, and standard proteins with known molecular weights were used for calibration.

BBM Phospholipase C (PLC) Assay

For PLC activity, we measured the formation of ^{3}H-inositol phosphates from BBM vesicles prelabeled with ^{3}H-myoinositol. BBM vesicles (5 mg/tube) were incubated in 1 ml of pre-equilibration medium containing also 10 mM LiCl and 5 μCi/ml ^{3}H-myoinositol (20 Ci/mmol) at 37°C for 120 min. After removing the nonincorporated ^{3}H-myoinositol, BBM vesicles were incubated with AII for varying times. The reaction was stopped by addition of 15% trichloro-acetic acid and washed with diethyl ether. The ^{3}H-inositol phosphates were then separated by ion-exchange chromatography [2] and the elution profile for the different inositol phosphates verified by using ^{3}H-labeled inositol phosphates. Radioactivity of the various fractions was determined by liquid scintillation counting corrected for backgrounds and expressed as dpm per mg protein.

BBM Phospholipase A_2 (PLA) Assay

BBM PLA activity was assayed by measuring the release of ^{3}H-arachidonic acid from prelabeled BBM. BBM labeling was achieved by incubating BBM vesicles (1.5 mg/tube) in 2 ml of pre-equilibration medium with 10 μM ^{3}H-arachidonic acid (95 Ci/mmol) added to the medium for 30 min at 37°C. Under these conditions, 70–85% of ^{3}H-arachidonic acid was incorporated. After the nonincorporated ^{3}H-arachidonic acid was removed by washing twice with the same medium containing 5 mg/ml of fatty acid-free bovine serum albumin, BBM vesicles were resuspended in 0.35 ml of the Na^+ uptake medium and incubated with or without testing agents for 15 min at 24°C. The reaction was stopped by adding 40 μl of ice-cold isosmotic solution containing mepacrine (final concentration 0.1 mM). The mixture was then centrifuged at 40,000 × g for 10 min, and the radioactivity of the supernatant was determined by liquid scintillation counting.

The effect of AII on PLA was further examined by analyzing the effect of AII on BBM content of lysophospholipids. Lipid extract of BBM (10 mg) [9] was evaporated to dryness under nitrogen and the residue resuspended in 150 μl of 2 : 1 chloroform : methanol. Individual phospholipid species were isolated by two-dimension thin-layer chromatography (TLC). 20 μl of lipid extract were spotted on silica gel TLC plates and developed in two dimensions. The first-dimension solvent system consists of chloroform : methanol : NH_4OH (65 : 25 : 5 vol/vol) and the second, chloroform : acetone : methanol : acetic acid : water (30 : 40 : 10 : 10 : 5 vol/vol). After development, chromatograms were dried and exposed to iodine vapors. Each phospholipid and its lyso-form were identified by comparison with authentic standards, and the spots were scraped into acid-washed tubes for phosphorus determination [21]. The recovery of phospholipids was monitored with phospholipid standards and averaged 90–95%.

Statistical Analysis

Data are presented as mean ± SE. Statistical significance was assessed by one-way analysis of variance (ANOVA).

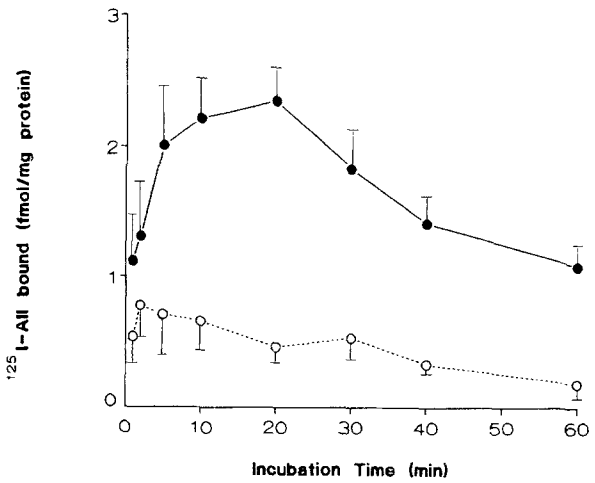

Fig. 1. Binding of ^{125}I-AII by BBM in the absence (●) and presence (○) of excess unlabeled AII. BBM vesicles were incubated with ^{125}I-AII for varying incubation times (*see* text). Binding generally peaked between 10–20 min incubation and the nonspecific binding (○) as determined in the presence of unlabeled AII was less than 30% of the total binding (●). (Mean ± SE; n = 3)

Materials

Radioisotopes were purchased from Amersham (Arlington Heights, IL), PTX from List Biological Lab. (Campbell, CA), Arachidonic acid from Cayman Chemical (Ann Arbor, MI), and all other chemicals were obtained from Sigma Chemical (St. Louis, MO).

Results and Discussion

Similar to previous reports in rat renal BBM [4, 5, 7], AII binding sites were also found in rabbit renal BBM. Figure 1 illustrates ^{125}I-AII binding by BBM as a function of time. Binding generally reached maximum after 10 to 20 min incubation and followed by a decline.[1] Nonspecific binding, as determined by ^{125}I-AII binding in the presence of excess nonlabeled AII, was less than 30% of the total binding. In separate studies, the binding was found to be saturable at AII concentrations higher than 10^{-8} M, and a linear increase of binding was observed with 50–200 μg membrane protein.

To examine if AII can directly affect BBM Na^+ transport through its binding sites on BBM, we stud-

[1] As previously reported with rat renal BBM [4], the decline of ^{125}I-AII binding after 20 min incubation is likely to result from AII degradation by BBM. This is inferred from the results of separate experiments where the incubation medium separated from BBM after 20 min incubation with ^{125}I-AII exhibited only half of the binding efficiency to fresh BBM as compared to that of the original incubation medium containing intact ^{125}I-AII.

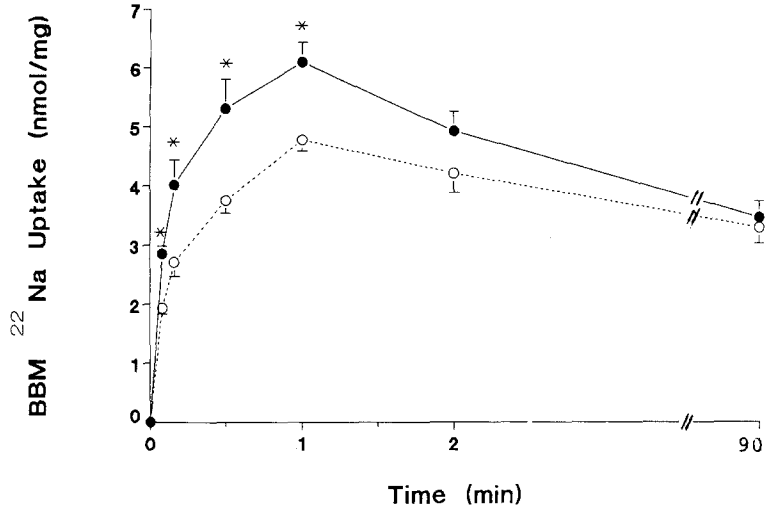

Fig. 2. Direct effect of AII (10^{-9} M) on BBM $^{22}Na^+$ uptake. BBM vesicles were incubated for 15 min with (●) or without (○) AII (10^{-9} M) prior to uptake measurement (*see* text). AII directly increased the early BBM $^{22}Na^+$ uptake. (Mean ± SE; $n = 4$; $^*P < 0.05$)

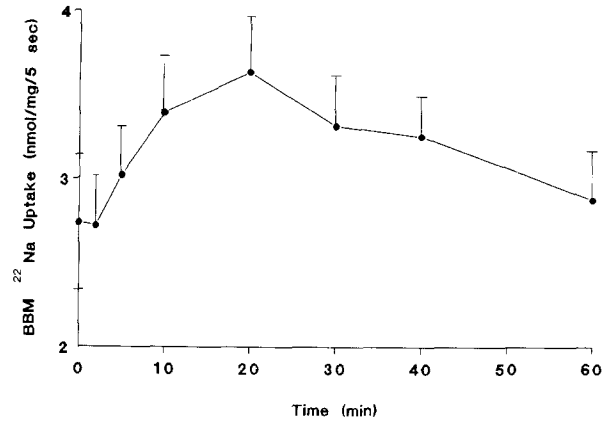

Fig. 3. Time course for AII effect on BBM Na^+ uptake. BBM vesicles were incubated with AII (10^{-9} M) for varying periods of time prior to uptake measurement. In parallel with ^{125}I-AII binding, the maximal AII effect was generally achieved between 10–20 min incubation. (Mean ± SE; $n = 3$)

Table 1. Effects of AII, mellitin and arachidonic acid on Na^+-dependent BBM ^{14}C-glucose uptake[a]

	5 sec	30 sec	1 min	90 min
	(pmol/mg)			
Control	402 ± 48	738 ± 138	561 ± 55	98 ± 4
AII (10^{-9} M)	397 ± 44	729 ± 103	528 ± 72	98 ± 7
Mellitin (500 ng/ml)	381 ± 46	697 ± 110	568 ± 78	93 ± 17
Arachidonic Acid (10^{-8} M)	377 ± 20	759 ± 92	588 ± 51	102 ± 8

[a] Mean ± M; $n = 3$ each.

ied the effect of AII on BBM $^{22}Na^+$ uptake. As is shown in Fig. 2, addition of AII (10^{-9} M) directly increased the initial BBM $^{22}Na^+$ uptake. In parallel with ^{125}I-AII binding results, the effect of AII on BBM $^{22}Na^+$ uptake generally reached maximum after 10 to 20 min incubation (Fig. 3). The following studies were thus performed with 15 min incubation with AII prior to uptake measurements.

Figure 4 shows the dose-dependent stimulatory effect of AII on BBM $^{22}Na^+$ uptake over the concentrations from 10^{-11} to 10^{-7} M. The stimulatory effect of AII on BBM Na^+ uptake was amiloride sensitive. With amiloride (1 mM) in the uptake medium, AII did not alter the remaining amiloride-insensitive Na^+ uptakes (hatched bars in Fig. 4). At 10^{-7} M concentration, AII increased the amiloride-sensitive BBM Na^+ uptake by almost twofold (3.54 ± 0.22 *vs.* 1.87 ± 0.19 nmol/mg/5 sec; $n = 6$; $P < 0.01$). In contrast to BBM Na^+ uptake, AII did not affect the Na^+-dependent BBM ^{14}C-glucose uptake (Table 1). These results thus suggest that AII stimulates BBM Na^+ uptake through Na^+/H^+ antiport system. Kinetic analysis for amiloride-sensitive BBM $^{22}Na^+$ uptake with Na^+ concentration varied from 0.5 to 20 mM by isosmotically replacing NaCl with mannitol, showed an effect of AII (10^{-9} M) to increase both K_m (6.53 ± 0.68 *vs.* 3.54 ± 0.46 mM; $n = 4$; $P < 0.05$) and V_{max} (23.5 ± 1.9 *vs.* 16.7 ± 2.7 nmol/mg/5 sec; $n = 4$; $P < 0.025$) (Fig. 5).

Since changes in BBM permeability to H^+ could affect Na^+/H^+ exchange activity, the effect of AII on BBM proton permeability was examined with acridine orange (AO) using the base-pulse technique [28]. Figure 6 depicts a representative recording of AO fluorescence. Vesicles were equilibrated at pH 6.0 in the presence of the probe. After a steady-state fluorescence was recorded, 10 mM Tris-base was added to the suspension (arrow, Fig. 6), which resulted in rapid quenching of AO fluorescence. The

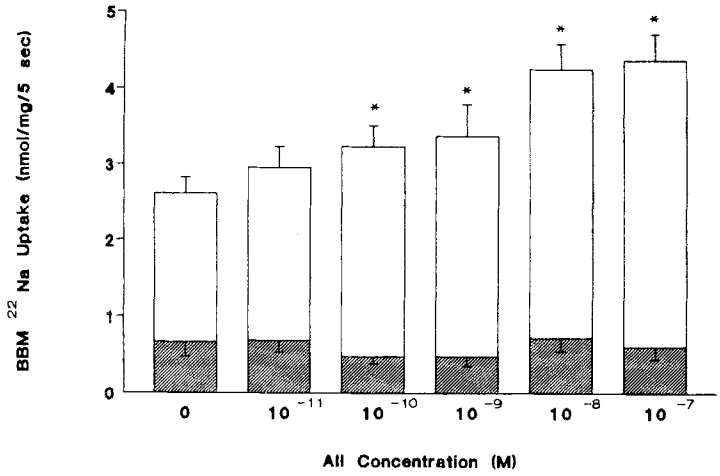

Fig. 4. Dose-dependent effect of AII on amiloride-sensitive (open bars) and insensitive (shaded bars) BBM $^{22}Na^+$ uptake. The amiloride-sensitive BBM $^{22}Na^+$ uptake was determined as the difference between the uptake in the absence and presence of 1 mM amiloride. AII at concentrations from 10^{-11} to 10^{-7} M caused a dose-dependent increase in amiloride-sensitive BBM $^{22}Na^+$ uptake. The amiloride-insensitive BBM $^{22}Na^+$ uptake was not affected. (Mean ± SE; $n = 6$; $^*P < 0.05$)

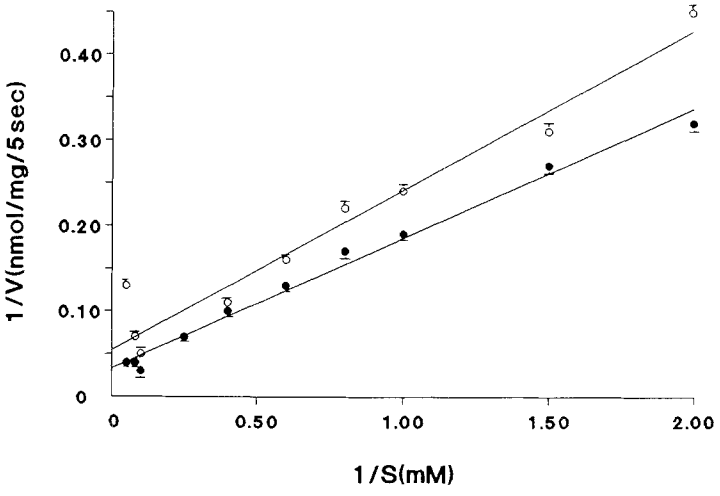

Fig. 5. Lineweaver-Burk plot for amiloride-sensitive BBM $^{22}Na^+$ uptake with (●) and without (○) AII (10^{-9} M). The amiloride-sensitive BBM $^{22}Na^+$ uptake was measured with Na^+ concentration varied from 0.5 to 20 mM by isosmotically replacing NaCl with mannitol in the uptake medium. AII increased both V_{max} and K_m for Na^+ (*see* text). (Mean ± SE; $n = 4$)

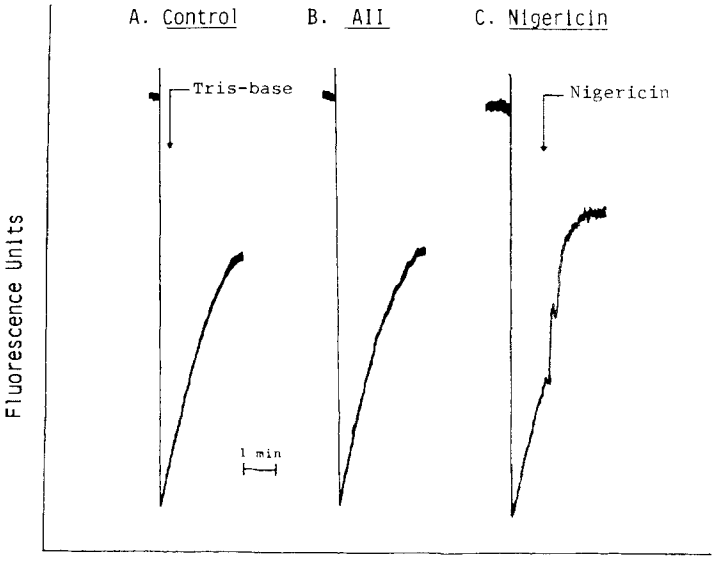

Fig. 6. Measurement of BBM proton permeability with acridine orange (AO). Membrane vesicles in pre-equilibration medium (100 μg/10 μl) were added to a cuvette containing 980 μl of the same medium to which 10 μM AO had been added. Steady-state fluorescence was continuously recorded at excitation 490 nm and emission 540 nm. After 3–5 min of steady-state tracing, 10 μl of 1 M Tris-base (final concentration 10 mM) was added to the cuvette (total volume 1 ml) and fluorescence tracing followed until a new steady state had been reached. *A* and *B* depict tracings from control vesicles and vesicles incubated with AII (10^{-8} M) for 15 min, respectively. In separate experiments (*C*), vesicles were pre-equilibrated with 5 mM KCl, and following the base pulse, 2 μl of nigericin (1 mg/ml) were added to the cuvette during the recovery phase and rapidly dissipitated the base-induced fluorescence quench, confirming that the recovery of fluorescence was due to dissipation of a H^+ gradient

Table 2. Effects of AII, GTP, GDP-βS, pertussis toxin (PTX) and arachidonic acid (AA) on BBM proton permeability[a]

Control	AII (10^{-9} M)	GTP (10^{-4} M)	GDP-βS (10^{-4} M)	PTX (500 ng/ml)	AA (10^{-8} M)
		(arbitrary fluorescence unit per min)			
17.6 ± 0.4	18.0 ± 1.1	18.8 ± 1.8	18.3 ± 0.79	18.1 ± 2.0	17.2 ± 1.4

[a] BBM proton permeability was measured by using the base-pulse technique with acridine orange. The initial rate of recovery of fluorescence was determined by computer-assisted curve fitting to a quadratic function and used as measure of proton permeability (*see* text). BBM were preincubated for 15 min with AII, GTP, GDP-βS or AA, and for 2 hr in ADP-ribosylation medium with PTX, prior to measurements. (Mean ± SE; $n = 4$ each.)

Table 3. BBM cAMP content[a]

	cAMP content (pmol/mg)
Control	23.3 ± 2.0
AII	28.6 ± 3.5

[a] BBM vesicles were incubated with or without AII (10^{-8} M) at 24°C for 20 min and precipitated by 90% N-propanol. The supernate was then collected, and the cAMP content was determined by cAMP ^{125}I-radioimmunoassay kit (New England Nuclear, MA). Incubation with AII did not affect the cAMP content in BBM vesicles. (Mean ± SE; $n = 5$; $P > 0.05$.)

Table 4. Effect of AII on inositol phosphate release from BBM prelabeled with ^{3}H-myoinositol[a]

	IP_1	IP_2	IP_3
Control	917 ± 166	119 ± 15	92 ± 11
AII	1142 ± 187	90 ± 13	94 ± 19

[a] Results shown are from experiments where BBM prelabeled with ^{3}H-myoinositol were incubated with AII (10^{-8} M) for 15 min. AII did not cause any detectable change in IP_1, IP_2 or IP_3 levels (cpm/mg). (Mean ± SE; $n = 4$.)

initial rate of recovery of intravesicular pH, quantitated by computer-assisted curve fitting to a quadratic function [16], was used as a measure of H^+ permeability. In these assays, all the solutions used were Na^+ free so that the dissipation of H^+ gradient could not be occurring via the Na^+/H^+ exchanger. As shown in Fig. 6 and Table 2, the initial rates of fluorescence recovery were not different between control and AII-pretreated BBM vesicles. As shown in Fig. 6, addition of protonophore, nigericin, in the presence of 5 mM KCl rapidly dissipated the base-induced fluorescence quench, confirming that the recovery of fluorescence was due to dissipation of H^+ gradient. Thus AII appears to increase BBM Na^+ uptake by stimulating Na^+/H^+ antiporter *per se* and not through altering membrane H^+ permeability. These results are similar to other reports which also showed a stimulatory effect of AII on proximal tubule Na^+/H^+ exchange activity [10, 18–20, 30]. However, the continuous stimulatory effect of AII on BBM Na^+ uptake up to 10^{-7} M contrasts the bimodal effect of peritubular AII previously reported in intact proximal tubules. While the reason for such difference is not clear, it remains to be examined if luminal AII similarly increases Na^+ transport over these concentrations in intact proximal tubules.

The direct effect of AII to stimulate BBM Na^+/H^+ antiport system thus underlines the functional significance of BBM AII binding sites and suggests the presence of local signal transduction mechanism. In this regard, membrane enzymes such as adenylate cyclase, phospholipase C (PLC) and phospholipase A_2 (PLA) have been implicated in mediating AII's actions [31, 35, 38]. In renal proximal tubule, the inhibition of adenylate cyclase and the decrease in cellular cAMP levels has been suggested to be related to the stimulatory effect of peritubular AII on Na^+ transport [20]. However, it is highly unlikely that the direct AII effect on BBM Na^+ transport observed in the present study was mediated through a decrease in cAMP levels because adenylate cyclase is a predominantly basolateral membrane enzyme and the addition of AII was found to have no effect on cAMP content in BBM vesicles (Table 3). We have therefore examined the effects of AII on BBM PLC and PLA. For PLC activity, we measured the formation of inositol phosphates from BBM vesicles prelabeled with ^{3}H-myoinositol. With varying AII concentrations (10^{-9}–10^{-7} M) for varying incubation times (15 sec–15 min), no significant changes in IP_3, IP_2, or IP_1 contents were found. Table 4 shows the results with 10^{-8} M AII for 15 min incubation. For PLA activity, the release of ^{3}H-arachidonic acid from prelabeled BBM vesicles was monitored. As is shown in Fig. 7, 15 min incubation with AII caused a dose-dependent increase in ^{3}H-arachidonic acid release. At 10^{-9} M concentration, AII increased the release of ^{3}H-arachidonic acid from 1258 ± 101 to 1597 ± 116 cpm/mg protein ($n = 6$; $P < 0.001$). The time course of AII (10^{-9} M) effect on ^{3}H-arachidonic acid release is shown in Table 5. While there was a cumulative increase in the spontaneous release of ^{3}H-arachidonic acid with time, the increase in ^{3}H-arachidonic acid release caused by AII reached maximum after 20 min incubation. This is similar to the time course observed for receptor binding (Fig. 1) and ^{22}Na uptake (Fig. 3). The estimated EC_{50} AII concentration

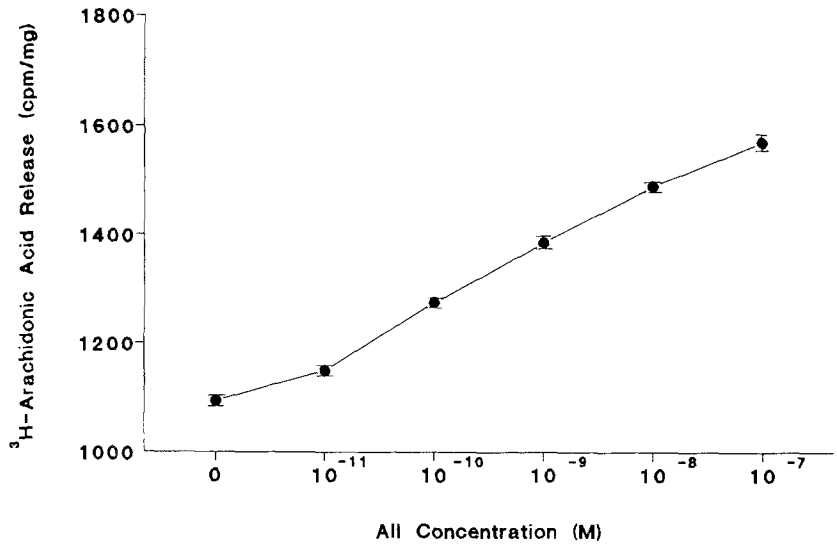

Fig. 7. Effect of AII on ^{3}H-arachidonic acid release from prelabeled BBM. Addition of AII (10^{-11}–10^{-7} M) caused a dose-dependent increase in the release of ^{3}H-arachidonic acid from prelabeled BBM vesicles. (Mean ± SE; $n = 3$)

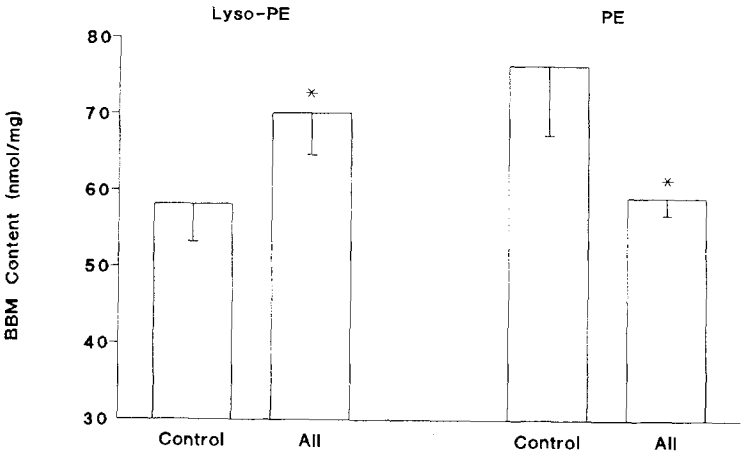

Fig. 8. Effect of AII on BBM content of lysophospholipid. Results of phosphotidylethanolamine (*PE*) and *lyso-PE* are shown. PE and lyso-PE were isolated from BBM lipid extract by two-dimensional thin-layer chromatography and quantitated by phosphorus determination. Addition of AII (10^{-9} M) increased BBM content of lyso-PE with comparable decrease in PE content. (Mean ± SE; $n = 4$; $^*P < 0.05$)

for PLA activation (7.0 nM) was also similar to that for ^{125}I-AII binding (8.9 nM) and ^{22}Na uptake (3.2 nM). To further confirm the effect of AII on PLA, we found that incubation with AII (10^{-9} M) for 15 min caused an increase in BBM content of lysophospholipid (Fig. 8). These data thus indicate that AII stimulates BBM PLA, but not PLC, and are in accordance with recent studies where AII was found to stimulate PLA but not PLC in cultured rabbit proximal tubular cells [35]. It is to be noted, however, that in the present study BBM vesicles were prepared in nominally calcium-free medium. The fact that AII was able to stimulate PLA under such condition may seem at odds with the conventional understanding that PLA activation is calcium dependent. In separate experiments, we also did not find any difference in either basal PLA activity or AII's effect to activate PLA when calcium-containing medium was used. Thus, these results may reflect either that BBM contains sufficient membrane bound calcium for PLA activation or that renal BBM PLA is calcium independent, as has been reported in other tissues such as heart [37], lung [23] and intestinal BBM [26].

Table 5. Time course of AII's effect on BBM PLA activity[a]

	Time (min)						
	5	10	15	20	30	40	60
Control	1404 ± 172	1462 ± 184	1533 ± 187	1665 ± 247	1748 ± 224	1780 ± 221	1910 ± 245
AII	1461 ± 199	1536 ± 191	1644 ± 240	1825 ± 238	1811 ± 202	1830 ± 287	1935 ± 299

[a] BBM vesicles prelabeled with ^{3}H-arachidonic acid were incubated with or without AII (10^{-9} M) for 5 to 60 min. The amount of ^{3}H-arachidonic acid released was determined as the PLA activity. While there was a time-dependent spontaneous release of ^{3}H-arachidonic acid, the increase in ^{3}H-arachidonic acid release caused by AII reached maximum after 20 min incubation. (cpm/mg; mean ± SE; $n = 5$.)

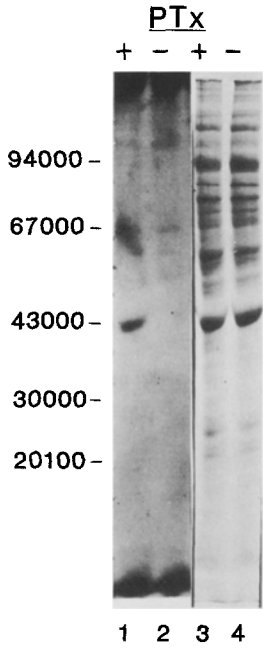

Fig. 9. Pertussis toxin-dependent ADP-ribosylation of BBM proteins. BBM vesicles were incubated in ADP-ribosylation medium containing ^{32}P-NAD with or without activated pertussis toxin (PTX, 500 ng/ml) at 24°C for 2 hr. Autoradiographic profiles of ^{32}P-labeled BBM proteins with or without PTX are depicted on lanes *1* and *2*, respectively. The SDS-PAGE stained for proteins by Coomassie blue of the same samples are depicted on lanes *3* and *4*. The same amount of protein was loaded on each lane, and standard proteins with known molecular weights were used for calibration. Lane *1* shows that PTX induced ADP-ribosylation of BBM proteins of approximately 42 kDa

Since G-protein has been widely proposed to couple the hormone receptor binding to its effectors, we further examined the presence and the involvement of BBM G-protein. Figure 9 shows that BBM contains proteins that are sensitive to PTX-catalyzed ADP-ribosylation. The molecular weight (42 kDa) of the major substrate corresponds to that of the α subunit of Gi regulatory component. In support of its involvement in AII's actions, it was found that addition of GTP potentiated, while GDP-βS or PTX abolished, the effects of AII on BBM PLA and $^{22}Na^+$ uptake (Fig. 10). Neither GTP nor GDP-βS or PTX affected BBM proton permeability (Table 2). These results thus demonstrate the presence of BBM PTX substrate G-protein and suggest its participation in AII's actions. In these studies, however, AII was able to exert its actions in the absence of added GTP. This apparent independence of AII's actions from GTP may reflect that AII's actions involve G-protein dependent and independent mechanisms, or that residual endogenous or contaminating GTP were present in BBM vesicles since GDP-βS or PTX abolished

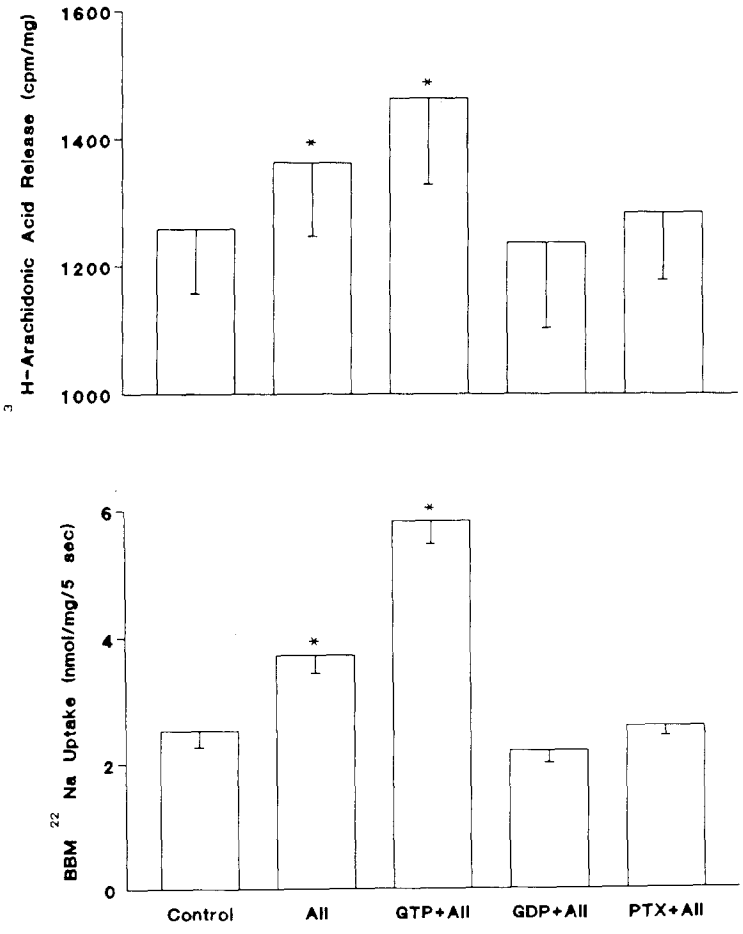

Fig. 10. Effects of GTP, GDP-βS and pertussis toxin (PTX) on the stimulatory effects of AII on BBM PLA and Na^+ uptake. BBM vesicles were incubated with AII (10^{-9} M) alone or together with GTP (10^{-4} M) or GDP-βS (10^{-4} M) for 15 min prior to measurements. For PTX, BBM vesicles were preincubated with PTX (500 ng/ml) in ADP-ribosylation medium for 2 hr prior to measurements. PLA activity was assessed by the release of prelabeled ^{3}H-arachidonic acid, and the amiloride-sensitive BBM Na^+ uptake was determined as the difference between BBM Na^+ uptake in the presence and absence of amiloride (1 mM). Addition of GTP potentiated, while GDP-βS or PTX abolished, the stimulatory effects of AII on BBM PLA and Na^+ uptake. (Mean ± SE; $n = 5$; $^*P < 0.05$)

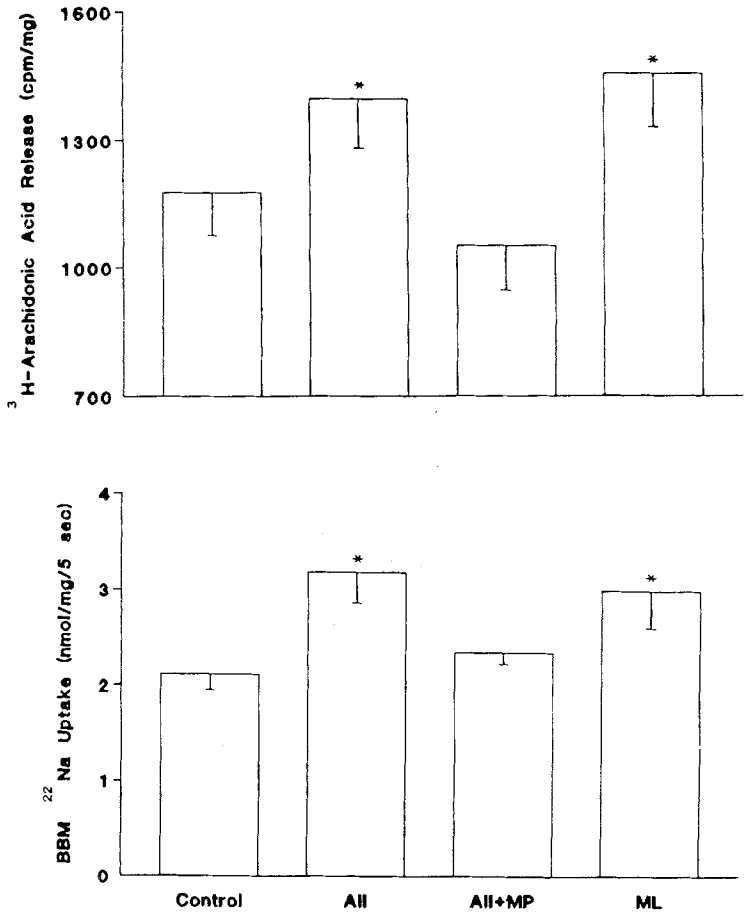

Fig. 11. Effects of AII, mepacrine (*MP*) and mellitin (*ML*) on BBM PLA and $^{22}Na^+$ uptake. PLA activity was assessed by the release of prelabeled ^{3}H-arachidonic acid, and the amiloride-sensitive BBM $^{22}Na^+$ uptake was determined as the difference between BBM $^{22}Na^+$ uptake in the presence and absence of amiloride (1 mM). Addition of MP (10^{-4} M) together with AII (10^{-9} M) abolished the stimulatory effects of AII on BBM PLA and $^{22}Na^+$ uptake. Similar to AII, addition of ML (500 ng/ml) increased BBM PLA and $^{22}Na^+$ uptake. (Mean ± SE; $n = 5$ each; $^*P < 0.05$)

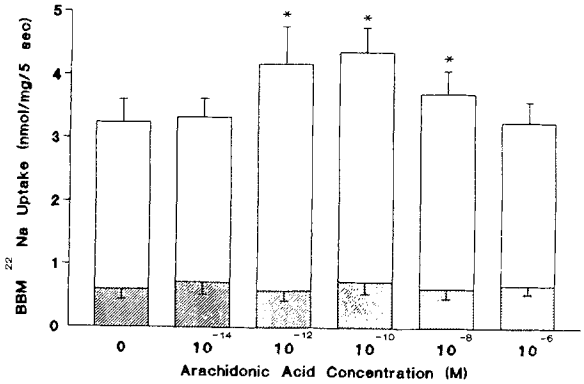

Fig. 12. Effect of arachidonic acid on amiloride-sensitive (open bars) and insensitive (shaded bars) BBM $^{22}Na^+$ uptake. The amiloride-sensitive BBM $^{22}Na^+$ uptake was determined as the difference between the uptake in the absence and presence of 1 mM amiloride. Addition of arachidonic acid (10^{-12}–10^{-8} M) increased the amiloride-sensitive BBM $^{22}Na^+$ uptake. (Mean ± SE; $n = 5$; $^*P < 0.05$)

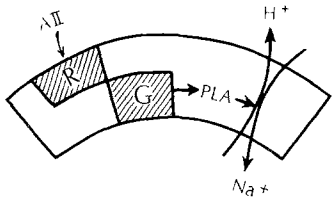

Fig. 13. Local BBM AII signal transduction mechanism. The figure represents a schematic drawing of the proposed signal transduction mechanism for AII's actions in renal BBM. AII binding to its BBM receptor (*R*) is coupled to a pertussis toxin sensitive G-protein (*G*) that leads to the activation of phospholipase A_2 (*PLA*) and subsequently stimulation of Na^+/H^+ exchanger in BBM

AII's actions. A similar observation has been reported recently which suggests the presence of residual GTP in allowing dopamine to activate renal BBM PLC [8].

The parallel changes in AII's effects on BBM PLA and Na^+ uptake caused by varying G-protein activity as shown in Fig. 10 suggest a possible role for PLA activation in mediating AII's effect on BBM Na^+ uptake. To examine this possibility, we studied the effect of PLA inhibitor, mepacrine. As shown in Fig. 11, addition of mepacrine (10^{-4} M) prevented AII to increase the release of ^{3}H-arachidonic acid and abolished its stimulatory effect on BBM $^{22}Na^+$ uptake. Although the autofluorescence of mepacrine made it difficult to assess its effect on BBM proton permeability, it is unlikely that mepacrine abolishes AII's effect on BBM Na^+ uptake through dissipation of BBM pH gradient because mepacrine (10^{-4} M) alone did not affect the amiloride-sensitive BBM Na^+ uptake (from 2.11 ± 0.1 to 2.54 ± 0.4 nmol/mg/5 sec; $n = 4$). On the other hand, PLA activation by another agent, mellitin (500 ng/ml), was also found to stimulate the amiloride-sensitive BBM $^{22}Na^+$ uptake (Fig. 11) without affecting BBM permeability to H^+ (Table 2) or Na^+-dependent BBM glucose uptake (Table 1). These results are thus consistent with the possibility that PLA activation may mediate the effect of AII on BBM Na^+ uptake. To further support the role of PLA, it was found that addition of arachidonic acid (10^{-12}–10^{-8} M) also increased the amiloride-sensitive BBM $^{22}Na^+$ uptake (Fig. 12) without affecting BBM proton permeability (Table 2) or Na^+-dependent BBM glucose uptake (Table 1).[2] Taken together, results of the present study indicate that a signal transduction mechanism for AII exists in renal BBM where AII binding is coupled to a PTX-sensitive G-protein, which activates PLA and stimulates BBM Na^+/H^+ antiport activity (Fig. 13).

Several considerations may be in order before any implication from these results to the intact proximal tubule. First, the possible contamination of BLM constitutes an inherent limitation in studies with BBM vesicles. Thus, despite the enrichment of enzyme markers suggests that our membrane preparation consists of mostly purified BBM vesicles, it remains uncertain to what extent BLM contamination in our vesicle preparation could have contributed to our findings. Nonetheless, the existence of a direct luminal AII effect, as has been demonstrated in intact proximal tubules [13, 19, 20], provides support for our conclusion. Secondly, in intact proximal tubule, the Na^+ transport process can be affected not only by the direct AII effect on BBM Na^+ transport but also by the intracellular processes which may occur subsequent to BBM PLA activation by AII. Thirdly, the physiological significance of these findings depends also on the in vivo AII concentration profile along the proximal tubule lumen. In this regard, it is of interest that local AII generating systems have recently been suggested to be present in glomerular and proximal tubular cells [15, 29, 39]. Thus, in addition to AII derived from circulation, locally produced AII from glomerular and early proximal tubular cells may also enter the proximal tubule lumen. Indeed a recent in vivo micropuncture study [34] reported a much higher AII concentration (nM) in the luminal fluid of the superficial proximal tubules than that in the circulation (pM). Considering the higher affinity for AII of BBM receptors than that of BBM angiotensinase [7], it is likely that the luminal AII can have access to its receptor before being degraded. The potential role for such a local luminal AII regulatory system in renal proximal tubule awaits further studies to be explored.

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[2] It is not clear from the results of these studies if arachidonic acid was further metabolized by BBM and if the effect of arachidonic acid to increase BBM $^{22}Na^+$ uptake was caused by arachidonic acid *per se* or was mediated by its metabolites.

References

1. Aronson, P.S., Sacktor, B. 1975. The Na gradient-dependent transport of D-glucose in renal brush border membrane vesicles. *J. Biol. Chem.* **250:**6032–6039
2. Berridge, M.J., Downes, C.P., Hanley, M.R. 1982. Lithium amplifies agonist-dependent phosphatidyl inositol responses in brain and salivary glands. *Biochem. J.* **206:**587–595
3. Biber, J., Stieger, B., Haase, W., Murer, H. 1981. A high yield preparation for rat kidney brush border membranes. Different behavior of lysosomal markers. *Biochim. Biophys. Acta* **772:**140–148
4. Brown, G.P., Douglas, J.G. 1982. Angiotensin II binding sites on isolated rat renal brush border membranes. *Endocrinology* **111:**1830–1836
5. Brown, G.P., Douglas, J.G. 1987. Influence of transmembrane potential differences of renal tubular cell on ANG II binding. *Am. J. Physiol.* **252:**F209–F214
6. Dominquez, J.H., Snowdowne, K.W., Freudenrich, C.C., Brown, T., Borle, A.B. 1987. Intracellular messenger for action of angiotensin II on fluid transport in rabbit proximal tubule. *Am. J. Physiol.* **252:**F423–F309
7. Douglas, J.G. 1987. Angiotensin receptor subtypes of the kidney cortex. *Am. J. Physiol.* **253:**F1–F7
8. Felder, C.C., Blecher, M., Jose, P.A. 1989. Dopamine-1-mediated stimulation of phospholipase C activity in rat renal cortical membranes. *J. Biol. Chem.* **264:**8739–8745

9. Folch, J., Lees, M., Stanley, G.H. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:**497–509
10. Gesek, F.A., Schoolwerth, A.C. 1990. Hormonal interactions with the proximal Na^+-H^+ exchanger. *Am. J. Physiol.* **258:**F514–F521
11. Harris, P.J. 1979. Stimulation of proximal tubular sodium reabsorption by ile^5 angiotensin II in the rat kidney. *Pfluegers Arch.* **381:**83–85
12. Harris, P.J., Navar, L.G. 1985. Tubular transport response to angiotensin. *Am. J. Physiol.* **248:**F621–F630
13. Harris, P.J., Young, J.A. 1977. Dose-dependent stimulation and inhibition of proximal tubular sodium reabsorption by angiotensin II in the rat kidney. *Pfluegers Arch.* **367:**295–297
14. Hubscher, G., West, G.R. 1965. Specific assays of some phosphotase in subcellular fractions of small intestinal mucosa. *Nature (London)* **205:**799–800
15. Ingelfinger, J.R., Zuo, W.M., Fon, E.A., Ellison, K.E., Dzau, V.J. 1990. In situ hybridization evidence for angiotensinogen messenger RNA in the rat proximal tubule. *J. Clin. Invest.* **85:**417–423
16. Jacquez, J.A. 1978. Tracers in the study of membrane processes. *In:* Membrane Physiology. T.E. Andreoli, J.F. Hoffman, and D.D. Fanestil, editors. pp. 157–159. Plenum, New York
17. Lansing, A.K., Belkhode, M.L., Lynch, W.E., Lieberman, I. 1967. Enzymes of plasma membranes of liver. *J. Biol. chem.* **242:**1772–1775
18. Liu, F.Y., Cogan, M.G. 1987. A potent regulator of acidification in the rat early proximal convoluted tubule. *J. Clin. Invest.* **80:**272–275
19. Liu, F.Y., Cogan, M.G. 1988. Angiotensin II stimulation of hydrogen ion secretion in the rat early proximal tubule. *J. Clin. Invest.* **82:**601–607
20. Liu, F.Y., Cogan, M.G. 1989. Angiotensin II stimulates early proximal bicarbonate absorption in the rat by decreasing cyclic adenosine monophosphate. *J. Clin. Invest.* **84:**83–91
21. Marinetti, G.V. 1962. Chromatographic separation, identification and analysis of phospholipids. *J. Lipid Res.* **3:**1–20
22. Mujais, S.K., Kauffman, S., Katz, A.I. 1986. Angiotensin II binding sites in individual segments of the rat nephron. *J. Clin. Invest.* **77:**315–318
23. Nijssen, J.G., Roosenboom, C.F.P., Van den Bosch, H. 1986. Identification of a calcium-independent phospholipase A2 in rat lung cytosol and differentiation from acetylhydrolase for PAF-acether. *Biochim. Biophys. Acta* **876:**611–618
24. Norman, J., Dezfooly, B.B., Nord, E.P., Kurtz, I., Schlosser, J., Chaudhari, A., Fine, L.G. 1987. EGF-induced mitogenesis in proximal tubular cells: Potentiation by angiotensin II. *Am. J. Physiol.* **253:**F299–F309
25. Peterson, D.R., Charbaszcz, G., Peterson, W.R., Oparil, S. 1979. Mechanism for renal tubular handling of angiotensin. *Am. J. Physiol.* **236:**F365–F372
26. Pind, S., Kuksis, A. 1987. Isolation of purified brush border membranes from rat jejunum containing a Ca-independent phospholipase A2 activity. *Biochim. Biophys. Acta* **901:**78–87
27. Quingley, J.P., Gotterer, G.S. 1969. Distribution of (Na + K)-stimulated ATPase activity in rat intestinal mucosa. *Biochim. Biophys. Acta* **173:**456–468
28. Rabon, E., Chang, H., Sachs, G. 1978. Quantitation of hydrogen ion and potential gradients in gastric plasma membrane vesicles. *Biochemistry* **17:**3345–3353
29. Rajaraman, S., Graves, K., Kunapuli, S. 1988. Identification of the sites of synthesis of angiotensin and renin in the kidney. *Kidney Int.* **33:**169A
30. Saccomani, G., Mitchell, K.D., Navar, L.G. 1990. Angiotensin II stimulation of Na^+-H^+ exchange in proximal tubule cells. *Am. J. Physiol.* **258:**F1188–F1195
31. Schlondorff, D., DeCandido, S., Satriano, J.A. 1987. Angiotensin II stimulates phospholipase C and A2 in cultured rat messangial cells. *Am. J. Physiol.* **253:**C113–C120
32. Schuster, V.L., Kokko, J.P., Jacobson, H.R. 1984. Angiotensin II directly stimulates sodium transport in rabbit proximal convoluted tubules. *J. Clin. Invest.* **73:**507–515
33. Sedmak, J.J., Grossberg, S.E. 1977. A rapid, sensitive and versatile assay for protein using Coomassie Brilliant blue G250. *Anal. Biochem.* **79:**544–552
34. Seikaly, M.G., Arant, B.S., Seney, F.D. 1990. Endogenous angiotensin concentrations in specific intrarenal fluid compartments of the rat. *J. Clin. Invest.* **86:**1352–1357
35. Welsh, C., Dubyak, J.G., Douglas, J.G. 1988. Relationship between phospholipase C activation and prostaglandin E2 and cyclic adenosine monophosphate production in rabbit tubular epithelial cells. Effects of angiotensin, bradykinin, and arginine vasopressin. *J. Clin. Invest.* **81:**710–719
36. Wharton, D.C., Tzgoloff, A. 1967. Cytochrome oxidase from beef heart mitochondria. *Methods Enzymol.* **10:**245–250
37. Wolf, R.A., Gross, R.W. 1985. Identification of neutral active phospholipase C which hydrolyzes choline glycerophospholipids and plasmalogen selective phospholipase A_2 in canine myocardium. *J. Biol. Chem.* **260:**7295–7303
38. Woodcock, E.A., Johnston, C.I. 1982. Inhibition of adenylate cyclase by angiotensin II in rat renal cortex. *Endocrinology* **111:**1687–1691
39. Yanagawa, N., Capparelli, A.W., Jo, O.D., Friedal, A., Barrett, J.D., Eggena, P. 1991. Production of angiotensinogen and renin-like activity by rabbit proximal tubular cells in culture. *Kidney Int. (in press)*

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