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Protein kinase C-induced phosphorylation modulates the Na^+-ATPase activity from proximal tubules

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Abstract

This study describes the modulation of the ouabain-insensitive Na^+-ATPase activity from renal proximal tubule basolateral membranes (BLM) by protein kinase C (PKC). Two PKC isoforms were identified in BLM, one of 75 kDa and the other of 135 kDa. The former correlates with the PKC isoforms described in the literature but the latter seems to be a novel isoform, not yet identified. Both PKC isoforms of BLM are functional since a protein kinase C activator, TPA, increased the total hydroxylamine-resistant $^{32}P_i$ incorporation from [γ-^{32}P]ATP into the BLM. In parallel, TPA stimulated the Na^+-ATPase activity from BLM in a dose-dependent manner, the effect being reversed by the PKC inhibitor sphingosine. The stimulatory effect of TPA on Na^+-ATPase involved an increase in the V_{max} (from 13.4 ± 0.6 nmol P_i mg^{-1} min^{-1} to 25.2 ± 1.4 nmol P_i mg^{-1} min^{-1}, in the presence of TPA, $P < 0.05$) but did not change the apparent affinity for Na^+ ($K_{0.5} = 14.5 \pm 2.1$ mM in control and 10.0 ± 2.1 mM in the presence of TPA, $P > 0.07$). PKC involvement was further confirmed by stimulation of the Na^+-ATPase activity by the catalytic subunit of PKC (PKC-M). Finally, the phosphorylation of an approx. 100 kDa protein in the BLM (the suggested molecular mass of Na^+-ATPase [1]) was induced by TPA. Taken together, these findings indicate that PKCs resident in BLM stimulate Na^+-ATPase activity which could represent an important mechanism of regulation of proximal tubule Na^+ reabsorption.

Keywords: Protein kinase C; Proximal tubule; Na^+-Adenosine 5′-triphosphatase; Basolateral membrane

1. Introduction

The modulation of Na^+ reabsorption in the kidney is critical for the maintenance of the extracellular compartment volume and for the long-term regulation of blood pressure. The principal site of renal Na^+ reabsorption is the proximal tubule, involving several transporters, including primary active pumping systems resident in the basolateral membrane of this segment of the nephron [2].

Two main active transporters are involved in Na^+ reabsorption in the proximal tubules: the ouabain-

Abbreviations: EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; Tris, tris(hydroxymethyl)aminomethane; ATP, adenosine triphosphate (sodium salt); PMSF, phenylmethylsulfonyl fluoride; PKC, protein kinase C; PKC-M, catalytic subunit of PKC; Sph, sphingosine; TPA, 12-*O*-tetradecanoylphorbol 13-acetate

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sensitive (Na^++K^+)-ATPase and the ouabain-insensitive, furosemide-sensitive Na^+-ATPase [3–6]. Several aspects about the structure and kinetic properties of the Na^+-ATPase remain unclear, but it is known that it transports Na^+ against its electrochemical gradient in a K^+-independent fashion [4,7]. Na^+-ATPase distribution is parallel to (Na^++K^+)-ATPase, but the catalytic activity of the former is about 10% that of the latter [3,5,7]. Recently, Marín and coworkers [1] demonstrated that Na^+-ATPase is a P-ATPase of approx. 100 kDa, able to form a phosphorylated intermediate during the catalytic cycle which is stimulated by furosemide and is insensitive to K^+.

In spite of several papers published on Na^+-ATPase, its physiological role remains to be elucidated. Initially, participation in cell volume regulation was proposed [7]. More recently, our laboratory showed that natriuretic compounds, like adenosine and bradykinin, inhibit the Na^+-ATPase activity of proximal tubule, while anti-natriuretic compounds, such as angiotensin II, stimulate the enzyme activity [8–10]. In the same conditions, the (Na^++K^+)-ATPase activity was not directly modulated by such substances. These observations suggest that the Na^+-ATPase of proximal tubule is also a target of action of natriuretic and anti-natriuretic compounds. It might be suggested that Na^+-ATPase is involved in fine tuning, whereas (Na^++K^+)-ATPase is responsible for most of the Na^+ reabsorption in the proximal tubule.

The role of protein kinase C (PKC) in modulating Na^+ reabsorption in proximal tubule is not clear. Liu and Cogan [11] demonstrated that the activation of PKC increases bicarbonate and water absorption in the S1 and S2 segments of the proximal tubule. Moreover, it has been shown that the action of several hormones, including angiotensin II (AG II), in proximal tubule is mediated by PKC [11,12]. The present work aimed to investigate the modulation of the Na^+-ATPase activity from basolateral membranes (BLM) by PKC. Data of this report indicate that PKC-induced phosphorylation activates the ouabain-insensitive Na^+-ATPase activity of BLM.

2. Materials and methods

2.1. Materials

ATP, ouabain, furosemide, azide, mannitol, phorbol ester TPA, sphingosine (Sph) and calphostin C (Calf) were purchased from Sigma (St. Louis, MO, USA). Percoll was obtained from Pharmacia (Uppsala, Sweden). The catalytic subunit of PKC (PKC-M) and rabbit anti-mouse PKC consensus sequence were purchased from Calbiochem (CA, USA). ECL-Plus chemiluminescent reagent was purchased from Amersham (IL, USA). All other reagents were of the highest purity available. [^{32}P]P_i was obtained from the Institute of Energetic and Nuclear Research (SP, Brazil).

All solutions were prepared with deionized glass-distilled water. [γ-^{32}P]ATP was prepared as described by Maia et al. [13].

2.2. Preparation of purified BLM

The BLM were prepared from adult pig kidney by the Percoll gradient method as described elsewhere [8–10,14]. Controls for enrichment and contaminant with other membranes were carried out as previously described [15]. The membrane preparation was resuspended in 250 mM sucrose at a final concentration of 10 mg of protein ml^{-1} and stored at −4°C. The (Na^++K^+)-ATPase activity, a marker for basolateral membranes, was 69.2 ± 7.2 nmol P_i mg^{-1} min^{-1}, 8.9 times higher than the activity found in cortex homogenate (7.8 ± 0.3 nmol P_i mg^{-1} min^{-1}). Protein concentration was determined by the Folin phenol method [16] using bovine serum albumin as a standard.

2.3. Western blotting

To determine the presence of PKC in the BLM we used Western blots to identify PKC isoforms. 50 μg of total protein from both cortex homogenates and BLM were separated by 10% SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were blocked for 1 h at room temperature in 5% non-fat dry milk plus TBS Tween 20 (TBST) 0.5%. After three ice-cold TBST 0.05% washes, the membranes were incubated for 3 h with polyclonal

antibody against a consensus sequence (Ac 543–549) of murine PKC isoforms (which also recognizes human, rat, and sheep PKCs isoforms) diluted 1/4000 in TBST 0.05% plus non-fat dry milk 5%. The membranes were then washed, incubated with goat anti-rabbit peroxidase conjugated secondary antibody diluted 1/10 000, and then washed three more times with TBST 0.05%. The blots were developed using the ECL-Plus kit (Amersham-Pharmacia) following the manufacturer's protocol, and exposed to film (Kodak X-Omat).

2.4. Measurement of hydroxylamine-resistant phosphorylation of BLM proteins

The incorporation of $^{32}P_i$ from [γ-^{32}P]ATP into isolated BLM was measured as the radioactivity bound to an insoluble protein fraction. The reaction was initiated by the addition of membrane preparation (final concentration of 1.5 mg ml^{-1}) to a reaction medium containing 1 mM [γ-^{32}P]ATP (disodium salt, 7 μCi μmol^{-1}), 10 mM $MgCl_2$, 20 mM HEPES-Tris (pH 7.0), 6 mM NaCl, 1.1 M hydroxylamine (pH 5.5) and 1 mM EGTA. Final osmolality was adjusted with mannitol to 300 mOsm/kg. After 20 min the reaction was stopped with 1.5 ml of an ice-cold solution (0.25 M perchloric acid, 1 mM ATP and 4 mM sodium phosphate, pH 7.0). The mixture was centrifuged for 20 min (3 times) at 2000×*g*, and the pellet was resuspended with 0.15 ml of an ice-cold solution (0.1 M NaOH, 2% (w/v) Na_2CO_3, 2% (w/v) SDS). The radioactivity was quantified by liquid scintillation counting.

2.5. Measurement of Na^+-ATPase activity

Except where otherwise noted, the standard assay medium (0.1 ml) contained: 10 mM $MgCl_2$, 5 mM [γ-^{32}P]ATP (0.75 μCi μmol^{-1}), 20 mM HEPES-Tris (pH 7.0) and 5 mM azide. The NaCl concentration used was 6 mM since in this concentration the effect of TPA (Na^+-ATPase activity increased 153%) is higher than in the presence of 120 mM NaCl (Na^+-ATPase activity increased 88%). Final osmolality was adjusted with mannitol to 300 mOsm/kg.

The ATPase activity was measured according to the method described by Grubmeyer and Penefsky [17]. The reaction was started by the addition of purified basolateral membranes to a final concentration of 0.3–0.5 mg ml^{-1}. The reaction was stopped after 30 min by the addition of 0.1 N HCl-activated charcoal. The $^{32}P_i$ released was measured in an aliquot of the supernatant obtained after centrifugation of the charcoal suspension for 10 min at 1900×*g* in a clinical centrifuge. Spontaneous hydrolysis of [γ-^{32}P]-ATP was measured simultaneously in tubes where the membranes were added after the acid. The Na^+-ATPase activity was calculated by the difference between the $^{32}P_i$ released in the absence and in the presence of 2 mM furosemide, both in the presence of 1 mM ouabain.

2.6. Electrophoresis analysis

SDS-PAGE gel (10%) was performed as described by Laemmli [18], with the following modifications: (1) the proteins were stained with Coomassie brilliant blue R-250 for 1 h; (2) the gel was destained with methanol 50% (v/v) and acetic acid 10% (v/v). The intensities of the bands in the autoradiograms were quantified by densitometry, using the Sigma-Gel program.

2.7. Statistical analysis

Data were analyzed by two-way analysis of variance (ANOVA), considering the treatments as factors. The significance of the differences was verified by Bonferroni's *t*-test.

3. Results and discussion

3.1. Identification and characterization of functionality of PKC isoforms present in the BLM

PKC isoforms, resident in the BLM, were identified by Western blotting assay. Fig. 1 shows the band profile of the autoradiograms obtained, in which two major bands were observed: a 75 kDa band and a 135 kDa band. The majority of the PKC isoforms expressed in several tissues have an apparent molecular mass of approx. 80 kDa [19]. This is the case of: (i) the conventional PKCs, activated by calcium and/or diacylglycerol (DAG), α (80–81 kDa), βI (79–80

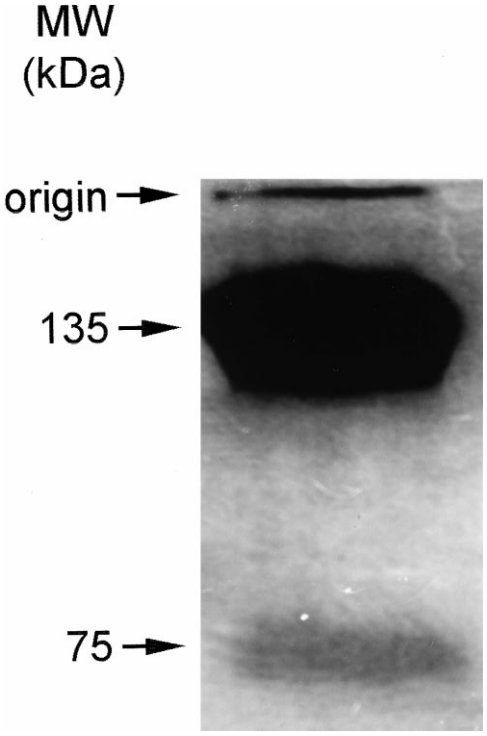

Fig. 1. Characterization of PKC isoforms in the BLM of proximal tubules. PKC isoforms were identified by Western blot assays, using a primary antibody reactive against a consensus sequence of PKC, as described in Section 2. Note two major bands in the autoradiogram: a 75 kDa band and a 135 kDa band. Control experiments were carried out in parallel in the absence of the primary antibody and in the presence of rabbit preimmune serum, assuring the specificity of the primary antibody ($n=5$).

kDa), βII (80 kDa), and γ (77–84 kDa), (ii) the novel PKC isoforms, calcium-independent and activated by DAG, δ (74–86 kDa), and θ (79 kDa), and (iii) the atypical isoform, calcium- and DAG-independent, ζ (76–80 kDa). The band of 75 kDa (Fig. 1) detected in BLM correlates with the PKC isoforms previously identified. However, the 135 kDa band observed in our experiments (Fig. 1) does not correlate with any of the PKC isoforms mentioned above. Since Western blotting assays were carried out in reducing gels, it cannot be ruled out that the 135 kDa band could be due to the dimerization of the 75 kDa protein. Another possibility is the occurrence of aggregation not involving disulfide bonds. Besides, control experiments performed in the absence of the primary antibody or in the presence of rabbit preimmune serum were executed in parallel, assuring that the bands observed in the autoradiograms were specific to the primary antibody and not a result of cross-reaction of the secondary antibody. The 135 kDa protein detected in our blotting assays can only be correlated to a protein kinase that presents the same consensus sequence identified by the primary antibody used. In agreement with this hypothesis, Damron and colleagues [20] identified the PKC isoform μ, also denominated PKD, with an apparent molecular mass of 115 kDa, indicating that PKC isoforms with higher molecular masses than that previously described can exist in some tissues.

The diverse distribution of PKC isoforms through the cells could explain, at least in part, the differences in the results concerning the regulation of Na^+ reabsorption by PKC. In this manner, the prevalence of one isoform over others in a specific cell type determines the final effect of PKC on renal Na^+ reabsorption. Supporting this hypothesis is the observation of Efendiev and coworkers [21] that showed opposing effects of PKC-β and PKC-ζ on rodent proximal tubule (Na^++K^+)-ATPase: PKC-β activates the enzyme whereas PKC-ζ inhibits it. So, it might be that BLM expresses PKC isoforms that result in the increase of proximal Na^+ reabsorption through the activation of the Na^+-ATPase activity.

The identification of PKC isoforms in BLM was followed by the characterization of the integrity of the enzyme, that was assayed by quantifying the total hydroxylamine-resistant $^{32}P_i$ incorporation from [γ-^{32}P]ATP into the BLM, in the presence of TPA.

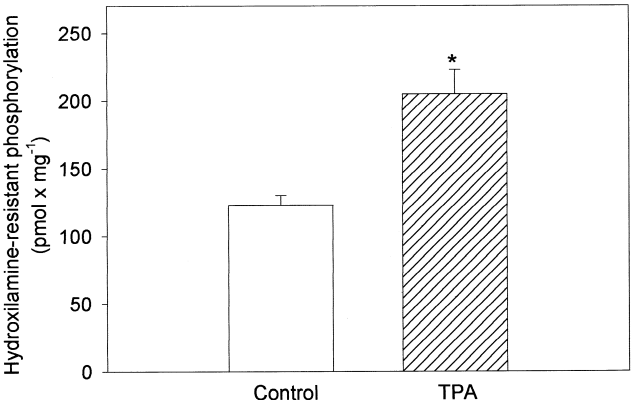

Fig. 2. Evidence for the phosphorylation of BLM proteins induced by PKC. Hydroxylamine-resistant phosphorylation of BLM proteins was measured in the presence or in the absence of 20 ng/ml TPA, as described in Section 2. *Statistically significant when compared to control ($n=6$, $P<0.05$).

The total hydroxylamine-resistant phosphorylation of BLM proteins increased from 123 ± 7 pmol P_i mg^{-1}, observed in the absence of TPA, to 198 ± 10 pmol P_i mg^{-1}, measured in the presence of 20 ng/ml TPA, corresponding to a 61% stimulation (Fig. 2). Mendez et al. [22] demonstrated that rat proximal tubule cells present both cytosolic and plasma membrane PKC. They also observed that PKC activity was translocatable to the membrane following phorbol ester treatment. These data indicate that PKC-mediated regulation of the Na^+ proximal tubule reabsorption by hormones involves translocation of PKC from cytosol to plasma membrane. However, our results suggest that the activation of a constitutive PKC of plasma membrane can mediate the actions of hormones in proximal tubule cells. Taken together these data indicate that the basolateral membrane preparation contains an activatable PKC, able to phosphorylate proteins of BLM.

3.2. Activation of the PKC pathway in BLM modulates the Na^+-ATPase activity

To verify a possible modulation of the Na^+-ATPase activity by the PKC pathway, experiments were performed in the presence of TPA and of the PKC inhibitor, Sph. The results are shown in Figs. 3 and 4. As the TPA concentration varied from 5 ng/ml to 40 ng/ml, the enzyme activity increased from 5.8 ± 0.7 nmol P_i mg^{-1} min^{-1} in the control to 10.5 ± 1.1 nmol P_i mg^{-1} min^{-1} in the presence of 20 ng/ml TPA, corresponding to a 90% stimulus (Fig. 3). Furthermore, the stimulatory effect of TPA on the Na^+-ATPase activity was completely abolished by the addition of Sph in a dose-dependent manner, with a maximal effect obtained at 50 ng/ml Sph (3.8 ± 0.6 nmol P_i mg^{-1} min^{-1}; Fig. 4). Studies of the kinetic properties of the TPA-mediated Na^+-ATPase activity stimulation revealed that 20 ng/ml TPA increased the V_{max} of the enzyme from 13.4 ± 0.6 nmol P_i mg^{-1} min^{-1} to 25.2 ± 1.4 nmol P_i mg^{-1} min^{-1}, whereas the apparent affinity for Na^+ ($K_{0.5}$) did not change ($K_{0.5}$ = 17.2 ± 4.3 mM in control and 13.6 ± 3.4 mM in the presence of TPA, $P > 0.5$) (Fig. 5).

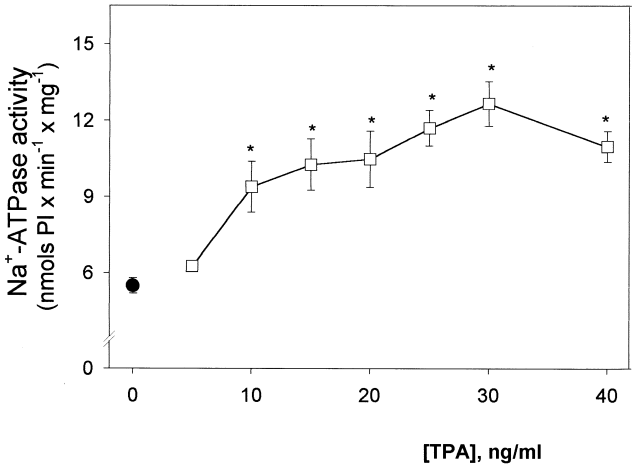

Fig. 3. Evidence for PKC-mediated stimulation of the Na^+-ATPase activity in proximal tubules. The ATPase activity was measured as described in Section 2, in the absence or in the presence of increasing concentrations of TPA (from 5 ng/ml to 40 ng/ml). *Statistically significant when compared to control ($n = 7$, $P < 0.05$).

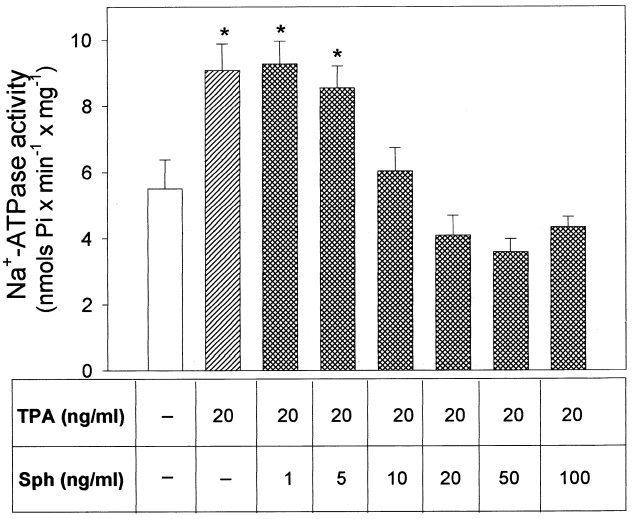

Fig. 4. Modulation of the stimulatory effect of TPA on the Na^+-ATPase activity by the PKC inhibitor Sph. ATPase activity was measured as described in Section 2, in the absence or in the presence of 20 ng/ml TPA and increasing concentrations of Sph (from 1 ng/ml to 100 ng/ml). *Statistically significant when compared to control ($n = 6$, $P < 0.05$).

The involvement of the PKC signal transduction pathway on the modulation of the Na^+-ATPase activity from BLM was further supported by the measurement of the enzyme activity in the presence of the catalytic subunit of PKC (PKC-M). The data summarized in Fig. 6 show that, in the presence of 2 nM PKC-M, the Na^+-ATPase activity of BLM increased from 5.0 ± 0.3 nmol P_i mg^{-1} min^{-1}, mea-

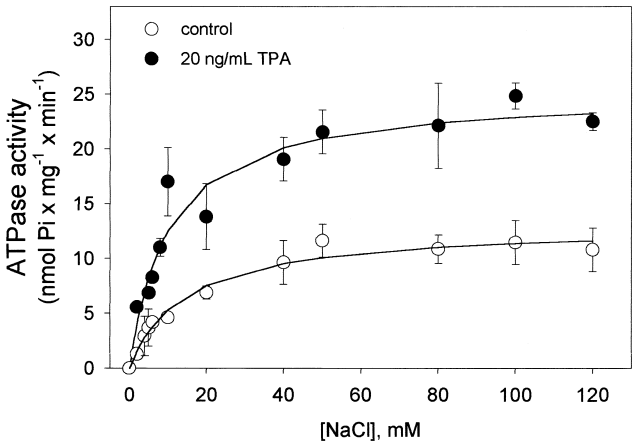

Fig. 5. Analysis of the kinetic parameters of Na^+-ATPase modulated by TPA. The Na^+-ATPase activity was measured in the absence or in the presence of increasing concentrations of Na^+ (from 1 mM to 120 mM). The ATPase activity was measured subtracting the values obtained with the same Na^+ concentration in the absence or in the presence of 20 ng/ml TPA, both in the presence of 1 mM ouabain. *Statistically significant when compared to control ($n=3$, $P<0.05$).

sured in the absence of PKC-M, to 8.7 ± 0.3 nmol P_i mg^{-1} min^{-1}, corresponding to a stimulation of 74% on the enzyme activity.

Finally, Fig. 7A shows the phosphorylation profile of BLM following incubation with [γ-^{32}P]ATP and 20 ng/ml TPA. These results were analyzed by the densitometry of the band intensities, shown in Fig. 7B. The intensity of the band of approx. 100 kDa

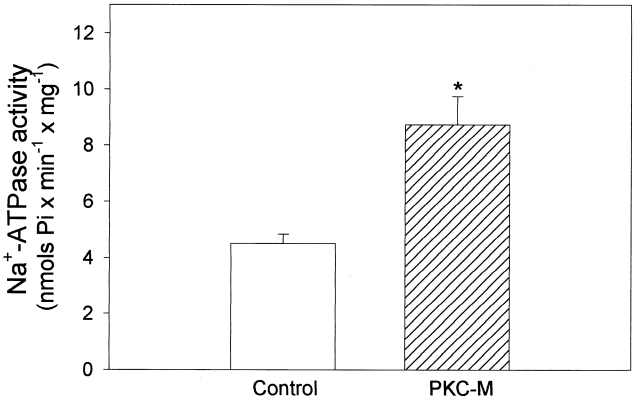

Fig. 6. Confirmation of the PKC-mediated stimulatory effect on the Na^+-ATPase activity of proximal tubules. The ATPase activity was measured as described in Section 2, in the absence or in the presence of 2 nM catalytic subunit of PKC (PKC-M). *Statistically significant when compared to control ($n=5$, $P<0.05$).

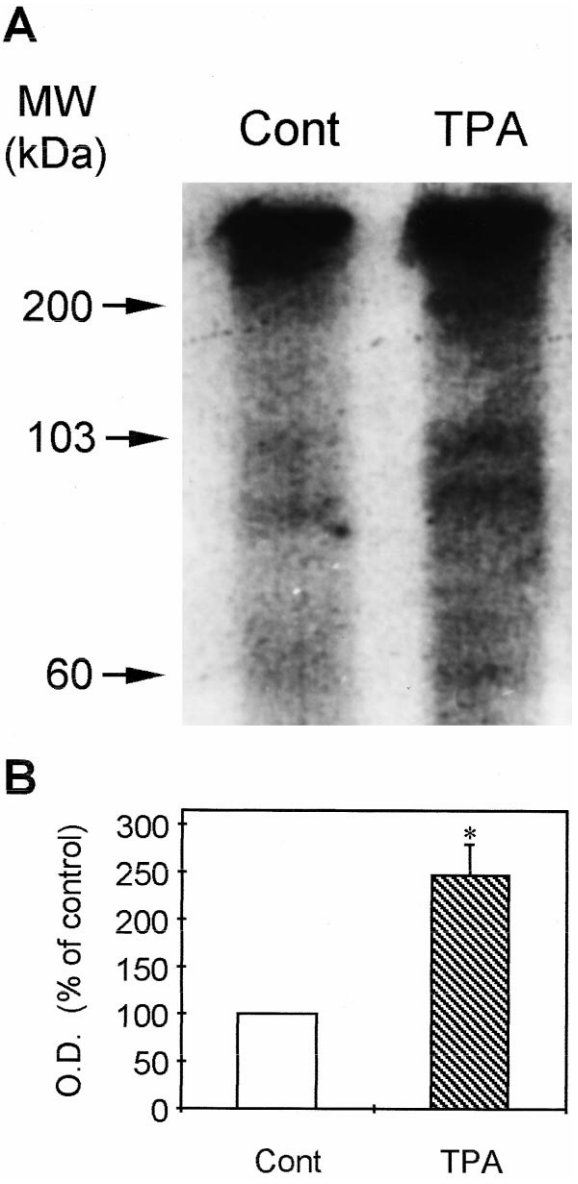

Fig. 7. (A) Analysis of protein phosphorylation profile by autoradiography after SDS-PAGE. Aliquots of phosphorylated proteins from BLM (50 μg of protein samples) were analyzed by SDS-PAGE. The molecular weight markers (myosin, phosphorylase β, bovine serum albumin, ovalbumin, and carbonic anhydrase) were electrophoresed in a neighboring lane of the same gel (not shown) and their corresponding positions are indicated on the autoradiogram. (B) Densitometry of the approx. 100 kDa band under the same conditions. *Statistically significant when compared to control ($n=3$).

increased 150% in the presence of 20 ng/ml TPA, when compared to control. These results indicate that PKC resident in the proximal tubule basolateral membrane is able to phosphorylate proteins present

in this membrane and, consequently, modulate their activities. Marín and coworkers [1] demonstrated that the molecular mass of Na^+-ATPase is approx. 100 kDa. Our data suggest that among the 100 kDa proteins phosphorylated by PKCs could be the Na^+-ATPase of proximal tubules, indicating that the ATPase is an effector protein for PKC regulation in BLM. Furthermore, considering that TPA mimics the effect of DAG on PKC activation, data presented in this report indicate that the Na^+-ATPase activity of proximal tubules is stimulated by a DAG-activated PKC isoform resident in BLM. Recently, our group demonstrated that the Na^+-ATPase activity of BLM is stimulated by the PKA pathway [23]. It has been shown that Na^+ reabsorption in proximal tubule involves the modulation of PKC and PKA pathways, which might regulate different transporters located in the apical and in the basolateral membranes. Furthermore, proximal Na^+ reabsorption mediated by Na^+-ATPase is upregulated by both pathways constitutive of BLM. These mechanisms of regulating the Na^+-ATPase activity could guarantee the fine adjustment of the Na^+ reabsorption, a possible physiological role for this enzyme in the proximal tubules.

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