

## Exposure of luminal membranes of LLC-PK<sub>1</sub> cells to ANG II induces dimerization of AT<sub>1</sub>/AT<sub>2</sub> receptors to activate SERCA and to promote Ca<sup>2+</sup> mobilization

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**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<sub>1</sub> cells to ANG II induces dimerization of AT<sub>1</sub>/AT<sub>2</sub> receptors to activate SERCA and to promote Ca<sup>2+</sup> 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<sub>1</sub> cells on Ca<sup>2+</sup>-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<sup>2+</sup> mobilization. Pretreatment with ANG II for 30 min enhanced the ANG II-induced Ca<sup>2+</sup> spark, demonstrating a positively self-sustained stimulus of Ca<sup>2+</sup> 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<sub>1</sub> and AT<sub>2</sub> receptors by 140%, and either losartan or PD12319 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<sup>2+</sup> stock in the reticulum to ensure a more efficient subsequent mobilization of Ca<sup>2+</sup>. This first report on the regulation of SERCA activity by ANG II shows a new mechanism for Ca<sup>2+</sup> homeostasis in renal cells and also for regulation of Ca<sup>2+</sup>-modulated fluid reabsorption in proximal tubules.

luminal effect of ANG II; Ca<sup>2+</sup> sparks; proximal tubule Ca<sup>2+</sup> 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<sup>2+</sup> 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<sup>2+</sup> is filtered by the kidneys, 99% of which is reabsorbed (70% of it in the proximal tubules) (19). ANG II regulates Ca<sup>2+</sup> reabsorption in both luminal and basolateral aspects of tubule membranes (4, 5, 13). In proximal tubule cells, intracellular Ca<sup>2+</sup> mobilization activates Ca<sup>2+</sup>-dependent intracellular signaling pathways, including those associated with ANG II-modulated Na<sup>+</sup> and water reabsorption mechanisms (8, 16, 17, 39, 42).

Active and passive transport mechanisms participate in Ca<sup>2+</sup> reabsorption in the proximal tubules (19, 48). Concerning primary active transporters, there are two main Ca<sup>2+</sup>-ATPase subfamilies in proximal tubule cells, the sarco(endo)plasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA) and the plasma membrane Ca<sup>2+</sup>-ATPase (PMCA) (38). These pumps control intracellular Ca<sup>2+</sup> concentration by regulating intracellular Ca<sup>2+</sup> stocks, fine-tuning cytosolic Ca<sup>2+</sup> 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<sub>1</sub>R/AT<sub>2</sub>R 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<sub>1</sub>R and AT<sub>2</sub>R 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).

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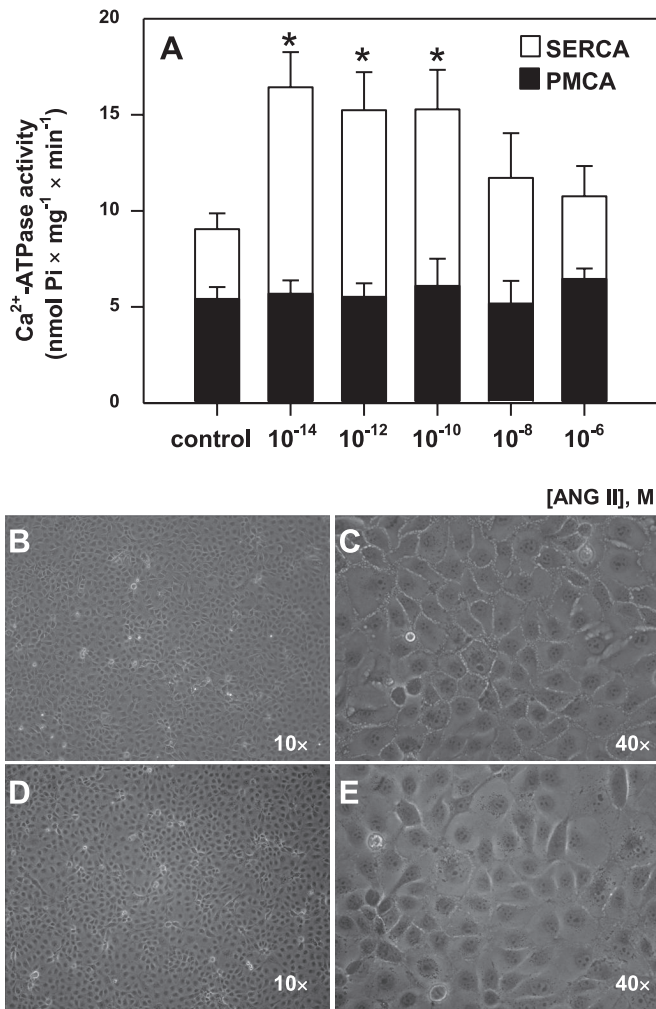


Fig. 1. A: ANG II effect via luminal membrane on calcium pumps in LLC-PK<sub>1</sub> cells. ANG II stimulates SERCA activity (top open bars) and does not modulate PMCA activity (bottom filled bars). Cells were incubated with different ANG II concentrations ( $10^{-14}$  to  $10^{-6}$  M) for 30 min and  $\text{Ca}^{2+}$ -ATPase activities were then measured. For details, see METHODS. Data bars indicate means  $\pm$  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<sub>1</sub> 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  $\text{Ca}^{2+}$  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<sub>1</sub> cells). We found that AT<sub>1</sub>R/AT<sub>2</sub>R 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  $\mu\text{g}/\text{ml}$ ), 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.  $^{32}\text{P}_i$  was obtained from the Brazilian Institute of Energy and Nuclear Research (IPEN, São Paulo, Brazil). Labeled [ $\gamma$ - $^{32}\text{P}$ ]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<sub>1</sub>R, and anti-AT<sub>2</sub>R antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Losartan was obtained from Merck Sharp & Dohme (Whitehouse Station, NJ). LLC-PK<sub>1</sub> 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<sub>1</sub> cells were grown in low-glucose DMEM (10% FBS) with antibiotic supplements in a humidified atmosphere of 5%  $\text{CO}_2$  in air at 37°C. Approximately  $5 \times 10^5$  cells were seeded in 25-cm<sup>2</sup> 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-Elvehjem 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  $\text{Ca}^{2+}$ -ATPase and PKC activities, and also for Western blotting analysis of AT<sub>1</sub>R and AT<sub>2</sub>R after immunoprecipitation with specific antibodies.

**Measurements of  $\text{Ca}^{2+}$ -ATPase activities (SERCA and PMCA).**  $\text{Ca}^{2+}$ -ATPase activity was measured by colorimetric quantification of inorganic phosphate ( $\text{P}_i$ ) released from ATP (46). Total  $\text{Ca}^{2+}$ -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  $\mu\text{M}$  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

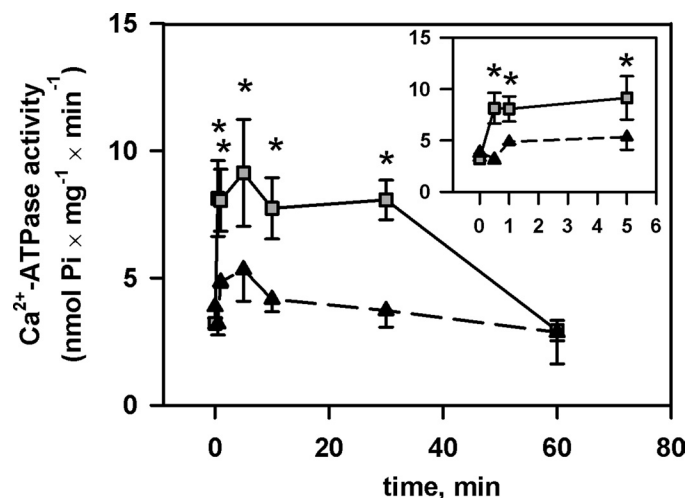


Fig. 2. Time course of ANG II effect on SERCA (□) and PMCA (▲) activities. The luminal membranes of LLC-PK<sub>1</sub> cells were exposed to  $10^{-10}$  M ANG II during the times indicated on the abscissa before  $\text{Ca}^{2+}$ -ATPase activity was determined. For details, see METHODS. Inset: ANG II effect SERCA (□) and PMCA (▲) activities during the first 5 min for a better visualization. Data points indicate means  $\pm$  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<sub>2</sub>, 10 mM NaN<sub>3</sub>, 120 mM KCl, 0.1 mM ouabain, 0.2 mM EGTA, and 7.5 μM free Ca<sup>2+</sup>. The total CaCl<sub>2</sub> needed for the desired free Ca<sup>2+</sup> 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<sub>i</sub> 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-<sup>32</sup>P]ATP into histone H8, which was used as substrate as described elsewhere (11). About 5 × 10<sup>5</sup> cells were incubated with different ANG II concentrations for 30 min, in the absence or presence of 5 × 10<sup>-8</sup> 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<sub>2</sub>, 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-<sup>32</sup>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-<sup>32</sup>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<sub>1</sub>R/AT<sub>2</sub>R heterodimers detection.** Immunoprecipitation and Western blotting analysis were performed as previously described (5), with slight modifications. LLC-PK<sub>1</sub> cells were incubated with different ANG II con-

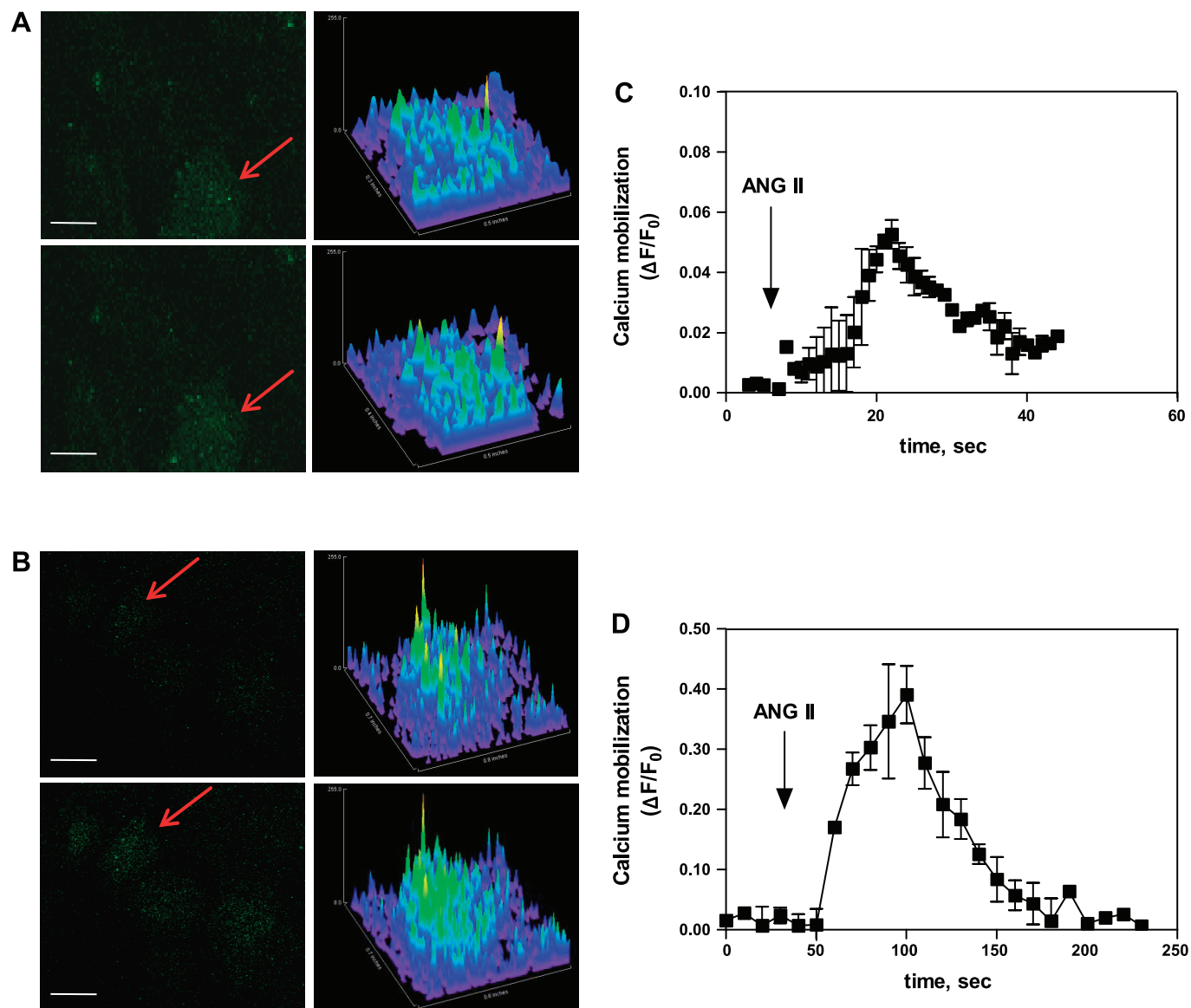


Fig. 3. Prior exposure of the luminal membrane of LLC-PK<sub>1</sub> cells to ANG II potentiates the ANG II-induced Ca<sup>2+</sup> mobilization from intracellular stores. Increases in cytosolic Ca<sup>2+</sup> were measured by confocal microscopy using the Fluo-3/AM indicator as described in METHODS. *A*: Ca<sup>2+</sup> 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<sup>-7</sup> M ANG II. *B*: same experiments as in *A*, except that the cells were preincubated with 10<sup>-9</sup> M ANG II before stimulation with 10<sup>-7</sup> M ANG II. *C* and *D*: averaged data (*n* = 6) for the Ca<sup>2+</sup> 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<sub>1</sub>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<sub>2</sub>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<sub>1</sub>R using a rabbit polyclonal antibody. The 45-kDa band in the immunoprecipitates detected with the AT<sub>1</sub>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<sub>2</sub>R/AT<sub>1</sub>R immunosignal ratio in the same gel indicated the relative amount of AT<sub>2</sub>R/AT<sub>1</sub>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<sub>1</sub> 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<sub>2</sub> in air at 37°C. The supernatants were subsequently analyzed by high-performance liquid chromatography (HPLC) as previously described (5).

**Measurement of intracellular Ca<sup>2+</sup> mobilization.** To assess cytosolic Ca<sup>2+</sup> mobilization, LLC-PK<sub>1</sub> cells were loaded with Fluo-3/AM (1 μM) at 37°C in a humidified incubator with 5% CO<sub>2</sub> in air for 30 min in the absence or presence of 10<sup>-9</sup> M ANG II. Cells were imaged in buffer (135 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM MgCl<sub>2</sub>, 2 mM glucose, 2 mM CaCl<sub>2</sub>; 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<sup>-7</sup> 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<sub>1</sub> cells cultures.** The activities of ACE, ACE2, neprilysin (NEP), and amino peptidases (AP) were measured using fluorescent substrates containing the fluorogenic group 4-methylcoumaryl-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<sub>1</sub> cells were plated in six-well plates at  $2 \times 10^5$  cells per well for 3 days in low-glucose DMEM (10% FBS) with antibiotic supplements in a humidified atmosphere of 5% CO<sub>2</sub> 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<sub>2</sub>, 0.63 mM MgSO<sub>4</sub>, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, and 6.1 mM glucose; pH 7.4 adjusted with HCl) containing 10 μM ZnCl<sub>2</sub>.

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),  $\lambda_{ex}$  and  $\lambda_{em}$  were 320 and 420 nm, respectively. For Phe-MCA (AP),  $\lambda_{ex}$  and  $\lambda_{em}$  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.

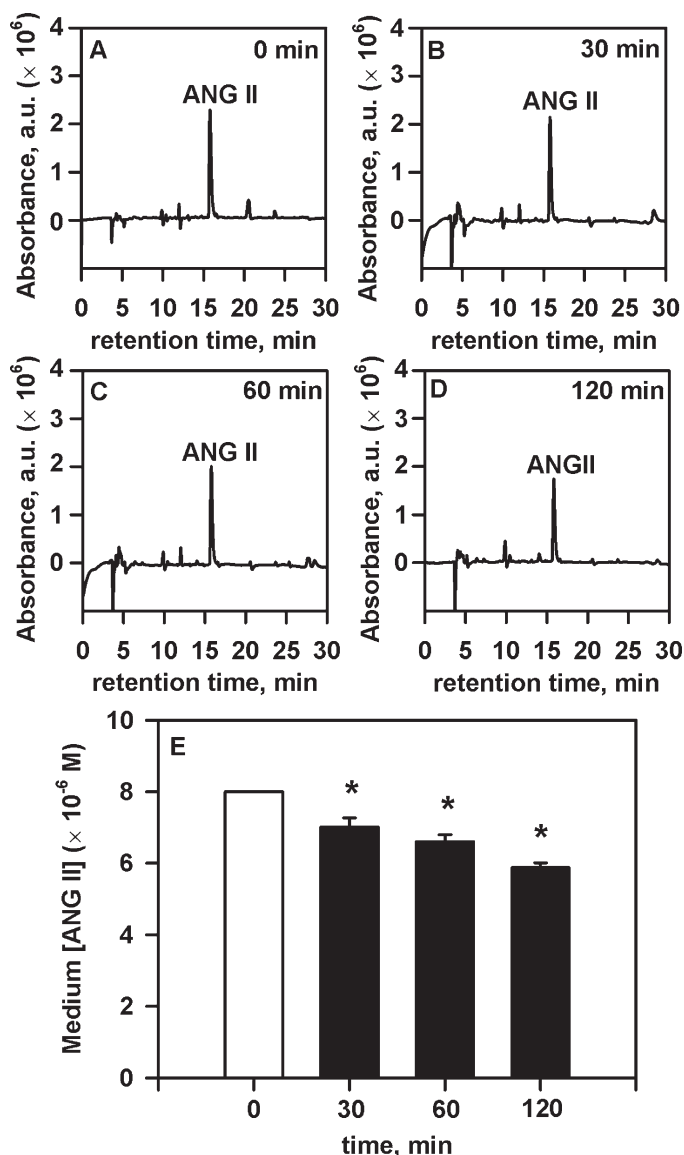


Fig. 4. ANG II is internalized by the luminal membrane of LLC-PK<sub>1</sub> cells. Cells firmly attached by their basolateral aspect (33) were exposed to 8 μM ANG II for different times and the medium was carefully collected and analyzed by high-performance liquid chromatography (HPLC), as described 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 1A shows SERCA and PMCA activities in membranes isolated 30 min after exposure of intact LLC-PK<sub>1</sub> 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  $\geq 100\%$ , while higher concentrations ( $10^{-8}$  and  $10^{-6}$  M) had no effect on pump catalysis (open bars in Fig. 1A). PMCA was unaffected by ANG II over the entire range of concentrations (filled bars in Fig. 1A).

Figure 1, B and C, shows the bright field microscopy of LLC-PK<sub>1</sub> cells at 90% of confluence, which was the experimental condition used in this study. It can be seen in a different field of view ( $\times 10$  and  $\times 40$ ) 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<sub>1</sub> 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<sub>1</sub> 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<sup>2+</sup> mobilization in LLC-PK<sub>1</sub> cells.* To investigate whether ANG II induces Ca<sup>2+</sup> mobilization in LLC-PK<sub>1</sub> cells, intracellular Ca<sup>2+</sup> was measured (Fig. 3). At  $10^{-7}$  M, ANG II induced a transient increase in cytosolic Ca<sup>2+</sup> (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<sub>1</sub> 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<sub>1</sub> cells were incubated with 8  $\mu$ M 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<sub>1</sub> 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. 5A), Phe-MCA for AP (Fig. 5B), MCA-rRL-EDDnp for NEP (Fig. 5C), and MCA-APK(Dnp)-OH for ACE2 (Fig. 5D), indicates the existence of active peptidases capable of hydrolyzing ANG II in the luminal membrane of LLC-PK<sub>1</sub> cells.

*AT<sub>1</sub> and AT<sub>2</sub> 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<sub>1</sub> cells were treated with AT<sub>1</sub>R and AT<sub>2</sub>R antagonists ( $10^{-10}$  M

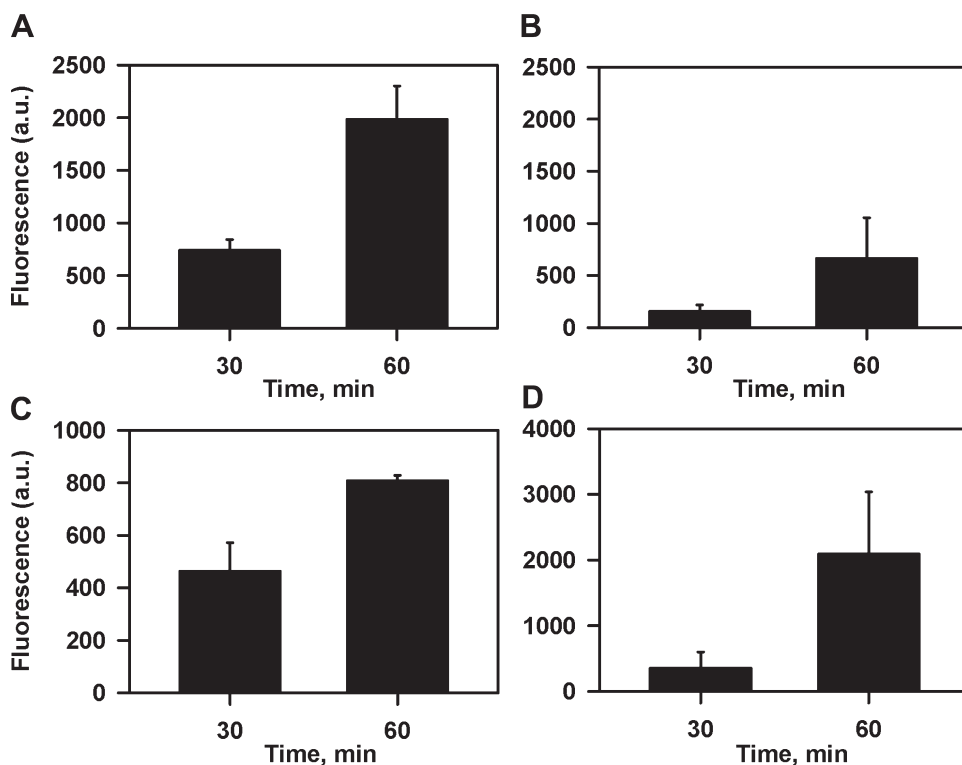


Fig. 5. Peptidase activities in the luminal-oriented side of LLC-PK<sub>1</sub> cells. The activities of angiotensin-converting enzyme (ACE; A), amino peptidases (AP; B), neprilysin (NEP; C), and ACE2 (D) were measured in the presence of their respective fluorescent substrates: 20  $\mu$ M Abz-FRK(Dnp)P (for ACE), 46.5  $\mu$ M Phe-MCA (for AP), 20  $\mu$ M MCA-rRL-EDDnp (for NEP), and 40  $\mu$ M 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  $\mu$ M lisinopril (for ACE), 10  $\mu$ M bestatin (for AP), 100 nM thiorphan (for NEP), and 10  $\mu$ M DX600 (for ACE2). Data bars indicate means  $\pm$  SE of at least 2 determinations.

losartan and 10<sup>-7</sup> 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<sub>1</sub>R/AT<sub>2</sub>R heterodimer formation that might explain why each antagonist alone blocked SERCA stimulation. Immunoprecipitation followed by Western blotting showed that the preexisting population of AT<sub>1</sub>R/AT<sub>2</sub>R heterodimers increased by 140% after exposure of LLC-PK<sub>1</sub> cells to 10<sup>-10</sup> and 10<sup>-6</sup> 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<sub>1</sub> 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<sup>-7</sup> and 10<sup>-6</sup> 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<sub>1</sub> cells was measured in the lysate after exposure of intact cells to ANG II, giving a biphasic response (Fig. 11). Whereas 10<sup>-10</sup> M ANG II significantly stimulated the basal PKC activity, there was a ~50% inhibition at 10<sup>-6</sup> M.

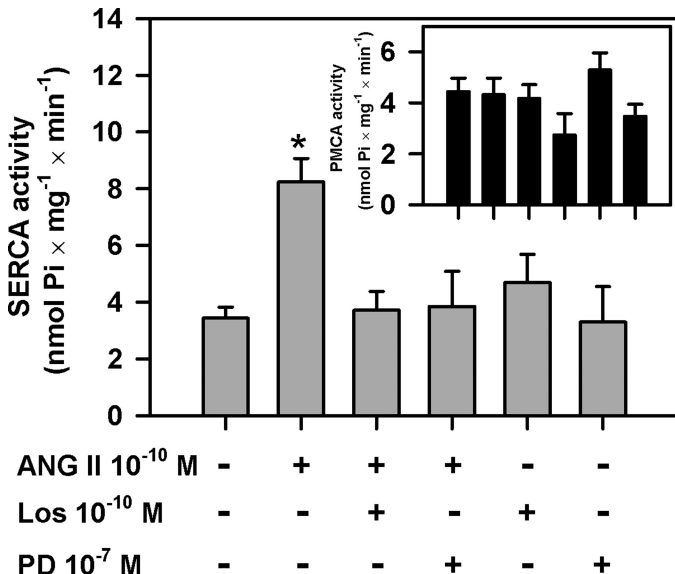


Fig. 6. ANG II effect on SERCA activity from LLC-PK<sub>1</sub> cells is mediated by AT<sub>1</sub> and AT<sub>2</sub> receptors. The luminal membranes of the cells were preincubated for 10 min with 10<sup>-10</sup> M losartan (Los) or 10<sup>-7</sup> M PD123319 (PD) receptor antagonists, before incubation with or without 10<sup>-10</sup> M ANG II for 30 min in the combinations shown on the abscissa; then, Ca<sup>2+</sup>-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).

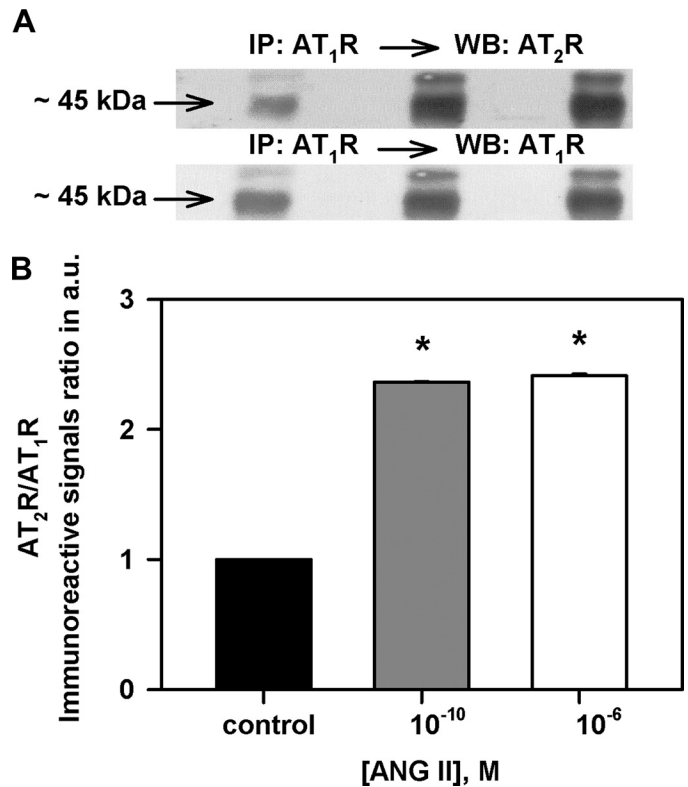


Fig. 7. ANG II promotes AT<sub>1</sub>R/AT<sub>2</sub>R heterodimers formation in LLC-PK<sub>1</sub> cells. **A**: cells were incubated with or without ANG II for 30 min. Cell lysates were immunoprecipitated with monoclonal anti-AT<sub>1</sub>R antibody and subsequently submitted to Western blotting using a polyclonal anti-AT<sub>2</sub>R antibody (IP: AT<sub>1</sub>R → WB: AT<sub>2</sub>R). After being stripped, membranes were probed with polyclonal anti-AT<sub>1</sub>R antibody (IP: AT<sub>1</sub>R → WB: AT<sub>1</sub>R). For details, see METHODS. **B**: densitometric representation of the immunoreactive signals ratio for AT<sub>2</sub>R and AT<sub>1</sub>R after immunoprecipitation of AT<sub>1</sub>R. 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<sup>2+</sup>-ATPase for luminal-oriented participation of ANG II in Ca<sup>2+</sup> homeostasis in a lineage of proximal tubule cells. With LLC-PK<sub>1</sub> 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<sup>2+</sup>-ATPase was modulated by luminal-oriented ANG II, with no influence on the basolateral plasma membrane Ca<sup>2+</sup>-ATPase, and 3) the stimulus of SERCA observed *in vitro* with the use of lysates (Fig. 1A) has a counterpart in the ANG II-induced transient increase in cytosolic Ca<sup>2+</sup> observed with intact cells (Fig. 3).

Even though LLC-PK<sub>1</sub> 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<sub>1</sub> cells to ANG II, at the physiological (nanomolar) concentrations described by Navar and Nishiyama (36) in renal tubules, promotes a

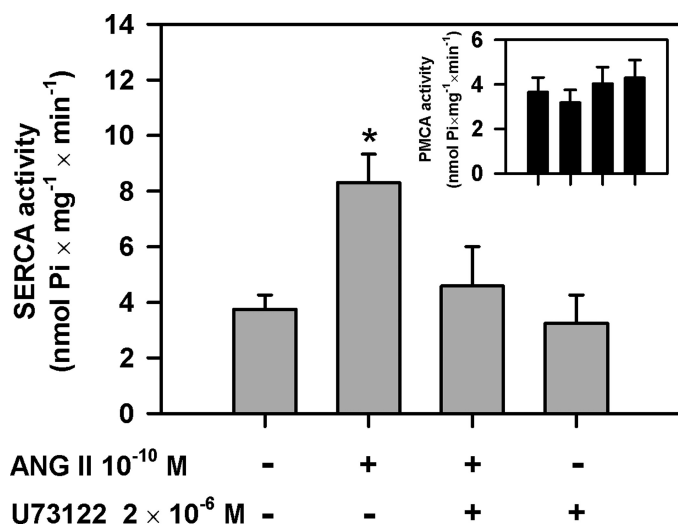


Fig. 8. Phospholipase C (PLC) is involved in the activation of SERCA by ANG II facing the luminal membrane. Cells were exposed to U73122 ( $2 \times 10^{-6}$  M) for 10 min before addition of  $10^{-10}$  M ANG II as indicated on the abscissa.  $\text{Ca}^{2+}$ -ATPase activities were measured after 30-min incubation. *Inset*: PMCA activity. For details, see METHODS. Data bars indicate means  $\pm$  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 ( $P < 0.05$ ).

rapid and persistent stimulus of SERCA activity within 30 s (Fig. 2). The earliness of the effect likely indicates a receptor-dependent  $\text{Ca}^{2+}$  release from endoplasmic reticulum (ER) rather than a  $\text{Ca}^{2+}$  leakage from the lumen of this organelle. The stimulus of SERCA activity, and of coupled  $\text{Ca}^{2+}$  transport toward the ER lumen, appears to follow a previous ANG II-induced  $\text{Ca}^{2+}$  release. Since SERCA is the ATPase that sustains  $\text{Ca}^{2+}$  stock within the reticulum (12), its fast stimulation by ANG II via luminal membrane may mean that

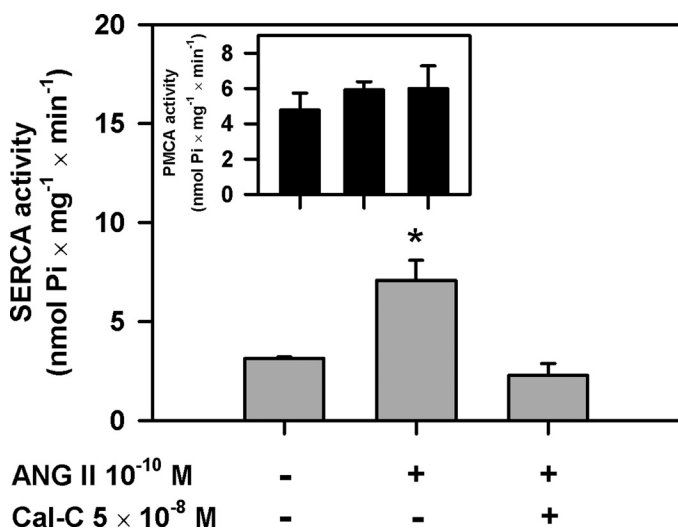


Fig. 9. Exposure of the luminal membrane to ANG II stimulates SERCA activity through a PKC signaling pathway. Cells were exposed to Calphostin C (Cal-C;  $5 \times 10^{-8}$  M) for 10 min, as shown. Then,  $10^{-10}$  M ANG II was added and 30 min later  $\text{Ca}^{2+}$ -ATPase activity was measured. *Inset*: PMCA activity. For details, see METHODS. Data bars indicate means  $\pm$  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 ( $P < 0.05$ ).

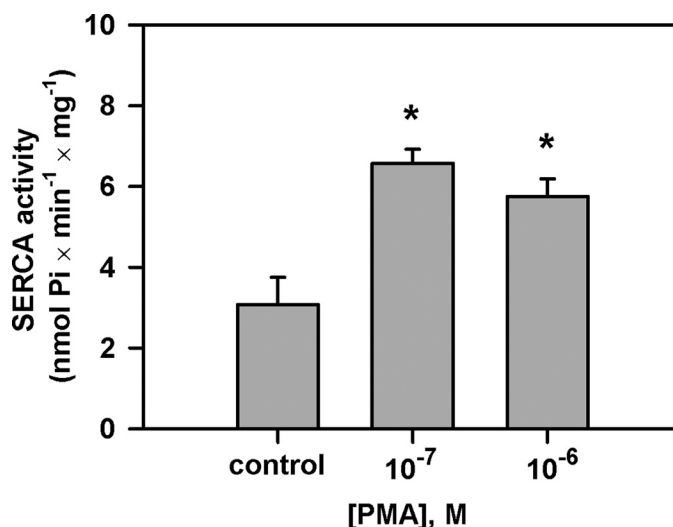


Fig. 10. PMA mimics stimulation of SERCA activity by ANG II. Cells were incubated with PMA ( $10^{-7}$  and  $10^{-6}$  M) for 30 min and  $\text{Ca}^{2+}$ -ATPase activity was then determined. Data bars indicate means  $\pm$  SE of at least 4 determinations in triplicate using different cell lysate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

SERCA activation increases the  $\text{Ca}^{2+}$  stock in the reticulum to ensure more efficient  $\text{Ca}^{2+}$  mobilization and, consequently, an enhanced cellular response facing a new stimulus of ANG II. This view is supported by measurements of intracellular  $\text{Ca}^{2+}$  mobilization (Fig. 3), which demonstrate that there is an increase of one order of magnitude in the peak of  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  waves and spikes promoted by tubular ANG II fluctuations result from modulation of SERCA turnover and, therefore, of the  $\text{Ca}^{2+}$  uptake/release cycles capable of amplifying

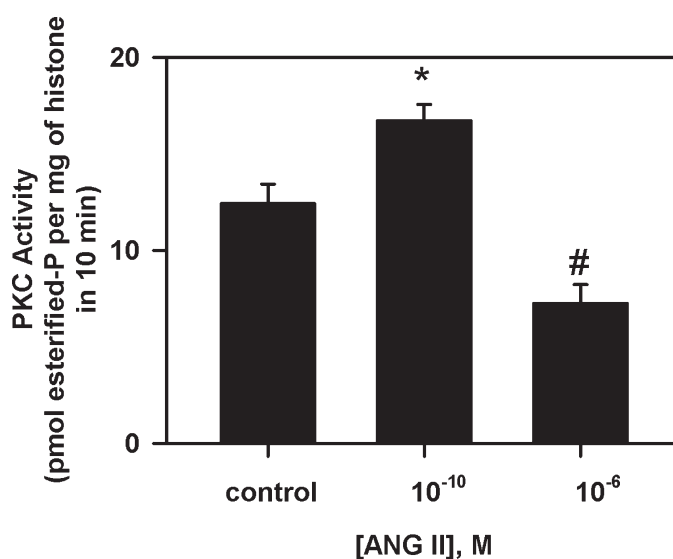


Fig. 11. Exposure of the luminal membrane to low concentration of ANG II stimulates PKC activity in LLC-PK<sub>1</sub> cells. ANG II ( $10^{-10}$  or  $10^{-6}$  M) was added to the culture for 30 min and PKC activity was measured as described in METHODS. Data bars indicate means  $\pm$  SE of at least 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<sup>2+</sup> mobilization that has been demonstrated seems to be an important way for luminal ANG II to regulate Ca<sup>2+</sup> homeostasis in renal cells, and also Ca<sup>2+</sup>-modulated fluid reabsorption in proximal tubules (17). Positive feedbacks involving tubular and interstitial ANG II have been proposed as key events in renal Na<sup>+</sup> (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<sup>2+</sup> homeostasis in proximal tubule cells, which appears to modulate Ca<sup>2+</sup> oscillations through different pathways. Whereas basolateral ANG II increases cytosolic Ca<sup>2+</sup> by inhibition of PMCA, the positively self-sustained luminal stimulus on SERCA provides a refined mechanism for further Ca<sup>2+</sup> control. It is noteworthy that, despite their polarity, the ANG II effect on Ca<sup>2+</sup> 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<sub>1</sub> 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<sub>1</sub>R/AT<sub>2</sub>R 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<sub>1</sub>R/AT<sub>2</sub>R 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<sub>1</sub>R and AT<sub>2</sub>R 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<sup>2+</sup>.

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<sup>-10</sup> and 10<sup>-6</sup> M ANG II (Fig. 7), the downregulation of SERCA stimulus at 10<sup>-6</sup> 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<sub>1</sub>R/AT<sub>2</sub>R dimerization and activation of the PLC/PKC signaling pathway. These observations and those we previously reported demonstrate that ANG II can modulate the Ca<sup>2+</sup> concentration in proximal tubule cells in a bidirectional manner from both the luminal and the basolateral side. Due to the relevance of Ca<sup>2+</sup> 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.

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#### 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.

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