Relationship between Phospholipase C Activation and Prostaglandin E_2 and Cyclic Adenosine Monophosphate Production in Rabbit Tubular Epithelial Cells

Effects of Angiotensin, Bradykinin, and Arginine Vasopressin

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Abstract

By employing early-passaged rabbit kidney epithelial cells in tissue culture, we demonstrated that angiotensin II (AII) has unique mechanisms of signal transduction. First, unlike its action in other target tissues, micromolar concentrations of AII are required to induce small rises in cytosolic calcium, $[Ca^{2+}]_i$, an action which is not accompanied by the release of inositol phosphates (IP). In contrast, nanomolar bradykinin (BK) mobilizes $[Ca^{2+}]_i$ through activation of phospholipase C and release of IP. Neither of these stimulated calcium responses exhibits pertussis toxin (PTx) sensitivity. Secondly, AII and BK at 10^{-9} to 10^{-7} M stimulate cAMP indirectly through PGE_2 production in distal cells. AII- and BK-stimulated PGE_2 release is PTx inhibitible, suggestive of the presence of a GTP binding protein mediating the response. By contrast, arginine vasopressin fails to elicit rises in $[Ca^{2+}]_i$ but exerts its primary effect on cAMP production in distal cells via direct coupling to a stimulatory GTP binding protein, as evidenced by uncoupling with cholera toxin. Regulation of PGE_2 synthesis appears to occur via phospholipase A_2, not C, by all three peptides.

Introduction

Angiotensin II (AII)[1] plays a major role in salt and volume homeostasis as evidenced in part by its important actions on sodium excretion through a variety of actions on the kidney. The peptide exerts effects on renal hemodynamics (1, 2), and also acts directly on tubular epithelium to influence salt and water transport (3). Such a direct transport effect is most likely mediated by hormone-receptor binding on the tubular epithelial cell (4, 5). Recently, this tubular site of action has been better delineated in studies demonstrating the presence of AII receptors on all segments of the nephron. However, by far the highest density occurs in the proximal convoluted tubule (6), a segment well documented to be a target for AII-induced electrolyte transport (3, 7).

Studies utilizing micropuncture and microperfusion techniques (3, 8) have demonstrated a dose-dependent bimodal effect of the peptide on tubular transport. Proximal tubular sodium and water reabsorption are stimulated by low concentrations (10^{-12} to 10^{-10} M) of AII, whereas higher doses (10^{-7} M) cause inhibition of transport, thus mediating a natriuresis. The intracellular mechanism underlying the effect of AII on sodium transport has been the subject of much recent investigation. Traditionally, studies of signal transduction in renal epithelial cells have involved the adenylate cyclase system with cyclic adenosine monophosphate (cAMP) functioning as a second messenger (9). Current evidence demonstrates that concentrations of AII in the picomolar to nanomolar range inhibit the production of cAMP in proximal tubular cells, an action that may be associated with antinatriuresis (10). This inhibition is not reversed with high doses of AII. By contrast in distal tubular epithelial cells, AII in nanomolar and greater concentrations stimulates prostaglandin E_2 (PGE_2) and cAMP production, actions that may promote natriuresis (10).

Little is known about the precise mechanism of AII-induced natriuresis in proximal tubules. Unlike cells of the distal tubule, proximal cells have little capacity to produce PGE_2 (11, 12), an autocoid known to stimulate cAMP (13, 14) and induce sodium excretion (15). Alternatively, studies of signal transduction in other target tissues such as glomerular mesangium (16) and vascular smooth muscle (17) have demonstrated that the primary actions of AII are mediated through phospholipase C-induced phosphoinositide metabolism and mobilization of intracellular calcium. Phospholipase C activation is also felt to be the primary pathway responsible for PGE_2 production by AII in other target tissues (16). In addition, bradykinin (BK), another natriuretic hormone, has been shown to catalyze the turnover of inositol lipids in the papillary collecting tubule (18). However, little is known about its mechanism of action on cortical tubular epithelial cells.

The present studies were designed to test the hypothesis that activation of phospholipase C may represent a mechanism of signal transduction for AII in cortical epithelial cells in addition to adenylate cyclase. We employed rabbit tubular epithelial cells in tissue culture to ascertain whether cytosolic calcium ($[Ca^{2+}]_i$) mobilization and phosphoinositide metabolism were linked to the AII, BK, and vasopressin receptors of cortical epithelial cells. Attempts were also made to determine whether phospholipase C activation accounted for hormonal activation of eicosanoid production. These studies reveal important differences in the mechanisms of hormonal action on tubular epithelial cells.

Methods

Cell isolation. Renal tubular epithelial (RTE) cells were isolated and cultured as described (manuscript submitted for publication). Briefly, 2-kg male New Zealand White rabbits were killed using nitrogen gas.

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1. *Abbreviations used in this paper:* AII, angiotensin II; BK, bradykinin; IP, inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; PIP_2, phosphatidylinositol bisphosphate; RTE, renal tubular epithelial (cells).

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The kidneys were removed and placed in cell culture medium during transport. Each kidney was perfused with 10–20 ml of Collins solution with 5 mM EGTA, until free of blood. The cortex was minced and homogenized (four strokes) with a Dounce homogenizer (loose pestle) as described (19). The homogenate was poured over a series of nylon mesh screens (from 200 to 20 μm) which removed glomeruli and intact tubules. The isolated cells, which passed through the 20-μm filter (average yield 25–40 $\times$ 10^7 cells), were pelleted by centrifugation at 200 *g* and layered on a discontinuous Percoll gradient of 30–60% (Pharmacia Fine Chemicals, Piscataway, NJ) which was spun at 1,500 *g* for 15 min. The resulting cell fractions (distal tubular cells less dense than proximal tubular cells) were placed in separate cultures.

Cell culture. Cell fractions from the gradient were cultured as described by Chung et al. (19), employing media containing a 50:50 mixture of Dulbecco's modified eagle's medium (DME, Gibco, Grand Island, NY) and Ham's F12 (Gibco) supplemented with 15 mM Hepes buffer (Sigma Chemical Co., St. Louis, MO), 1.2 mg/ml sodium bicarbonate, 192 IU/ml penicillin, 200 μg/ml streptomycin, 5 μg/ml bovine insulin, 5 μg/ml human transferrin, and 5 $\times$ 10^{-8} M hydrocortisone, with the addition of 5% fetal bovine serum. Freshly isolated cells were plated at a high density, 0.2 $\times$ 10^6 cells/cm^2, to discourage dedifferentiation and maintained in an humidified incubator 5% CO_2/95% air at 37°C. Primary, first- or second-passage cells were used after being grown to confluency. Cells were washed and placed in serum-free media for 12–24 h prior to each experiment. In the designated experiments, cells were pretreated with one of the following substances, 400 ng/ml pertussis toxin (List Biological, Campbell, CA) for 16 h, 10 μg/ml flurbiprofen (Upjohn Co., Kalamazoo, MI) for 30 min, or the phorbol ester, phorbol myristate acetate (PMA), at 0.1 μg/ml for 30 min. Cells used in suspension were harvested by trypsinization (trypsin EDTA, Gibco) for 2–3 min, and pelleted by centrifugation at 250 *g* for 4 min. Fluorometric determination of cytosolic Ca^{2+} employed cells in suspension or grown as monolayers on Aclar square plastic coverslips (Allied Engineered Plastics, Pottsville, PA) cut to fit into the fluorimeter cuvette. PGE_2/cAMP and phosphoinositide-labeling studies employed cells grown in 24- (2 cm^2) and 12-well (9 cm^2) Costar culture dishes (Costar, Cambridge, MA), respectively. The peptide hormones, angiotensin II, bradykinin, and arginine vasopressin (AVP) were purchased from U. S. Biochemical Corp., Cleveland, OH.

Cyclic AMP and PGE_2 radioimmunoassays (RIAs). The cells were preincubated for 30 min at 37°C in serum-free media as described above, and containing 250 μM 3-isobutyl-1-methylxanthine and 1% bovine serum albumin (Sigma Chemical Co.), essential fatty acid free. Dose–response relationships were obtained by incubating the cells for exactly 5 min at 37°C in triplicate wells for each concentration of peptide hormone employed. The supernatant was aspirated for the PGE_2 RIAs and the remaining intracellular component was extracted with 0.1 N HCl (for 24 h) for cAMP determination. PGE_2 RIAs employed antisera purchased from Institut Pasteur, Paris and [^{3}H]PGE_2 (100–200 μCi/mmol), New England Nuclear, Boston, MA). The 50% intercept averaged 30 pg. Cross-reactivity with other eicosanoids was low, as published (20). Cyclic AMP RIAs employed the acetylation technique as described (21).

Fura 2 loading and cytosolic Ca^{2+} measurement. All studies were performed using a balanced salt solution (BSS) containing 120 mM NaCl, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM Hepes, adjusted to pH 7.40 with concentrated NaOH. Cells were washed twice with BSS spun at 250 *g* for 4 min and diluted to a final concentration of $\sim$ 1 $\times$ 10^6 cells/ml in BSS containing 10 mM D-glucose, and 0.1% albumin. The cells were warmed at 37°C for 10 min, followed by incubation with 1.0 μM fura 2 acetoxymethylester (Molecular Probes, Inc., Eugene, OR) for 20 or 30 min, for suspensions and monolayers, respectively. After an additional wash with fresh BSS, the cells were again incubated for 10 min to allow complete hydrolysis and entrapment of the esters. Loading was terminated by washing with ice-cold BSS. Suspensions were diluted to a final concentration of $\sim$ 0.25–0.50 $\times$ 10^6 cells/ml BSS with glucose and albumin. Fluorescence was monitored using a fluorometer designed by the Johnson Foundation Biomedical Instrumentation Group, Philadelphia, PA, as previously described (22). Aliquots of cell suspensions added to the cuvette were maintained at 37°C with constant mixing. Peptide hormones were added from concentrated stock solutions. Fluorescence was measured using an excitation wavelength of 339 nm and an emission wavelength of 500 nm. In cell suspensions, calibration of fura 2 fluorescence as a function of $[Ca^{2+}]_i$ was performed using digitonin (20 μg/ml) permeabilization in the presence of 1.2 mM Ca^{2+} to achieve maximal fluorescence (F_{max}). This was followed by chelation of extracellular Ca^{2+} with 10 mM EGTA and alkalinization with 70 mM Tris HCl, pH 10.5, to achieve minimal fluorescence (F_{min}). Monolayers were calibrated using the nonfluorescent divalent cation ionophore, ionomycin (10 μM) (Calbiochem-Behring Corp., San Diego, CA) followed by 1.0 mM $MnCl_2$ to quench fura 2 fluorescence. In this case, the calcium-dependent fluorescence was approximated at 70% of the total Mn-quenchable fluorescence based on in vitro measurements with fura 2 standards. $[Ca^{2+}]_i$ was then calculated using a previously described formula (23). The effective K_d for Ca^{2+} binding to fura 2 was assumed to be 224 nM.

Phosphoinositide labeling, extraction, and analysis. Epithelial cells were grown to confluence in 9-cm^2 culture dishes. 2–3 d prior to an experiment, the growth medium in each well was replaced with 0.6 ml of serum-free inositol-free DME supplemented with penicillin 100 U/ml, streptomycin 100 μg/ml, insulin 0.25 μg/ml, transferrin 0.25 μg/ml, selenium 5 ng/ml, and 4 μCi/ml of myo-[2-^{3}H(N)]-inositol (16.5 Ci/mmol, New England Nuclear). The cells were incubated 48–72 h in an humidified atmosphere of 90% air/10% CO_2. The monolayers were then washed with ice-cold, isotope-free BSS. Prior to stimulation with agonists, 600 μl of BSS supplemented with 10 mM LiCl were added and the cells were preincubated for 10 min at 37°C. Incubations were performed in triplicate for each dose of peptide hormone agonist and terminated by the addition of 120 μl of 3.3 N perchloric acid (Sigma Chemical Co.). The monolayers were scraped and acid-extracted for 15 min at 4°C. The extracts (720 μl) were removed and the wells rinsed with 480 μl of 0.55 N PCA. The pooled extract plus rinse was centrifuged for 5 min at 10,000 *g*. 1 ml of the supernatant was neutralized was 55 μl of 10 N KOH and iced for 15 min. The precipitated perchloric acid was pelleted by centrifugation. 1 ml of the neutralized supernatant was then diluted with 9 ml of 5 mM Na borate and applied to a 0.8-ml column of AG 1 $\times$ 8 (200–400 mesh) anion exchange resin (Bio-Rad Laboratories, Richmond, CA). The loaded columns were then washed with 24 ml of 60 mM Na formate/5 mM Na borate. For measurements of total inositol phosphates elutions were performed with 12 ml of 1 M NH_4 formate/0.1 M formic acid. For measurements of individual inositol phosphates, three fractions, respectively containing mono-, bis-, and tris/tetraphosphate esters, were collected from each column by successive elution with 12 ml each of (*a*) 0.2 M ammonium formate/0.1 formic acid, (*b*) 0.4 M ammonium formate/0.1 M formic acid, and (*c*) 1 M ammonium formate/0.01 M formic acid. Between collection of fractions 2 and 3, the column was rinsed with an additional 8 ml aliquot (which was discarded) of 0.4 M ammonium formate/0.1 M formic acid. Each 12-ml eluate was placed in a 20-ml scintillation vial and evaporated to dryness and partially desalted by vacuum-centrifugation (Speed-Vac 200, Savant Instruments, Inc., Hicksville, NY) for at least 18 h. The dried, desalted residues of fractions 1 and 2 as well as that for the total inositol phosphate measurement were dissolved in 1 ml H_2O and counted in 10 ml of scintillation fluid (Formula 963, New England Nuclear). The residue of fraction 3 was dissolved in 4 ml H_2O and counted in 14 ml of scintillation fluid.

Statistical analysis. Results are expressed as the mean±SEM and statistical significance determined using Student's *t* test for unpaired data.

Results

Cellular characteristics. Electron micrographs are demonstrated from acutely isolated cells in Fig. 1, displaying charac-

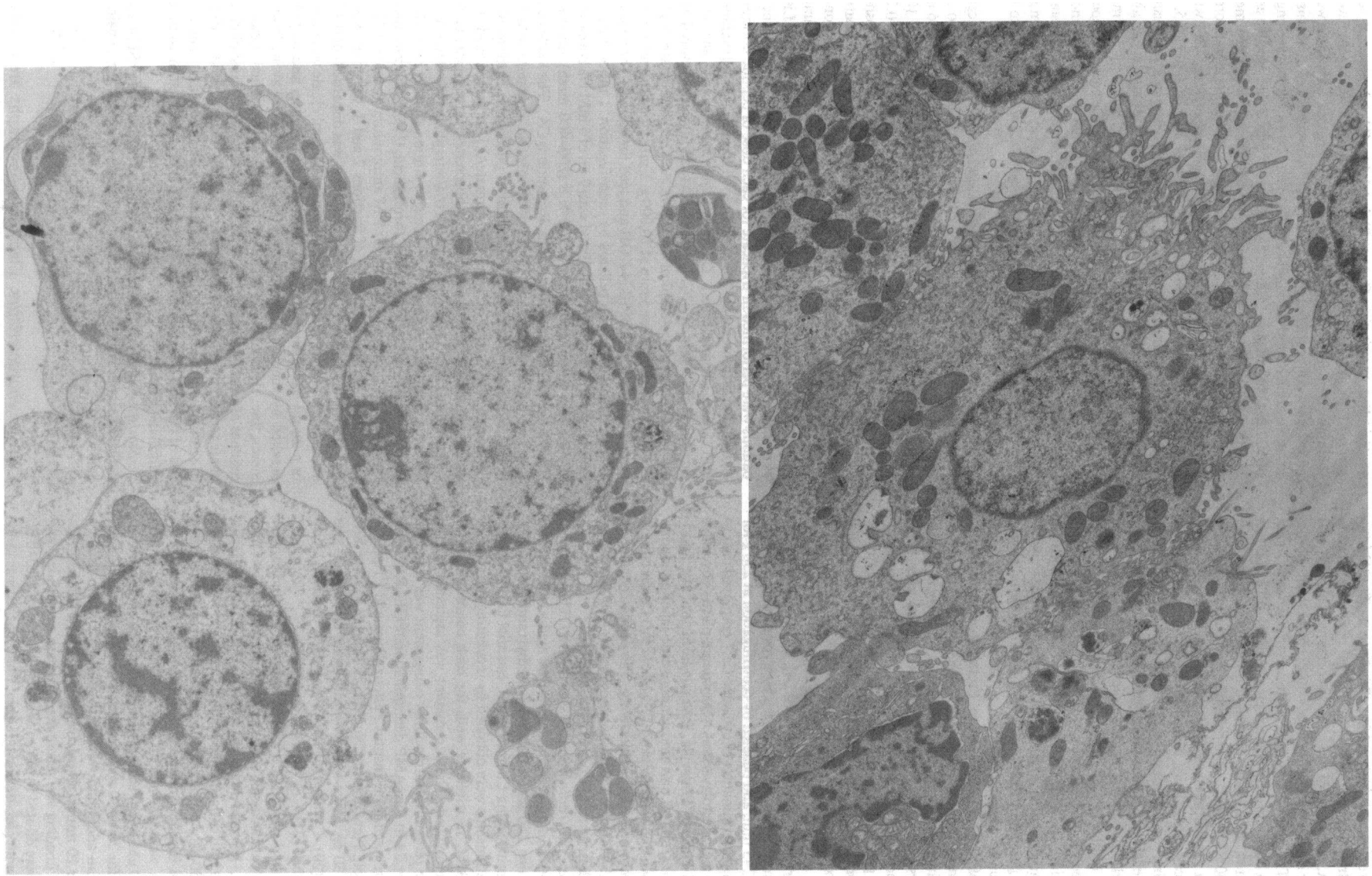

Figure 1. Electron micrographs. (*Left*) Distal tubular epithelial cells after acute isolation and gradient centrifugation (× 2,700). Cells are recovered at a density of 1.019–1.023 g/ml. Note the absence of microvilli, large nucleus to cytoplasmic ratio, and fewer mitochondria than proximally. (*Right*) Polarized proximal tubular epithelial cell (× 5,000) with brush border and long mitochondria. Density of these cells was 1.028 g/ml.

teristics as previously described by Evaloff et al. (24). Distal tubular cells display no microvilli, a large nucleus to cytoplasmic ratio, large round nucleus, relatively few mitochondria, and relatively few small vesicles in the cytoplasm. Distal tubular cells are recovered on the gradient at a density of 1.019–1.023 g/ml as determined employing a digital densitometer. Proximal tubular cells (Fig. 1, *right*) display polarized microvilli, basal-lateral infolding (appearing as large cytoplasmic vacuoles), and abundant long mitochondria. The density of proximal cells ranges from 1.026 to 1.034 g/ml. A detailed characterization of enzyme distribution, hormone receptors, and morphology has been presented elsewhere (24, manuscript submitted for publication). Cells have been maintained in tissue culture as described (19).

Mobilization of intracellular Ca^{2+} stores in renal tubular epithelial cells. Activation of phospholipase C and subsequent rises in cytosolic Ca^{2+} have been shown to be the primary mechanisms of signal transduction for AII in various target tissues including vascular smooth muscle and glomerular mesangium (16, 17). In addition, calcium has been shown to be an important modulator of epithelial ion permeability and, in the kidney, rises in cytosolic calcium have been shown to inhibit sodium reabsorption in the proximal tubule (25). Therefore, experiments were performed studying the effect of AII on cytosolic calcium levels in proximal and distal RTE cells. Comparisons were made to BK and AVP, other hormones known to be modulators of fluid transport.

Fig. 2 illustrates the Ca^{2+} transients elicited by the indicated concentrations of BK, AII, and AVP employing cells maintained in tissue culture. The cells were most sensitive to BK, which produced a rapid rise in cytosolic calcium in a dose-dependent fashion. The response plateaued at a 10- and 4-fold rise with 10 nM BK in proximal and distal cells, respectively. Occasionally, responses could be elicited by 0.01 nM BK in the proximal cells but this finding was inconsistent. Concentrations of AII in the physiological range consistently produced no rise in cytosolic Ca^{2+}. A concentration of at least 0.1 μM was required to produce even a small transient (Fig. 2).

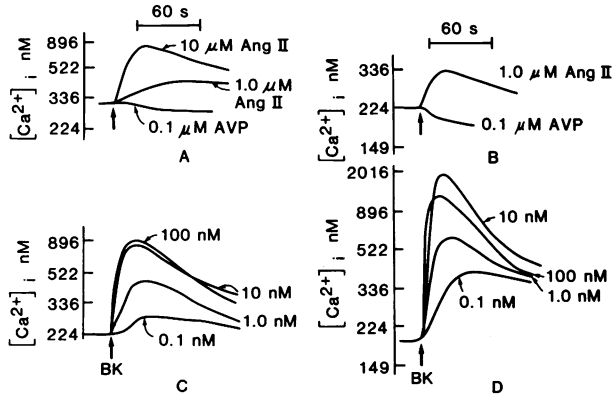

Figure 2. Dose-dependent effects of AII, BK, and AVP on rises in cytosolic calcium in monolayers of RTE cells of the (*A, C*) distal and (*B, D*) proximal tubule. Each superimposed curve represents the tracing obtained from stimulation of cells grown on separate coverslips by the indicated concentrations of hormone and measured by fura 2 fluorescence. Each set of tracings is a representative sample of experiments performed on the same day, using cells from the same culture and passage.

10 μM AII produced a 2.5–3.0-fold rise in $[Ca^{2+}]_i$. With both BK and AII, not only was the magnitude of the $[Ca^{2+}]_i$ rise greater with increasing concentrations of peptide, the rate of rise was much more rapid as well, indicating dose-dependent kinetics of the calcium response. AVP consistently produced no rise in cytosolic Ca^{2+}, as expected, since its mode of action in kidney epithelial cells is linked to stimulation of adenylate cyclase (9).

The proximal and distal tubular epithelial cells produced similar qualitative results, with the proximal cells appearing to be more responsive. For example, when stimulated with BK, these cells produced higher absolute levels of $[Ca^{2+}]_i$ (1,834 nM vs. 878 nM) and larger percent increases (876% proximally vs. 300% distally). In addition, proximal cells responded to lower concentrations of peptides, e.g., 0.01 nM BK and 0.1 μM AII. We found no significant differences in either hormone-induced rise in $[Ca^{2+}]_i$ between cell suspensions and cells grown on coverslips as monolayers with respect to potency or magnitude of response. Thus, the brief trypsinization used to obtain suspensions appeared not to have a significant effect on receptor number or integrity of signal transduction as detected by measuring increases in cytosolic calcium.

Contribution of $[Ca^{2+}]_i$ and Ca^{2+} influx. To determine whether the observed rises in cytosolic calcium were indeed the result of calcium release from intracellular stores as opposed to calcium influx from the extracellular pool, we studied the effects of manipulating voltage-sensitive calcium channels. First, the calcium-entry blocking agent nifedipine was added prior to stimulation with the peptide agonists in order to inhibit calcium influx. The results indicate that nifedipine did not significantly alter agonist-induced Ca^{2+} transients with respect to magnitude or kinetics (data not shown). Similarly, when these channels were activated by depolarization of the cells with 40 mM KCl, no $[Ca^{2+}]_i$ rise was elicited. Thus, the observed rise in cytosolic Ca^{2+} did not depend on calcium influx into the cell through voltage-sensitive channels.

Nonetheless, when the extracellular calcium was chelated with 3 mM EGTA prior to agonist stimulation, marked alterations in the calcium response were observed, as shown in Fig. 3. In particular, the AII response was sluggish in the presence of EGTA, peaked well below the maximal response observed with AII alone, and returned quickly to basal values. On the other hand, even in the presence of EGTA, BK produced a response of equal magnitude and rapidity but which also returned quickly to baseline. This finding suggests that this slow

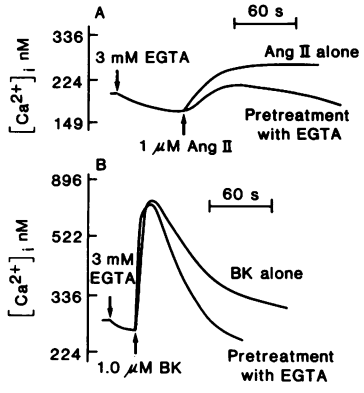

Figure 3. Effect on chelation of the extracellular calcium on the $[Ca^{2+}]_i$ rises induced by (*A*) AII and (*B*) BK in cell suspensions. Comparison tracings are superimposed and represent stimulation with the indicated concentration of peptide hormone alone and after chelation of extracellular calcium with 3 mM EGTA.

secondary phase depends significantly on Ca^{2+} influx for both BK- and AII-stimulated transients. Similar results have been described for other cells (22, 26). Moreover, influx of extracellular Ca^{2+} appears to contribute far more to the overall magnitude of AII-induced Ca^{2+} response. As previously indicated, the influx is via channels which are distinct from voltage-sensitive channels and may represent a type of receptor-operated calcium channel for AII.

Effect of pretreatment with PMA, pertussis toxin, and flurbiprofen. To better characterize the mechanism of the hormone-induced $[Ca^{2+}]_i$ rise, the cells were pretreated with various inhibitory agents. The effect of preincubation of the cells with the phorbol ester PMA (0.1 μg/ml) for 30 min is illustrated in Fig. 4. As with other phorbol esters, PMA is a potent stimulator of a Ca^{2+}- and phospholipid-sensitive protein kinase, protein kinase C, which stimulates protein phosphorylation (27). In this respect it mimicks the action of endogenous diacylglycerol (27), one of the by-products of phospholipase C activation. As shown, such treatment produced a marked blunting of the stimulation usually induced by BK, with a shift of the dose-response curve to the right (maximal $[Ca^{2+}]_i$ value 685 nM vs. 1,176 nM). In addition, PMA pretreatment almost completely abolished the Ca^{2+} transient usually elicited by 10 μM AII. The same pretreatment for only 2 min instead of the longer 30-min incubation had no effect on either BK- or AII-induced stimulation (data not shown). This result is in contrast to observations of short-term PMA effects in other tissues studied (28). Possible explanations may involve low levels of protein kinase C in RTE cells or alternative pathways of activation.

PGE_2 and other eicosanoids have been shown to mobilize cytosolic calcium in glomerular mesangium (29). Therefore, studies were conducted to determine their role in AII- and BK-induced rises in $[Ca^{2+}]_i$ in tubular epithelial cells. Preincubation of the cells with the potent cyclooxygenase inhibitor flurbiprofen 10 μg/ml for 30 min had no effect on basal, AII-, or BK-stimulated $[Ca^{2+}]_i$ levels, either in proximal or distal renal tubular cells. The same dose and length of incubation has been shown to drastically reduce the levels of PGE_2 in these cells (see data below).

Additional studies with glomerular mesangium have reported that AII-induced $[Ca^{2+}]_i$ rises are inhibited by pertussis toxin pretreatment (16). Pertussis toxin is an agent that ADP-ribosylates subunits of specific GTP binding proteins, membrane proteins that couple receptor stimulation to enzyme activation (30, 31). Our data indicate that in both proximal and distal epithelial cells, preincubation with 400 ng/ml pertussis toxin for 14 h also had no effect on basal, AII- or BK-stimulated $[Ca^{2+}]_i$ values (data not shown). Thus, rises in cytosolic calcium induced by peptide hormone agonists appear to be mediated neither through stimulation of eicosanoids nor via a pertussis toxin-sensitive, GTP binding protein. However, activation of protein kinase C markedly blunts stimulated $[Ca^{2+}]_i$ rises without affecting basal values.

Phosphoinositide turnover. In general, the receptor-mediated rises in $[Ca^{2+}]_i$ observed in a variety of organs occurs secondary to hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) and the generation of inositol phosphates, in particular inositol (1,4,5-) trisphosphate (IP_3) (32). PIP_2 hydrolysis has been associated with AII stimulation of various tissues, including vascular smooth muscle (17, 33), hepatocytes (34), and mesangial cells (16). To determine whether the described rises in cytosolic calcium are associated with such processes, we measured total inositol phosphates generated in response to the peptide agonists AII and BK in both proximal and distal cells. As shown in Fig. 5, increasing doses of BK stimulated significantly increased levels of inositol phosphates. Proximal cells were more sensitive and produced highly significant increases in response to the lowest concentration of BK used, 10^{-9} M. On the other hand, AII failed to produce any detectable increase in inositol phosphate levels at doses that reproducibly produced a rise in cytololic calcium suggesting an action of AII on receptor-operated Ca^{2+} channels of epithelial cells. Similarly, vasopressin, a hormone not associated with stimulated rises in $[Ca^{2+}]_i$ in these cells, failed to alter levels of inositol phosphate (data not shown). Experiments were performed in the presence of 10 mM LiCl to inhibit terminal phosphatase activity and thus allow measurement of accumulated inositol phosphates, if any, produced during stimulation with agonists.

Additional experiments were performed to confirm that the observed effects of AII and BK on cytosolic calcium were a consequence of their primary ability (or inability) to generate IP_3 specifically. Fig. 6 shows the amount of inositol phosphate released as the mono-, bis-, and trisphosphate esters, IP, IP_2,

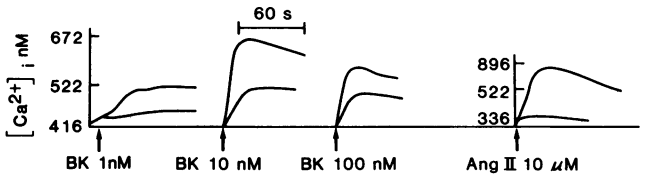

Figure 4. Effect of PMA on dose-dependent stimulation of $[Ca^{2+}]_i$ rises induced by BK and AII in cell suspensions. The upper tracing of each pair represents the transient rise in cytosolic calcium stimulated by the indicated concentration of BK or AII alone. The lower tracing represents the same experimental conditions except for pretreatment with 0.1 μg/ml PMA for 30 min prior to addition of the hormone.

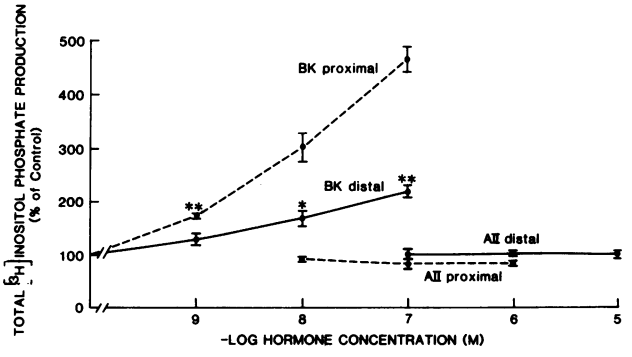

Figure 5. Measurement of total [^{3}H]inositol phosphate production in response to BK and AII in proximal and distal RTE cells. Cells were labeled for 48–72 h with [^{3}H]myoinositol (4 μCi/ml) and incubated with the indicated concentrations of agonist for 15 min in the presence of 10 mM LiCl. Each value represents the mean±SE of triplicate determinations. $*P < 0.05$, $**P < 0.005$, and for subsequent higher doses, comparisons made to basal, unstimulated values.

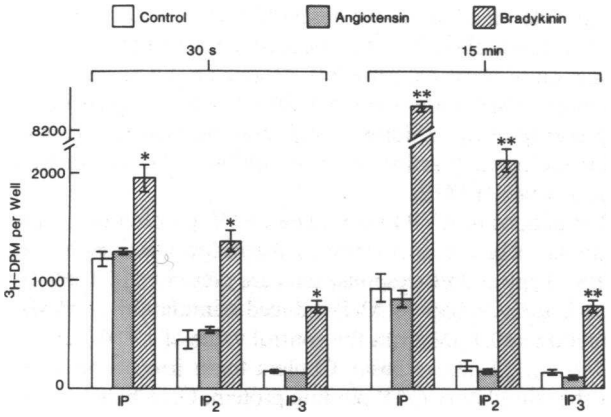

Figure 6. [^{3}H]inositol phosphate accumulation at 30 s (*left*) and 15 min (*right*) after stimulation with AII and BK in distal RTE cells. Cell monolayers were labelled for 48 h with [^{3}H]myoinositol (2 μCi/ml). After washing and preincubation with 10 μM LiCl for 10 min, the cells were exposed to 10^{-5} M AII or BK, or vehicle for the indicated time periods. Individual inositol phosphates were extracted and analyzed as described. Each value represents the mean±SE of duplicate determinations. *$P < 0.05$, **$P < 0.005$ comparisons made to corresponding control values.

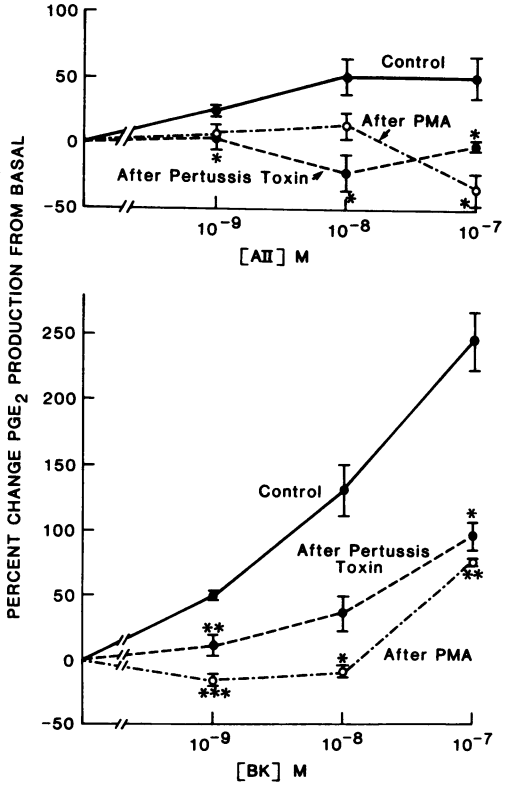

Figure 7. Stimulated production of PGE_2 by AII (*upper panel*) and BK (*lower panel*) in distal tubular epithelial cells. Control curves (*solid lines*) represent the percent change from basal production of 26.8±1.3 ng/mg protein (n = 28) for the indicated concentrations of either AII or BK alone. Pertussis toxin curves represent the percent change from basal production of 43.8±5.6 ng/mg protein (n = 8) for the same experimental conditions except for pretreatment with 400 ng/ml toxin for 16 h prior to stimulation with peptide hormones. PMA curves represent the percent change from basal production of 18.2±2.1 ng/mg protein (n = 5) for the same conditions except for pretreatment with 0.1 μg/ml PMA for 30 min prior to stimulation. Each point represents the mean±SE, where n = 3–16. *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$, comparisons made to control values at the same concentration of hormone.

and IP_3, respectively. Again, no significant release of inositolphosphates was observed after stimulation with 10^{-5} AII, either at 30 s or 15 min. Conversely, BK produced a nearly 5- and 16-fold increase in IP_3 at 30 s and 15 min, respectively. Such data are consistent with the previous observations of the greater magnitude and sensitivity which BK exhibits in eliciting cytosolic Ca^{2+} transients in kidney epithelial cells.

Pharmacologic characterization of peptide hormone receptors. Peptide analogues and antagonists were used to help characterize the BK and AII receptors to which the stimulated Ca^{2+} responses were presumably linked. The bradykinin analogue Lys-bradykinin (1.0 μM) produced a rise in cytosolic calcium that was 73% of the response observed with the same concentration of BK. Alternatively, the specific B-1 receptor agonist des-Arg^9 BK and antagonist des Arg Leu^8 BK had no significant effects. These findings were consistent with an action of BK mediated through a B-2 receptor, as occurs in most target tissues. It is intriguing to note that proximal cells are more sensitive to the actions of BK than cells of the distal tubule, despite documentation by binding studies that receptor density is greatest distally (35). With regards to the AII receptor, pretreatment with the antagonist [Sar^{1}Leu^8] at a concentration of 10 μM completely abolished the $[Ca^{2+}]_i$ rise induced by 1.0 μM AII. However, we found that inhibition of the response required a 10-fold higher concentration of antagonist than agonist and there was no effect if [Sar^{1}Leu^8] was added after the AII. These findings are consistent with AII action mediated through a specific receptor.

PGE_2 and cAMP. In a series of experiments parallel to those characterizing changes in calcium homeostasis, we measured the effect of pertussis toxin, flurbiprofen, and PMA on the peptide-stimulated production of PGE_2 and cAMP, other cellular messengers known to affect sodium transport in these cells.

As shown in Fig. 7, BK and AII stimulate the production of PGE_2 in distal renal tubular cells in a dose-dependent fashion with 10^{-7} M BK and AII resulting respectively, in a 247±23% and 45±11% increase, above basal production of 27±13 ng/mg protein. A possible mechanism for hormone-induced PGE_2 production in distal tubular cells is activation of phospholipase C with release of diacylglycerol. Diacylglyerol can then be cleaved by diglyceride lipase resulting in arachidonic acid release. An alternative pathway for PGE_2 production is via activation of phospholipase A_2, resulting in direct release of arachidonic acid. The former pathway is felt to be responsible for the bulk of AII-induced eicocanoid production in other tissues (16).

Pretreatment of the cells with pertussis toxin (400 ng/ml) increased basal PGE_2 production to 43.8±5.6 ng/mg protein (n = 8, $P < .001$ compared with control) but produced a significant decrease in stimulated PGE_2 release. The observation that pertussis toxin fails to affect changes in $[Ca^{2+}]_i$ but is able to inhibit agonist-stimulated PGE_2 production suggests that AII and BK may stimulate phospholipase A_2 via a pertussis-sensitive GTP binding protein analogous to α_1-adrenergic stimula-

tion of thyroid cells (36). Moreover, pretreatment with PMA (0.1 μg/ml) not only shifted the BK dose–response curve to the right, but produced inhibition of PGE_2 to below basal levels of 18.2±2.1 ng/mg protein (n = 5, P = 0.011 compared with control) for both BK and AII. This inhibition was not overcome by raising the dose of AII to as high 10 μM and was most likely due to receptor desensitization or downregulation. Pretreatment with the potent cyclooxygenase inhibitor flurbiprofen (10 μg/ml) resulted in nearly complete abolishment of both basal and stimulated PGE_2 production in these cells, in agreement with previous reports (10). For example, flurbiprofen inhibited basal PGE_2 production from a control value of 27±1.3 ng/mg protein (n = 28) to 1.3±0.2 ng/mg protein (n = 6, $P < 0.001$) in the presence of flurbiprofen. In addition, PGE_2 stimulation by 10^{-7} M BK was decreased from 109±12 ng/mg protein 1.7±0.3 ng/mg protein ($n = 6$, $P < 0.001$) in the presence of flurbiprofen.

The production of cAMP in distal tubular cells paralleled that of PGE_2 with dose-dependent stimulation by AII and BK (Table I). Similarly, pretreatment with pertussis toxin or PMA produced significant decreases in the agonist-stimulated production of cAMP. The percent increase in cAMP levels stimulated by BK 0.1 μM dropped from 134% to 38% after pertussis toxin and to 33% after PMA. Similarly, 0.1 μM AII alone produced a 31% increase in cAMP which was reduced to 7% after pertussis toxin (NS) and to −26% after PMA. In addition, in order to determine the role of prostaglandins in cAMP stimulation, we abolished PGE_2 production with flurbiprofen prior to addition of the agonist. This resulted in hormone-induced inhibition of cAMP to subbasal values of −22% and −20% with BK and AII, respectively. Thus, the ability of these peptides to stimulate cAMP production appears to be dependent initially on their ability to stimulate PGE_2. Blocking such actions with a cyclooxygenase inhibitor reveals their primary inhibitory actions on cAMP production.

Proximal tubular cells, on the other hand, have very little capacity for PGE_2 production and therefore, provide a relatively prostaglandin-free model of tubular epithelial cells in which to observe changes in cAMP production. It has been shown that picomolar concentrations of AII inhibit the production of cAMP in primary cultures of proximal tubular cells (10, 37), similar to that observed in flurbiprofen-treated distal tubular cells. In addition, we observed that pretreatment with pertussis toxin abolished AII-induced decrements in cAMP production in proximal cells (37). Moreover, AII caused stimulation of cAMP under these conditions. This is suggestive of the presence of an inhibitory GTP binding protein, G_i, mediating the primary action of AII to inhibit cAMP production in the absence of PGE_2.

Table I. Hormonal Effects on cAMP in Distal Tubular Epithelial Cells as a Percent Change from Basal

	Basal	AII (0.01–0.1 μM)	BK (0.1 μM)
	pmol/mg protein	%	
Control	18±1 (5)	31±8 (6)	134±22 (4)
Pertussis toxin	21±1 (3)	7±8 (3)	38±15* (3)
PMA	16±1 (3)	−26±4* (3)	33±14* (3)
Flurbiprofen	15±1 (3)	−20±9.8* (5)	−22±6* (3)

The number of experiments is given in parentheses.
* $P < 0.05$ as compared with the corresponding control value.

The actions of AVP to stimulate cAMP production in distal tubular cells are in contrast to the indirect actions of AII and BK. Typical dose–response data are presented in Table II. Pertussis toxin enhances AVP-induced stimulation of cAMP production at 0.1 μM from the control value of 1,150% above basal to 3,255% above basal. Cholera toxin irreversibly activates the stimulatory GTP binding protein, G_s. Cholera toxin pretreatment of the epithelial cells at a dose of 400 ng/ml for 12 h markedly diminished AVP-induced stimulation of cAMP consistent with an action of AVP on cAMP via coupling to G_s. AVP, like AII and BK, stimulates PGE_2 release in distal tubular epithelial cells, e.g., 120±20% (n = 3) increase at 10^{-9} M and 415±106% (n = 3) increase at 10^{-8} M above basal PGE_2 release of 4.5±0.7 ng/mg protein (n = 4). However, inhibition of PGE_2 production with flurbiprofen enhances AVP-induced stimulation of cAMP production (Table II). This confirms the direct effect of AVP on cAMP production, in contrast to the indirect actions of AII and BK to stimulate cAMP through PGE_2 release.

Discussion

The present studies demonstrate that kidney epithelial cells possess unique mechanisms of signal transduction for AII that distinguish them from other target issues. Although phospholipase C mediates the majority of actions of AII in sites such as glomerular mesangium and vascular smooth muscle, it appears to play little role in mediating the actions of AII on tubular epithelium. The strongest evidence for this is derived from the inability of the peptide to hydrolyze phosphoinositides to inositol phosphates, specifically IP_3, even in the presence of large doses of AII (10^{-5} M). In addition, AII is relatively impotent in its ability to elicit transient rises in cytosolic calcium, requiring micromolar concentrations, in contrast to other tissues, where nanomolar concentrations of AII produce both rises in $[Ca^{2+}]_i$ and IP_3 formation. Finally, the small rise in calcium stimulated by AII in epithelial cells was, in large part, a result of calcium influx from the extracellular pool through nonvoltage-sensitive channels.

Using BK as a positive control, however, we demonstrated that the mechanism for phospholipase C activation and hydrolysis of phosphoinositides is intact in kidney epithelial cell cultures. Specifically, BK produced large, rapid rises in $[Ca^{2+}]_i$, primarily from intracellular stores, as well as rises in inositol phosphates in both proximal and distal cells. Thus, the inability of AII to stimulate phospholipase C cannot be explained by the absence of such a system in cell culture. In addition, the integrity of AII receptors appeared to be intact, as evidenced by its ability to affect changes in other messengers such as cAMP and PGE_2 at low concentrations of hormone. Taken together, the data is more suggestive of the direct coupling of the AII receptor to a calcium channel rather than to phospholipase C. The mechanism and significance of such a receptor-operated calcium channel require further study.

Table II. Actions of AVP on cAMP in Distal Tubular Epithelial Cells

	Control	Pertussis toxin	Cholera toxin	Flurbiprofen
Basal (*pmol/mg protein*)	15±4 (3)	30.6±1 (3)	1,014±388 (4)	53±4.9 (3)
		% change from basal		
AVP 10^{-9}	264±31 (5)	—	—	—
AVP 10^{-8}	654±196 (6)	1,525±288 (2)	−0.75±4.3* (4)	1,209±81 (3)
AVP 10^{-7}	1,150±122 (5)	3,255±498* (3)	33±8 (3)	1,678±18* (2)

The number of experiments is given in parentheses. * Significantly different from the corresponding control value at $P < 0.05$.

A more sensitive mechanism of signal transduction for AII appeared to be stimulation of PGE_2 production in cells of the distal tubule. In contrast to other target tissues in which AII-induced arachidonic acid release is felt to occur via the action of diglyceride lipase, eicosanoid production in RTE cells appears to occur via a pathway which is distinct from phospholipase C activation. Several lines of evidence support this concept. AII-stimulated PGE_2 production, but not rises in cytosolic calcium, were inhibited by pertussis toxin pretreatment. In addition, AII-induced PGE_2 release was significantly increased at 10^{-9} M but rises in $[Ca^{2+}]_i$ required micromolar concentrations. Finally, as stated above, phospholipase C plays little role in mediating the actions of AII in RTE cells. Similarly, BK-induced PGE_2 production was dissociated from phospholipase C, as evidenced by differential pertussis toxin sensitivity. The phorbol ester, PMA, did not appear to be a useful tool for making a distinction between various pathways of signal transduction since it indiscriminately inhibited cAMP and PGE_2 production by all three agonists, as well as rises in $[Ca^{2+}]$; by AII and BK. Thus AII and BK receptors may be coupled to phospholipase A_2 via a pertussis-sensitive G protein. Similar results have been described in thyroid cells, where adrenergic receptors are coupled to phospholipases C and A_2 via different GTP binding proteins (36). AII-stimulated eicosanoid production provides a possible mechanism for the natriuretic effect observed at higher concentrations of hormone in this segment of the nephron.

Another important and highly sensitive mechanism of signal transduction for AII in kidney epithelial cells appears to be linked to adenylate cyclase. Picomolar concentrations of AII inhibit cAMP production in cells of the proximal tubule (10, 37), while nanomolar concentrations stimulate cAMP distally, through PGE_2 production. The regional differences appear to be, at least in part, due to differences in PGE_2 biosynthetic capacity and influence of AII on PGE_2 levels. PGE_2 production in the proximal tubule is low compared with the distal and AII has no known stimulatory effect on PGE_2 proximally. Indeed, when PGE_2 production was abolished with flurbiprofen, AII was shown to inhibit cAMP production in the distal tubule, as well. Such attenuation of cAMP production suggests the action of an inhibitory GTP-binding protein in the adenylate cyclase complex (G_i) of kidney epithelial cells, as described in other tissues (38). Consistent with this possibility was the observation that AII-induced decrements in cAMP production in proximal cells were significantly reversed by pretreatment with pertussis toxin. Since cAMP has been shown to inhibit volume reabsorption in the isolated perfused proximal tubule (39), the decrements in cAMP levels induced by AII provide a possible mechanism for the sodium reabsorption observed with picomolar AII in proximal tubular cells.

In contrast to the indirect actions of AII and BK to stimulate cAMP via PGE_2 production in distal cells, AVP appeared to stimulate cAMP directly. Inhibition of cyclooxygenase activity and PGE_2 production actually enhanced cAMP formation by AVP. In addition, cholera toxin uncoupled AVP-stimulated cAMP production, consistent with the presence of a stimulatory GTP binding protein (G_s) mediating the AVP response.

The physiologic relevance of AII-induced rises in cytosolic calcium are as yet unclear but several observations are of interest. First, rises in cytosolic calcium induced by calcium ionophore or quinidine inhibits sodium transport in the proximal tubule, thereby inducing a natriuresis. Micropuncture and microperfusion studies have demonstrated that AII inhibits sodium reabsorption at concentrations of 2×10^{-8} to 10^{-5}, which correlate with AII stimulation of cytosolic calcium. Dominguez et al. (40) have recently reported studies in which 10^{-8} and 10^{-6} M AII inhibit fluid transport in the proximal tubule and stimulate very small increases in $[Ca^{2+}]_i$, 13% and 32%, respectively, above basal. In addition, BK, a very potent natriuretic hormone, also potently stimulated transient rises in calcium in these cells. However, the relative importance, if any, which stimulated rises in $[Ca^{2+}]_i$ have in mediating AII-induced natriuresis remains to be determined, especially considering the very large dose required.

In summary, we used early cultures of proximal and distal renal tubular epithelial cells which retain many of the morphologic and biochemical properties of in vivo systems (manuscript submitted for publication). We have found AII to have unique and complex mechanisms of signal transduction which are distinct from its actions in other target tissues, as well as from other hormones in the same cells. In proximal and distal cells, nanomolar BK and micromolar AII exert an effect through pertussis toxin-insensitive rises in cytosolic calcium. BK stimulates inositol phosphate formation and calcium mobilization of intracellular stores consistent with activation of phospholipase C. AII depends to a greater extent on calcium influx and fails to stimulate inositol formation consistent with

activation of a receptor-operated Ca channel. In contrast to AII and BK, AVP does not affect phospholipase C or Ca^{2+} metabolism and exerts its primary effect on distal tubular cAMP production via coupling to a stimulatory GTP binding protein (G_s). Nanomolar concentrations of AII and BK stimulate cAMP in distal cells, through primary stimulation of PGE_2. Regulation of PGE_2 synthesis by all three peptides appears to occur through activation of phospholipase A_2, not C. We also provide evidence to suggest that a pertussis toxin-inhibitable, GTP binding protein may also be involved in the actions of AII and BK to stimulate PGE_2 release. The applicability of such varied systems to fluid and electrolyte transport in systems in vivo requires further study.

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