View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Repositório Institucional UNIFESP

First published January 4, 2012; doi:10.1152/ajprenal.00381.2011.

Exposure of luminal membranes of LLC-PK_1 cells to ANG II induces dimerization of AT_1/AT_2 receptors to activate SERCA and to promote Ca^{2+} mobilization

Fernanda M. Ferrão,[1,2] Lucienne S. Lara,[2,3] Flavia Axelband,[1,2] Juliana Dias,[1,2] Adriana K. Carmona,[4] Rosana I. Reis,[5] Claudio M. Costa-Neto,[5] Adalberto Vieyra,[1,2] and Jennifer Lowe[1,2]

[1]*Instituto de Biofísica Carlos Chagas Filho,* [3]*Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Rio de Janeiro;* [2]*Instituto Nacional de Ciência e Tecnologia de Biologia Estrutural e Bioimagem, Rio de Janeiro;* [4]*Department of Biophysics, Federal University of São Paulo, São Paulo; and* [5]*Department of Biochemistry and Immunology, School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil*

Submitted 12 July 2011; accepted in final form 29 December 2011

Ferrão FM, Lara LS, Axelband F, Dias J, Carmona AK, Reis RI, Costa-Neto CM, Vieyra A, Lowe J. Exposure of luminal membranes of LLC-PK_1 cells to ANG II induces dimerization of AT_1/AT_2 receptors to activate SERCA and to promote Ca^{2+} mobilization. *Am J Physiol Renal Physiol* 302: F875–F883, 2012. First published January 4, 2012; doi:10.1152/ajprenal.00381.2011.—ANG II is secreted into the lumens of proximal tubules where it is also synthesized, thus increasing the local concentration of the peptide to levels of potential physiological relevance. In the present work, we studied the effect of ANG II via the luminal membranes of LLC-PK_1 cells on Ca^{2+}-ATPase of the sarco(endo)plasmic reticulum (SERCA) and plasma membrane (PMCA). ANG II (at concentrations found in the lumen) stimulated rapid (30 s) and persistent (30 min) SERCA activity by more than 100% and increased Ca^{2+} mobilization. Pretreatment with ANG II for 30 min enhanced the ANG II-induced Ca^{2+} spark, demonstrating a positively self-sustained stimulus of Ca^{2+} mobilization by ANG II. ANG II in the medium facing the luminal side of the cells decreased with time with no formation of metabolites, indicating peptide internalization. ANG II increased heterodimerization of AT_1 and AT_2 receptors by 140%, and either losartan or PD123319 completely blocked the stimulation of SERCA by ANG II. Using the PLC inhibitor U73122, PMA, and calphostin C, it was possible to demonstrate the involvement of a PLC→DAG(PMA)→PKC pathway in the stimulation of SERCA by ANG II with no effect on PMCA. We conclude that ANG II triggers SERCA activation via the luminal membrane, increasing the Ca^{2+} stock in the reticulum to ensure a more efficient subsequent mobilization of Ca^{2+}. This first report on the regulation of SERCA activity by ANG II shows a new mechanism for Ca^{2+} homeostasis in renal cells and also for regulation of Ca^{2+}-modulated fluid reabsorption in proximal tubules.

luminal effect of ANG II; Ca^{2+} sparks; proximal tubule Ca^{2+} homeostasis; fluid reabsorption

THE KIDNEY IS an essential organ in homeostasis and body fluid regulation. Its functions are modulated by different hormones and autacoids that act on hydroelectrolytic balance, extracellular volume, and blood pressure, such as angiotensin II (ANG II) (24). ANG II plays a crucial role in renal Ca^{2+} handling, one of the main cell messengers (4, 5, 13, 51), which is involved in the fine tuning of fluid reabsorption in different segments of the nephron (17). Approximately 60% of the plasma Ca^{2+} is filtered by the kidneys, 99% of which is reabsorbed (70% of it in the proximal tubules) (19). ANG II regulates Ca^{2+} reabsorption in both luminal and basolateral aspects of tubule membranes (4, 5, 13). In proximal tubule cells, intracellular Ca^{2+} mobilization activates Ca^{2+}-dependent intracellular signaling pathways, including those associated with ANG II-modulated Na^+ and water reabsorption mechanisms (8, 16, 17, 39, 42).

Active and passive transport mechanisms participate in Ca^{2+} reabsorption in the proximal tubules (19, 48). Concerning primary active transporters, there are two main Ca^{2+}-ATPase subfamilies in proximal tubule cells, the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) and the plasma membrane Ca^{2+}-ATPase (PMCA) (38). These pumps control intracellular Ca^{2+} concentration by regulating intracellular Ca^{2+} stocks, fine-tuning cytosolic Ca^{2+} activity in many cell types (12). Therefore, renal SERCA and PMCA are excellent targets for hormones, including ANG II and angiotensin-derived peptides (6). We found that picomolar ANG II inhibits PMCA activity via a PLC/protein kinase C (PKC) pathway triggered by the peptide binding to AT_1R/AT_2R heterodimers (4, 5). Micromolar ANG II is metabolized by peptidases associated with the basolateral membrane, generating Ang-(3–4) that reactivates PMCA (5, 6).

Classically, ANG II was described to be formed only systemically. Different studies in the last two decades found that ANG II is formed in different tissues such as brain, heart, and adipose tissue (7, 18, 34) and, especially, in kidney, where local renin-angiotensin system was first described (10, 20, 25, 26, 28, 50). Because of this local synthesis, ANG II levels are much higher in the kidney than in plasma (37, 43), being found in proximal tubule fluid in the nanomolar range (9, 23, 36, 43). This tubular fluid ANG II is not only derived from the filtrate but is also secreted into the lumen by proximal tubule cells (37). Alternatively, angiotensinogen or ANG I may be secreted into the proximal tubule and converted to ANG II by the brush-border angiotensin-converting enzyme (ACE) (15, 27, 35, 37, 40). Intratubular ANG II synthesis indicates that luminal ANG II effects are also as important as its interstitial effects. ANG II AT_1R and AT_2R are also expressed in different nephron segments, including the basolateral and luminal membranes of proximal tubule cells (32, 44, 49), and in organelles of renal cells (14, 52).

Address for reprint requests and other correspondence: J. Lowe, Instituto de Biofísica Carlos Chagas Filho, Laboratório de Físico-Química Biológica Aída Hassón-Voloch, Universidade Federal do Rio de Janeiro, Avenida Carlos Chagas Filho, 373-CCS-Bloco G-Sala 37, Cidade Universitária 21.941-902 Rio de Janeiro, Brazil (e-mail: lowe@biof.ufrj.br).

1931-857X/12 Copyright © 2012 the American Physiological Society

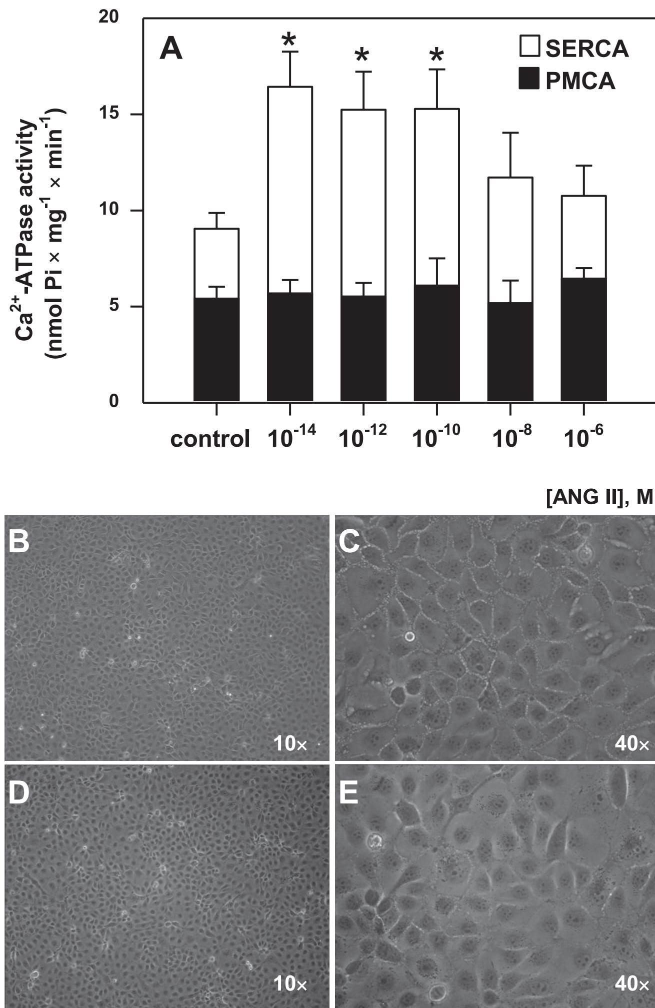

Fig. 1. *A*: ANG II effect via luminal membrane on calcium pumps in LLC-PK_1 cells. ANG II stimulates SERCA activity (*top* open bars) and does not modulate PMCA activity (*bottom* filled bars). Cells were incubated with different ANG II concentrations (10^{-14} to 10^{-6} M) for 30 min and Ca^{2+}-ATPase activities were then measured. For details, see METHODS. Data bars indicate means ± SE of at least 4 determinations in triplicate using different cell lysate preparations. *SERCA activities statistically different from the control without ANG II ($P < 0.05$). *B–E*: bright field microscopy of LLC-PK_1 cells at 90% of confluence (*B* and *C*) and at 100% of confluence (*D* and *E*).

Due to the high ANG II concentrations in proximal luminal fluid and the importance of Ca^{2+} in water and solutes in proximal reabsorption, we investigated whether exposition of the luminal membrane side to ANG II modulates SERCA and PMCA activities in an immortalized lineage of proximal tubule cells from pig kidney (LLC-PK_1 cells). We found that AT_1R/AT_2R heterodimers participate in an ANG II-mediated signaling pathway starting from the luminal side that culminates in an increase in SERCA turnover.

METHODS

Material. A Neubauer chamber (Boeco, Hamburg, Germany) was used for cell counting. Low-glucose DMEM supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml), FBS, and trypsin solution (0.25% trypsin plus 0.04% EDTA-tetrasodium salt) was obtained from GIBCO (Invitrogen Life Technologies, Carlsbad, CA). Distilled water deionized through Milli-Q resins (Millipore) was used to prepare solutions. $^{32}P_i$ was obtained from the Brazilian Institute of Energy and Nuclear Research (IPEN, São Paulo, Brazil). Labeled [γ-^{32}P]ATP was obtained as in Ref. 30. Acetonitrile and trifluoroacetic acid were from TEDIA Brasil (Rio de Janeiro, RJ). Protein A/G-agarose, anti-AT_1R, and anti-AT_2R antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Losartan was obtained from Merck Sharp & Dohme (Whitehouse Station, NJ). LLC-PK_1 cells (American Type Culture Collection) were kindly provided by Dr. Celso Caruso-Neves. Trypan blue solution 1% (wt/vol), trypsin inhibitor (type II-S soybean), bovine serum albumin, ouabain, PD123319, buffers, and ANG II were obtained from Sigma (St. Louis, MO). All other reagents were of the highest purity available.

Renal epithelial cell culture. LLC-PK_1 cells were grown in low-glucose DMEM (10% FBS) with antibiotic supplements in a humidified atmosphere of 5% CO_2 in air at 37°C. Approximately 5×10^5 cells were seeded in 25-cm^2 culture flasks and used after reaching 90% confluence, typically in 3 days (see Fig. 1, *B* and *C*). The cultures maintained polarity with cells attached to the plastic by their basolateral aspect (33), thus allowing ANG II access only to their luminal surface. Cells were washed with PBS, pH 7.5, and immediately incubated with DMEM in the absence of FBS and the different compounds that are indicated in the figures. Cells were harvested and lysed in a buffer containing 1 mM EDTA, 20 mM HEPES-Tris (pH 7.0), 250 mM sucrose, and 0.15 mg/ml trypsin inhibitor, using a Potter-Elvejhem homogenizer with a teflon pestle. After protein concentration determination by the Lowry assay (29), lysates were kept on ice and the further assays were carried out the same day for Ca^{2+}-ATPase and PKC activities, and also for Western blotting analysis of AT_1R and AT_2R after immunoprecipitation with specific antibodies.

Measurements of Ca^{2+}-ATPase activities (SERCA and PMCA). Ca^{2+}-ATPase activity was measured by colorimetric quantification of inorganic phosphate (P_i) released from ATP (46). Total Ca^{2+}-ATPase was calculated as the difference between the total activity and that determined in the presence of 2 mM EGTA. SERCA activity was taken as the fraction of total activity that was inhibited by 1 μM thapsigargin (the specific SERCA inhibitor), the remaining activity being considered as PMCA catalysis. Activity was measured in a solution containing 50 mM bis-Tris-propane buffer (pH 7.4), 5 mM

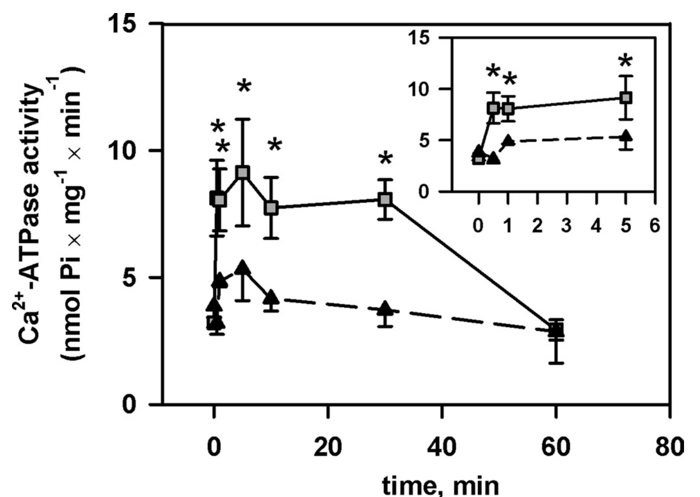

Fig. 2. Time course of ANG II effect on SERCA (■) and PMCA (▲) activities. The luminal membranes of LLC-PK_1 cells were exposed to 10^{-10} M ANG II during the times indicated on the abscissa before Ca^{2+}-ATPase activity was determined. For details, see METHODS. *Inset*: ANG II effect SERCA (■) and PMCA (▲) activities during the first 5 min for a better visualization. Data points indicate means ± SE of at least 4 determinations in triplicate using different cell lysate preparations. *Statistically different from the control without ANG II ($P < 0.05$).

ATP, 5 mM $MgCl_2$, 10 mM NaN_3, 120 mM KCl, 0.1 mM ouabain, 0.2 mM EGTA, and 7.5 μM free Ca^{2+}. The total $CaCl_2$ needed for the desired free Ca^{2+} concentration was calculated as in Ref. 45. Assays were started by adding cell lysate (0.1 mg/ml protein) at 37°C and stopped after 20 min by addition of 0.75 ml of activated charcoal in 0.1 M HCl. After centrifugation at 18,000 *g* to sediment the charcoal, the amount of P_i released was measured in aliquots of 500 μl of the supernatants.

Determination of PKC activity. PKC activity was measured by the incorporation of the γ-phosphoryl group from [γ-^{32}P]ATP into histone H8, which was used as substrate as described elsewhere (11). About 5 × 10^5 cells were incubated with different ANG II concentrations for 30 min, in the absence or presence of 5 × 10^{-8} M calphostin C (PKC inhibitor) that had been added to the culture 10 min before ANG II. After treatment, cell lysates and protein assays were done as described above. PKC activity was measured in a solution containing 4 mM $MgCl_2$, 20 mM HEPES-Tris (pH 7.0), 12 mM NaF, 1 mM ouabain, 1.5 mg/ml histone H8, and 0.7 mg/ml protein of cell lysates. Reaction was initiated by adding [γ-^{32}P]ATP (∼10 Ci/mmol). After 10 min at 37°C, the reaction was stopped by adding 0.1 ml trichloroacetic acid (40% wt/vol). The content of each tube was filtered through 0.45-μm-pore Millipore filters, which were successively washed with 20% trichloroacetic acid and phosphate buffer (2 mM, pH 7.0) to remove unused [γ-^{32}P]ATP. The radioactivity incorporated into the histone was determined in a liquid scintillation counter. PKC activity was calculated by the difference between the radioactivity incorporated into histone in the presence of lysates originated in cells untreated or treated with calphostin C.

Immunoprecipitation and Western blotting for AT_1R/AT_2R heterodimers detection. Immunoprecipitation and Western blotting analysis were performed as previously described (5), with slight modifications. LLC-PK$_1$ cells were incubated with different ANG II con-

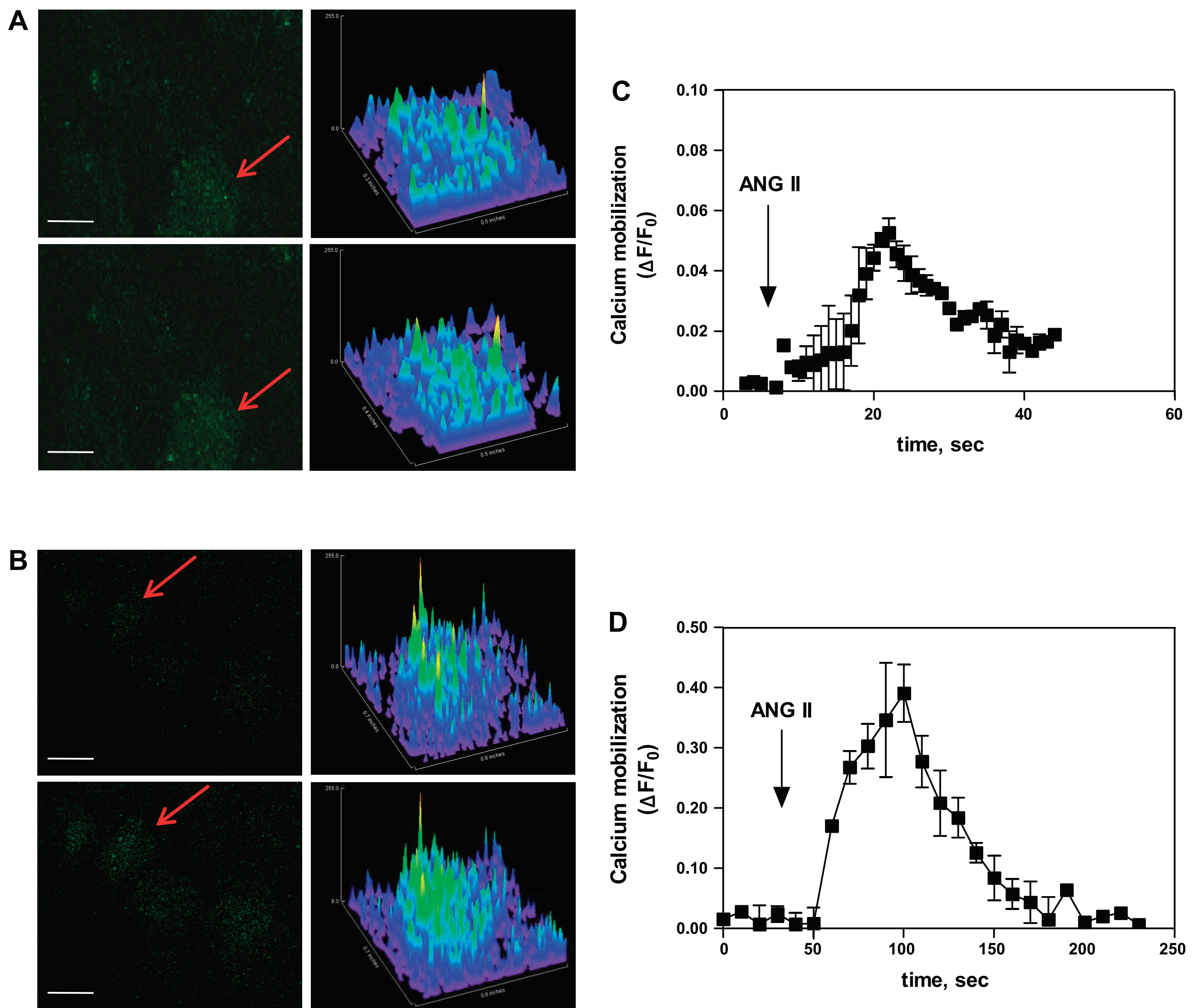

Fig. 3. Prior exposure of the luminal membrane of LLC-PK$_1$ cells to ANG II potentiates the ANG II-induced Ca^{2+} mobilization from intracellular stores. Increases in cytosolic Ca^{2+} were measured by confocal microscopy using the Fluo-3/AM indicator as described in METHODS. *A*: Ca^{2+} signals (*left* images) and ImageJ analysis (*right* images) of the single cell indicated by red arrow at *left*, before (*top*) and after (*bottom*) exposure of the cells to 10^{-7} M ANG II. *B*: same experiments as in *A*, except that the cells were preincubated with 10^{-9} M ANG II before stimulation with 10^{-7} M ANG II. *C* and *D*: averaged data (n = 6) for the Ca^{2+} mobilization illustrated in *A* and *B*, respectively. The white bar represents 50 μm. The points represent means ± SE of the acquired fluorescence values (see METHODS).

centrations for 30 min at 37°C and cell lysates were obtained as described above. Protein (1 mg/ml) was solubilized at room temperature in 0.01% (wt/vol) CHAPS for 30 min, followed by incubation with a mouse anti-AT$_1$R monoclonal antibody (1:200) for 60 min at 4°C. Protein A/G-agarose was added and the mixture was left overnight at 4°C under gentle agitation. Supernatants were separated from the immunoprecipitates by centrifugation at 1,000 *g* for 5 min at 4°C, and the resulting sediments were washed three times with Tris-buffered saline (pH 7.6), mixed with Laemmli buffer, and heated at 100°C for 4 min to remove antibodies and protein A/G-agarose. After centrifugation (16,100 *g* for 2 min at 4°C), supernatants containing the receptors were submitted to electrophoresis (10% SDS-PAGE), transferred to nitrocellulose membranes, and probed with a rabbit polyclonal anti-AT$_2$R antibody (1:500). The total protein was controlled using Ponceau red staining after transfer to nitrocellulose membranes to verify whether the same quantity of protein is present in all lanes. After being stripped, the same membrane was probed for AT$_1$R using a rabbit polyclonal antibody. The 45-kDa band in the immunoprecipitates detected with the AT$_1$R polyclonal antibody was considered to correspond to 100% of the receptors and this was the loading internal control of receptors in each experiment. The AT$_2$R/AT$_1$R immunosignal ratio in the same gel indicated the relative amount of AT$_2$R/AT$_1$R heterodimers. The tiny band of 50 kDa probably corresponds to the IgG heavy chain and no other bands were observed in the full membrane.

Quantification of ANG II by high-performance liquid chromatography. ANG II was measured after incubation with LLC-PK$_1$ cells attached to the culture flasks. ANG II (8 μM) was incubated with cells for different times (0, 30, 60, and 120 min) in a humidified atmosphere of 5% CO_2 in air at 37°C. The supernatants were subsequently analyzed by high-performance liquid chromatography (HPLC) as previously described (5).

Measurement of intracellular Ca^{2+} mobilization. To assess cytosolic Ca^{2+} mobilization, LLC-PK$_1$ cells were loaded with Fluo-3/AM (1 μM) at 37°C in a humidified incubator with 5% CO_2 in air for 30 min in the absence or presence of 10^{-9} M ANG II. Cells were imaged in buffer (135 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 2 mM glucose, 2 mM $CaCl_2$; pH 7.2). Fluorescence imaging involved the use of a scanning laser confocal microscope Leica SP5 (Leica) with a ×63 water immersion objective. Fluo-3 fluorescence dye was excited at 488 nm with an argon ion laser, and the emitted fluorescence was measured at 510 nm. Digital image analyses were made using ImageJ (available at http://rsb.info.nih.gov/ij). Time course software was used to capture three-dimensional volume images of the cells (zyt) in the Live Data Mode acquisition. Fluorescence values were reported as $\Delta F/F_0$, ($\Delta F = F_t - F_0$), where F_t is the observed fluorescence at time *t* after stimulus with 10^{-7} M ANG II, and F_0 is the fluorescence at $t = 0$. F_t and F_0 were computed by adding the intensity of the pixels in a circular area enclosing a single cell. Experiments were carried out at room temperature (23–25°C).

Measurement of ACE, ACE2, neprilysin, and amino peptidases activities in LLC-PK$_1$ cells cultures. The activities of ACE, ACE2, neprilysin (NEP), and amino peptidases (AP) were measured using fluorescent substrates containing the fluorogenic group 4-methyl-coumaryl-7-amide (MCA) or ortho-amino benzoic acid (Abz) and the fluorescent suppressor groups dinitrophenyl (Dnp) or 2,4-dinitrophenyl-ethylendiamino (EDDnp), in the absence or presence of the inhibitors lisinopril (for ACE), thiorphan (for NEP), DX600 (for ACE2), and bestatin (for AP), as previously described (6, 41). Briefly, hydrolysis of substrates was monitored by spectrofluorimetry (Hitachi F-4500, Tokyo, Japan). LLC-PK$_1$ cells were plated in six-well plates at 2×10^5 cells per well for 3 days in low-glucose DMEM (10% FBS) with antibiotic supplements in a humidified atmosphere of 5% CO_2 in air at 37°C. After careful removal of the medium, the layers of 90% confluent cells were supplied with HBSS buffer (140 mM NaCl, 5 mM KCl, 0.1 mM $CaCl_2$, 0.63 mM $MgSO_4$, 1 mM Na_2HPO_4, and 6.1 mM glucose; pH 7.4 adjusted with HCl) containing 10 μM $ZnCl_2$. The fluorescent substrates were then added in absence or presence of their inhibitors and incubated for different times and aliquots were removed for the fluorimetric measurements. For assays using MCA-APK(Dnp)-OH (ECA2), Abz-FRK(Dnp)P (ECA), and MCA-rRL-EDDnp (NEP), λ_{ex} and λ_{em} were 320 and 420 nm, respectively. For Phe-MCA (AP), λ_{ex} and λ_{em} were 380 and 460 nm, respectively. The peptidase activities (in arbitrary units) are presented as the difference between the hydrolysis in the absence and presence of the corresponding inhibitors.

Statistical analysis. Statistical analyses used ANOVA with the Newman-Keuls posttest. Statistical significance was set at $P < 0.05$. Data were analyzed using the GraphPad Prism 5.0 program.

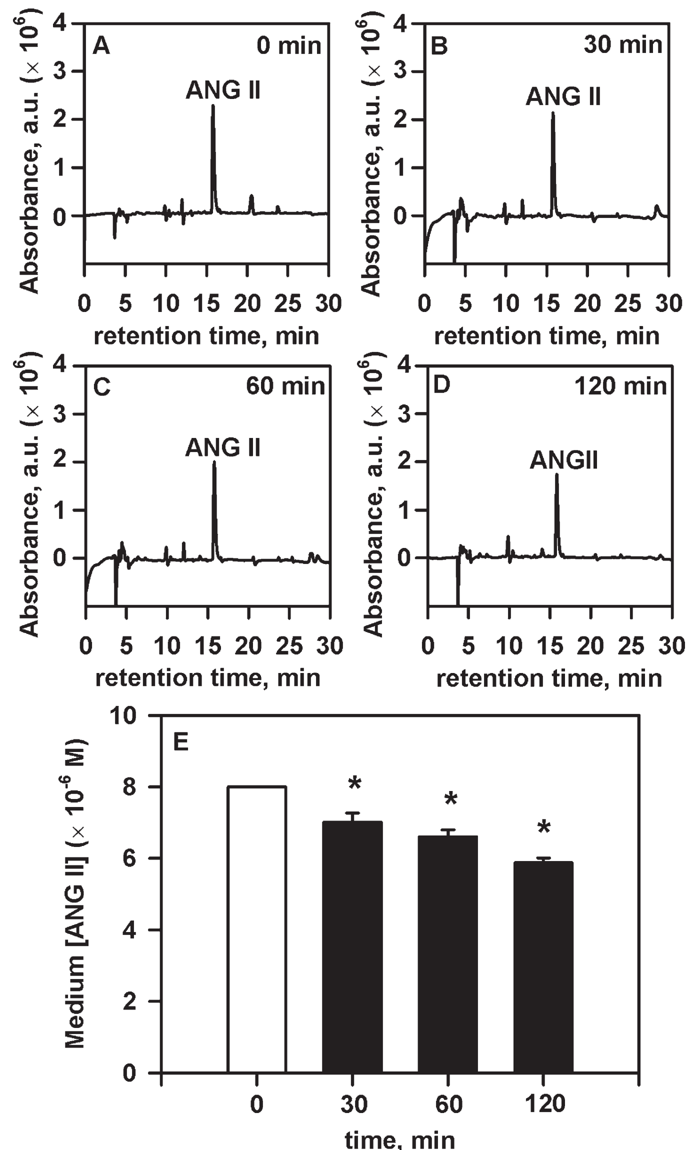

Fig. 4. ANG II is internalized by the luminal membrane of LLC-PK$_1$ cells. Cells firmly attached by their basolateral aspect (33) were exposed to 8 μM ANG II for different times and the medium was carefully collected and analyzed by high-performance liquid chromatography (HPLC), as described in METHODS. *A–D*: representative HPLC chromatograms showing the decay of ANG II without formation of shorter peptides. *E*: graphic representation of remaining ANG II in culture medium after incubation with the cells at the indicated times. Data points indicate means ± SE. *Statistically different from the starting ANG II concentration ($P < 0.05$).

RESULTS

Activation of SERCA by exposure of the luminal membrane to ANG II. Figure 1*A* shows SERCA and PMCA activities in membranes isolated 30 min after exposure of intact LLC-PK_1 cells to ANG II from the luminal side. Lower concentrations of ANG II (10^{-14} to 10^{-10} M) led to an increase of SERCA activity by ≥100%, while higher concentrations (10^{-8} and 10^{-6} M) had no effect on pump catalysis (open bars in Fig. 1*A*). PMCA was unaffected by ANG II over the entire range of concentrations (filled bars in Fig. 1*A*).

Figure 1, *B* and *C*, shows the bright field microscopy of LLC-PK_1 cells at 90% of confluence, which was the experimental condition used in this study. It can be seen in a different field of view (×10 and ×40) that they present the same preserved monolayer structure with the same well-established tight junctions encountered with 100% of confluence (Fig. 1, *D* and *E*), thus preventing ANG II-containing medium from accessing the basolateral membranes. The stimulatory effect of 10^{-10} M ANG II on SERCA activity was exactly the same as that found with 90% confluent cells with no influence on PMCA (data not shown).

Time course of the luminal effect of ANG II. Activation of SERCA by luminal exposure of LLC-PK_1 cells to ANG II (10^{-10} M) is time-dependent (Fig. 2). After a short exposure of 30 s, ANG II had already stimulated SERCA activity, as measured after isolation of membranes; this increase persisted until 30 min of incubation. With luminal membranes of the cells exposed to ANG II for 1 h, activation no longer occurred (gray symbols). Except when otherwise noted, the following experiments were therefore undertaken in membranes isolated after exposure of LLC-PK_1 cells to 10^{-10} M ANG II for 30 min. PMCA was unaffected by ANG II at any of the incubation times (Fig. 2, black symbols).

ANG II increases Ca^{2+} mobilization in LLC-PK_1 cells. To investigate whether ANG II induces Ca^{2+} mobilization in LLC-PK_1 cells, intracellular Ca^{2+} was measured (Fig. 3). At 10^{-7} M, ANG II induced a transient increase in cytosolic Ca^{2+} (Fig. 3, *A* and *C*). When the cells were pretreated with 10^{-9} M ANG II for 30 min before stimulation with 10^{-7} M, the increase was 10-fold higher, which was sustained for 3-fold longer than in the absence of prior preincubation with 10^{-9} M ANG II (Fig. 3, *B* and *D*).

ANG II is internalized by LLC-PK_1 cells via luminal membrane. A possible mechanism for ANG II-mediated SERCA activation is the internalization of the peptide (36, 47) or the formation of small ANG II-derived activating peptides (5, 6). To test the hypothesis that ANG II is internalized or partially metabolized, LLC-PK_1 cells were incubated with 8 μM ANG II for different times and the resulting supernatants were analyzed by HPLC (Fig. 4). Representative HPLC chromatograms show a time-dependent decrease of medium ANG II without the formation of metabolites.

ACE, ACE2, NEP, and AP are active in luminal membranes of LLC-PK_1 cells. Since there was a decrease in ANG II concentration in the medium associated with no formation of derived peptides, the activities of ACE, ACE2, NEP, and AP were measured (Fig. 5). The hydrolysis of each corresponding fluorescent substrates, Abz-FRK(Dnp)P for ACE (Fig. 5*A*), Phe-MCA for AP (Fig. 5*B*), MCA-rRL-EDDnp for NEP (Fig. 5*C*), and MCA-APK(Dnp)-OH for ACE2 (Fig. 5*D*), indicates the existence of active peptidases capable of hydrolyzing ANG II in the luminal membrane of LLC-PK_1 cells.

AT_1 and AT_2 receptors heterodimerize and are involved in ANG II effect on SERCA. To determine which receptor is involved in the ANG II effect on SERCA activity, LLC-PK_1 cells were treated with AT_1R and AT_2R antagonists (10^{-10} M

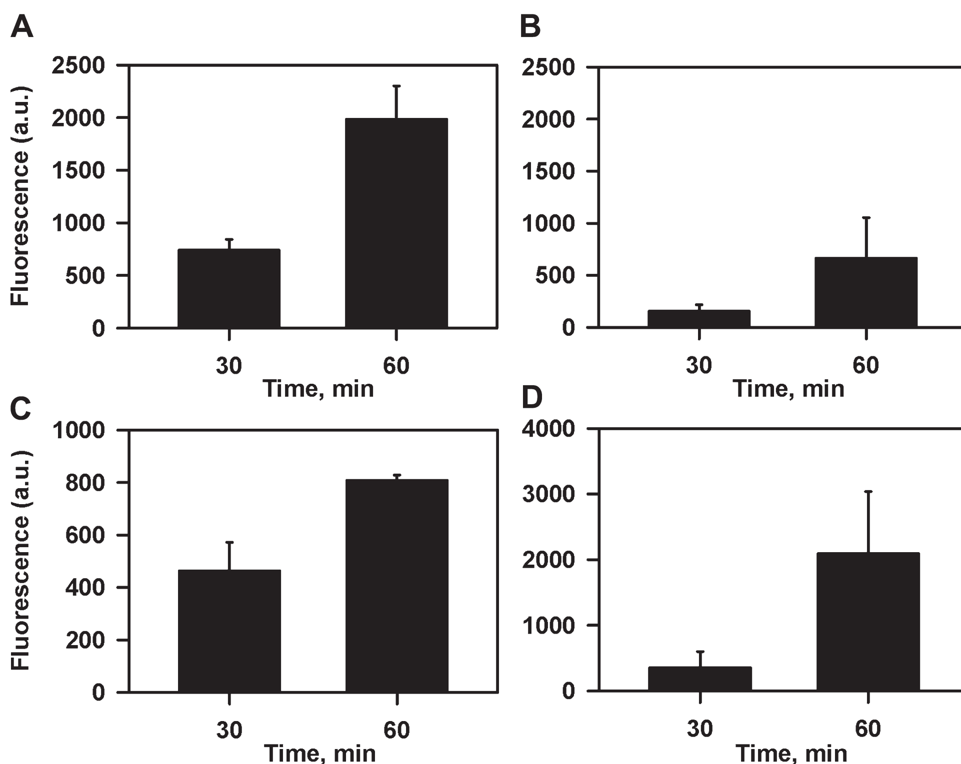

Fig. 5. Peptidase activities in the luminal-oriented side of LLC-PK_1 cells. The activities of angiotensin-converting enzyme (ACE; *A*), amino peptidases (AP; *B*), neprilysin (NEP; *C*), and ACE2 (*D*) were measured in the presence of their respective fluorescent substrates: 20 μM Abz-FRK(Dnp)P (for ACE), 46.5 μM Phe-MCA (for AP), 20 μM MCA-rRL-EDDnp (for NEP), and 40 μM MCA-APK(Dnp)-OH (for ACE2). The substrates and inhibitors were added to the medium bathing the 90% confluent cells and aliquots were removed at 30 and 60 min for the fluorimetric assays as described under METHODS. The obtained values are expressed in fluorescence arbitrary units and represent the difference between the hydrolysis in the absence and presence of their respective inhibitors: 1 μM lisinopril (for ACE), 10 μM bestatin (for AP), 100 nM thiorphan (for NEP), and 10 μM DX600 (for ACE2). Data bars indicate means ± SE of at least 2 determinations.

losartan and 10^{-7} M PD123319, respectively), before incubation with ANG II (Fig. 6). Both agents blocked the ANG II effect on SERCA. The two antagonists per se had no effect and, as expected from the previous results, PMCA was not modulated by any treatment (Fig. 6, *inset*). Thus, we investigated whether ANG II via luminal membranes led to AT_1R/AT_2R heterodimer formation that might explain why each antagonist alone blocked SERCA stimulation. Immunoprecipitation followed by Western blotting showed that the preexisting population of AT_1R/AT_2R heterodimers increased by 140% after exposure of LLC-PK$_1$ cells to 10^{-10} and 10^{-6} M ANG II (Fig. 7).

Signaling pathway stimulated by ANG II. The main signaling pathway stimulated by ANG II in renal cells is the PLCβ/PKC pathway (16, 39). To investigate whether these enzymes are involved in the stimulation of SERCA mediated by ANG II via luminal membranes of LLC-PK$_1$ cells, the PLC inhibitor U73122 and the PKC inhibitor calphostin C were added to the culture medium 10 min before exposure of cells to ANG II. U73122 and calphostin C blocked stimulation of SERCA activity by ANG II (Figs. 8 and 9). PMCA was unaffected by the inhibitors (Figs. 8, *inset*, and 9, *inset*). Furthermore, SERCA activity was stimulated by 10^{-7} and 10^{-6} M PMA (Fig. 10), mimicking to the same extent the ANG II effect, an observation that confirms the participation of PKC in the luminally triggered ANG II signaling pathway. PKC activity from LLC-PK$_1$ cells was measured in the lysate after exposure of intact cells to ANG II, giving a biphasic response (Fig. 11). Whereas 10^{-10} M ANG II significantly stimulated the basal PKC activity, there was a ~50% inhibition at 10^{-6} M.

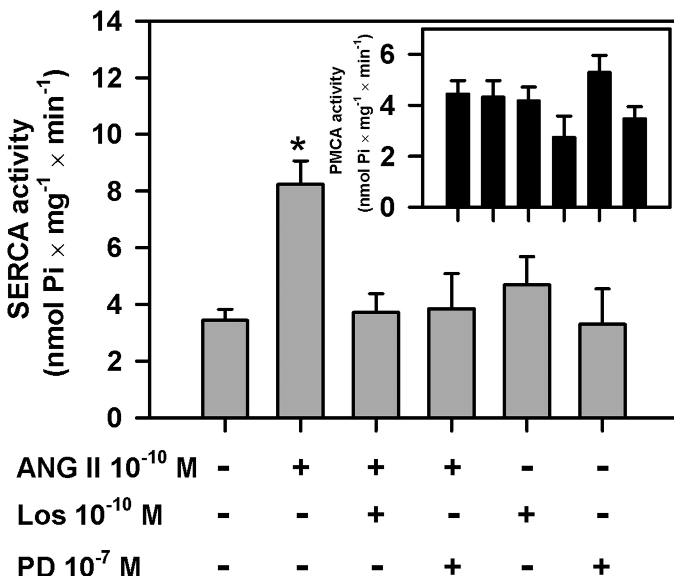

Fig. 6. ANG II effect on SERCA activity from LLC-PK$_1$ cells is mediated by AT_1 and AT_2 receptors. The luminal membranes of the cells were preincubated for 10 min with 10^{-10} M losartan (Los) or 10^{-7} M PD123319 (PD) receptor antagonists, before incubation with or without 10^{-10} M ANG II for 30 min in the combinations shown on the abscissa; then, Ca^{2+}-ATPase activity was determined. *Inset*: PMCA activity. Data bars indicate means ± SE of at least 4 determinations in triplicate using different cell lysate preparations. *Statistically different from control in the absence of ANG II and from treatment with ANG II + losartan or ANG II + PD123319 ($P < 0.05$).

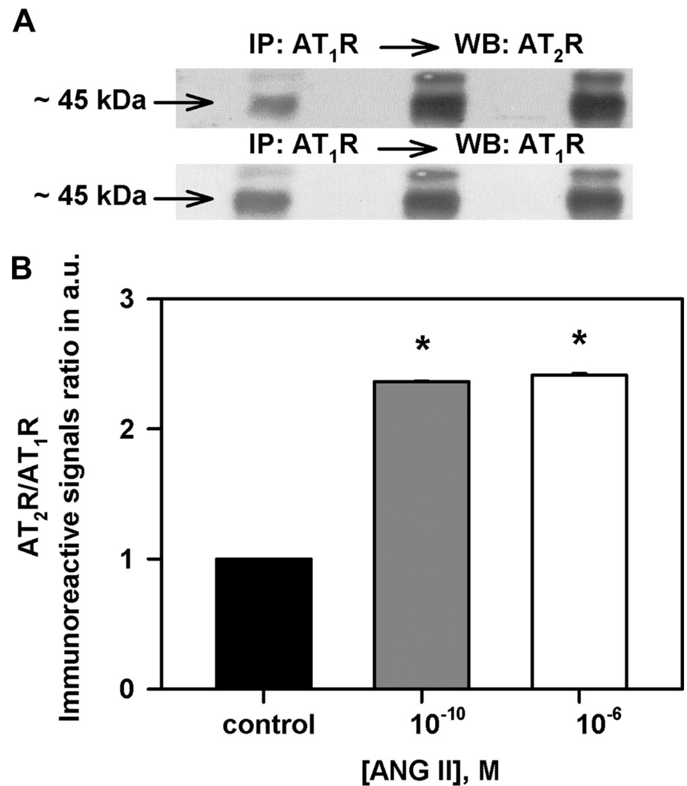

Fig. 7. ANG II promotes AT_1R/AT_2R heterodimers formation in LLC-PK$_1$ cells. *A*: cells were incubated with or without ANG II for 30 min. Cell lysates were immunoprecipitated with monoclonal anti-AT_1R antibody and subsequently submitted to Western blotting using a polyclonal anti-AT_2R antibody (IP: AT_1R → WB: AT_2R). After being stripped, membranes were probed with polyclonal anti-AT_1R antibody (IP: AT_1R → WB: AT_1R). For details, see METHODS. *B*: densitometric representation of the immunoreactive signals ratio for AT_2R and AT_1R after immunoprecipitation of AT_1R. Data bars indicate means ± SE using different cell lysate preparations. *Statistically different from the control without ANG II ($P < 0.05$).

DISCUSSION

We demonstrated that SERCA is the target Ca^{2+}-ATPase for luminal-oriented participation of ANG II in Ca^{2+} homeostasis in a lineage of proximal tubule cells. With LLC-PK$_1$ cells firmly and totally adherent to the wall of culture flasks or to coverslips inside culture plates by their basolateral surfaces (33), exposure to ANG II could only have been to their luminal surfaces. The main initial points to be considered are *1*) the response elicited by ANG II on intact cells was preserved after the 2-h period had elapsed from the end of incubation to the recovery of lysates, *2*) only the intracellular Ca^{2+}-ATPase was modulated by luminal-oriented ANG II, with no influence on the basolateral plasma membrane Ca^{2+}-ATPase, and *3*) the stimulus of SERCA observed in vitro with the use of lysates (Fig. 1*A*) has a counterpart in the ANG II-induced transient increase in cytosolic Ca^{2+} observed with intact cells (Fig. 3).

Even though LLC-PK$_1$ cell cultures are luminally oriented without access of the medium to the basolateral aspect (Fig. 1, *B–E*), they are not tubular structures. Despite this limitation, the experiments here described also clearly demonstrate that exposure of the luminal membrane of LLC-PK$_1$ cells to ANG II, at the physiological (nanomolar) concentrations described by Navar and Nishiyama (36) in renal tubules, promotes a

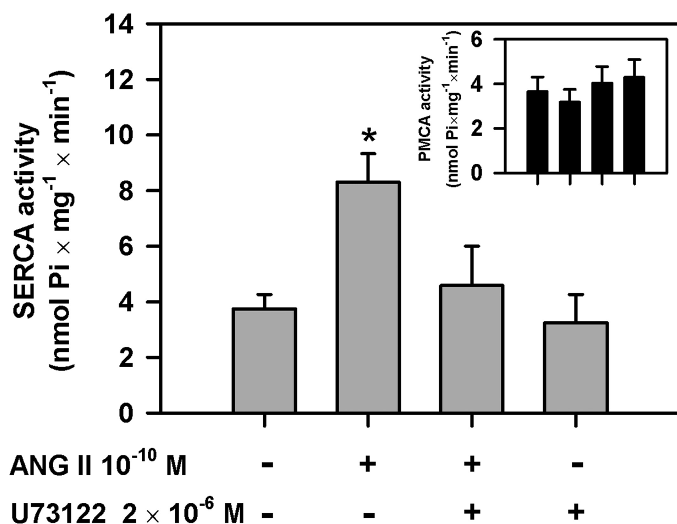

Fig. 8. Phospholipase C (PLC) is involved in the activation of SERCA by ANG II facing the luminal membrane. Cells were exposed to U73122 (2×10^{-6} M) for 10 min before addition of 10^{-10} M ANG II as indicated on the abscissa. Ca^{2+}-ATPase activities were measured after 30-min incubation. *Inset*: PMCA activity. For details, see METHODS. Data bars indicate means ± SE of at least 5 determinations in triplicate using different cell lysate preparations. *Statistically different from the control in absence of ANG II and from treatment with ANG II + U73122 ($P < 0.05$).

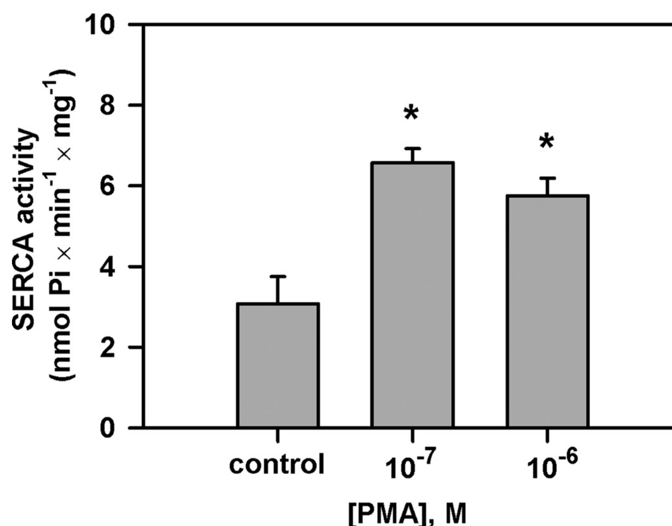

Fig. 10. PMA mimics stimulation of SERCA activity by ANG II. Cells were incubated with PMA (10^{-7} and 10^{-6} M) for 30 min and Ca^{2+}-ATPase activity was then determined. Data bars indicate means ± SE of at least 4 determinations in triplicate using different cell lysate preparations. *Statistically different from the control without PMA ($P < 0.05$).

rapid and persistent stimulus of SERCA activity within 30 s (Fig. 2). The earliness of the effect likely indicates a receptor-dependent Ca^{2+} release from endoplasmic reticulum (ER) rather than a Ca^{2+} leakage from the lumen of this organelle. The stimulus of SERCA activity, and of coupled Ca^{2+} transport toward the ER lumen, appears to follow a previous ANG II-induced Ca^{2+} release. Since SERCA is the ATPase that sustains Ca^{2+} stock within the reticulum (12), its fast stimulation by ANG II via luminal membrane may mean that SERCA activation increases the Ca^{2+} stock in the reticulum to ensure more efficient Ca^{2+} mobilization and, consequently, an enhanced cellular response facing a new stimulus of ANG II. This view is supported by measurements of intracellular Ca^{2+} mobilization (Fig. 3), which demonstrate that there is an increase of one order of magnitude in the peak of Ca^{2+} mobilization induced by 10^{-7} M ANG II after a pretreatment with 10^{-9} M ANG II (compare Fig. 3, *A* and *C*, with Fig. 3, *B* and *D*). Therefore, we propose that enhanced intracellular Ca^{2+} waves and spikes promoted by tubular ANG II fluctuations result from modulation of SERCA turnover and, therefore, of the Ca^{2+} uptake/release cycles capable of amplifying

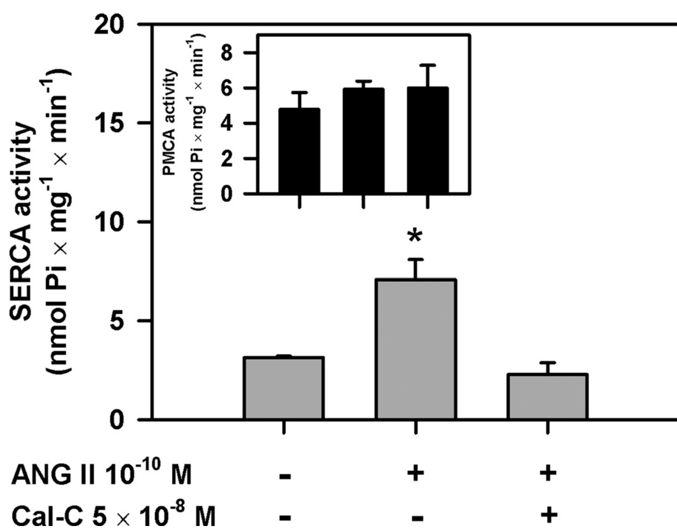

Fig. 9. Exposure of the luminal membrane to ANG II stimulates SERCA activity through a PKC signaling pathway. Cells were exposed to Calphostin C (Cal-C; 5×10^{-8} M) for 10 min, as shown. Then, 10^{-10} M ANG II was added and 30 min later Ca^{2+}-ATPase activity was measured. *Inset*: PMCA activity. For details, see METHODS. Data bars indicate means ± SE of at least 6 determinations in triplicate using different cell lysate preparations. *Statistically different from the control and from treatment with ANG II + Cal-C ($P < 0.05$).

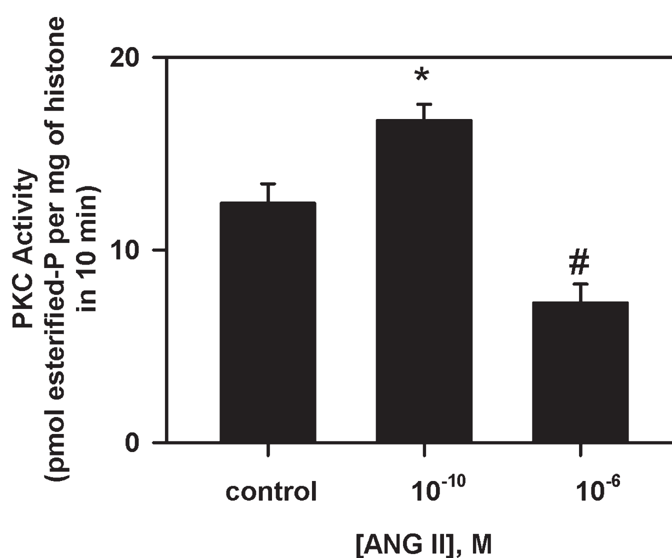

Fig. 11. Exposure of the luminal membrane to low concentration of ANG II stimulates PKC activity in LLC-PK$_1$ cells. ANG II (10^{-10} or 10^{-6} M) was added to the culture for 30 min and PKC activity was measured as described in METHODS. Data bars indicate means ± SE of at least 4 determinations in triplicate using different cell lysate preparations. *$P < 0.05$ with respect to control without ANG II. #$P < 0.05$ with respect to 10^{-10} M ANG II.

the efficiency of the regulatory responses triggered by ANG II. The positive amplification of ANG II Ca^{2+} mobilization that has been demonstrated seems to be an important way for luminal ANG II to regulate Ca^{2+} homeostasis in renal cells, and also Ca^{2+}-modulated fluid reabsorption in proximal tubules (17). Positive feedbacks involving tubular and interstitial ANG II have been proposed as key events in renal Na^+ (and fluid) balance (36).

The lack of a luminal effect of ANG II on PMCA activity (Figs. 1 and 2), combined with our previous studies showing inhibition of basolateral PMCA (4−6), demonstrates the sidedness of ANG II effects on Ca^{2+} homeostasis in proximal tubule cells, which appears to modulate Ca^{2+} oscillations through different pathways. Whereas basolateral ANG II increases cytosolic Ca^{2+} by inhibition of PMCA, the positively self-sustained luminal stimulus on SERCA provides a refined mechanism for further Ca^{2+} control. It is noteworthy that, despite their polarity, the ANG II effect on Ca^{2+} homeostasis in proximal tubule cells is biphasic concerning the agonist concentration (5, 6) (Fig. 1); however, the processes involved seem to be different. While counteracting effects on PMCA are associated with limited proteolysis of ANG II with formation of Ang-(3–4) (5), progressive suppression of luminal activation appears to be linked to internalization of the peptide because its time-dependent decrease (Fig. 4) in the "luminal compartment" occurs without detectable ANG II metabolites, despite the presence of different active peptidases (ACE, ACE2, NEP, and AP) in the luminal side of the LLC-PK_1 cells (Fig. 5). An important related observation is probably that the biphasic and dose-dependent ANG II regulation on both luminal and peritubular sides of proximal tubule has also been described with respect to proximal reabsorption of solutes and fluid (17, 21, 23, 43).

This work provides evidence that the activation of SERCA by luminal-oriented ANG II is sensitive to losartan and PD123319 (Fig. 6), and therefore ANG receptors are involved. This response is mediated by AT_1R/AT_2R heterodimers, a formation that is increased by $>100\%$, either at nano- or micromolar ANG II concentrations (Fig. 7). Previous results of our group showed that ANG II also acts via the AT_1R/AT_2R heterodimer to initiate the specific signaling pathway that culminates in inhibition of PMCA activity in basolateral membranes (5). This seems to be a more widespread and relevant phenomenon than previously thought. Dimerization may be a necessary prerequisite in the induction of cell responses mediated by G protein-coupled receptors (22, 31) involving ANG II and bradykinin (1−3, 31), although the functionality of ANG II receptor dimerization is not completely understood. After binding to luminal receptors, internalized ANG II could rapidly interact with its receptors in the ER (14) to stimulate SERCA activity. Thus, it seems plausible that AT_1R and AT_2R in the luminal and basolateral membranes of proximal tubule cells (44, 49) are functionally connected with those found in organelles (14, 52) to ensure the fine-tuning of cytosolic Ca^{2+}.

As in the case of inhibition of the basolateral PMCA (4, 5), ANG II also activates the U73122-sensitive PLC → DAG/PMA-stimulated → calphostin-inhibited PKC pathway (Figs. 8−11). However, in this case ANG II facing the luminal membrane leads to the stimulus of SERCA pumping activity. The experiments in Fig. 11 shed some light to the possible mechanism by which the basal SERCA activity is recovered at high ANG II concentrations (Fig. 1). Because heterodimer formation is identical at 10^{-10} and 10^{-6} M ANG II (Fig. 7), the downregulation of SERCA stimulus at 10^{-6} M ANG II occurs downstream of the receptors and may be due to a decrease in PKC activity (Fig. 11).

In conclusion, exposure of the luminal membrane to ANG II stimulates SERCA pumping activity in proximal tubule cells by a mechanism that involves AT_1R/AT_2R dimerization and activation of the PLC/PKC signaling pathway. These observations and those we previously reported demonstrate that ANG II can modulate the Ca^{2+} concentration in proximal tubule cells in a bidirectional manner from both the luminal and the basolateral side. Due to the relevance of Ca^{2+} oscillations in the control of fluid reabsorption, the concerted ANG II-mediated effects on SERCA and PMCA in proximal tubules emerge as a key feature in body fluid homeostasis.

ACKNOWLEDGMENTS

The technical support by Andreia Abrantes, Alexandre Abrantes, and Glória Costa-Sarmento is acknowledged. The manuscript has been corrected for its English presentation by BioMedES (UK).

GRANTS

This work was supported by grants from the Brazilian National Research Council (CNPq), Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), the Fundação de Amparo a Pesquisa do Estado de São Paulo, and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: F.M.F., L.S.L., A.K.C., C.M.C.-N., A.V., and J.L. conception and design of research; F.M.F., L.S.L., F.A., J.D., R.I.R., and J.L. performed experiments; F.M.F., L.S.L., F.A., J.D., A.K.C., R.I.R., C.M.C.-N., A.V., and J.L. analyzed data; F.M.F., L.S.L., F.A., J.D., A.K.C., R.I.R., C.M.C.-N., A.V., and J.L. interpreted results of experiments; F.M.F., L.S.L., F.A., J.D., R.I.R., A.V., and J.L. prepared figures; F.M.F., L.S.L., A.V., and J.L. drafted manuscript; F.M.F., L.S.L., A.K.C., R.I.R., C.M.C.-N., A.V., and J.L. edited and revised manuscript; F.M.F., L.S.L., F.A., J.D., A.K.C., R.I.R., C.M.C.-N., A.V., and J.L. approved final version of manuscript.

REFERENCES

1. **Abadir PM, Periasamy A, Carey RM, Siragy HM.** Angiotensin II type 2 receptor-bradykinin B_2 receptor functional heterodimerization. *Hypertension* 48: 316–322, 2006.
2. **AbdAlla S, Lother H, Quitterer U.** AT_1 receptor heterodimers show enhanced Gprotein activation and altered receptor sequestration. *Nature* 407: 94–98, 2000.
3. **AbdAlla S, Lother H, Abdel-tawab AM, Quitterer U.** The angiotensin AT_2 receptor is an AT_1 receptor antagonist. *J Biol Chem* 276: 39721–39726, 2001.
4. **Assunção-Miranda I, Guilherme AL, Reis-Silva C, Costa-Sarmento G, Oliveira MM, Vieyra A.** Protein kinase C-mediated inhibition of renal Ca^{2+} ATPase by physiological concentrations of angiotensin II is reversed by AT_1- and AT_2-receptor antagonists. *Regul Pept* 127: 151–157, 2005.
5. **Axelband F, Assunção-Miranda I, de Paula IR, Ferrão FM, Dias J, Miranda A, Miranda F, Lara LS, Vieyra A.** Ang-(3–4) suppresses inhibition of renal plasma membrane calcium pump by Ang II. *Regul Pept* 155: 81–90, 2009.
6. **Axelband F, Dias J, Miranda F, Ferrão FM, Barros NM, Carmona AK, Lara LS, Vieyra A.** A scrutiny of the biochemical pathways from Ang II to Ang-(3–4) in renal basolateral membranes. *Regul Pept* 158: 47–56, 2009.
7. **Bader M, Peters J, Baltatu O, Muller DN, Luft FC, Ganten D.** Tissue rennin-angiotensin systems: new insights from experimental animal models in hypertension research. *J Mol Med* 79: 76–102, 2001.

8. **Bernstein KE, Sayeski PP, Doan T, Farmer PK, Ali MS.** Signal transduction pathways of angiotensin II in the kidney. *Contrib Nephrol* 135: 16–33, 2001.
9. **Braam B, Mitchell KD, Fox J, Navar LG.** Proximal tubular secretion of angiotensin II in rats. *Am J Physiol Renal Fluid Electrolyte Physiol* 264: F891–F898, 1993.
10. **Bruneval P, Hinglais N, Alhenc-Gelas F, Tricottet V, Corvol P, Menard J, Camilleri JP, Bariety J.** Angiotensin I converting enzyme in human intestine and kidney. Ultra-structural and imunohistochemical localization. *Histochemistry* 85: 73–80, 1986.
11. **Cabral LM, Wengert M, da Ressurreição AA, Feres-Elias PH, Almeida FG, Vieyra A, Caruso-Neves C, Einicker-Lamas M.** Ceramide is a potent activator of plasma membrane Ca^{2+}-ATPase from kidney-promixal tubule cells with protein kinase A as an intermediate. *J Biol Chem* 282: 24599–24606, 2007.
12. **Carafoli E.** Intracellular calcium homeostasis. *Annu Rev Biochem* 56: 395–433, 1987.
13. **Charbonneau A, Leclerc M, Brunette MG.** Effect of angiotensin II on calcium reabsorption by the luminal membranes of the nephron. *Am J Physiol Endocrinol Metab* 280: E928–E936, 2001.
14. **Cook JL, Re R, Alam J, Hart M, Zhang Z.** Intracellular angiotensin II fusion protein alters AT_1 receptor fusion protein distribution and activates CREB. *J Mol Cell Cardiol* 36: 75–90, 2004.
15. **Darby IA, Sernia C.** In situ hybridization and immunohistochemistry of renal angiotensinogen in neonatal and adult rat kidneys. *Cell Tissue Res* 281: 197–206, 1995.
16. **Dinh DT, Frauman AG, Johnston CI, Fabiani ME.** Angiotensin receptors: distribution, signalling and function. *Clin Sci (Lond)* 100: 481–492, 2001.
17. **Du Z, Ferguson W, Wang T.** Role of PKC and calcium in modulation of effects of angiotensin II on sodium transport in proximal tubule. *Am J Physiol Renal Physiol* 284: F688–F692, 2003.
18. **Engeli S, Negrel R, Sharma AM.** Physiology and pathophysiology of the adipose tissue renin-angiotensin system. *Hypertension* 35: 1270–1277, 2000.
19. **Féraille E, Doucet A.** Sodium-potassium-adenosinetriphosphatase-depedent. Sodium transport in the kidney: hormonal control. *Physiol Rev* 81: 345–417, 2001.
20. **Gomez RA, Lynch KR, Chevalier RL, Wilfong N, Everett A, Carey RM, Peach MJ.** Renin and angiotensinogen gene expression in the maturing rat kidney. *Am J Physiol Renal Fluid Electrolyte Physiol* 254: F582–F587, 1988.
21. **Harris PJ, Hiranyachattada S, Antoine AM, Walker L, Reilly AM, Eitle E.** Regulation of renal tubular sodium transport by angiotensin II and atrial natriuretic factor. *Clin Exp Pharmacol Physiol Suppl* 3: S112–S118, 1996.
22. **Hebert TE, Moffett S, Morello JP, Loisel TP, Bichet DG, Barret C, Bouvier M.** A peptide derived from a β_2-adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation. *J Biol Chem* 271: 16384–16392, 1996.
23. **Hiranyachattada S, Harris PJ.** Regulation of renal proximal fluid uptake by luminal and peritubular angiotensin II. *J Renin Angiotensin Aldosterone Syst* 5: 89–92, 2004.
24. **Ichihara A, Kobori H, Nishiyama A, Navar LG.** Renal renin-angiotensin system. *Contrib Nephrol* 143: 117–130, 2004.
25. **Ingelfinger J, Zuo WM, Fon EA, Ellison KE, Dzau VJ.** In situ hybridization evidence for angiotensinogen messenger RNA in the rat proximal tubule. *J Clin Invest* 85: 417–423, 1990.
26. **Kimbrough HM Jr, Vaughan ED Jr, Carey RM, Ayers CR.** Effect of intrarenal angiotensin II on renal function in conscious dogs. *Circ Res* 40: 174–178, 1977.
27. **Lalouel JM, Rohrwasser A, Terreros D, Morgan T, Ward K.** Angiotensinogen in essential hypertension: from genetics to nephrology. *J Am Soc Nephrol* 12: 606–615, 2001.
28. **Levens NR, Freedlender AE, Peach MJ, Carey RM.** Control of renal function by intrarenal angiotensin II. *Endocrinology* 112: 43–49, 1983.
29. **Lowry OH, Rosebrough NJ, Farr AL, Randall RJ.** Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265–275, 1951.
30. **Maia JCC, Gomes SL, Juliani MH.** Preparation of (γ-^{32}P) and (α-^{32}P)-nucleoside triphosphates with high specific activity. In: *Genes and Antigenes of Parasites, A Laboratory Manual Proceedings*. Rio de Janeiro, Brazil: Editora Fundação Oswaldo Cruz, 1983, p. 146–147.
31. **Miura S, Matsuo Y, Kiya Y, Karnik SS, Saku K.** Molecular mechanisms of the antagonistic action between AT_1 and AT_2 receptors. *Biochem Biophys Res Commun* 391: 85–90, 2010.
32. **Miyata N, Park F, Li XF, Cowley AW Jr.** Distribution of angiotensin AT_1 and AT_2 receptor subtypes in the rat kidney. *Am J Physiol Renal Physiol* 277: F437–F446, 1999.
33. **Montrose MH, Friedrich T, Murer H.** Measurements of intracellular pH in single LLC-PK_1 cells: recovery from an acid load via basolateral Na^+/H^+ exchange. *J Membr Biol* 97: 63–78, 1987.
34. **Morimoto S, Sigmund CD.** Angiotensin mutant mice: a focus on the brain renin-angiotensin system. *Neuropeptides* 36: 194–200, 2002.
35. **Navar LG, Imig JD, Zou L, Wang CT.** Intrarenal production of angiotensin II. *Semin Nephrol* 17: 412–422, 1997.
36. **Navar LG, Nishiyama A.** Why are angiotensin concentrations so high in the kidney? *Curr Opin Nephrol Hypertens* 13: 107–115, 2004.
37. **Navar LG, Lewis L, Hymel A, Braam B, Mitchell KD.** Tubular fluid concentrations and kidney contents of angiotensins I and II in anesthetized rats. *J Am Soc Nephrol* 5: 1153–1158, 1994.
38. **Parys JB, De Smedt H, Borghgraef R.** Calcium transport systems in the LLC-PK_1 renal epithelial established cell line. *Biochim Biophys Acta* 888: 70–81, 1986.
39. **Rangel LB, Caruso-Neves C, Lara LS, Lopes AG.** Angiotensin II stimulates renal proximal tubule Na^+-ATPase activity through the activation of protein kinase C. *Biochim Biophys Acta* 1564: 310–316, 2002.
40. **Rohrwasser A, Morgan T, Dillon HF, Zhao L, Callaway CW, Hillas E, Zhang S, Cheng T, Inagami T, Ward K, Terreros DA, Lalouel JM.** Elements of a paracrine tubular renin-angiotensin system along the entire nephron. *Hypertension* 34: 1265–1274, 1999.
41. **Sabatini RA, Bersanetti PA, Farias SL, Juliano L, Juliano MA, Casarini DE, Carmona AK, Paiva AC, Pesquero JB.** Determination of angiotensin I-converting enzyme activity in cell culture using fluorescence resonance energy transfer peptides. *Anal Biochem* 363: 255–262, 2007.
42. **Schelling JR, Singh H, Marzec R, Linas SL.** Angiotensin II dependent proximal tubule sodium transport is mediated by cAMP modulation of phospholipase C. *Am J Physiol Cell Physiol* 267: C1239–C1245, 1994.
43. **Seikaly MG, Arant BS Jr, Seney FD Jr.** Endogenous angiotensin concentrations in specific intrarenal fluid compartments of the rat. *J Clin Invest* 86: 1352–1357, 1990.
44. **Sharma M, Sharma R, Greene AS, McCarthy ET, Savin VJ.** Documentation of angiotensin II receptors in glomerular epithelial cells. *Am J Physiol Renal Physiol* 274: F623–F627, 1998.
45. **Sorenson MM, Coelho HS, Reuben JP.** Caffeine inhibition of calcium accumulation by the sarcoplasmic reticulum in mammalian skinned fibers. *J Membr Biol* 90: 219–230, 1986.
46. **Taussky HH, Shorr E.** A microcolorimetric method for the determination of inorganic phosphorus. *J Biol Chem* 202: 675–682, 1953.
47. **Thekkumkara T, Linas SL.** Role of internalization in AT_{1A} receptor function in proximal tubule epithelium. *Am J Physiol Renal Physiol* 282: F623–F629, 2002.
48. **Tinel H, Kinne-Saffran E, Kinne RK.** Calcium signalling during RVD of kidney cells. *Cell Physiol Biochem* 10: 297–302, 2000.
49. **Wang ZQ, Millatt LJ, Heiderstadt NT, Siragy HM, Johns RA, Carey RM.** Differential regulation of renal angiotensin subtype AT_{1A} and AT_2 receptor protein in rats with angiotensin-dependent hypertension. *Hypertension* 33: 96–101, 1999.
50. **Yanagawa N, Capparelli AW, Jo OD, Friedal A, Barrett JD, Eggena P.** Production of angiotensinogen and renin-like activity by rabbit proximal tubular cells in culture. *Kidney Int* 39: 938–941, 1991.
51. **Yucha C, Guthrie D.** Renal homeostasis of calcium. *Nephrol Nurs J* 30: 622–628, 2003.
52. **Zhuo JL, Li XC, Garvin JL, Navar LG, Carretero OA.** Intracellular ANG II induces cytosolic Ca^{2+} mobilization by stimulating intracellular AT_1 receptors in proximal tubule cells. *Am J Physiol Renal Physiol* 290: F1382–F1390, 2006.