Biochimica et Biophysica Acta 1838 (2014) 1003–1009

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Biphasic regulation of type II phosphatidylinositol-4 kinase by sphingosine: Cross talk between glycero- and sphingolipids in the kidney

Thiago Lemos [a], Karine S. Verdoorn [a,b], Luciana Nogaroli [a], Thiago Britto-Borges [a], Thaís A. Bonilha [a], Pilar A.M. Moreno [a], Osman Feitosa Silva [a], Giovane G. Tortelote [a], Marcelo Einicker-Lamas [a,b,*]

[a] Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, 21949–902, Ilha do Fundão, Rio de Janeiro, Brazil
[b] Instituto Nacional de Ciência e Tecnologia de Biologia Estrutural e Bioimagem, Brazil

ARTICLE INFO

Article history:
Received 26 August 2013
Received in revised form 9 December 2013
Accepted 12 December 2013
Available online 19 December 2013

Keywords:
Phosphatidylinositol-4 kinase
Sphingosine kinase
Basolateral plasma membrane
Kidney
Signaling

ABSTRACT

Phosphatidylinositol-4 kinase (PI-4K) is responsible for the generation of phosphatidylinositol-4 phosphate (PtdIns(4)P), a bioactive signaling molecule involved in several biological functions. In this study, we show that sphingosine modulates the activity of the PI-4K isoform associated with the basolateral membranes (BLM) from kidney proximal tubules. Immunoblotting with an anti-α subunit PI-4K polyclonal antibody revealed the presence of two bands of 57 and 62 kDa in the BLM. BLM-PI-4K activity retains noteworthy biochemical properties; it is adenosine-sensitive, not altered by wortmanin, and significantly inhibited by Ca^{2+} at the μM range. Together, these observations indicate the presence of a type II PI-4K. Endogenous phosphatidylinositol (PI) alone reaches PI-4K half-maximal activity, revealing that even slight modifications in PI levels at the membrane environment promote significant variations in BLM-associated-PI-4K activity. ATP-dependence assays suggested that the $Mg.ATP^{2-}$ complex is the true substrate of the enzyme and that free Mg^{2+} is an essential cofactor. Another observation indicated that higher concentrations of free ATP are inhibitory. BLM-associated-PI-4K activity was ~3-fold stimulated in the presence of increasing concentration of sphingosine, while in concentrations higher than 0.4 mM, in which S1P is pronouncedly formed, there was an inhibitory effect on PtdIns(4)P formation. We propose that a tightly coupled regulatory network involving phosphoinositides and sphingolipids participate in the regulation of key physiological processes in renal BLM carried out by PI-4K.

1. Introduction

Membrane phospholipids participate in a variety of cellular processes. They establish a hydrophobic barrier, provide a matrix for catalytic processes and influence the functional properties of membrane-associated events [1]. It is also well documented that plasma membrane lipids are implicated in signal transduction events in response to extracellular stimuli. Phosphatidylinositol (PtdIns) and other inositol-containing lipids (phosphoinositides) represent a small fraction of the total glycerolipids present in all cellular membranes. These phosphoinositides are known to regulate distinct cell signaling pathways, which in turn modulate important cell functions such as proliferation, differentiation, membrane trafficking and cytoskeletal organization [2,3]. Receptor-stimulated phosphoinositide turnover has drawn much attention from researchers for over 50 years, since a striking increase in ^{32}P-labeling in both PtdIns and phosphatidic acid (PA) was observed after cell stimulation [4]. The synthesis, degradation, and subsequent resynthesis of the phosphoinositides form a crucial cellular metabolic cycle known as the phosphoinositide cycle, and whose proper function is required for cell viability [5,6]. A primary step in the synthesis of the different phosphoinositides is the phosphorylation of PtdIns at the D4 position of the inositol ring. This produces phosphatidylinositol-4 phosphate (PtdIns(4)P), which is the substrate for another phosphoinositide kinase that catalyzes the phosphorylation of PtdIns(4)P in the D5 position and yields phosphatidylinositol-4,5 bisphosphate (PtdIns(4,5)P_2).

PtdIns(4,5)P_2 is further converted by phospholipase C (PLC) into two second messengers, namely, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). The discovery of this step was a major breakthrough in phosphoinositide metabolism due to its importance in the activation of intracellular Ca^{2+} transients through receptor-stimulated events. The hydrophilic headgroup IP_3 also mobilizes Ca^{2+} from internal stores while the hydrophobic backbone DAG activates protein kinase C (PKC) and initiates a variety of Ca^{2+}-dependent, lipid-dependent cell responses [6–8].

In the last twenty years, it has become clear that phosphoinositides act as more than second messenger precursors. For instance, PtdIns(4)P

Abbreviations: BLM, kidney basolateral membranes; BLM-PI4K, phosphatidylinositol-4 kinase from kidney basolateral membrane; DAG, diacylglycerol; DGK, diacylglycerol kinase; EGTA, ethylene glycol bis(β-amino-ethyl ether)*N,N,N′,N′*-tetracetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid; HPLC, high performance liquid chromatography; NaN_3, sodium azide; PA, phosphatidic acid; [$^{32}P_i$]P_i, radioactive orthophosphate; PMSF, phenylmethylsulfonyl fluoride; PtdIns(4)P, phosphatidylinositol-4-phosphate; PtdIns(4)^{32}P, radioactive phosphatidylinositol-4-phosphate; TLC, thin-layer chromatography

* Corresponding author at: Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, 21949–902, Ilha do Fundão, Rio de Janeiro, Brazil. Tel.: +55 21 2562 6520; fax: +55 21 2280 8193.
E-mail address: einicker@biof.ufrj.br (M. Einicker-Lamas).

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http://dx.doi.org/10.1016/j.bbamem.2013.12.007

has been implicated as an important activator of both Ca^{2+}-ATPases [9–11] and ion channels [12], which suggests that this acidic phospholipid is a direct modulator of Ca^{2+} homeostasis and perhaps even most of the Ca^{2+}-dependent cell functions. Thus, enzymes that phosphorylate PtdIns on the D4 position of the inositol ring, which are referred to as PtdIns-4 kinases (PI-4K), now are critical components of cell biology metabolism [13].

PI-4Ks have two major classifications based on their catalytic properties [14]. The type II enzymes are membrane-bound proteins that have been purified from different sources with molecular weights in the range of 55–60 kDa. They have a high affinity for ATP (K_m in the 10–50 μM range) and thus high sensitivity to inhibition by adenosine (K_i in the 10–100 μM range) [14]. Their activity is greatly stimulated by detergents, requires Mg^{2+} (or Mn^{2+}) and is inhibited in the presence of micromolar concentrations of Ca^{2+} (for review see [14–16]). On the other hand, type III PI-4K are larger proteins with molecular weights ranging from 90 to 230 KDa. They have low affinity for ATP (K_m in the 400–800 μM range), low sensitivity to adenosine (K_i above 1 mM) and are not commonly associated to membranes [3,14]. The type I PtdIns kinase refers to the PI-3 K family [17–19].

Basolateral membranes (BLM) from kidney proximal tubules constitute an interesting model for the study of phosphoinositide turnover and its physiological roles, because they preserve a variety of different enzymes and receptors involved with this process, including surface receptors, trimeric G-proteins, protein kinases, phospholipases and different ion transporters [20–22]. Previous results from our laboratory demonstrated the formation of PtdIns(4)P in phosphorylation assays of purified BLM from pig kidney [11,23,24]. The results indicated the presence of a mechanism involved in the metabolism of phosphoinositides and suggested that this signaling lipid is involved in the regulatory network associated with this membrane. In addition, we demonstrated the existence of crosstalk between phosphoinositides and sphingolipid metabolism, suggesting their potential involvement in the regulation of kidney BLM functions [23].

The main goal of this paper is to describe the influence of sphingolipid metabolism on BLM-associated-PI-4K activity, and to better characterize the biochemical properties of this enzyme. Both bioactive lipid classes (phosphoinositides and sphingolipids) have important roles in transepithelial ion fluxes by locally controlling the levels of important lipid mediators such as PtdIns(4)P, diacylglycerol (DAG) and sphingosine-1 phosphate (S1P), which further modulate the ion pumps and other transporters based on the BLM [20–22].

2. Material and methods

2.1. Material

Buffers, bovine serum albumin and protease inhibitors were obtained from Sigma Chemical Co. (Saint Louis, MO). Percoll was from Pharmacia (Uppsala, Sweden). Thin layer chromatography (TLC) plates and all the reagents used in the chromatographic procedures were provided by Merck (Darmstaadt, Germany). All the solutions and films used in autoradiograms were from Kodak (Resende, Brazil). Distilled water deionized with the Milli-Q system of resins (Millipore Corp., Marlborough, MA) was used to prepare all solutions. [$^{32}P_i$]P_i was obtained from IPEN (São Paulo, Brazil). [γ-^{32}P]ATP was prepared as described [25]. The antibody anti-PI-4K α-subunit was purchased from Santa Cruz Research Antibodies (Santa Cruz, CA). All the reagents used for SDS-PAGE and the nitrocellulose membranes, ECL kit and hyperfilm used for the immunoblotting assay were provided by Amersham Bioscience (Buckinghamshire, UK).

Pig kidneys were obtained from a slaughterhouse that operates under the supervision of licensed veterinarians. Kidneys were promptly removed after the animals had been killed, and transported in a chilled solution containing 250 mM sucrose, 10 mM Hepes-Tris (pH 7.6), 2 mM EDTA, 1 mM PMSF, and 0.15 mg/ml of soybean trypsin inhibitor. The external portion of the cortex was carefully removed and immediately used for membrane preparation.

2.2. Isolation of basolateral membranes

Purified basolateral membranes derived from kidney proximal tubules were prepared using the Percoll gradient method [26]. Controls for contamination with other membranes were carried out as previously described [20,27]. The specific activity of the basolateral membrane marker $(Na^+ + K^+)$ATPase (163,8 ± 1.3 nmol × mg^{-1} × min^{-1}) was enriched six-fold when compared to that measured in the starting kidney cortex homogenate (not shown). The membranes were stored in 250 mM sucrose in liquid N_2, which preserved marker activity for at least 5 months.

2.3. Phosphorylation assay

We used the procedure previously described [11,23,24] with slight modifications. Except when otherwise noted, the standard assay medium (1 ml) contained 30 mM MES-Tris (pH 7.0), 1 mM [γ-^{32}P]ATP (specific activity 10^5 cpm/nmol), 1.1 mM $MgCl_2$ or 1 mM EDTA for Mg^{2+}-free conditions, 10 mM NaN_3, 0.5 mM ouabain, and 0.5 mM EGTA. The reaction was started by the addition of [γ-^{32}P]ATP to the membranes (0.1 mg protein/ml) and the incubation time was 10 min at 37 °C. Except when noted, the substances to be tested under each experimental condition were added immediately before addition of [γ-^{32}P]ATP.

2.4. Lipid analysis

Total lipids were extracted by adding 5 ml of chloroform/methanol/HCl (2:1:0.075, v/v) as described in [28] after incubation of the membranes. The resulting organic phase was dried under N_2, reconstituted in 90 μl of chloroform/methanol/H_2O (7.5:2.5:0.2 v/v), and spotted onto heat-activated silica-gel 60 TLC plates. The plates were developed in chloroform/acetone/methanol/acetic acid/water (120:45:39:36:24, v/v), and the lipids that were phosphorylated by [γ-^{32}P]ATP were detected in the autoradiograms of the TLC plates as described elsewhere [11,23,24].

2.5. Measurement of phosphatidylinositol-4 kinase activity

The PtdIns(4)^{32}P spots detected by autoradiography were scraped from the TLC plate, put in a vial and counted in a liquid scintillation counter. The activity was expressed as pmol PtdIns(4)P × mg^{-1} × min^{-1}.

2.6. Determination of free ATP and the $MgATP^{2-}$ complex concentrations

Free ATP and $MgATP^{2-}$ at different ATP and $MgCl_2$ concentrations were calculated using software that took into account the different species involved in the equilibria between EGTA, contaminant Ca^{2+} (10 μM), ATP, Mg^{2+} and H^+, and the influence of ionic strength on the association constants for ATP and Mg^{2+} [29].

2.7. Protein determination assay

A 10 μl aliquot (in triplicate) was used for the protein determination assay, which was performed using the Folin phenol method described by Lowry et al. [30] with the addition of 5% SDS in the samples, and bovine serum albumin as standard.

2.8. SDS-PAGE and immunoblotting

The BLM fractions were solubilized in a small volume of sample buffer to a final concentration of 2 μg/μl. The maximal volume/slot was 50 μl. The total BLM proteins were separated and identified in 12.5% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using a Mini PROTEAN®3 System (Bio-Rad Laboratories, Hércules, CA)

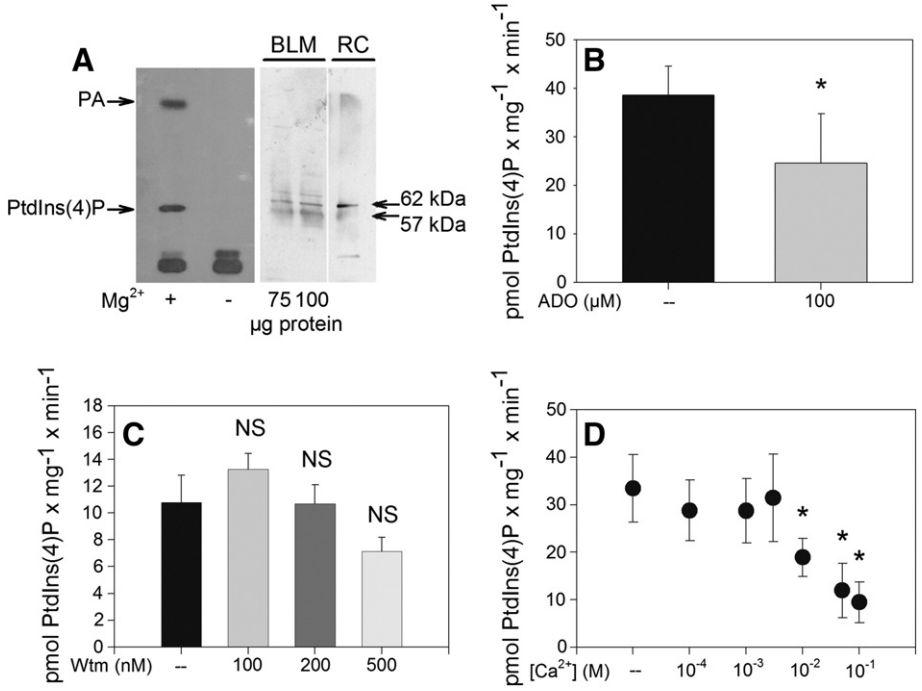

Fig. 1. Evidence for the presence of a PI-4K type II in BLM from kidney proximal tubules. A, left panel. Autoradiogram of a TLC plate showing that only PtdIns(4)^{32}P and ^{32}PA are formed in a Mg^{2+}-dependent fashion after the phosphorylation assay carried out as described in Section 2 [24]. A, right panel. Immunoblotting detection of the PI-4K type II α-subunit. Total proteins from BLM fraction were separated by SDS-PAGE (10% gel) and PI-4K was further identified by immunoblotting as described in Section 2.8, using aliquots of 75 or 100 µg BLM protein as indicated. BLM = basolateral membranes; RC = rat cerebellum. B. Adenosine-sensitivity of BLM-PI-4K activity. The activity was assayed at 120 µM ATP in the absence (black bar) or presence (gray bar) of 100 µM adenosine. Results express means ± SEM from different experiments done in triplicate (n = 5). * Statistically significant with $p < 0,05$. C. BLM-associated-PI-4K activity was insensitive to wortmannin (Wtm). BLM-associated-PI-4K activity was performed as described in Section 2.5, in the absence or presence of different concentrations of Wtm, as shown in the abscissa. NS = not statistically significant. D. The BLM-associated-PI-4K activity was significantly inhibited by high Ca^{2+} concentrations (mM range). BLM-associated-PI-4K activity was performed as described in Section 2.5, in the absence or presence of different concentrations of free Ca^{2+}, as shown in the abscissa. Results are expressed as means ± SEM from different experiments done in triplicate (n = 5). * Statistically different ($p < 0,05$).

at 60 mA/gel. The proteins were transferred at 350 mA to a Hybond™ nitrocellulose membrane (Amersham Bioscience, Uppsala, Sweden) using the same Bio-Rad system described above for approximately 90 min. The nitrocellulose membrane containing the immobilized proteins was first blocked with non-fat dry milk (5%) plus BSA (1%) in Tris buffered saline containing Tween (0.1%) (TBS-T), for 90 min. After the blockage, the membrane was washed twice in TBS-T under constant stirring for 3 min. Next, the membrane was incubated with the polyclonal antibody anti-PI-4K α-subunit (1:300) under constant stirring for 2 h at room temperature. The membrane was washed again (five times, 3 min each) with TBS-T. The secondary antibody used was an anti-mouse HRP (1:2000) which was incubated with the membrane for 90 min at room temperature under constant stirring with a sequential wash (five times, 3 min each) with TBS-T. The PI-4K was detected using the chemiluminescence ECL™ system and Hyperfilm. The molecular weight was determined by using pre-stained molecular weight standards.

2.9. Statistical analysis

The results were expressed as means ± SEM of at least three separate determinations that were performed in triplicate with different membrane preparations. Where appropriate, data was compared by analysis of variance (ANOVA) using the statistical software package SPSS 8.0.

3. Results

3.1. BLM-associated-PI-4K identification and activity

Prior observations from our group reported the detection of a PI-4K activity associated to the BLM from kidney proximal tubule cells [11,23,24]. Here we explored the main requirements for the BLM-associated-PI-4K activity in order to provide evidence that the enzyme in question is a type II PI-4K. Fig. 1A (left panel) shows the formation of PtdIns(4)^{32}P, after pre-incubation of the BLM fraction in the presence of [γ-^{32}P]ATP, followed by lipid extraction and separation by TLC as described in the Methods section of this paper. In spite of the presence of different phospholipids revealed after the exposure of the TLC plates to iodine vapors (data not shown), only two phospholipids were significantly labeled with the use of a specific activity of 10^5 cpm/nmol [γ-^{32}P]ATP,[1] as revealed by the autoradiogram of the respective TLC plate: PtdIns(4)^{32}P and ^{32}P-PA (see also [24]). The left panel for Fig. 1A also shows that the PI-4K activity was completely abolished when Mg^{2+} was removed from the reaction medium. The presence of a PI-4K associated with the BLM was further confirmed by immunoblotting with the use of a polyclonal antibody anti-PI-4K α-subunit, which recognizes different PI-4K α-subunits. Fig. 1A (right panel) reveals that the antibody recognized two main bands at ≈57 and 62 kDa, which are also present in rat cerebellum (RC), used here as a positive control, and correspond to the type II PI-4K α-subunit. It is also possible to observe other weakly detected bands of higher molecular weight suggesting the existence of other PI-4K isoforms in the BLM.

3.2. BLM-associated-PI-4K features

The low molecular weight detected after immunoblotting suggested that the BLM-associated-PI-4K activity should be ascribed as a type II enzyme. Supplementary Figures show basic assays that attempt to standardize the preferred conditions under which to measure the

[1] PtdIns(4,5)P_2 is detected at highest specific activity (10^{11} cpm/nmol) [31].

BLM-associated-PI4-K activity. This analysis includes a time-course for the formation of PtdIns(4)P that shows a linearity in the PI-4K activity within the time range spanned in the assay (up to 10 min; Suppl. Fig. 1, top panel). We also tested different protein concentrations (Suppl. Fig. 1, bottom panel) as well as the temperature dependence and pH effect for PI-4K activity. Suppl. Fig. 2 shows that the BLM-PI-4K activity gradually increased from 17 °C reaching its maximal value at 37 °C (main panel). The inset to Suppl. Fig. 2 shows that a single Arrhenius function adjusts to the experimental points in the temperature interval 17–37 °C, with an energy of activation value of 58.6 kJ × mol^{-1}. The optimal pH for the BLM-PI-4K activity was in the 6.9 − 7.1 range (Suppl. Fig. 3).

The functional characterization of a type II PI-4K was accomplished through standard experiments. Fig. 1B shows that the BLM-PI-4K activity is highly sensitive to adenosine at a micromolar range (100 μM) and completely insensitive to the PI-3K inhibitor wortmanin, (even if at higher concentrations known to inhibit type III PI-4K (Fig. 1C)). Furthermore, Fig. 1D shows the increased concentrations of Ca^{2+} vastly inhibit BLM-PI-4K at the micro molar range. These results in addition to the molecular weight of the immunodetected protein (Fig. 1A, right panel) clearly indicate that the enzyme in question is a Type II PI-4K associated to the BLM.

3.3. Substrate dependence for the BLM-PI-4K activity

PI-4K has two substrates (the phosphorylatable PtdIns and the phosphorylating ATP) and a cofactor (Mg^{2+}). Fig. 2A shows that endogenous BLM PtdIns is enough to promote the half maximal activation of the enzyme in non-stimulated condition, and Fig. 2B and C show that the true phosphorylating substrate is the $Mg.ATP^{2-}$ complex, since (i) the BLM-associated-PI-4K activity increases (main panel in Fig. 2B) in parallel with the raise in the concentration of $Mg.ATP^{2-}$ (see dashed line in the inset) and (ii) the correlation of PtdIns(4)P formation vs BLM-associated-PI-4K activity/[substrate] decreases according to $Mg.ATP^{2-}$ complex consumption (Fig. 2C) (half maximal activation in the energy–donor substrate curve was found at 24 μM $Mg.ATP^{2-}$). Moreover, Table 1 clearly shows that free ATP is an inhibitor of the enzyme, probably by competition with the $MgATP^{2-}$ complex. When the kinetics of BL-PI-4K activation is examined as a function of total $MgCl_2$, a sigmoidal profile is obtained (Fig. 2B). The cooperativity of the total magnesium curve ($n \approx 2$) suggests that two sites for the cation are present in BLM-PI-4K.

3.4. Crosstalk between glycero- and sphingolipids in the BLM

In previous papers, we demonstrated reciprocal modulation of glycero- and sphingolipids in the BLM [23,24]. Attenuation of the PA synthesis was observed upon addition of sphingosine (Sph) in phosphorylation assays similar to those used here [24], and a ~3-fold stimulation of PI-4K activity was detected with addition of 0.1 mM Sph or 0.4 μM $S1^{32}P$ [23]. In the present study we further characterized the influence of Sph on the BLM-associated-PI-4K (Fig. 3). A ~3-fold augment in BLM-associated-PI-4K activity was detected with increasing Sph concentration, up to 100 μM, followed by an apparent return to the basal activity at higher concentrations (Fig. 3A). Under these higher Sph concentrations, a strong phosphorylation of Sph to sphingosine-1 phosphate (S1P) occurs (Fig. 3B and as previously described in [23]-Fig. 3C). Interestingly, the excitatory effect of Sph on BLM-PI-4K activity seems to be independent of PtdIns levels, given that the addition of this substrate promotes only slight additive effect on PtdIns(4)P generation, compared with the endogenous lipid content (Fig. 3C).

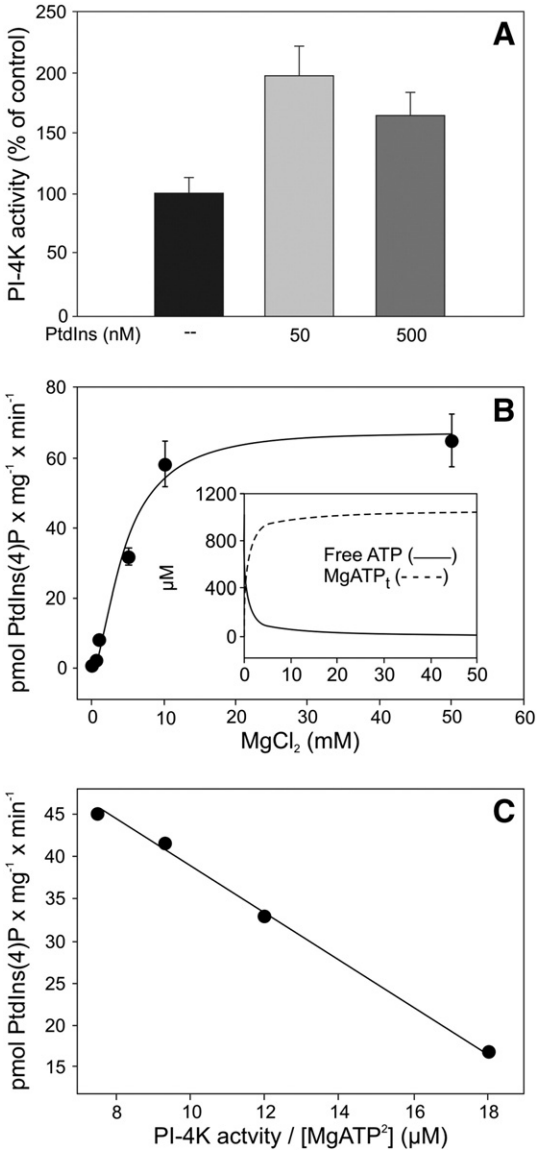

Fig. 2. Substrates-dependency for the BLM-associated-PI-4K activity. A. The BLM-PI-4K activity was performed as described in Section 2.5, without (black bar) and with (gray bars) exogenous PtdIns at 50 or 500 nM, as depicted in the abscissa. Results are expressed as mean ± SEM of four different experiments done in triplicate. B. Correlation between the $Mg.ATP^{2-}$ complex formation according to addition of increased Mg^{2+} and the raise in the BLM-associated-PI-4K activity. PtdIns(4)P formation increases according to the augment in Mg^{2+} up to 15 mM (main panel). The increase in the BLM-associated-PI-4K activity is simultaneously to the formation of the $Mg.ATP^{2-}$ complex (see dashed line in the inset), while it reaches a plateau concomitantly to the consumption of free ATP (see solid line in the inset). The BLM-associated-PI-4K activity was measured at the total $MgCl_2$ concentrations shown on the abscissa in a fixed concentration of ATP 1 mM and the other standard conditions described in Section 2.5. The smooth curve was adjusted to the experimental points by non-linear regression using the equation BLM-associated-PI-4K activity = $V_{max} \times [MgCl_2]^n / \{(K_{0.5\ Mg})^n + [MgCl_2]^n\}$. $K_{0.5Mg}$ = 4.7 mM is the concentration of $MgCl_2$ that gives half-maximal velocity (V_{max} = 67.5 pmol × $mg^{-1} \times min^{-1}$) and n is a cooperativity index (1.85). Inset: variation in the concentrations of the $MgATP^{2-}$ complex and of free ATP with increasing $MgCl_2$ concentrations. C. Abscissa: BLM-associated-PI-4K activity/$[MgATP^{2-}]$; ordinate: BLM-associated-PI-4K activity. The straight line was adjusted to the data by least squares using the equation BLM-associated-PI-4K = $V_{max} - b \times [MgATP^{2-}]$. Values of V_{max} = 65.9 pmol $mg^{-1} \times min^{-1}$ and $K_{m\ MgATP}$ = 24 μM were calculated from the y- and x-intercepts, respectively. The concentrations of $MgATP^{2-}$ complex (1 mM total ATP, varying $MgCl_2$ concentrations) were calculated as described in Material and Methods.

4. Discussion

Phosphatidylinositol kinase activity has been reported to be present in a variety of cellular membranes while different isozymes of PI-4K are present within the cell — suggesting that the product of its reaction may have more than one biological function as well as different mechanisms for their regulation [5]. The detection of PI-4K activity associated to the BLM has already been reported in previous work from our laboratory [11,23,24]. Here, we added new insights and detailed biochemical characterization of the BLM-associated-PI-4K function and regulation. The

Table 1
Free ATP inhibits BLM-associated-PI-4K activity[a].

Total ATP (mM)	$MgATP^{2-}$/free ATP ratio	PI-4K activity (pmol × mg^{-1} × min^{-1})
0.7	5.0	44.9
1.0	3.2	32.7
1.5	1.6	24.0
2.0	1.0	14.9
5.0	0.3	9.0

[a] BLM-associated-PI-4K activity was measured under the standard conditions described in Material and Methods at the total [γ-^{32}P]ATP concentrations shown. Free ATP and $MgATP^{2-}$ concentrations (obtained with 1.1 mM $MgCl_2$ and varying the total ATP) were calculated as described in Material and Methods [29].

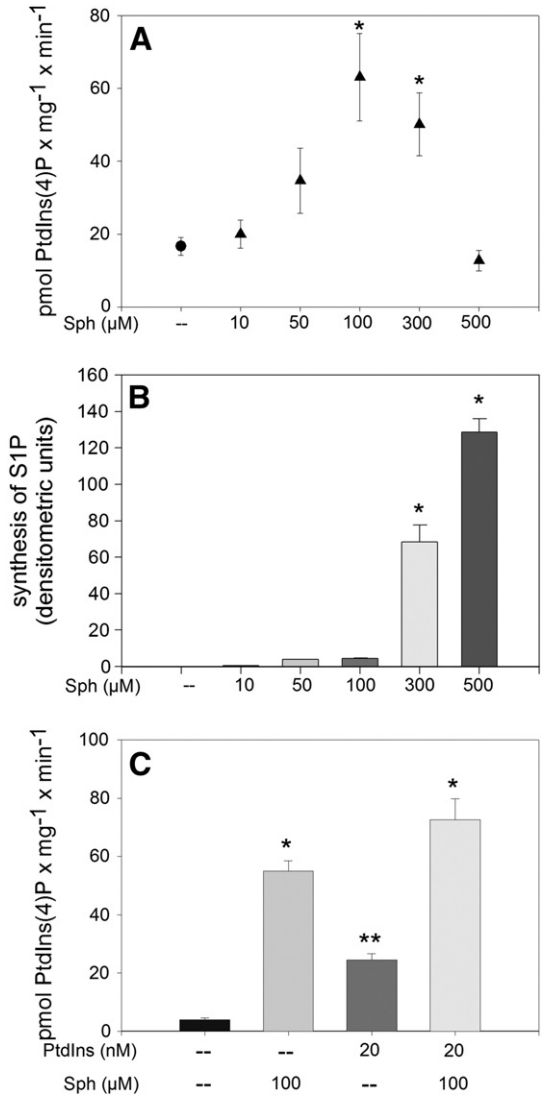

Fig. 3. BLM-associated-PI-4K activity is influenced by the presence of Sph and by its phosphorylated derivative S1P. A. PtdIns(4)P formation is activated by increasing concentration of exogenous Sph up to 100 μM. The BLM-associated-PI-4K activity was performed as described in Section 2.5, without and with exogenous Sph, as depicted in the abscissa. Results are expressed as mean ± SEM of four different experiments done in triplicate. B. Increased $S1^{32}P$ synthesis in BLM preparation incubated with a high μM range of exogenous Sph (from 300 to 500 μM). Phosphorylation assays and lipid extraction were conducted as described in Sections 2.3 and 2.4. $S1^{32}P$ and $PtdIns(4)^{32}P$ formation were measured in the same assays carried out with increasing concentrations of Sph, as shown in the abscissa. Results expressed as means ± SEM from four different experiments done in triplicate (n = 4). * Statistically different ($p < 0.01$). C. The BLM-associated-PI-4K activity was performed as described in Section 2.5, without or with the addition of exogenous PtdIns at 20 nM, or Sph at 100 μM, as depicted in the abscissa. Results are expressed as mean ± SEM of four different experiments done in triplicate. * Statistically different with $p < 0.05$; ** $p < 0.01$.

immunodetection of two bands of ≈57 and 62 kDa in the BLM is related to the type II PI-4K, which are known to be associated with plasma membranes and were already purified from different sources with similar molecular weight [14–16]. The $K_{mMg.ATP}$ found (24 μM) is also compatible with that described for type II PI-4K [3,14]. The existence of two Mg^{2+} sites, revealed by the cooperativity of the $MgCl_2$ curve ($n = 1.85$; Fig. 2B) and supported by the observation that the $Mg.ATP^{2-}$ complex is the true energy-donor substrate at a $K_{Mg} = 4.7$ mM in the extracellular concentration range, is compatible with a model in which BLM PI-4K has a cytosolic active site (for the complex $Mg.ATP^{2-}$) and a regulatory site for free Mg^{2+} that faces the interstitial space.

The importance of phosphoinositides in cell signaling events of the kidney has been studied for several years [32,33] and our group showed evidences of glycero- and sphingolipids interaction [24], especially activation of PI-4K by Sph [23]. In the present study, detailed substrate characterization of the BLM-associated PI-4K of proximal tubule cells is provided and for the first time the biphasic modulation of PI-4K by Sph is presented. Analyzing these and our previous results [23], it is possible to hypothesize two mechanisms of Sph-mediated PI-4K activity regulation. At first, the trade-off between PI-4K activity and S1P formation (Fig. 3A and B, respectively) is suggestive of an antagonistic role of Sph and its phosphorylated derivative: while Sph acts stimulating PtdIns(4)P formation in BLM, higher availability of S1P inhibits this process. The role of Sph on cell metabolism usually involves its action as an intracellular messenger, modulating the activity of other important cell signaling kinases, such as PKC. In addition, much of its physiological effect seems to be counterbalanced (at least partially) by the formation of S1P. The second potential mechanism accounting for the biphasic effect of Sph on PI-4K relies on our previous results showing an agonistic effect of BLM-derived S1P on PI-4K activity (see Fig. 6 from [23]). In this hypothesis the enhancement of the kinase's activity with 100 μM Sph could be due to the low level S1P formation (note that increased activity of PI-4K in [23] was observed with nanomolar levels of S1P). Conversely, higher levels of this phophorylated sphingolipid could have the opposite effect on PtdInd(4)P formation. Whatever the mechanism involved, the balance between Sph/S1P level should be considered critical for regulation of PI-4K activity in BLM of kidney cells.

The physiological relevance of PI-4K in BLM can be directly associated with ion transport across the renal epithelium, judging by the modulatory effect of PtdIns(4)P on different plasma membrane ion transporters and channels [9,11,12]. The generation of PtdIns(4)P in the BLM of proximal tubule cells can also be important for other cell processes [34], as well as being converted to $PtdIns(4,5)P_2$ [35] or $PtdIns(3,4)P_2$, which are involved in a myriad of cellular events including ion transport [13,36]. Different points of convergence among these distinct signaling cascades can play important physiological roles in the BLM [23,37–40]. The first product of PI-4K that is described here can be considered as the first messenger of the network that modulates relevant function in these cells, such as the reabsorption of large amounts of fluid.

PtdIns(4)P and PA are the only products simultaneously phosphorylated at picomolar stoichiometric amounts at the same rate at pH 7.0, 37 °C, 1 mM ATP and 1.1 mM $MgCl_2$ (Supplementary material) suggesting that PI-4K (presented here) and diacylglycerol kinase (DGK) activities [24] are tightly coupled in BLM. This could also be important in the regulation of transport events, since the substrate for DGK, the DAG molecule, is an activator of PKC, which in turn is a potent inhibitor of Ca^{2+} transport [20,41] and an activator of Na^+ transport [31,42]. Different thermal behavior of PI-4K and DGK (Fig. S3; compare with Fig. 3 in [24]) and the contrasting response to Sph in conditions in which the phosphorylated form of this sphingolipid prevails [23], indicate that alterations in the neighboring lipid microenvironment of the two glycerolipid kinases could be important in the balance of PtdIns(4)P/PA within the renal membranes. The non-additive effects of PtdIns and Sph on PI-4K activity (Fig. 3C) may indicate that the

efficient modulation triggered via sphingolipids can occur independently of local PtdIns fluctuation.

The opposite effect of Sph on DGK (see [24]) and BLM-PI4-K (present study) deserve further mention due to their potential physiological relevance. It can be hypothesized that under conditions in which ceramidase activity and Sph release are stimulated above the normal levels [43,44] with preservation of high DAG levels (as in the case of a rise in circulating angiotensin II and concomitant activation of PLC [41]) the stimulation of PtdIns(4)P formation by Sph (Fig. 3A) could compensate for uncontrolled increase in intracellular Ca^{2+} in kidney cells. Plasma membrane Ca^{2+}-ATPase activity, which is considered responsible for fine tuning cytosolic Ca^{2+} levels [45,46] can strongly be stimulated by PtdIns(4)P [9,11], and could attenuate the DAG-stimulated PKC inhibition of Ca^{2+}-ATPase. Since the present work was carried out in the absence of any agonist, it can be postulated that different hormones and/or autacoids that are able to activate PKC, such as adenosine and angiotensin II [20,41], are physiological cognates in the modulation of PI-4K and in their associated regulatory networks in kidney cells.

Thorough comprehension of the biochemical properties of these enzymes could lead to new drug development that would be aimed at targeting the production of glycero- and sphingolipid-derived second messengers inside kidney cells and thereby regulating the hydro- and electrolytic balance of the kidney.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2013.12.007.

Acknowledgments

The authors would like to thank Mr. Alexandre Abrantes for helpful technical assistance. AA was the recipient of a technical assistance fellowship from Fundação Carlos Chagas Filho de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ). TAB and PAMM were undergraduate students of the PINC/Federal University of Rio de Janeiro Medical School Program and fellows of the CNPq PROFIX Program. AV and ME-L are fellowships from CNPq and from the Program Cientistas de Nosso Estado – FAPERJ. The authors are thankful to Dr. Evald Muraj for critical review of this manuscript.

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