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Research Article

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Cytoskeleton-dependent Endocytosis Is Required for Apical Type 1 Angiotensin II Receptor-mediated Phospholipase C Activation in Cultured Rat Proximal Tubule Cells

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Abstract

Renal proximal tubule sodium reabsorption is enhanced by apical or basolateral angiotensin II (AII). Although AII activates phospholipase C (PLC) in other tissues, AII coupling to PLC on either apical or basolateral surfaces of proximal tubule cells is unclear. To determine if AII causes PLC activation, and the differences between apical and basolateral AII receptor function, receptors were unilaterally activated in rat proximal tubule cells cultured on permeable, collagen-coated supports. Apical AII incubation resulted in concentration- and time-dependent inositol trisphosphate (IP₃) formation. Basolateral AII caused greater IP₃ responses. Apical AII-induced IP₃ generation was inhibited by DuP 753, suggesting that the type 1 AII receptor subtype mediated proximal tubule PLC activation. Apical AII signaling did not result from paracellular ligand leak to basolateral receptors since AII-induced PLC activation occurred when basolateral AII receptors were occupied by Sar-Leu AII or DuP 753. Inhibition of endocytosis with phenylarsine oxide prevented apical (but not basolateral) AII-induced IP₃ formation. Cytoskeletal disruption with colchicine or cytochalasin D also prevented apical AII-induced IP₃ generation. These results demonstrate that in cultured rat proximal tubule cells, AII is coupled to PLC via type 1 AII receptors and cytoskeleton-dependent endocytosis is required for apical (but not basolateral) AII receptor-mediated PLC activation. (*J. Clin. Invest.* 1992. 90:2472–2480.) Key words: angiotensin II • proximal tubule • apical • signaling • endocytosis

Introduction

Angiotensin II (AII)¹ has multiple physiological effects on sodium and fluid volume homeostasis. Although some of these effects are indirectly mediated by AII-induced aldosterone secretion and AII-induced contraction of the systemic and renal vasculature (1, 2), recent studies have revealed that AII di-

rectly enhances renal proximal tubule sodium reabsorption (3–7). Moreover, increased bicarbonate absorption was observed when AII was applied to either the apical (AP) or basolateral (BL) surface of isolated perfused proximal tubules (4). The BL AII response was anticipated because AII receptors (8, 9) and signaling enzymes (10) are present on BL membranes. The AP AII response was somewhat unexpected because AII was assumed to primarily activate proximal tubules at BL sites, as filtered AII concentrations were thought to be low due to the presence of angiotensinases in brush border membranes. Furthermore, AII receptors on the AP membrane have been postulated to mediate ligand salvage, as well as receptor recycling and degradation (11), rather than signaling. This limited function of AP AII receptors also seemed logical since signaling enzymes appeared to be present on the BL proximal tubule cell membrane (10).

The signaling pathways that transduce AII activity in proximal tubule cells are controversial. Although microperfusion of rat proximal tubules with AII resulted in decreased adenylyl cyclase activity (12), the effect of AII on phospholipase C (PLC) is less clear. AII elicits PLC signals in most systems (13, 14), but the ability of AII to generate PLC signals in proximal tubule cells is uncertain. Some investigators have observed increased protein kinase C activity (15), which may indicate prior PLC activation. Other studies have demonstrated no PLC response to AII in proximal tubule cells (16, 17).

Proximal tubule cells are polarized and contain AP and BL membranes with unique lipid, protein, and organelle distribution. Although AII receptors have been identified on AP and BL proximal tubule cell membranes prepared from intact kidneys (8, 9, 18), the specific receptor (AP or BL) that could couple AII to PLC is not known. Furthermore, AII receptor-mediated endocytosis is required for AII activity in vascular smooth muscle (19) and adrenal glomerulosa (20) cells, but the role of endocytosis in AII-induced signal transduction from AP and BL receptors in proximal tubule cells is not known.

Until recently it has not been possible to selectively study AP versus BL receptor function because culture conditions favoring growth and differentiation of epithelial cells were not ideal. The principal problem had been that cells were grown on impermeable supports, such as plastic. Under these conditions, selective access to the BL surface was not possible because cells attach to supports along the BL membrane. Since the support surface was impermeable, agonists could not reach BL receptors. Furthermore, proximal tubule cells cultured on plastic exhibit impaired growth and differentiation because nutrient uptake occurs predominantly along the BL membrane (21). These technical problems have recently been overcome by the technique of culturing epithelial cells on permeable supports (21, 22). This method yielded proximal tubule cells with well-differentiated AP and BL membranes and provided the opportunity to unilaterally activate AII receptors by selectively exposing agonist to one membrane, but not the other.

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1. Abbreviations used in this paper: AII, angiotensin II; AP, apical; AT₁, type 1 AII receptor; BL, basolateral; DME F-12, DME and Ham's nutrient mixture F-12; IP₃, inositol trisphosphate; PLC, phospholipase C.

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The purposes of our study, therefore, were to determine whether AII is coupled to PLC in cultured rat proximal tubule cells, to identify whether AII-induced PLC activation was mediated by AP or BL AII receptors, and to establish differences between AP and BL AII receptor requirements for signaling.

Methods

Proximal tubule cell isolation and culture. The technique of proximal tubule cell isolation was derived from Vinay et al. (23) with few modifications. Kidneys were excised from male Sprague-Dawley rats without perfusion, minced, and digested with collagenase (272 U/ml) for 30 min at 37°C. The supernatant was discarded and digestion was terminated by incubating the pellet with 2.5% albumin in isolation solution (135 mM NaCl, 5 mM KCl, 0.8 mM MgSO₄, 0.3 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 1 mM MgCl₂, 2.5 mM CaCl₂, 17 mM glucose, 10 mM Hepes, 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, pH 7.20) on ice. Glomeruli were excluded from the cell suspension by sieving through sterile wire mesh. Proximal tubule cells were separated from the remainder of the pellet by Percoll gradient centrifugation. The proximal tubule cell band was extracted and washed with sterile isolation solution and then mixed with DME and Ham's nutrient mixture F-12 (DME F-12) containing 6.5 µM transferrin, 87 µM insulin, 10 µM dexamethasone, 1.7 nM epidermal growth factor, 14.3 mM NaHCO₃, 10% bovine calf serum, 100 U/ml penicillin G, 100 µg/ml streptomycin, and 5 mM MEM nonessential amino acid solution, pH 7.4. Cells were plated on 24.5-mm, 0.4-µm pore-permeable supports (3412; Costar Corp., Cambridge, MA), which were prewetted with type I collagen and suspended in six-well, 35-mm cell culture cluster dishes (3416; Costar Corp.). The BL surface was bathed with 10% bovine calf serum in DME F-12. Attempts to culture cells on Matrigel yielded monolayers that never reached confluence. Media was changed every 3–4 d, and cells achieved confluence within 5–7 d.

Electron microscopy. Confluent proximal tubule cell monolayers on permeable supports were prepared for transmission electron microscopy by fixing with 2% glutaraldehyde in 0.1 M cacodylate (pH 7.35) for 1 h at 21°C. Cells were then washed twice with 0.1 M cacodylate for 15 min and fixed in 1% osmium tetroxide/0.1 M cacodylate for 30 min at 21°C. The fixed specimen was washed twice with deionized water for 10 min, dehydrated in ethanol, and imbedded in epon. Ultrathin sections were made and counterstained with uranyl acetate and lead citrate. Cells were observed with an electron microscope (Philips Electron Instruments, Co., Mahwah, NJ).

Measurement of sodium transport. Unidirectional AP to BL sodium transport was determined by adding ²²Na (2 µCi/ml) with or without 10⁻⁷ M AII to buffer solution containing the following (mM): 117 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.8 MgSO₄, 0.9 NaH₂PO₄, 5.6 glucose, 25 NaHCO₃, 10 Hepes, 1.0 PMSF, pH 7.40, 21°C), which was then incubated with the AP surface. The BL surface was bathed in the same buffer solution without PMSF or ligand. The contents of the entire plate were rocked by platform mixer at 100 rpm. Time zero was defined as the time when AP buffer was added. Aliquots of 5 µl were sampled by microsyringe from the BL buffer at 5-, 10-, 20-, and 30-min intervals. ²²Na was quantified by liquid scintillation and ²²Na mass was calculated from a simultaneously determined standard curve. ²²Na transport was determined by dividing ²²Na mass by permeable support surface area. Results were expressed in fmol/mm².

Measurement of cyclic AMP. Cyclic AMP concentrations were determined by a competitive binding assay. After agonist incubation for 7 min at 21°C, proximal tubule cells cultured on 6.5-mm, 0.4-µm pore permeable supports (3427; Costar Corp.) were lysed with 0.01 N HCl. The supernatant was then incubated with 100 µl ¹²⁵I-cyclic AMP and 100 µl antiserum at 4°C for 3 h. The suspension was then incubated with a second antibody (which is bound to polymer particles) at 21°C for 10 min and centrifuged at 1,500 g for 10 min. The supernatant was discarded, leaving the antibody-bound fraction. ¹²⁵I was determined by gamma scintillation, and cyclic AMP values were calculated from a

simultaneously determined standard curve. Results were reported in picograms per milligram protein. Total protein was determined by the Lowry method (24). The final protein value was determined by subtracting protein content of collagen-coated wells without cells from total protein.

Measurement of inositol trisphosphate (IP₃). IP₃ was determined as previously reported by our laboratory for vascular smooth muscle cells (25). When proximal tubule cell monolayers were almost confluent, medium was changed to 10% bovine calf serum in inositol-deficient growth medium with 2.4 µCi/ml myo-[2-³H]inositol (2 ml/well) for 48 h at 37°C. Preliminary studies revealed that incorporation of myo-[2-³H]inositol equilibrated at 48 h. After this incubation period, wells were washed with phosphate buffered saline at 21°C to remove unincorporated tracer. Agonists were dissolved in buffer (50 mM Tris base, 50 mM Tris hydrochloride, 100 mM NaCl, 10 mM KCl, 10 mM MgCl₂, 1% albumin, 10,000 U/ml bacitracin, pH 7.40) and added to AP or BL surfaces for indicated times at 21°C. Reactions were terminated by addition of equal volumes of 20% trichloroacetic acid. The resulting suspension was removed by scraping the permeable support surface and centrifuging the contents at 400 g for 10 min. The supernatant was extracted three times with equal volumes of diethyl ether and the aqueous phase was frozen at -20°C. Samples were thawed, pH was adjusted to 7 with 50 mM Tris base, and samples were applied to 2-cm AG1-X8 anion exchange resin columns at 21°C. Inositol phosphates were measured by anion-exchange column chromatography according to the method of Berridge et al. (26) with sequential elutions with 0.2 M ammonium formate in 0.1 M formic acid (IP₁), 0.5 M ammonium formate in 0.1 M formic acid (IP₂), and 1.0 M ammonium formate in 0.1 M formic acid (IP₃). Each fraction was quantified for ³H by liquid scintillation. IP₃ values were expressed as percent of baseline (the ratio of IP₃ content in agonist-exposed cells to IP₃ content in cells exposed only to Tris buffer).

Materials. Materials were obtained from the following sources: Sigma Chemical Co. (St. Louis, MO), DME F-12, Tris base, Tris hydrochloride, AII, Sar-Leu AII, type I collagen, wire mesh, arginine vasopressin, colchicine, cytochalasin D, trichloroacetic acid, sodium formate, transferrin, insulin, dexamethasone, epidermal growth factor, penicillin G/streptomycin, MEM nonessential amino acid solution, ammonium formate, formic acid, sodium chloride, potassium chloride, magnesium sulfate, sodium phosphate, potassium phosphate, magnesium chloride, calcium chloride, glucose, Hepes, sodium bicarbonate, and PMSF; SASSCO (Omaha, NE), Sprague-Dawley rats; Worthington Biochemical Corp. (Freehold, NJ), collagenase; Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden), Percoll; Hazelton Research Products (Denver, PA), inositol-deficient DME; Collaborative Research (Bedford, MA), Matrigel; Fluka Chemical Corp. (Ronkonkoma, NY), phenylarsine oxide; Fisher Scientific (Fair Lawn, NJ), sodium borate, diethyl ether; Hyclone Laboratories Inc. (Logan, UT), bovine calf serum; American Radiolabeled Chem. Inc. (St. Louis, MO), myo-[2-³H]inositol; Amersham Corp. (Arlington Heights, IL), cyclic AMP assay kit, ²²Na, [¹⁴C]inulin; Barnstead/Thermolyne Corp. (Dubuque, IA), RotoMix platform mixer, type 48200; Hamilton Co. (Reno, NV), microsyringe 1700; Bio-Rad Laboratories (Richmond, CA), Dowex AG1-X8 anion exchange resin; Packard Instrument Co., Inc., United Technologies (Downers Grove, IL), Optifluor scintillation fluid. DuP 753 was a gift from Ronald D. Smith, E.I. DuPont de Nemours (Wilmington, DE).

Statistics. Results are expressed as the mean ± SE. Comparison of ²²Na transport between AII and control groups was made by the unpaired, two-tailed Student's *t* test. All other comparisons were made by one-way analysis of variance with the Scheffe test for multiple comparisons. Statistical significance is defined as *P* < 0.05.

Results

Initial studies were performed to confirm the presence of pure proximal tubule cell monolayers. Fig. 1 is a representative elec-

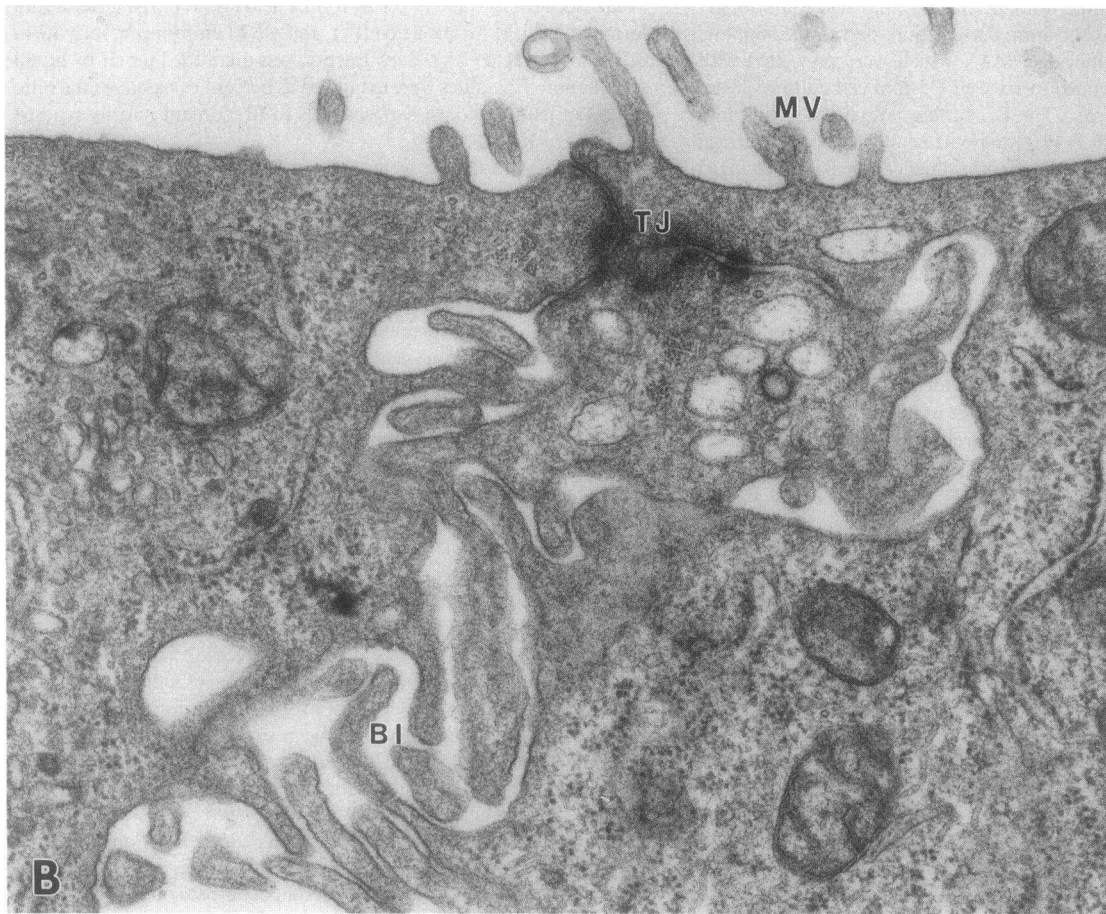
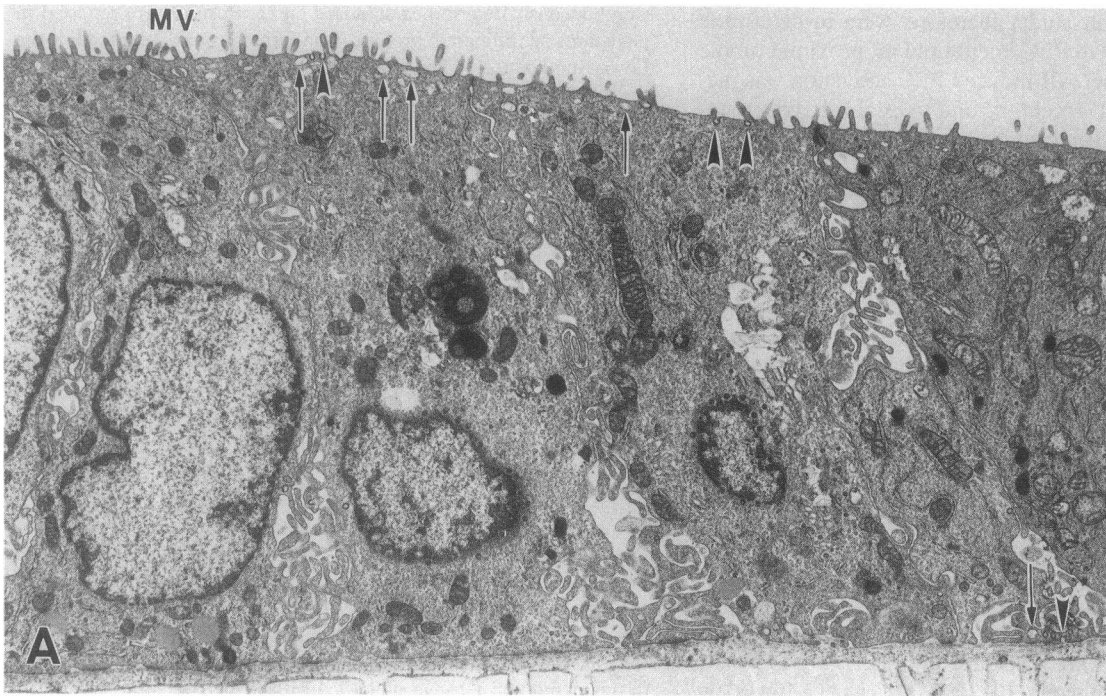


Figure 1. Representative transmission electron micrographs of confluent proximal tubule cell monolayers on permeable supports at 5 d of growth. (A) $\times 9,000$, MV, apical microvilli, arrowheads, coated pits, \uparrow , endosomes. (B) $\times 60,000$, MV, apical microvilli, TJ, tight junctions, BI, basolateral infoldings.

tron micrograph of rat proximal tubule cells cultured on permeable supports for 5 d. Characteristic proximal tubule cell features include cuboidal to columnar cells with AP microvilli, BL infoldings, and bipolar organelle distribution. Of note is the relatively greater density of AP-coated pits and endosomes, as well as the presence of distinct intercellular tight junctions. To characterize the functional properties of the cells, cyclic AMP levels were measured after AP incubation with various hormones (Fig. 2). PTH caused concentration-dependent cyclic AMP increases (10^{-9} M PTH: $P < 0.01$ compared with control), arginine vasopressin caused no change in cyclic AMP, and AII elicited concentration-dependent cyclic AMP decreases (10^{-6} M AII: $P = 0.09$ compared with control). These data were consistent with known proximal tubule cell responses to each hormone (12, 27–29). Further elucidation of proximal tubule cell function was provided by ^{22}Na transport studies. Fig. 3 demonstrates that coinubation of AP 10^{-7} M AII with tracer amounts of ^{22}Na resulted in time-dependent ^{22}Na flux in an AP to BL direction ($P < 0.05$ compared with control at 20 and 30 min).

Our next studies were designed to determine if AII generated PLC signals in proximal tubule cells. Fig. 4 demonstrates that AP or BL addition of AII resulted in PLC signals, as evidenced by increases in IP_3 . There were concentration-dependent effects of AP AII, with a threshold of 10^{-8} M AII and a $14.5 \pm 2.8\%$ IP_3 increase with 10^{-7} M AII ($P < 0.01$ compared with baseline, baseline = 544.3 ± 52.7 cpm per well). BL addition of AII also elicited concentration-dependent PLC signals, with a $26.6 \pm 6.1\%$ increase in IP_3 at 10^{-7} M AII ($P < 0.05$ compared with baseline, baseline = 595.0 ± 69.1 cpm per well). Fig. 5 demonstrates the time dependency of AP AII-induced IP_3 signaling. The peak response was observed at 30 s ($15.7 \pm 5.9\%$ IP_3 increase above baseline, baseline = 613.9 ± 72.1 cpm per well). Thereafter, the IP_3 response slowly declined by 60 and 90 s of AII incubation. By 10 min, IP_3 levels were less than baseline values. These data suggest that AII receptors are coupled to PLC in proximal tubule cells.

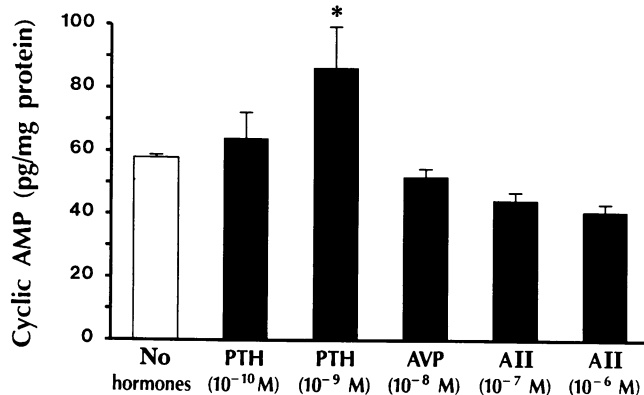


Figure 2. Effect of AP parathyroid hormone (PTH), arginine vasopressin (AVP), and angiotensin II (AII) on proximal tubule cell cyclic AMP concentration. Hormones (10^{-9} M PTH and 10^{-10} M PTH, 10^{-8} M AVP, 10^{-6} M AII, and 10^{-7} M AII) or Tris buffer were incubated with the AP proximal tubule cell surface for 7 min at 37°C . Reactions were terminated by addition of 0.01 N HCl. Cyclic AMP levels were determined by competitive antibody binding assay as described in Methods. Results are means of four experiments. * $P < 0.01$ compared with group with no hormone incubation.

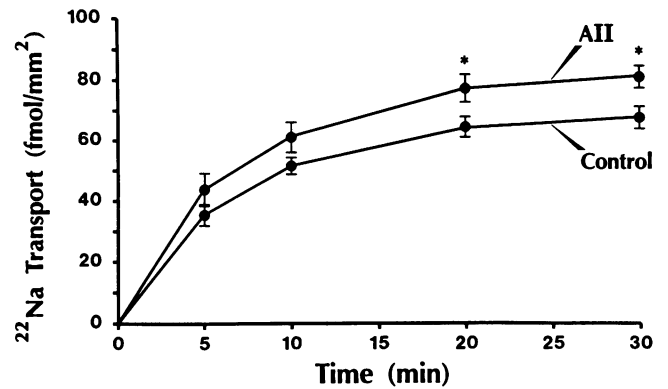


Figure 3. Effect of AP AII on proximal tubule ^{22}Na transport. Unidirectional AP to BL ^{22}Na transport was determined in the presence or absence of 10^{-7} M AP AII as described in Methods. Results are means of nine experiments. * $P < 0.05$ compared with controls.

To determine whether AII receptors were functionally type 1 AII (AT_1) receptors, proximal tubule cells were exposed to AII in the presence of the specific nonpeptide AT_1 antagonist DuP 753. Fig. 6 demonstrates that preincubation with DuP 753 in the AP buffer prevented AP AII-induced IP_3 formation (baseline = 731.2 ± 84.6 cpm per well). The data suggest that AT_1 receptors transduce PLC activation in proximal tubule cells.

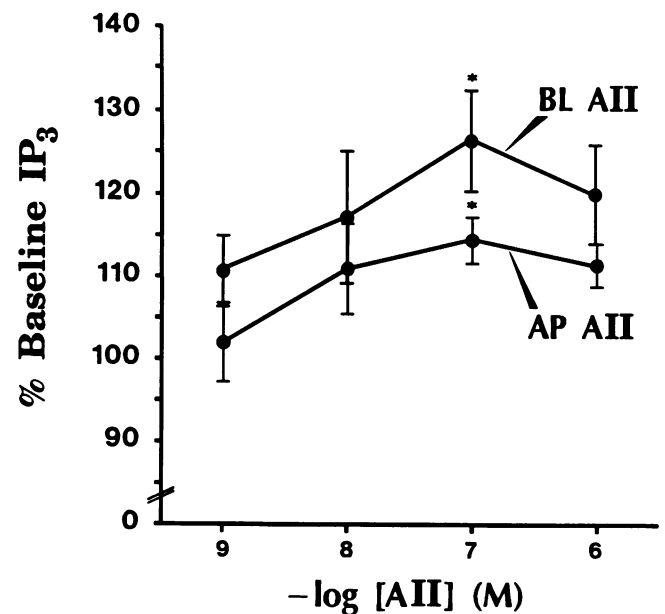


Figure 4. Effect of AII concentration on IP_3 formation. Subconfluent proximal tubule cell monolayers on permeable supports were incubated with 10% bovine calf serum in inositol-deficient media containing myo- $[2\text{-}^3\text{H}]$ inositol for 48 h at 37°C as described in Methods. Confluent cell monolayers were washed with phosphate-buffered saline and exposed to the indicated concentrations of AP or BL AII versus Tris buffer for 30 s at 21°C . Reactions were terminated by addition of 20% trichloroacetic acid. Cell suspensions were extracted in diethyl ether and the aqueous phase was applied to anion-exchange columns. Inositol phosphates were serially eluted with 0.2, 0.4, and 1.0 M ammonium formate in 0.1 M formic acid and quantified for ^3H by liquid scintillation. Results are means of four to eight experiments. * $P < 0.01$ compared with baseline (Tris buffer without AII).

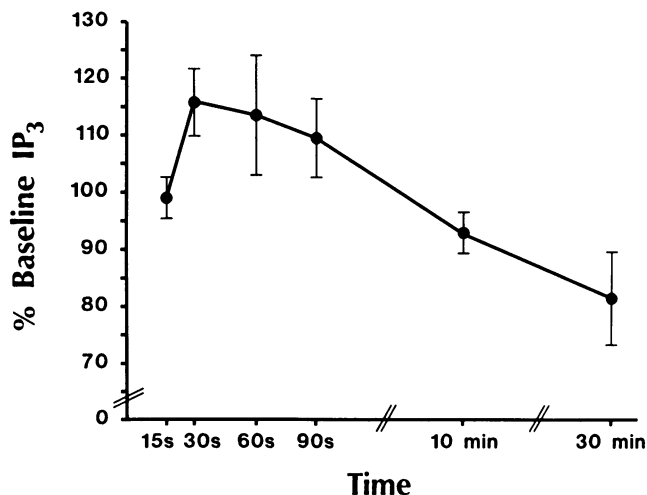


Figure 5. Effect of incubation time with AP AII on IP₃ formation. Subconfluent proximal tubule cell monolayers on permeable supports were incorporated with myo-[2-³H]inositol as described in Methods. The AP proximal tubule cell surface was incubated for the indicated times with 10⁻⁷ M AII at 21°C. IP₃ was extracted and measured as described in Methods. Results are means of three to seven experiments.

Although AII receptors have been identified on AP membranes prepared from proximal tubules (8, 9), AP receptors have previously been noted to be primarily degradative receptors (11). Since AII elicited PLC signals from AP as well as BL sites, we next sought to determine whether the AP signal was mediated by AP receptors or by paracellular leak of AII from AP to BL sites. Fig. 1 demonstrates the presence of well-defined tight junctions between adjacent PT cells, thereby making paracellular leak unlikely. Additional evidence against paracellular ligand leak is shown in Table I. When [¹⁴C]inulin or [³H]-

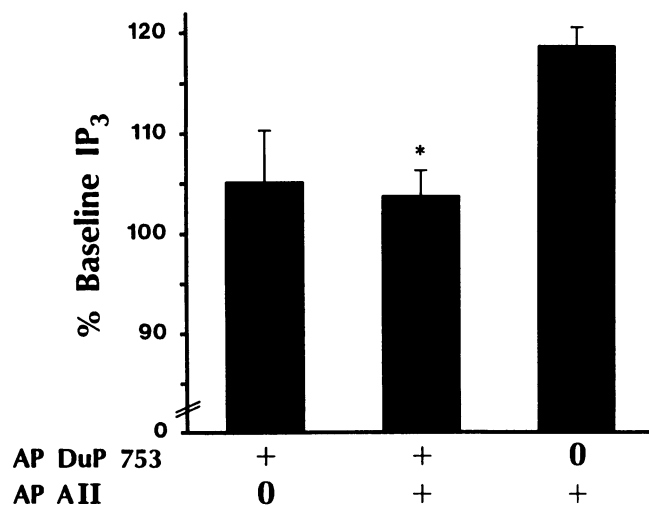


Figure 6. Effect of AP DuP 753 preincubation on AP AII-induced IP₃ formation. Confluent proximal tubule cell monolayers on permeable supports that were previously incorporated with myo-[2-³H]inositol (see Methods) and then incubated with AP 10⁻⁶ M DuP 753 or Tris buffer for 20 min at 21°C. Cells were then washed with phosphate-buffered saline and exposed to AP 10⁻⁷ M AII or Tris buffer for 30 s at 21°C. IP₃ was extracted and measured as described in Methods. Results are means of five or six experiments. **P* < 0.05 compared with AP AII alone.

Table I. Percent [¹⁴C]inulin and [³H]inositol in BL Buffer after Addition to AP Buffer

Time	Percent AP ¹⁴ C dose (n = 5)	Percent AP ³ H dose (n = 4)
min		
0	0	0
30	0.5±0.5	3.5±0.6
60	1.7±0.5	5.8±1.2
90	2.4±1.3	7.9±1.8
120	3.2±1.8	ND

BL, basolateral; AP, apical.

inositol was added to the AP buffer, negligible amounts of radioisotope were identified in the BL buffer after 90 min of incubation. This degree of leak was considered particularly insignificant because AP AII incubations were only 30 s in duration. To further exclude the possibility of paracellular AII leak, the IP₃ response to AP AII was determined in the presence of BL AII receptor occupancy with the peptide AII antagonist Sar-Leu AII (Fig. 7 A) or the nonpeptide antagonist DuP 753 (Fig. 7 B). When 10⁻⁷ M Sar-Leu AII was exposed to the BL proximal tubule cell surface, small increases (6.2±4.1% above baseline, baseline = 555.2±44.6 cpm per well) in IP₃ were observed (Fig. 7 A). This partial agonist effect was more pronounced at higher concentrations of Sar-Leu AII (data not shown). Nevertheless, even in the presence of BL Sar-Leu AII, AP AII incubation resulted in additional increases in IP₃ formation (*P* < 0.05 compared with baseline). There was no significant difference between AP AII-induced IP₃ generation in the presence or absence of BL Sar-Leu AII. Because Sar-Leu AII elicited a small agonist effect, we performed studies with a

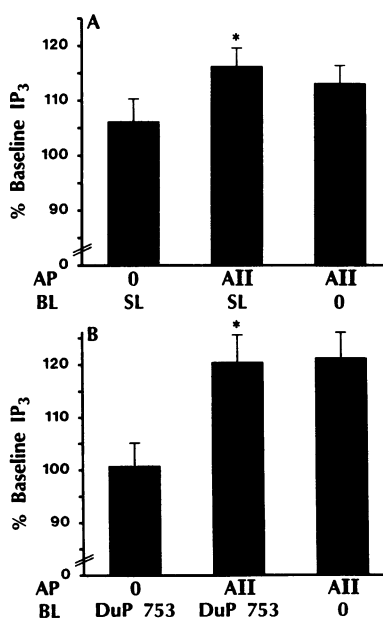


Figure 7. Effect of basolateral (BL) Sar-Leu AII (SL) or BL DuP 753 on AP AII stimulation of IP₃. Proximal tubule cells were incorporated with myo-[2-³H]inositol as described in Methods. In A, the BL surfaces of confluent proximal tubule cell monolayers on permeable supports were incubated with 10⁻⁷ M Sar-Leu AII or Tris buffer for 30 min at 21°C. AP and BL surfaces were washed with phosphate-buffered saline and the AP proximal tubule cell surface was then incubated with 10⁻⁷ M AII or Tris buffer for 30 s at 21°C.

IP₃ was extracted and measured as described in Methods. Results are the means of three to seven experiments. B is similar to A except for preincubation with BL 10⁻⁶ M DuP 753 (instead of Sar-Leu AII) or Tris buffer for 20 min at 21°C. Results are means of three to five experiments. **P* < 0.05 compared with baseline.

structurally dissimilar nonpeptide antagonist. Fig. 7 *B* shows that BL DuP 753 did not elicit IP₃ signals. Moreover, in the presence of BL DuP 753, AP AII caused a 19.7% IP₃ increase ($P < 0.05$ compared with baseline), which was comparable to the IP₃ signal from the AP surface without the BL antagonist (20.5% IP₃ increase above baseline, baseline = 678.0±66.3 cpm per well). Taken together, these data exclude paracellular leakage of AP ligand to BL sites and suggest that AP AII receptors activate PLC.

Since AII signaling appears to require AII receptor-mediated endocytosis in vascular smooth muscle (19) and adrenal glomerulosa cells (20), we questioned whether AP AII receptors directly activated PLC or required endocytosis before activation of PLC. Fig. 8 demonstrates the effect of phenylarsine oxide (a trivalent oxidizing agent that prevents AII receptor-mediated endocytosis by binding to receptor sulfhydryl groups [19, 20, 30]) on AP and BL AII receptor-mediated PLC activation. When the AP proximal tubule surface was preincubated with phenylarsine oxide (Fig. 8 *A*), complete inhibition of AP AII-induced IP₃ formation was observed (3.2±5.4% below baseline, $P < 0.05$ compared with AII alone, baseline = 596.4±55.3 cpm per well). In contrast to the AP surface, addition of phenylarsine oxide to the BL buffer (Fig. 8 *B*) did not prevent BL AII-induced IP₃ formation (25.3±3.9 vs. 21.3±5.4% above baseline, baseline = 648.4±46.4 cpm per well). Since BL AII-induced IP₃ formation was preserved in the presence of phenylarsine oxide, these data demonstrated that phenylarsine oxide was not toxic. Furthermore, the studies also demonstrated a disparity between AP and BL AII receptor function. Whereas BL receptors were directly coupled to PLC, AP AII or AII receptors required endocytosis before generating PLC signals.

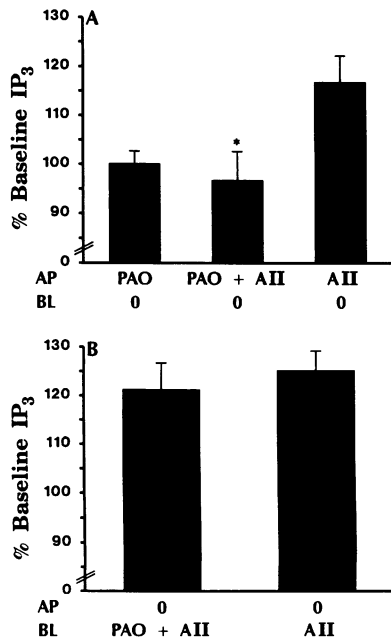


Figure 8. Effect of AP or BL phenylarsine oxide (PAO) on ipsilateral AII-stimulated IP₃ formation. Proximal tubule cells were incorporated with myo-[2-³H]inositol as described in Methods. In *A*, the AP surfaces of confluent proximal tubule cell monolayers on permeable supports were incubated with 10⁻⁴ M PAO or Tris buffer for 20 min at 21°C, washed, and exposed to AP 10⁻⁷ M AII or Tris buffer for 30 s at 21°C. The BL surface was incubated with Tris buffer throughout the experiment. IP₃ was extracted and assayed as described in Methods. Results are means of four or five experiments. In *B*, the BL surfaces of confluent proximal tubule cell monolayers were incubated with 10⁻⁴ M PAO or Tris buffer for 20 min at 21°C, washed, and exposed to BL 10⁻⁷ M AII or Tris buffer for 30 s at 21°C. The AP surface was incubated with Tris buffer throughout the experiment. Results are means of three experiments. * $P < 0.05$ compared with AII alone.

Results are means of four or five experiments. In *B*, the BL surfaces of confluent proximal tubule cell monolayers were incubated with 10⁻⁴ M PAO or Tris buffer for 20 min at 21°C, washed, and exposed to BL 10⁻⁷ M AII or Tris buffer for 30 s at 21°C. The AP surface was incubated with Tris buffer throughout the experiment. Results are means of three experiments. * $P < 0.05$ compared with AII alone.

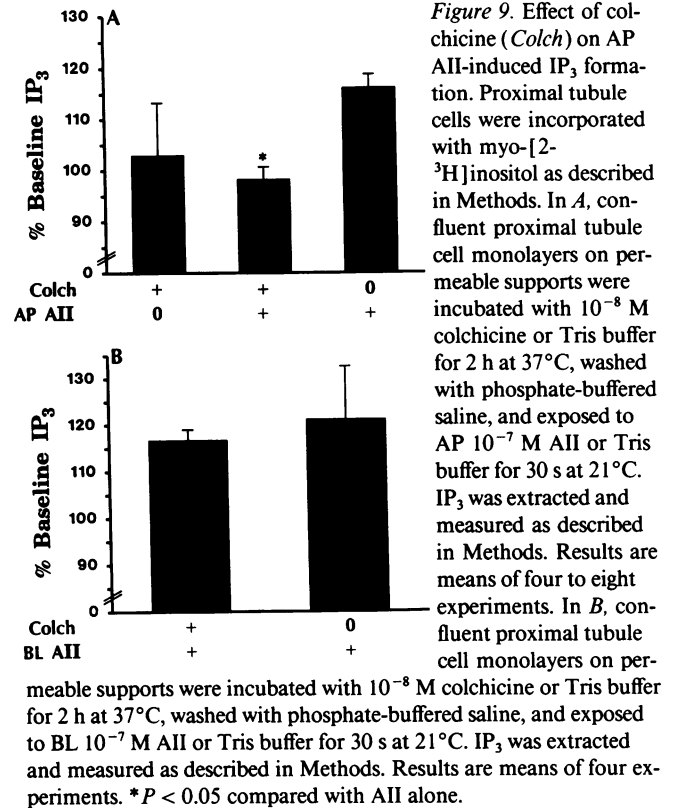


Figure 9. Effect of colchicine (Colch) on AP AII-induced IP₃ formation. Proximal tubule cells were incorporated with myo-[2-³H]inositol as described in Methods. In *A*, confluent proximal tubule cell monolayers on permeable supports were incubated with 10⁻⁸ M colchicine or Tris buffer for 2 h at 37°C, washed with phosphate-buffered saline, and exposed to AP 10⁻⁷ M AII or Tris buffer for 30 s at 21°C. IP₃ was extracted and measured as described in Methods. Results are means of four to eight experiments. In *B*, confluent proximal tubule cell monolayers on permeable supports were incubated with 10⁻⁸ M colchicine or Tris buffer for 2 h at 37°C, washed with phosphate-buffered saline, and exposed to BL 10⁻⁷ M AII or Tris buffer for 30 s at 21°C. IP₃ was extracted and measured as described in Methods. Results are means of four to eight experiments. * $P < 0.05$ compared with AII alone.

Many ligands and receptors are transported within endosomes after binding (31), and the AP membrane is associated with a highly developed cytoskeleton (32), which may facilitate endosome trafficking. To investigate the role of cytoskeletal structures in AP AII receptor signaling, we determined the effect of two agents that interfere with cytoskeletal function at different sites: colchicine (an agent that disrupts microtubules [33]) and cytochalasin D (an agent that disrupts microfilaments [34]). Fig. 9 *A* reveals that colchicine did not alter basal IP₃ levels (baseline = 572.0±54.3 cpm per well). However, colchicine preincubation prevented AP AII-mediated IP₃ formation (Fig. 9 *A*) but had no effect on BL AII-mediated IP₃ formation (Fig. 9 *B*, baseline = 415.8±38.5 cpm per well). Fig. 10 demonstrates that cytochalasin D inhibited AP AII-mediated IP₃ formation (Fig. 10 *A*, baseline = 558.9±35.2 cpm per well) and had no effect on BL AII-mediated IP₃ formation (Fig. 10 *B*, baseline = 570.3±76.8 cpm per well). These data indicate that neither colchicine nor cytochalasin D were toxic in the concentrations used in these studies and that microtubules and microfilaments are required for AP AII-induced PLC activation.

Discussion

The results of the present study demonstrated that rat proximal tubule cells cultured on permeable supports retained the morphological, biochemical, and functional characteristics of proximal tubule cells in vivo. Proximal tubule cells were unlikely to permit paracellular ligand leakage as evidenced by electron microscopy and impediment to paracellular inulin or inositol movement. Exposure of either AP or BL AII receptors to AII resulted in PLC activation, as indicated by increased IP₃ forma-

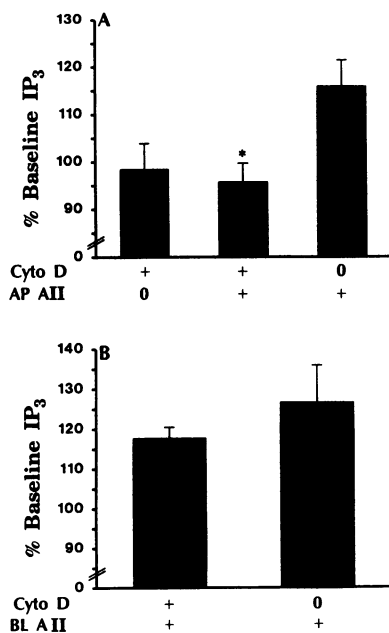


Figure 10. Effect of cytochalasin D (*Cyto D*) on AII-induced IP₃ formation. Proximal tubule cells were incorporated with myo-[2-³H]inositol as described in Methods. In *A*, confluent proximal tubule cell monolayers on permeable supports were incubated with 2 μ M cytochalasin D for 30 min at 37°C, washed with phosphate-buffered saline, and exposed to AP 10⁻⁷ M AII or Tris buffer for 30 min at 21°C. IP₃ was extracted and measured as described in Methods. Results are means of four experiments. In *B*, confluent proximal tubule

cell monolayers on permeable supports were incubated with 2 μ M cytochalasin D or Tris buffer for 30 min at 37°C, washed with phosphate-buffered saline, and exposed to BL 10⁻⁷ M AII or Tris buffer for 30 s at 21°C. IP₃ was extracted and measured as described in Methods. Results are means of four experiments. **P* < 0.05 compared with AII alone.

tion. AII-induced PLC activation was mediated by specific AT₁ receptors since AII-induced IP₃ generation was prevented by the nonpeptide antagonist DuP 753. Furthermore, the AP signal did not result from paracellular ligand leak to BL receptors, as AP addition of AII elicited PLC signals in the presence of BL receptor inhibition with the peptide antagonist Sar-Leu AII or DuP 753. Finally, agents that inhibited endocytosis or cytoskeletal function inhibited AP, but not BL PLC activation.

Results from *in vivo* experiments have demonstrated a biphasic effect of AII on proximal tubule sodium absorption, with picomolar AII concentrations stimulating sodium absorption and micromolar AII concentrations causing decreased sodium absorption (1, 3). Our results indicate that AP 10⁻⁷ M AII enhanced sodium absorption in cultured proximal tubule cells (Fig. 3).

Our data revealed that AII, at concentrations that enhanced ²²Na transport, increased IP₃ by up to 26.6% above baseline in cultured proximal tubule cells. It is unclear, however, whether the early (30-s AII incubation) IP₃ increase was the predominant signal that caused the ²²Na transport that occurred 20–30 min later. Although studies by Liu and Cogan (15) suggest that AII-induced bicarbonate flux in microperfused rat proximal tubules may have been associated with activation of PLC, other investigators have not identified AII-mediated activation of PLC in proximal tubule cells (16, 17). Although the explanation for this discrepancy is not readily apparent, species differences (rat vs. rabbit) could be one factor. A more likely explanation, however, is that experimental conditions have varied between studies. Whereas prior studies were conducted with proximal tubule cell suspensions (17) or proximal tubule cells grown on impermeable surfaces (16), we used proximal tubule cells that were cultured on permeable supports. In contrast to cells cultured on impermeable supports, there is enhanced

growth and differentiation of proximal tubule cells cultured on permeable supports (21).

We were concerned by the modest IP₃ response to AP AII, as studies with AII in other tissues have yielded greater IP₃ responses (13, 14, 25, 35). One potential explanation for the weaker IP₃ signal in proximal tubule cells is that AII may elicit stronger PLC signals in S₁ proximal tubule segments, since this segment demonstrates both the greatest AII receptor density and the largest bicarbonate flux in response to AII (4). Because our method of isolating proximal tubules also included S₂ and S₃ segments, the PLC signal could be “diluted” by inclusion of less active S₂ and S₃ portions. Another possibility for the modest IP₃ signal in proximal tubule cells is that IP₃ degradation by phosphatases may be enhanced in these cells. In support of this contention, we noted an additional 15.2% increase in AP AII-induced IP₃ levels after 20 min incubation with 10 mM lithium chloride (data not shown), an agent that inhibits myo-inositol-1-phosphatase activity (36). Although the use of lithium would have resulted in consistently higher inositol phosphate values, we chose to perform studies without lithium because this agent may also affect other interrelated, AII-responsive enzymes, such as adenylyl cyclase (37, 38). However, because only small changes in IP₃ are necessary to cause intracellular calcium mobilization (39), we therefore considered the observed AII-induced IP₃ increase to be biologically relevant.

The relation between duration of AII exposure and peak IP₃ formation in proximal tubule cells is similar to the time dependency observed in vascular smooth muscle cells (13, 40). In both systems there is an early IP₃ response that decays over 30–60 s, with no subsequent increases. In contrast, the time dependency between AII and IP₃ differs in adrenal glomerulosa cells, in which there is a biphasic AII response (14). In these cells there is an early IP₃ peak (similar to vascular smooth muscle and proximal tubule cells), followed by a second IP₃ peak, which is characterized by sustained IP₃ formation for \geq 30 min (14, 41).

To be certain that there was specific coupling of proximal tubule AII receptors to PLC, we performed studies with AII receptor antagonists. Incubation with either the peptide antagonist Sar-Leu AII (data not shown) or the nonpeptide inhibitor DuP 753 prevented AII-induced IP₃ formation (Fig. 6). These data confirm that there is specific coupling between AII receptors and PLC in cultured rat proximal tubule cells. Although previous studies with AP and BL proximal tubule membrane preparations documented more than one AII receptor subtype (9), inhibition of AII-generated IP₃ with DuP 753 suggested that the PLC signal was mediated by AT₁ receptors (42, 43).

Our studies also demonstrated that AII-induced PLC signals were elicited by both AP and BL receptors. These results are consistent with studies demonstrating that AII receptors have been identified on both the AP and BL proximal tubule cell membranes (8, 9, 18), and AII elicits bicarbonate absorption when added to AP or BL sites of microperfused proximal tubules (4). Although BL receptors are exposed to AII from circulating plasma, the source of AII that could activate AP receptors has only recently been confirmed. Seikaly et al. (44) have shown that AII undergoes glomerular filtration and is present in the proximal tubule lumen in concentrations that were comparable to the AP AII concentrations used in our studies. Furthermore, we observed that separate AP and BL incubations with equivalent concentrations of AII resulted in greater BL IP₃ formation.

The results of three studies indicate that the PLC signal elicited from AP AII receptors was mediated by AP AII receptors rather than by paracellular ligand leak to BL sites. In this regard, transmission electron micrographs revealed proximal tubule cell monolayers with well-defined tight junctions. In addition, significant inulin or inositol leak from AP to BL buffer was not observed. Furthermore, exposure of AP receptors to AII when the BL receptors were occupied by AII receptor antagonists still resulted in PLC signaling. These data confirm that the AP AII receptor is coupled to PLC.

Since AP AII receptors have been shown to be degradative receptors (11) and since AII receptors require endocytosis for signaling in vascular smooth muscle (19) and adrenal glomerulosa (20) cells, we questioned whether there was an endocytosis requirement for signaling on the AP proximal tubule surface. To answer this question, we compared AP and BL responses with AII in the presence of phenylarsine oxide, colchicine, and cytochalasin D. The results indicated that receptor-mediated endocytosis through a cytoskeletal domain is required for AII-induced PLC activation from AP, but not BL sites. This conclusion is consistent with recently described data that the cytoskeleton is not merely a structural cell component but also plays a dynamic role in protein and glycoprotein targeting (45–48). In addition, differences between the cytoskeletal dependence of AP and BL AII receptor endocytosis are consistent with morphological findings that demonstrate highly developed cytoskeletal components in the AP domain of polarized epithelial cells (32).

A requirement for cytoskeleton-dependent endocytosis is not unique for AP AII receptor-mediated PLC activation. Other AP functions such as epidermal growth factor receptor-mediated ricin uptake (49), glucose (50–53), and water (54–57) transport also require cytoskeleton-dependent endocytosis. In the case of glucose transport, microfilament disruption with cytochalasin B resulted in impaired glucose transporter trafficking and diminished glucose uptake (50–53). Toad bladder studies have revealed that water transport is dependent upon water channel movement through the cytoskeleton, since water transport was inhibited after disruption of either microfilaments (54–57) or microtubules (54, 55, 57). Although endocytosis through cytoskeletal domains appears to be required for a number of AP functions, there is little information about the role of the cytoskeleton in receptor-mediated signal transduction. Our data revealed that the cytoskeleton was not required for BL AII receptor activation of PLC, but AP activation of PLC was cytoskeleton dependent in proximal tubule cells.

Although our studies do not indicate the site to which AP AII or AII receptors are directed, it is unlikely that PLC is activated after ligand transcytosis and binding to BL receptors, since AP AII incubation was associated with IP₃ formation in the presence of BL receptor inhibition (Fig. 7). Since endocytosis-dependent signal transduction was observed in vascular smooth muscle (19) and adrenal glomerulosa (20) cells, it has been speculated that IP₃ may be formed after activation of endosomal membrane-bound PLC (20). Such a mechanism is also plausible in proximal tubule cells. In conclusion, our studies suggest that the mechanism of AII function in proximal tubule cells is more complex than previously recognized. In cultured rat proximal tubule cells AII is coupled to PLC, binding to either AP or BL AII receptors results in PLC activation, and cytoskeleton-dependent endocytosis is required for AP, but not BL, AII receptor-mediated PLC activation.

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