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The stimulatory effect of angiotensin II on Na^+-ATPase activity involves sequential activation of phospholipases and sustained PKC activity

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ABSTRACT

Angiotensin II (Ang II) stimulates the proximal tubule Na^+-ATPase through the AT_1 receptor/phosphoinositide phospholipase Cβ (PI-PLCβ)/protein kinase C (PKC) pathway. However, this pathway alone does not explain the sustained effect of Ang II on Na^+-ATPase activity for 30 min. The aim of the present work was to elucidate the molecular mechanisms involved in the sustained effect of Ang II on Na^+-ATPase activity. Ang II induced fast and correlated activation of Na^+-ATPase and PKC activities with the maximal effect (115%) observed at 1 min and sustained for 30 min, indicating a pivotal role of PKC in the modulation of Na^+-ATPase by Ang II. We observed that the sustained activation of PKC by Ang II depended on the sequential activation of phospholipase D and Ca^{2+}-insensitive phospholipase A_2, forming phosphatidic acid and lysophosphatidic acid, respectively. The results indicate that PKC could be the final target and an integrator molecule of different signaling pathways triggered by Ang II, which could explain the sustained activation of Na^+-ATPase by Ang II.

1. Introduction

Sodium reabsorption in proximal tubule cells is a crucial step in the determination of renal sodium excretion [1,2]. The electrochemical sodium gradient in these cells depends on two sodium pumps located in the basolateral membrane (BLM): classic ouabain-sensitive ($Na^+ + K^+$) ATPase and ouabain-insensitive, furosemide-sensitive Na^+-ATPase, called the second sodium pump [3–5]. The observation that Na^+-ATPase is about 10 times less active than ($Na^+ + K^+$)ATPase [5,6] suggests that this enzyme may be involved in fine tuning, whereas ($Na^+ + K^+$)ATPase is responsible for most of the Na^+ reabsorption in the proximal tubule.

It has been shown that proximal tubule Na^+-ATPase is a target for several hormones and autacoids involved in the modulation of renal sodium excretion, including angiotensin II (Ang II) [7]. Usually, the effects of Ang II are mediated by two G protein coupled receptor subtypes: AT_1 and AT_2 [8]. The AT_1 receptors can be coupled to multiple cellular signaling cascades with distinct temporal characteristics [9]. It has been shown that activation of the AT_1 receptor in a variety of cell types induces several pathways, such as phospholipases and kinases [9,10]. However, the role of these pathways in the modulation of Na^+-ATPase by Ang II is not completely understood.

In a previous work, we showed that Ang II stimulates Na^+-ATPase of proximal tubule BLM via AT_1 receptors, in a process that involves PKC and the transient activation of Gq-protein-mediated activation of phosphoinositide phospholipase Cβ (PI-PLCβ) [7,11,12]. The effect of Ang II on Na^+-ATPase activity was observed even after 30 min of incubation, although the activation of PI-PLCβ was maximal after 30 s and returned to basal values after 1 min, suggesting a possible involvement of other signaling pathways in Ang II-mediated stimulation of Na^+-ATPase.

The aim of this work was to elucidate the molecular mechanisms involved in the sustained effect of Ang II on this enzyme activity. The possible role of phospholipase D and phospholipase A_2 was evaluated. Isolated BLMs of proximal tubules were used because, in this preparation, Ang II modulates Na^+-ATPase activity without changes in ($Na^+ + K^+$)ATPase activity [7]. We observed that Ang II promotes sequential activation of the phospholipase D (PLD) and phospholipase A_2 (PLA_2) pathways, after fast activation of PI-PLCβ, leading to phosphatidic acid (PA)/lysophosphatidic acid (LPA) production, maintaining activation of PKC. This has several physiologic implications within a complex interacting network that is crucial to the regulation of body homeostasis control.

2. Materials and methods

2.1. Materials

ATP (sodium salt), N^6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate (d-cAMP, sodium salt), phorbol myristate acetate (PMA),

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ouabain, ethylenediaminetetraacetic acid (EDTA), *N*-2-hydroxyethylpiperazine *N*′-2-ethanesulfonic acid (HEPES), tris(trishydroxymethyl)-aminomethane (Tris), Ang II and histone were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Percoll was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Palmitoyl trifluoromethyl ketone ($PACOCF_3$), quinacrine, bromoenol lactone (BEL) and calphostin C were purchased from Calbiochem (California, USA). All other reagents were of the highest purity available. [^{32}P]Pi was obtained from the Institute of Energetic and Nuclear Research, São Paulo, SP, Brazil and [γ-^{32}P]ATP was prepared as described by Maia et al. [13]. All solutions were prepared with deionized glass-distilled water.

2.2. Preparation of isolated basolateral membranes

BLMs were prepared from the outer cortex of adult pig kidneys. The kidneys were obtained from a commercial slaughter house immediately after the death of the animals and maintained in a cold solution containing (mM): sucrose 250, Hepes–Tris (pH 7.6) 10, EDTA 2 and phenylmethylsulfonyl fluoride (PMSF) 1. Thin slices of outer cortex were removed using a scalpel. After dissection, the slices were homogenized in the same cold solution with a Teflon and glass homogenizer. The homogenate was centrifuged at 3000 rpm in a SCR20B centrifuge using an RP12-2 rotor (Hitachi) for 10 min at 4 °C. The supernatant was collected and stored at 4 °C. The fraction enriched in BLMs was isolated by the Percoll gradient method [14]. The membrane preparation was resuspended in 250 mM sucrose at a final concentration of 5–10 mg protein ml^{-1} and stored at −20 °C. The ($Na^+ + K^+$)ATPase activity, a marker for BLMs, was 7–8 times higher than the activity found in the cortex. The same enrichment was observed for Na^+-ATPase specific activity. On the other hand, the specific activities of alkaline phosphatase and 5′-nucleotidase, markers of luminal membrane, were only enriched 1.2- and 0.25-fold, respectively. Residual contamination with other subcellular membrane fractions was minimal [15,16]. The protein concentration was determined by the Folin phenol method [17] using bovine serum albumin as a standard.

2.3. Measurement of ATPase activity

ATPase activity was measured according to the method described by Grubmeyer and Penefsky [18]. The composition of the assay medium for the measurement of Na^+-ATPase activity (0.1 ml) was 4 mM $MgCl_2$, 4 mM ATP (specific activity of approx. 10^4 Bq nmol^{-1} of [γ-^{32}P]ATP), 20 mM Hepes/Tris, pH 7.0, 90 mM NaCl, and 1 mM ouabain. The specific inhibitors, antagonists and/or activator were added when indicated. The reaction was started by adding protein to a final concentration of 0.3–0.5 mg/ml, and was stopped after 10 min by adding 0.1 M HCl-activated charcoal. The [^{32}P]P_i released was measured in an aliquot of 0.2 ml of the supernatant obtained after centrifugation of the charcoal suspension for 5 min at 1500 *g* in a clinical centrifuge. Spontaneous hydrolysis of [γ-^{32}P]ATP was measured simultaneously in tubes to which protein was added after the acid. The Na^+-ATPase activity was calculated from the difference between the [^{32}P]P_i released in the absence and in the presence of 2 mM furosemide, both in the presence of 1 mM ouabain. The possibility that there is vesicle formation in our preparation can be ruled out because: (1) the isolated BLM ($Na^+ + K^+$)ATPase activity in the presence of alamethicin (0.05 mg/mg protein), a permeabilizing agent that provides free access for ions, ATP, and other reactants to the interior of sealed vesicles, was not significantly different from that obtained in the absence of the drug (data not shown); (2) sodium- and potassium-stimulated ($Na^+ + K^+$)ATPase activities were not significantly different (less than 10% of difference) (data not shown). Probably the low percentage of inside-out vesicles is due to storage of the preparation at −4 °C instead of −80 °C for at least 1 week.

2.4. Protein kinase C activity assay

The PKC activity of isolated BLMs was measured by PKC-calphostin-sensitive incorporation of [^{32}P]Pi from [γ^{32}-P]ATP (7 μCi μmol^{-1}), using histone as substrate. The composition of the reaction medium was: 4 mM $MgCl_2$, 20 mM HEPES–Tris (pH 7.0), 1.5 mg/ml histone and 0.7 mg/ml protein. After 10 min, the reaction was stopped with 40% trichloroacetic acid (TCA) and the sample immediately placed on ice. An aliquot (0.1 ml) was filtered through a Millipore filter (0.45 μm) and washed with ice-cold 20% TCA solution and 0.1 M phosphate buffer (pH 7.0). The radioactivity was quantified by liquid scintillation counting (Packard Tri-Carb 2100 TR). The specific PKC was calculated from the difference between the activity in the absence and in the presence of 10^{-8} M calphostin C. Phorbol myristate acetate (PMA) was used as PKC activator.

2.5. Measurement of phospholipase activity

PLD activity was measured as described by Chalifa et al. [19], quantifying the [^{14}C]phosphatidic acid (PA) or [^{14}C]phosphatidyl ethanol (PEt) released using [^{14}C]phosphatidylcholine (PC) as substrate. The substrate was sonicated in medium containing 1-hexadecyl-2[^{14}C]arachdonyl-*sn*-glycerol-3-phosphocholine, sodium oleate 25 mM and cold PC to a specific activity of 420 mCi/mol. The reaction was started by the addition of the substrate to the medium assay with: $MgCl_2$ 1 mM, Hepes-Na 50 mM (pH 7.2), EGTA 1 mM, ethanol 2% (when indicated) and 0.3 mg/ml BLM protein. After 15 min at 37 °C the reaction was stopped by the addition of chloroform/methanol/HCl (1:1:0.006, v/v/v). The lipid fraction was extracted and separated using thin layer chromatography (TLC) on silica gel plates. The eluent mixture used was ethyl acetate/isooctane/acetic acid/water (13:2:3:10, v/v/v/v). Iodide vapor was then used to reveal the bands. The bands corresponding to PA or PEt were scraped and counted using liquid scintillation (Packard Tri-Carb 2100TR, Illinois, USA).

The PLA activity was measured as described by Yang et al. [20] using 100 μM dipalmitoylphosphatidylcholine (containing 5.0×10^5 cpm 1-palmitoyl-2-[1-^{14}C]palmitoyl L-3-phosphatidylcholine) in 0.8 mM Triton X-100, micelles in 200 mM Hepes (pH 7.0), 5 mM EDTA, and 2 mM 1 mM dithiothreitol (DTT). The reaction was started by the addition of BLM protein (0.1–0.3 mg/ml) at 37 °C. The lipid extraction was performed as described by Horowitz and Perlman [21], and modified by Malaquias and Oliveira [22]. The lipid samples were applied to TLC silica gel plates using two solvent systems: $CHCl_3$/CH_3OH/H_2O (65:35:5, v/v/v) and C_6H_{14}/$CH_3CH_2OCH_2CH_3$/CHOOH (90:60:4, v/v/v). The region corresponding to free fatty acids was identified after exposure with iodide vapor, scraped and submitted to liquid scintillation counting (Packard Tri-Carb 2100TR, Illinois, USA).

2.6. Data analysis

The means were compared by one-way analysis of variance (ANOVA) taking into account the treatment of the experimental groups. The magnitudes of the differences were evaluated using the multiple comparative Bonferroni test. The data are presented as the mean ± standard error.

3. Results and discussion

3.1. Correlation between Na^+-ATPase and PKC activities

To test for a possible temporal correlation between activation of Na^+-ATPase and PKC activities by Ang II, isolated BLM was incubated with Ang II 10^{-8} M for different times (30 s up to 30 min). The Na^+-ATPase and PKC activities were then measured in parallel (Fig. 1). Ang II induced fast and correlated activation of both Na^+-ATPase and PKC

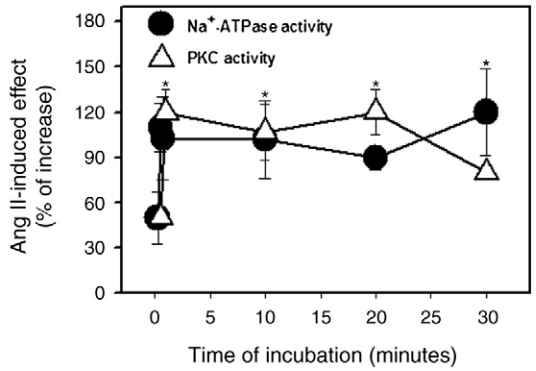

Fig. 1. The effect of time of Ang II incubation on Na^+-ATPase (closed circles) and PKC (open triangles) activity. BLM was incubated with 10^{-8} M Ang II and the enzyme activities were measured at different times as described in Section 2 ($n=6$). The results are expressed as the mean ± SE. The Na^+-ATPase and PKC activities in the absence of Ang II were 24.3 ± 3.1 nmol Pi mg^{-1} min^{-1} and 41.2 ± 5.5 nmol P-histone mg^{-1} min^{-1}, respectively. *Statistically significant when compared to controls ($p<0.05$).

activities with the maximal effect observed at 1 min. Under these conditions, the enzyme activities were increased by around 115% and this was sustained for 30 min. These data agree with those observed in a previous work where Ang II increased Na^+-ATPase activity through activation of a Ca^{2+}-insensitive, PMA-sensitive PKC, probably belonging to a novel family subtype [11]. Usually members of this family are activated by an increase in the level of diacylglycerol (DAG), which can be generated by activation of PI-PLCβ. We showed that 10^{-8} M Ang II activated PI-PLCβ activity in a fast and transient way; the maximal effect occurred after 1 min of incubation and then returned to the control level [12]. Together these results lead us to postulate that PKC could be the final target and an integrator molecule of different signaling pathways triggered by Ang II, which could explain the sustained activation of Na^+-ATPase by Ang II.

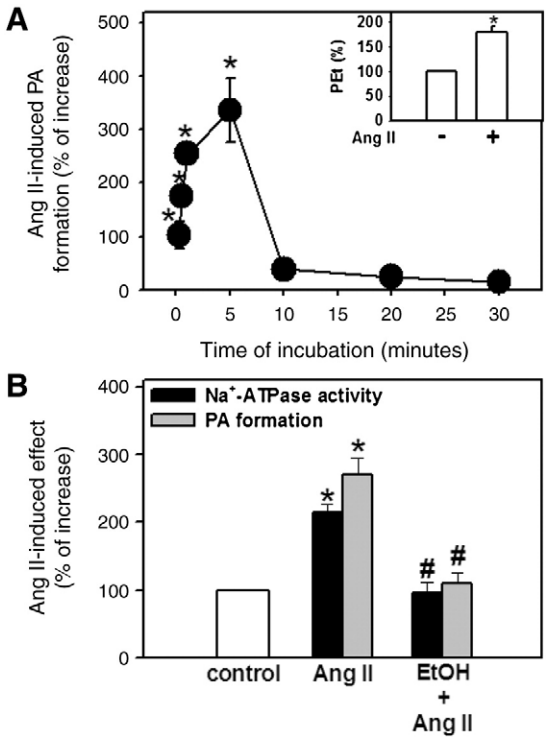

Fig. 2. Involvement of PLD on the modulation of Na^+-ATPase activity by Ang II. (A) Time course of PA. BLM was incubated with 10^{-8} M Ang II for different times and the formation of PA was measured by TLC as described in Section 2 ($n=4$). Inset: the effect of Ang II on PEt formation. BLM was incubated with 10^{-8} M Ang II for 5 min ($n=3$). (B) Effect of Ang II on Na^+-ATPase activity (black bars) and PA formation (gray bars). Open bar represents the control conditions taken as 100% for each reaction. BLM was incubated with 10^{-8} M Ang II for 30 or 5 min to measure the Na^+-ATPase activity or PA formation, respectively ($n=8$). The results are expressed as mean ± SE. *Statistically significant when compared to controls. #Statistically significant when compared to the enzyme activity obtained in the presence of 10^{-8} M Ang II. $p<0.05$ under all conditions.

3.2. PLD/PA and iPLA$_2$/LPA pathways are involved in the sustained effect of Ang II on Na^+-ATPase activity

Based on phospholipid metabolism, it is known that PLD may be involved in the sustained effect of a previous and transiently activated PI-PLCβ pathway [23]. However, the activation of PLD in BLM of proximal tubular cells has not yet been demonstrated. The physiologic product of PLD action is phosphatidic acid (PA) [23]. We therefore investigated whether Ang II stimulates PA synthesis in the BLMs of proximal tubule from pig kidney. As depicted in Fig. 2A, Ang II 10^{-8} M stimulates PA production with maximal effect at 6 min (200%), then returning to the control value. One unique characteristic of PLD is its ability to catalyze a particular reaction, called transphosphatidylation, in which the polar head of PC is changed by a primary alcohol, such as ethanol (EtOH), giving PEt and choline [24]. Incubation of isolated BLMs for 5 min with 10^{-8} M Ang II increased the formation of PEt (Fig. 2A, inset) confirming the activation of PLD by Ang II. Because transphosphatidylation occurs rather than hydrolysis reaction, the addition of primary alcohols to the assay medium blocks PA synthesis by PLD [24]. In this way, the stimulatory effect of 10^{-8} M Ang II on Na^+-ATPase activity and PA formation was inhibited by 2% EtOH (Fig. 2B). Ethanol (2%) alone did not change the pump activity. These results indicate that PLD could mediate the effect of Ang II in proximal tubule cells. Rothman et al. [25] showed that 2% EtOH inhibits Na^+-ATPase activity after the treatment of BLM with 1.5 μg SDS/μg of protein. Because we did not use SDS in our reaction medium, the difference between our results and Rothman's results could be due to the influence of the Na^+-ATPase phospholipid environment. To test this hypothesis we carried out simultaneous experiments in the presence or in the absence of SDS (0.4 μg SDS/μg protein). It was observed that 2% EtOH inhibited the enzyme activity in 52% in the presence of SDS and did not change the enzyme activity in the absence of it (data not shown). Furthermore, Table 3 of Rothman et al. showed that the Na^+-ATPase activity of homogenates from rat kidney cortex slices pre-incubated in an isotonic medium did not change with the addition of 2% ethanol. On the other hand, when the rat kidney cortex slices were incubated with hypotonic medium, inhibition of the Na^+-ATPase activity was observed. These results show that the effect of ethanol depends on previous swelling of the cells and is probably not a direct effect on the enzyme activity. Because we use isolated basolateral membranes, it is not possible that the swelling of the cells occurred.

Fig. 3A shows the effect of PA on Na^+-ATPase activity. The dose–response curve of PA (10^{-13} to 10^{-8} g/ml) on Na^+-ATPase shows a progressive enhancement of the enzyme activity, with the maximum effect (100%) achieved at 10^{-11} g/ml. The stimulatory effect of PA was already observed at concentrations as low as 10^{-13} g/l. Furthermore, the simultaneous addition of 10^{-9} g/l PA and 10^{-8} M Ang II to the reaction medium stimulates Na^+-ATPase activity in a similar and non-additive manner (Fig. 3B). Based on our hypothesis that PKC could be the final target and the integrator of different signaling pathways triggered by Ang II, we investigated whether PKC is involved in the effect of PA on Na^+-ATPase activity. Fig. 3B shows that PA-stimulated or Ang II-stimulated Na^+-ATPase activity were completely abolished in the presence of 10^{-8} M PKC inhibitor, calphostin C, indicating that PKC is involved in this process. Furthermore, 10^{-8} M calphostin C alone did not change the Na^+-ATPase activity.

So far our results show the involvement of the PI-PLC/PLD pathway, mediating the effect of Ang II on Na^+-ATPase activity. However, the sequential activation of PI-PLCβ and PLD does not

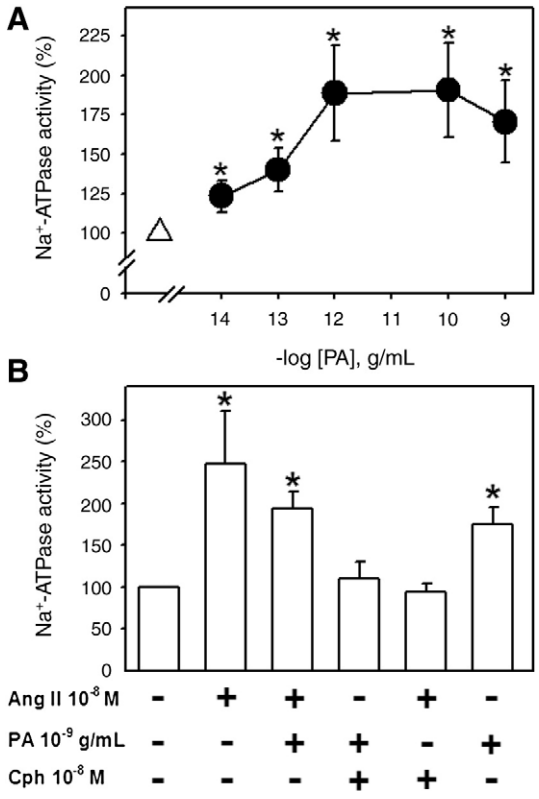

Fig. 3. PA modulation of Na^+-ATPase activity involves PKC. (A) Dose–response of incubation with PA on Na^+-ATPase activity (10^{-13}–10^{-8} g/ml). BLM was incubated with (closed circle) or without (open triangle) PA for 5 min and ATPase activity was measured ($n=5$). (B) Modulation of the effect of PA on Na^+-ATPase activity by 10^{-8} M calphostin C (Cph). BLM was incubated with 10^{-8} M Ang II, 10^{-9} g/ml PA and/or 10^{-8} M Cph for 5 min when indicated and enzyme activity was measured ($n=6$). The results are expressed as the mean ± SE. *Statistically significant when compared to controls. #Statistically significant when compared to the enzyme activity obtained in the presence of 10^{-9} g/ml PA. $p<0.05$ under all conditions.

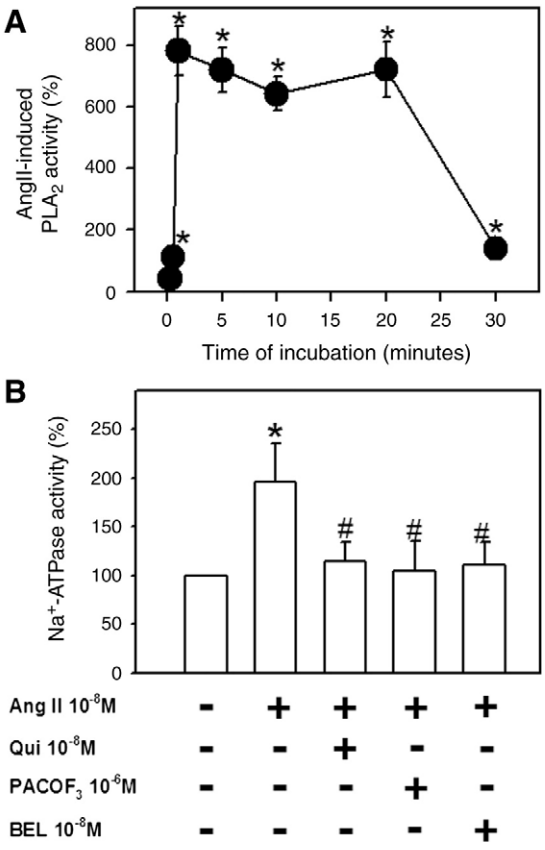

Fig. 4. Involvement of PLA_2 on the modulation of Na^+-ATPase activity by Ang II. (A) Time course of the effect of Ang II on PLA_2 activity. BLM was incubated with 10^{-8} M Ang II for different times and the PLA_2 activity was measured as described in Section 2 ($n=5$). (B) Modulation of the effect of Ang II on Na^+-ATPase activity by PLA_2 inhibitors. When indicated, BLM was incubated with 10^{-8} M Ang II, 10^{-8} M quinacrine (Qui), 10^{-6} M $PACOF_3$ and/or 10^{-8} M BEL for 5 min and the enzyme activity was measured ($n=4$). The results are expressed as the mean ± SE. *Statistically significant when compared to controls. #Statistically significant when compared to the enzyme activity obtained in the presence of 10^{-8} M Ang II. $p<0.05$ under all conditions.

completely explain the activation of Na^+-ATPase for 30 min because after 10 min, PI-PLCβ and PLD activities are not stimulated by Ang II, while the activation of PKC activity is still observed. It is widely known that Ang II stimulates PLA_2 in some cellular types such as endothelium and vasculature cells [26,27]. In our study, we investigated PLA_2 activation by Ang II. Isolated BLMs were incubated with 10^{-8} M Ang II for different times. After the reaction, the phospholipids were separated using a TLC technique and PLA_2 activity was quantified. The temporal course shows an 8-fold increase in PLA_2 activity in the presence of 10^{-8} M Ang II, with the maximum effect reached at 10 min (Fig. 4A). This stimulus was sustained for 20 min and decreased after 30 min. Even after 30 min, an increase in PLA_2 activity of 138% could still be observed. When PA synthesis is decreasing, the release of palmitic acid, a product of PLA_2 activity, is increasing, showing a correlation between the decrease in substrate level (PA) and the increase in product formation (palmitic acid and LPA). The level of LPC did not change after the treatments (data not shown).

In a recent paper, we observed that isolated BLM presents constitutive Ca^{2+}-insensitive PLA_2 ($iPLA_2$) inhibited by quinacrine, $PACOCF_3$ and a specific inhibitor, BEL [28]. Fig. 4B shows that with the addition of 10^{-8} M quinacrine, 10^{-6} M $PACOCF_3$ or 10^{-6} M BEL, the stimulatory effect of 10^{-8} M Ang II on Na^+-ATPase activity did not occur. It is well known that $iPLA_2$ can metabolize PA generating LPA [29], which exerts several biologic effects [30]. We investigated whether LPA could modulate Na^+-ATPase activity. The addition of increasing doses of LPA (10^{-8} to 10^{-5} M) promoted a stimulatory effect on the enzyme activity in a dose-dependent manner, with the maximum value (120%) reached at 10^{-11} M (Fig. 5A). The correlation between the effect of LPA and PKC activity was shown by the observation that the stimulatory effect of 10^{-8} M LPA on the enzyme activity was reversed by 10^{-8} M PKC inhibitor, calphostin C (Fig. 5B).

In the next step we tested whether the effect of PA on Na^+-ATPase activity depends on $iPLA_2$ activity. Fig. 6A shows that the stimulatory effect of PA on the enzyme activity is completely abolished by the addition of 10^{-6} M $PACOF_3$ or 10^{-6} M BEL. Furthermore, this effect was overcome by simultaneous addition of LPA, a product of the breakdown of PA by $iPLA_2$. Similar effects were observed when 10^{-8} M Ang II was added instead of PA (Fig. 6B).

The results shown in the present work and previous observations [7,11,12] suggest that Ang II modulation of proximal tubule Na^+-ATPase activity involves the activation of PI-PLCβ/PLD/$iPLA_2$ and the PKC of plasma membrane is the final target and the integrating component of these pathways. Studies on the role of PKC in the modulation of renal sodium transporters have indicated that the tissue specific distribution of different PKC isoforms is the determinant of the final effect of this kinase on renal sodium transporters [31].

We have already reported the presence of several components of different signaling cascades such as PI-PLC/DAG/PKC and adenylyl cyclase/cAMP/PKA and their functionality in isolated BLM. In addition, we have shown that these pathways are coupled to several receptors including angiotensin receptors, AT_1 and AT_2 [7,11,12,15,28]. The presence of complete signaling complexes in plasma membrane, from receptors and all intermediary elements to final membrane targets, illustrates that isolated BLM is a suitable model for investigation of the

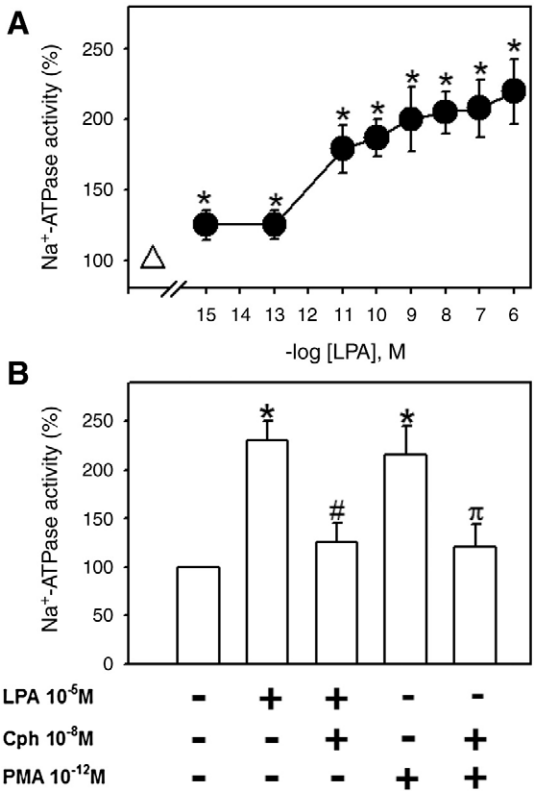

Fig. 5. PKC mediated the modulation of Na^+-ATPase activity by LPA. (A) Dose–response of incubation with LPA on Na^+-ATPase activity (10^{-15}–10^{-6} M). BLM was incubated with (closed circle) or without (open triangle) LPA for 10 min and the Na^+-ATPase activity was measured ($n=5$). (B) Modulation of the effect of 10^{-8} M LPA or 10^{-12} M phorbol ester (PMA) on Na^+-ATPase activity by 10^{-8} M calphostin C (Cph). BLM was incubated with 10^{-8} M LPA or 10^{-12} M PMA in the presence or in the absence of 10^{-8} M Cph for 10 min when indicated and the enzyme activity was measured ($n=6$). The results are expressed as the mean ± SE. *Statistically significant when compared to controls. #Statistically significant when compared to the enzyme activity obtained in the presence of 10^{-8} M LPA. πStatistically significant when compared to the enzyme activity obtained in the presence of 10^{-12} M PMA. $p<0.05$ under all conditions.

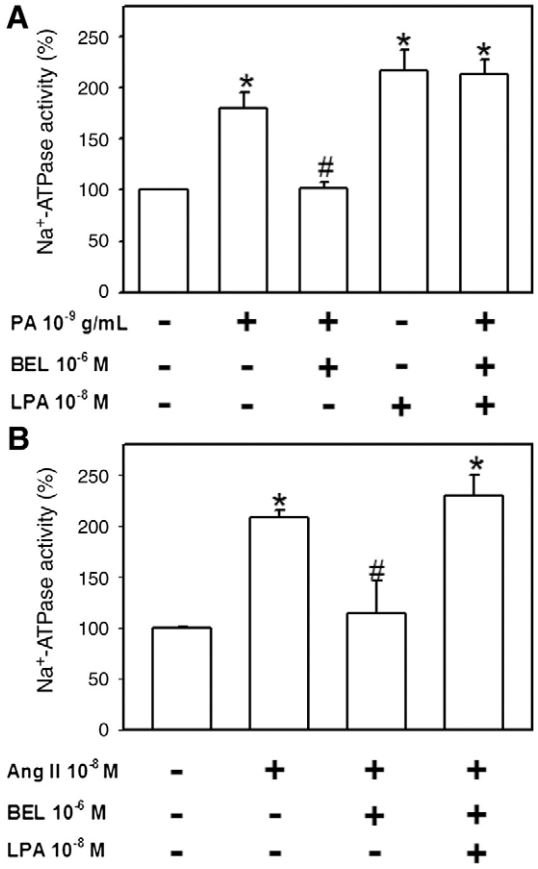

Fig. 6. The effect of PA on Na^+-ATPase is mediated for LPA. (A) The effect of PA is mediated by $iPLA_2$; BLM was incubated with 10^{-9} g/ml PA, 10^{-6} M BEL and/or 10^{-8} M LPA for 10 min when indicated and the Na^+-ATPase activity was assayed as described in Section 2 ($n=5$). (B) The effect of Ang II is mediated by $iPLA_2$. BLM was incubated with 10^{-8} M Ang II, 10^{-6} M BEL and/or 10^{-8} M LPA for 10 min when indicated and the Na^+-ATPase activity was assayed as described in Section 2 ($n=6$). The results are expressed as the mean ± SE. *Statistically significant when compared to controls. #Statistically significant when compared to the enzyme activity obtained in the presence of 10^{-8} M Ang II.

signaling pathways associated with plasma membrane involved in the modulation of Na^+-ATPase. In the present study, we could correlate the sustained activation of Na^+-ATPase by Ang II with the activation of a novel PKC isoform based on the following evidence: (1) the assay medium was calcium-free with EGTA added, ruling out the involvement of a conventional PKC; (2) as shown previously, DAG and phorbol ester stimulates the PKC present in our preparation, ruling out the involvement of an atypical PKC.

In a previous paper, we showed that the effect of Ang II on Na^+-ATPase and PKC activity is mediated by AT_1 receptor [11]. Thus, it is plausible to postulate that Ang II increases PLD activity of BLM through AT_1 receptor. This hypothesis agrees with the observations in several works, in which AT_1 receptor mediates the activation of PLD by Ang II in smooth muscle and endothelial cells [32]. It is well established that there are close interactions between PKC and PLD in the regulation of PLD by PKC, an issue that is still incompletely understood [33]. Evidence suggests that PLD activation by PKC requires an initial hydrolysis of PI lipids and DAG generation. In this way, it is possible that previously PI-PLCβ-activated PKC could act as the PLD activator. Our results show that the time course of PKC and PLD activation supports PI-PLCβ/PKC-mediated PLD activation by Ang II.

It has been reported that PA itself could act as an intracellular second messenger [23]. In vitro studies showed that PA is able to substitute PS in the activation of PKC [34]. On the other hand, PA could be converted to DAG by PA phosphohydrolase leading to the activation of PKC [23]. However, this possibility can be ruled out because: (1) the stimulatory effect of PA is completely reverted by $iPLA_2$ inhibitors, indicating that the effect PA depends on the further activation of $iPLA_2$; (2) LPA, the product of PA hydrolysis by $iPLA_2$, stimulates Na^+-ATPase in a similar way to PA and Ang II. It has been reported that PA could also be synthesized from DAG phosphorylation by a DAG kinase (DGK); however, this does not seems to be the case since PA synthesis is completely blocked by the addition of ethanol, a PLD inhibitor.

The molecular mechanisms involved in the activation of $iPLA_2$ are poorly understood. In a previous study, we observed that PKC activation led to an increase in $iPLA_2$ activity [28]. In a similar way, using membrane fractions of ventricular myocytes, Steer et al. [35] showed that PMA increases $iPLA_2$ through activation of a resident pool of PKC. Although no phosphorylation consensus sequences have been described in $iPLA_2$, different studies suggest the involvement of phosphorylation in the regulation of this enzyme [36–38]. These results indicate that activation of PKC by previous activation of PI-PLCβ and PLD by Ang II could mediate the activation of $iPLA_2$ by this peptide. The activation of these loops by PKC could represent an important positive feedback within the Ang II-induced signaling pathways involved in maintenance of the stimulatory effect on Na^+-ATPase activity.

The Na^+-ATPase activity was always measured in the presence of 1 mM ouabain and in the absence of KCl. Under these conditions, the $(Na^+ + K^+)$ATPase activity is completely inhibited, even the partial reactions when K is absent. These results rule out the possibility that

the ouabain-insensitive Na^+-ATPase activity measured could be correlated with $(Na^+ + K^+)$ATPase or even with a partial reaction of the catalytic cycle of this enzyme. Furthermore, it has been demonstrated previously that 2 mM furosemide does not affect the activities of other ATPases, such as $(Na^+ + K^+)$ATPase, Mg^{2+}-ATPase, Ca^{2+}-ATPase and Ecto-ATPase [3–6].

The link between Na^+-stimulated ATPase activity and Na^+ transport was also shown by Malnic et al. [39]. They observed that furosemide inhibits Na^+ reabsorption in the proximal tubule. It is well known that Ang II increases proximal tubule sodium reabsorption, but the molecular mechanism is not completely understood. Furthermore, it was shown that PKC activation leads to an increase in proximal tubule sodium reabsorption [31]. Thus, it is possible to postulate that the effect of Ang II on Na^+-ATPase activity is mediated, at least in part, by the effect of Ang II on sodium reabsorption in proximal tubule cells through an AT_1R/PI-PLCβ/PLD/iPLA$_2$ pathway, which is integrated by activation of PKC.

The results obtained in this work, together with previous data from the literature, suggest extensive cross-talk between cellular signaling pathways triggered by a single initial effector, Ang II. Ang II is a crucial peptide to body homeostasis, and exerts multiple functions as hormone, cytosine and autacoid. Determination of the molecular mechanisms involved in Ang II modulation of sodium transporters is fundamental to our comprehension of physiologic extracellular volume regulation and opens new possibilities for possible pharmacologic interventions in pathologic conditions such as hypertension.

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