J. Biochem. **134**, 529–536 (2003)
DOI: 10.1093/jb/mvg170

Sphingosine-1-Phosphate Formation Activates Phosphatidylinositol-4 Kinase in Basolateral Membranes from Kidney Cells: Crosstalk in Cell Signaling through Sphingolipids and Phospholipids

Marcelo Einicker-Lamas[*,1], Leandro D. Wenceslau[*,1], Robson R. Bernardo[1], Luciana Nogaroli[1], Adilson Guilherme[2], Mecia M. Oliveira[1] and Adalberto Vieyra[†,1]

[1]Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21941-590, Brazil; and [2]University of Massachusetts Medical School, Worcester, MA 01605, USA

Received May 1, 2003; accepted July 19, 2003

Sphingosine-1-phosphate (S1P) and phosphatidylinositol-4 phosphate [PtdIns(4)P] are important second messengers in various cellular processes. Here, we show that S1P and PtdIns(4)P are formed in purified basolateral membranes (BLM) derived from kidney proximal tubules, indicating the presence of a plasma membrane associated SPK (BLM-SPK) and phosphatidylinositol-4 kinase (PI-4K). We observed that S1P synthesis is linear with time, dependent on protein concentration, and saturable in the presence of increasing concentrations of sphingosine. Different from the observations on cytosolic SPKs, the formation of S1P by BLM-SPK is Mg^{2+}-independent and insensitive to the classical inhibitor of the cytosolic SPKs, DL-*threo*-dihydrosphingosine. With sphingosine as substrate, the enzyme shows cooperative kinetics (n = 3.4) with a $K_{0.5}$ value of 0.12 mM, suggesting that BLM-SPK is different from the previously characterized cytosolic SPK. The formation of PtdIns(4)P markedly inhibits BLM-SPK activity. Conversely, a strong activation of PtdIns(4)P synthesis by the formation of S1P is observed. Taken together, these results indicate that (i) basolateral membranes from kidney cells harbor a SPK activity that potentially regulates renal epithelium function, and (ii) the formation of S1P mediated by SPK enhances PI-4K activity, while PtdIns(4)P in turn inhibits SPK, suggesting an interplay between these lipid signaling molecules. These findings suggest the possibility of crosstalk between sphingolipids and glycerolipids, which might be involved in the regulation of transepithelial fluxes across the BLM of kidney cells.

Key words: basolateral membranes, kidney, phosphatidylinositol-4 kinase, sphingosine kinase, sphingosine-1-phosphate.

Abbreviations: BLM, kidney basolateral membranes; BLM-SPK, sphingosine kinase from kidney basolateral membranes; EGTA, ethylene glycol bis(β-amino-ethyl ether)*N,N,N′,N′*-tetracetic acid; GroPtdIns, glyceroinositols; Hepes, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid; HPLC, high performance liquid chromatography; NaN_3, sodium azide; PI-4K, phosphatidylinositol-4 kinase; PtdIns(3)P, phosphatidylinositol-3-phosphate; PtdIns(4)P, phosphatidylinositol-4-phosphate; $^{32}P_i$, radioactive orthophosphate; PMSF, phenylmethylsulfonyl fluoride; PKC, protein kinase C; Sph, sphingosine; SPK, sphingosine kinase; S1P, sphingosine-1-phosphate; TLC, thin-layer chromatography.

Sphingolipids are well-recognized components of virtually all vertebrate and many invertebrate cells, and for many years were viewed only as structural components of cellular membranes. Nowadays, sphingolipids represent another growing class of lipid mediators that include ceramides, sphingosine (Sph) and its phosphorylated derivative sphingosine-1-P (S1P) that are reported to have important cellular functions through different cell signaling pathways (*1–5*). Earlier studies have demonstrated the presence of cytosolic and microsomal bound sphingosine kinase (SPK) activities in different tissues (*6*, *7*). Later, S1P was shown to be a potent signaling molecule since it promotes the rapid release of Ca^{2+} from internal stores in an IP_3-insensitive manner (*1*, *8*, *9*). S1P also participates in many different cellular events including cell proliferation (*4*, *10*, *11*), suppression of apoptosis (*12*, *13*), modulation of cell motility, tumor invasiveness (*14*, *15*), neurite retraction (*16*) and angiogenic vascular maturation (*17*). This broad variety of physiological effects is associated with the localization of the enzyme in both the cytosolic and membrane compartments (and also in the extracellular compartment), where different forms of SPK exist (*17–19*).

Under basal conditions, S1P levels are very low; however, upon stimulation by growth factors or other agonists, a transient increase in S1P levels can be detected (*2*, *20*). As it is a second messenger, its intracellular levels must be under rigorous control. In addition to the participation of SPKs (*17–19*), the synthesis of ceramide *via* the fumonisin B1-sensitive ceramide synthase pathway may contribute to the regulation of S1P levels (*2*), and at least

[*]M.E-L. holds a PROFIX fellowship from CNPq-Brazil. L.D.W. is a recipient of a fellowship from CNPq/PIBIC (PINC Program, School of Medicine).
[†]To whom correspondence should be adressed. Fax: +55-21-22808193, E-mail: avieyra@biof.ufrj.br

two other enzymes are involved in the degradation of S1P: a specific phosphatase and a lyase (*21–24*). The discovery of a G-protein coupled S1P receptor superfamily, called endothelial differentiation gene 1 receptors (Edgs) (*5*, *25–30*), strongly suggests that S1P can act as a dual messenger: an extracellular ligand for cell surface receptors as well as an intracellular signaling molecule (*25–31*).

Phosphoinositides are glycerophospholipids that are also involved in different cell signaling pathways in almost all cell types (for review see Ref. *32*). In the last fifteen years, it has become increasingly clear that polyphosphoinositides cover a wider variety of functions other than just their role as second messenger precursors (*33*, *34*). Such is the case of phosphatidylinositol-3-phosphate (*35*) and phosphatidylinositol-4-phosphate [PtdIns(4)P] (for review see Ref. *34*).

Renal SPK was purified and characterized from total homogenates of rat kidneys (*36*). Recently, Gijsbers *et al.* (*19*) showed separated cytosolic and membrane-bound SPK activities in rat kidney. In the present work, we show the formation of S1P and PtdIns(4)P in purified basolateral membranes obtained from pig kidney proximal tubules in which 75–80% of the glomerular filtrate is reabsorbed. Our results also show crosstalk between the SPK and PI-4K signaling pathways, indicating not only a new biosynthetic pathway for S1P, but also suggesting the possibility of new physiological roles for SPK/S1P and PI-4K/PtdIns(4)P in the regulation of enzymes and transporters present in renal basolateral plasma membranes.

EXPERIMENTAL PROCEDURES

Materials—Sphingosine [the naturally occurring D(+)-*erytro-trans*-isomer], S1P standard, sphingosine kinase inhibitor (DL-*threo*-dihydrosphingosine), 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 reagents used in the chromatographic procedures were from Merck (Darmstadt, Germany). All solutions and films used in autoradiograms were from Kodak (Resende, Brazil). Distilled water deionized with the Milli-Q system of resins (Millipore, Marlborough, MA) was employed in the preparation of all solutions. $^{32}P_i$ was obtained from IPEN (São Paulo, Brazil). [γ-^{32}P]ATP was prepared as described by Maia *et al.* (*37*).

Pig kidneys were obtained from various slaughterhouses under the supervision of licensed veterinarians. Kidneys were rapidly removed after the animals had been killed, cut into small pieces and transported in a chilled solution of 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.

Basolateral Membrane Isolation—Purified basolateral membranes derived from proximal tubules were prepared using the Percoll gradient method (*38*). The orientations of the isolated membranes were right-side-out ≈60%, inside-out ≈8% and unsealed fragments ≈32% (*39*). Controls for enrichment and against contamination with other membranes were carried out as previously described (*40*, *41*). The specific activity of the basolateral membrane marker (Na^++K^+)ATPase measured in preparations selected at random (370 ± 10.6 nmol mg^{-1} min^{-1}) was ≈ 40-fold enriched as compared to that measured in the starting kidney cortex homogenate. The membranes were stored in 250 mM sucrose in liquid N_2, which preserved marker activity for at least five months. Membrane protein content was determined by the phenol Folin reagent using bovine serum albumin as standard.

Phosphorylation Assay—We used the procedure previously described (*42*) except that detergents were not employed. Briefly, the assay medium (1 ml) contained 30 mM MES-Tris (pH 7.0), 1 mM [γ-^{32}P]ATP (specific activity ≈2.2×10^8 cpm/μmol), 1.1 mM $MgCl_2$ or 1 mM EDTA for Mg^{2+}-free conditions (when stated), 10 mM NaN_3, 1 mM ouabain, and 0.5 mM EGTA. The reaction was started by adding [γ-^{32}P]ATP to the membranes (0.2 mg protein/ml). Sph was dissolved in dimethyl sulfoxide (Me_2SO) and added directly to the reaction medium under intense vortexing. The maximal Me_2SO concentration in the assay (at 400 μM Sph) was 3%. DL-*threo*-dihydrosphingosine was also dissolved in Me_2SO. Except when noted, the different substances to be tested under each experimental condition were added at the beginning of the reaction.

Lipid Analysis by TLC—Total lipids were extracted after incubation of the membranes by adding 5 ml of chloroform:methanol:HCl (2:1:0.075, v/v) as described by Horwitz and Perlman (*43*). Lipids were separated and first identified by TLC using chloroform:acetone:methanol:acetic acid:water (120:45:39:36:24, v/v). Lipids phosphorylated by [γ-^{32}P]ATP were detected in autoradiograms of the TLC plates (*42*). Bidimensional chromatography was performed as described (*44*). In the experiments in which the phosphorylated lipids were added back to the membranes, the lipids were eluted from the silica with the same solvent employed for lipid extraction (see above), dried under N_2, and dissolved in chloroform to prepare a solution of known concentration. Aliquots were put in test tubes, dried again to remove the solvent and sequentially supplied with reaction medium and membranes under vigorous vortexing. The recovery after elution (determined from the residual radioactivity in the silica powder) ranged between 80 and 90%.

Identification of PtdIns(4)P and S1P by HPLC Analysis—High performance liquid chromatography (HPLC) was performed using a SCL-10AVP System (Shimadzu, Tokyo) connected to a 20-μl loop (model LC-10AT). Radiolabeled PtdIns(4)^{32}P detected on the TLC plates was recovered by scraping the corresponding spots and re-extracting from the silica as described above, deacylated, and analyzed by HPLC according to Serunian *et al.* (*45*). The retention time was compared with those of deacylated [^{3}H]PtdIns(4)P and PtdIns(3)^{32}P standards prepared as previously described (*46*). The column used was a Partisphere Sax column (Whatman, Cliffton, NJ) and the mobile phase was 1 M $(NH_4)_2HPO_4$ (pH adjusted to 3.8 with phosphoric acid) (*47*). Samples were collected at 30 s intervals and radioactivity was measured in a liquid scintillation counter.

To confirm the identity of S1^{32}P, the spot obtained after bidimensional TLC was recovered by scraping and re-extracting. HPLC analysis of this sample was carried out

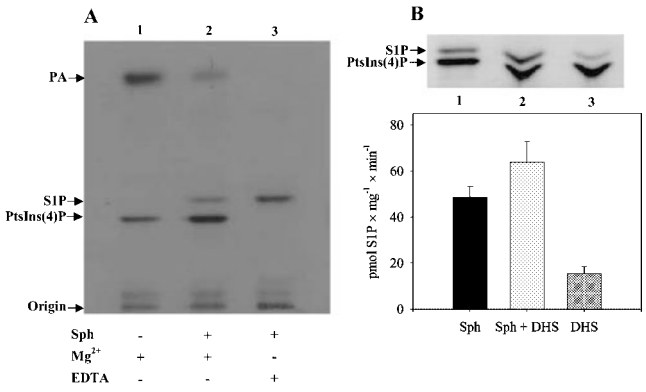

Fig. 1. **Presence of a sphingosine kinase (SPK) in basolateral membranes (BLM) of kidney proximal tubules.** A: Autoradiogram of a TLC plate showing the lipid phosphorylation pattern. Lane 1: control; lane 2: BLM incubated with 0.1 mM Sph; lane 3: as in lane 2 without Mg^{2+}. B: Autoradiogram of BLM incubated with 0.1 mM Sph in the absence (lane 1) or presence of the inhibitor DL-*threo*-dihydrosphingosine (1 mM) (lane 2). Lane 3 is a control to which only the inhibitor was added. The data are representative of TLC experiments on three different membrane preparations performed in duplicate. The graph shows the fold increase in SPK activity under the different conditions. SE was calculated from the absolute values. PA: phosphatidic acid; S1P: sphingosine-1-phosphate; PtdIns(4)P: phosphatidylinositol-4-phosphate.

using a reverse phase column (LiChrosphere® 60 RP-18, 12.5 × 0.4 cm I.D., 5 μm, Merck, Darmstadt, Germany) (*47*), eluted with an isocratic mobile phase (acetonitrile: isopropanol: methanol:water, 50:27:18:5, v/v) at a flow rate of 1.0 ml/min. The peaks corresponding to the standard S1P (Sigma Chemical Co., St. Louis, MO) or the S1^{32}P synthesized by BLM were detected by absorbance at 205 nm. Samples were collected at 1 min intervals and radioactivity was measured in a liquid scintillation counter.

Measurement of Sphingosine Kinase and Phosphatidylinositol-4 Kinase Activities—The S1^{32}P and PtdIns(4)^{32}P spots were detected by autoradiography, scraped from the TLC plates, placed in vials, and measured in a liquid scintillation counter. The kinase activities are expressed as pmol [of S1P or PtdIns(4)P] [mg of membrane protein]$^{-1}$ min^{-1}.

Measurement of Remaining [γ-^{32}P]ATP—Aliquots of samples were removed at different times, centrifuged to sediment the membranes (15,000 ×*g*), and quenched with activated charcoal to adsorb the nonhydrolyzed [γ-^{32}P]ATP. The liberated $^{32}P_i$ was measured in a scintillation counter. The remaining [γ-^{32}P]ATP is expressed as percentage of the control (at time zero).

RESULTS

Formation of Sphingosine-1-Phosphate mediated by Sphingosine Kinase Activity in Basolateral Membranes of Kidney Proximal Tubules—The presence of different lipid kinase activities in purified BLM derived from proximal tubule kidney cells has been reported (*42*). SPK has been purified from rat kidney homogenates (*36*) and, more recently, this activity was demonstrated in vesicles derived from the endoplasmic reticulum and plasma membrane of rat kidney (*19*). To determine whether the purified BLM of kidney proximal tubules contain a SPK activity, initial experiments were conducted to establish assay conditions for quantifying S1P formation in these membranes. As shown in Fig. 1, incubation of purified BLM in the presence of 1.1 mM $MgCl_2$ and 1 mM [γ-^{32}P]ATP for 20 min led to the formation of PtdIns(4)^{32}P (Fig. 1A, lane 1). The presence of S1^{32}P, even in low amounts (<1.0 pmol [mg protein]$^{-1}$ min^{-1}) was also detected by TLC analysis of the reaction products. However, when 0.1 mM of the naturally occurring D(+)-*erythro-trans*-isomer sphingosine was added to the assay (Fig. 1A, lane 2), exogenous Sph was recognized by membrane-associated SPK as judged by the significantly increased formation of S1^{32}P (48.6 ± 4.6 pmol [mg protein]$^{-1}$ min^{-1}) (n = 4). Under experimental conditions (no Sph added), the phosphorylation of endogenous phosphatidylinositol reached 10.3 ± 1.1 pmol [mg protein]$^{-1}$ min^{-1} (n = 4). Interestingly, when 0.1 mM Sph was added, the formation of PtdIns(4)P increased to 32.5 ± 2.4 pmol [mg protein]$^{-1}$ min^{-1} (n = 4) (see also below Fig. 6B).

Consistent with a previous report that PI-4Ks are dependent on $MgCl_2$ in the assay buffer (*48*, *49*), the removal of Mg^{2+} by adding 5 mM EDTA completely prevented the formation of PtdIns(4)^{32}P. In contrast, as seen in Fig. 1A, lane 3, no inhibitory effect on SPK activity was observed after the removal of Mg^{2+}; indeed, the formation

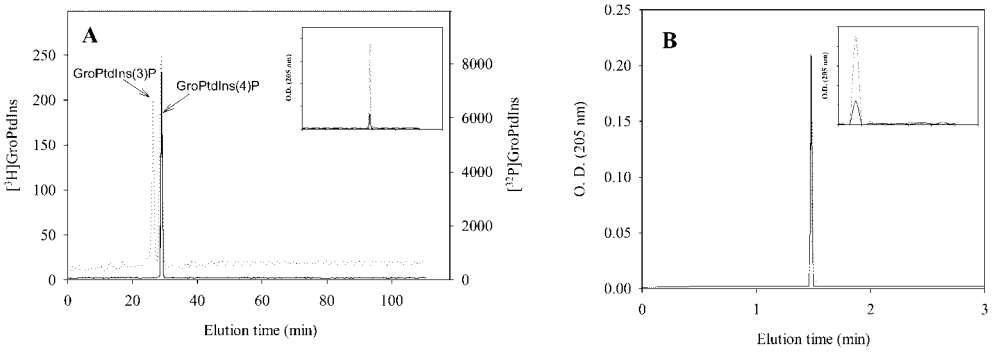

Fig. 2. **Identification of PtdIns(4)P and S1P by HPLC.** Radiolabeled lipids that co-migrated with the PtdIns(4)P and S1P standards on TLC plates were eluted, recovered, and analyzed by HPLC as described under Experimental Procedures. A: PtdIns(4)P. The continous line shows the deacylated product of the reaction catalyzed by BLM-PI-4K obtained as described under "EXPERIMENTAL PROCEDURES." The single peak was detected by scintillation counting. Dotted line: glycero-inositol groups (Gro) of deacylated PtdIns(3)^{32}P and [^{3}H]PtdIns(4)P standards. Inset: PtdIns(4)P levels in BLM before (continous line) or after (dashed line) incubation with ATP, as detected by absorbance at 205 nm. B: S1P. Continous line corresponds to the synthesized S1P detected by absorbance at 205 nm. Dotted line: S1P standard. Inset: S1P levels in BLM before (continous line) or after incubation with ATP (dashed line).

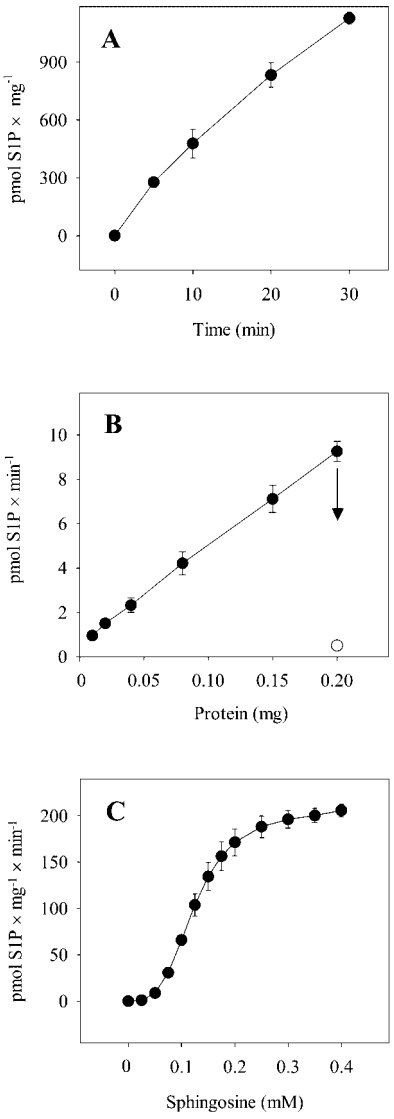

Fig. 3. **Time course, protein concentration dependence and Sph concentration dependence of BLM-SPK activity.** A: Purified basolateral membranes were assayed as described in "EXPERIMENTAL PROCEDURES." Aliquots were removed at the times indicated on the abscissa to measure the amount of S1^{32}P formed. B: Samples containing 0.1 mM Sph were incubated for 20 min in the presence of the membrane protein at the concentrations shown on the abscissa; open circle: boiled membranes. C: Samples containing 0.1 mg protein/ml were incubated for 20 min with the Sph concentrations shown on the abscissa. The line was fitted by nonlinear regression according to the equation $v = V_{max}\,[Sph]^n/(K'_m + [Sph]^n)$, in which $n = 3.4$ is an index of cooperativity, and $[Sph]_{0.5} = K'_m{}^{1/n} = 0.12$ mM is the concentration of Sph that gives half-maximal velocity. Results are expressed as the mean ± SE of at least three different experiments performed in triplicate.

of S1^{32}P was enhanced two-fold (Fig. 1A, lane 3). Thus, the data indicate that basolateral membranes from kidney cells phosphorylate Sph in the absence of Mg^{2+}. The enzyme activity was also insensitive to a classical inhibitor of cytosolic SPK, DL-*threo*-dihydrosphingosine (*19*, *20*, *36*, *50*), even at a high concentration (1 mM) as shown (Fig. 1B), since it had no effect on BLM-SPK from kidney cells (Fig. 1B, lanes 2 and 3).

To confirm the molecular identity of PtdIns(4)^{32}P and S1^{32}P obtained under the experimental conditions detailed in Fig. 1A, the regions corresponding to their migration (new radioactive region that appeared upon the addition of 0.1 mM Sph) on unidimensional TLC plates were scraped off, and the material was eluted from the silica and separated by bidimensional chromatography. The radioactive lipids purified by this procedure were re-extracted from the silica and analyzed by HPLC as described in "EXPERIMENTAL PROCEDURES" (Fig. 2). Figure 2A shows that only PtdIns(4)^{32}P was formed, since neither radioactivity counting nor optical detection revealed a product with a retention time corresponding to that of the PtdIns(3)^{32}P standard. Only a single radioactive peak matched the commercial [^{3}H]PtdIns(4)P standard. In addition, the inset to Fig. 2A shows that the amount of PtdIns(4)P present in the membranes before incubation with ATP is barely detectable.

Both the eluted sample with the greater mobility in TLC and the standard S1P showed identical retention times (Fig. 2B), confirming it to be the product of a BLM-SPK. The single peak eluted from the HPLC column was the only source of radioactivity. All other samples collected during elution showed only background radioactivity. As in the case of PtdIns(4)P, there was a very small peak of non-radioactive S1P in the membranes before the phosphorylation assay (inset to Fig. 2B). Taken together, the data from Fig. 1 and Fig. 2B show that a Mg^{2+}-independent SPK activity, unrelated to previously described SPKs, mediates S1P formation in BLM of kidney proximal tubules.

Characterization of the Sphingosine-Kinase Activity in Basolateral Membranes—To characterize the SPK activity in purified renal BLM, a number of experiments were carried out to examine the enzyme kinetics. Fig. 3A shows a linear time-dependent increase in BLM-SPK activity up to 30 min. BLM-SPK activity was also dependent on protein concentration, as shown in Fig. 3B, with the maximal protein concentration tested being 0.15 mg/ml. It is important to note that the activity was completely lost after boiling, as expected (Fig. 3B). Interestingly, as depicted in Fig. 3C, SPK activity with D(+)-*erythro*-sphingosine as a lipid substrate showed typical cooperative kinetics with $K_{0.5}$ = 0.12 mM and a Hill number (n) of 3.4. The latter kinetic property is also different from the Michaelian kinetics described for other SPKs (*36*, *51*).

Evidence of Crosstalk between the Sphingosine Kinase and Phosphatidylinositol-4 Kinase Activities in Basolateral Membranes—As the action of BLM-SPK in phosphorylating Sph was more pronounced in the absence of Mg^{2+} and the opposite behavior was observed for PI-4K (Fig. 1A), we proceeded to investigate further the role of Mg^{2+} on the modulation of these two enzymes. Figure 4A shows that the addition of 1.1 mM $MgCl_2$ to a membrane preparation preincubated for 10 min with [γ-^{32}P]ATP led to an immediate activation of PI-4K, with a consequent yield of PtdIns(4)P. The formation of PtdIns(4)P was accompanied by a decrease in S1P levels. It is important to note that the observed decrease in S1P formation was not due to a shortage of [γ-^{32}P]ATP as >80% remained available at the end of the assay time, as depicted in Fig. 4B. The latter indicates that the decrease in S1P forma-

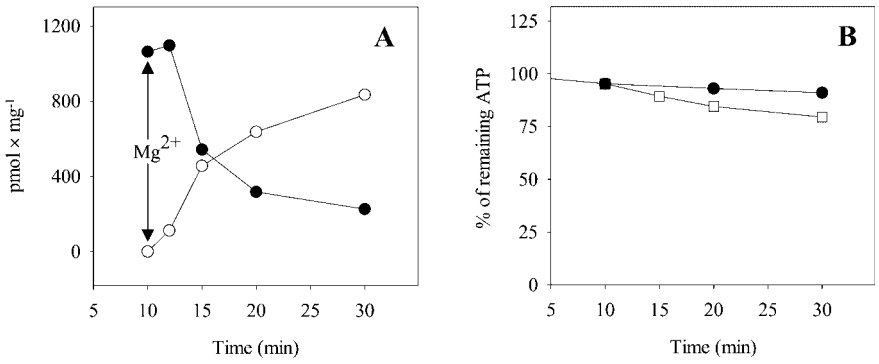

Fig. 4. **Effect of Mg^{2+} on PI-4K and SPK activities.** A: Time course of PI-4K and SPK activities. Mg^{2+} was added 10 min after the start of the reaction. Aliquots were removed at the times shown on the abscissa and the PI-4K (open circles) and SPK (λ) products were measured. B: ATP levels during the assay time. The data are representative of three different experiments that agreed within 10%. solid circles: no Mg^{2+}; open squares: 1.1 mM $MgCl_2$.

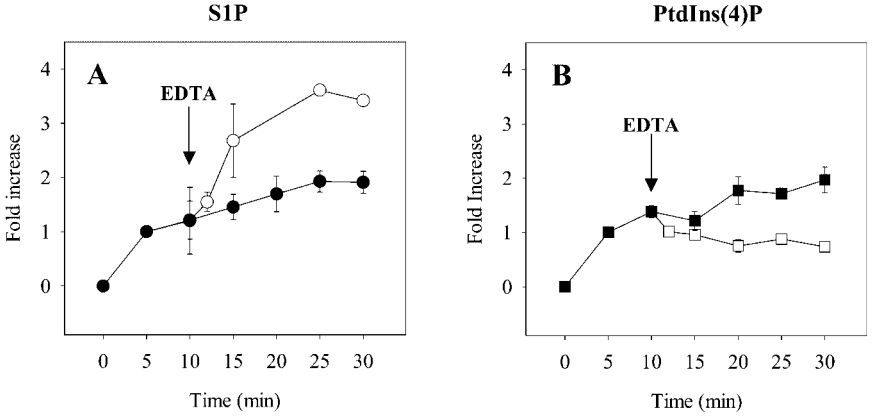

Fig. 5. **Effect of an EDTA chase on the PI-4K and SPK activities.** A: SPK activity (solid circles) was measured in the presence of Mg^{2+} for 10 min as described in "EXPERIMENTAL PROCEDURES." After this period, 5 mM EDTA was added to the assay medium and the reaction was followed for an additional 20 min (open circles). B: PI-4K activity (solid and open squares) was measured under the same conditions as described for 5A. Results are expressed as mean ± SE of the results of three different experiments performed in triplicate. Standard errors were calculated from the absolute values.

tion was not due to a decrease in ATP after the activation of Mg^{2+}-ATPases.

The role of Mg^{2+} was further investigated in a set of experiments in which Mg^{2+} was removed by adding 5 mM EDTA after a 10-min preincubation of the membranes with [γ-^{32}P]ATP. Figure 5A shows that the EDTA chase resulted in an increase in S1P production, while it decreased the yield of PtdIns(4)P (Fig. 5B).

Next, we addressed the question of whether the observed Mg^{2+} modulation of BLM-SPK and PI-4K was effected by their products. Freshly phosphorylated PtdIns(4)^{32}P and S1^{32}P were collected by scraping from TLC plates, as described in "EXPERIMENTAL PROCEDURES," re-extracted from the silica, and added directly to the assay medium to determine their effects on the phosphorylation of plasma membrane lipids. The eluted lipids were used instead of commercially obtained products to ensure they would have exactly the same fatty acid composition as those present in the original membranes. Figure 6A shows that the addition of PtdIns(4)P to the reaction led to a strong inhibition of SPK, while the addition of S1P to the Mg^{2+}-containing phosphorylation assay led to a significant increase in the PI-4K (Fig. 6B), suggesting crosstalk between these two signaling cascades.

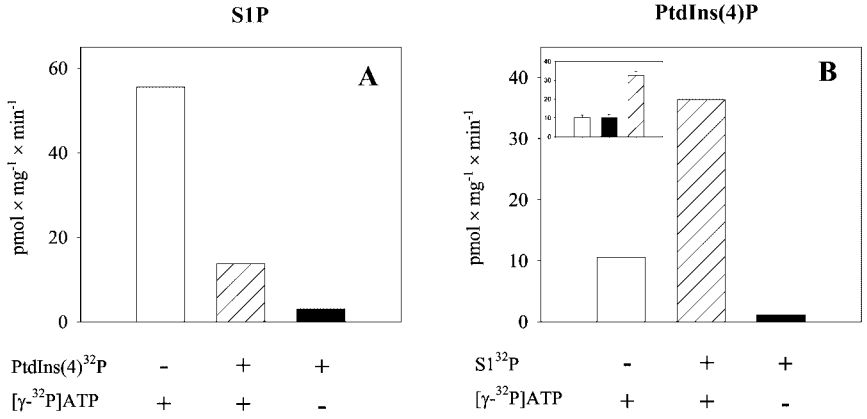

Fig. 6. **Evidence of crosstalk between glycero- and sphingolipids in the renal basolateral plasma membrane.** PtdIns(4)^{32}P or S1^{32}P produced in the basolateral membrane fractions purified from kidney cells were re-extracted from the silica plates (see "EXPERIMENTAL PROCEDURES") and [γ-^{32}P]ATP was added in the combinations as indicated on the abscissa. SPK (A; no Mg^{2+}) and PI-4K (B; with Mg^{2+}) activities were assayed in the presence or absence of [γ-^{32}P]ATP, PtdIns(4)^{32}P and S1^{32}P in the combinations specified on the abscissa as described under Experimental Procedures. The concentrations of added PtdIns(4)^{32}P and S1^{32}P in the corresponding assays were 300 and 400 nM, respectively. Since there were very small peaks of non-radioactive Ptdins(4)P and S1P in non-incubated membranes (insets to Figs. 2, A and B), endogenous lipids were not considered in calculating the concentrations in the reconstituted assays. Results are expressed as the means of three determinations performed in duplicate, using different membrane preparations and lipid extracts. The corresponding measurements agreed within 10%. The inset in B shows PtdIns(4)P formation by BLM in the absence (empty bar) and presence of 0.4 μM (filled bar) or 0.1 mM (hatched bar) Sph.

DISCUSSION

In this work, we used purified basolateral membranes from proximal kidney tubule cells to demonstrate the presence of membrane-associated SPK and PI-4K, as judged by the formation of $S1^{32}P$ and $PtdIns(4)^{32}P$ in the phosphorylation assay described in Fig. 1. The BLM-SPK described here displays properties different from those of SPKs studied in other membrane fractions (*18*, *52*) and, especially, from that found in the cytosol, which has been well characterized (*36*, *53*). The phosphorylation of Sph to S1P by the BLM-SPK can occur in the absence of Mg^{2+} (Fig. 1A, lane 3), an obligatory cofactor for the cytosolic enzyme from different sources (*36*). This observation means that different amounts of S1P can be readily formed as a result of a local increase in Sph levels independent of fluctuations in Mg^{2+}.

Another unusual feature of kidney BLM-SPK is its insensitivity (Fig. 1B, lane 2) to 1 mM DL-*threo*-dihydrosphingosine, a potent competitive inhibitor of the cytosolic form of SPK (*19*, *20*, *36*, *50*), which usually displays a significant inhibitory effect at 10–20 μM. This analog also was not a substrate for BLM-SPK (Fig. 1B, lane 3). These results clearly show that the saturated isomer is not recognized by the membrane-associated enzyme in a productive form and reveal a fine-tuned, stereospecific recognition mechanism not seen with the cytosolic kinase. In several human SPKs cascades, DL-*threo*-dihydrosphingosine is also an effective inhibitor in the micromolar concentration range (*18*, *51–53*). The disparate responses to the inhibitor and Mg^{2+} found in this work suggest the possibility that the renal basolateral membrane-associated enzyme is not simply an isoform of a broad SPK family.

An interesting feature of the BLM-SPK activity shown in this study is the strong cooperativity of the substrate curve (n = 3.4; Fig. 3C), suggesting that more than one molecule of Sph is involved at several enzyme sites that interact during catalysis, whereas other SPKs display a typical Michaelian sphingosine dependence (*36*, *51*). Therefore, the cooperativity found for the renal enzyme may indicate oligomeric association within the membrane as described for other membrane-bound enzymatic systems (*54*, *55*).

A membrane localization would add support to the view that S1P also plays an autocrine role (*5*, *26*, *31*). After its formation within the membrane moiety, S1P could easily be released to the external milieu, thus allowing a faster interaction with its receptors. This view is supported by two different kinds of evidence: (i) the extracellular formation of S1P has been demonstrated (*17*); (ii) extracellular G-coupled receptors for S1P have been shown to exist in cell cultures and implicated in different physiological processes (*5*, *25–30*). These receptors have been described in a kidney cell line, where they appear to be involved in transient cytosolic Ca^{2+} mobilization (*56*), a phenomenon that plays a crucial role in the regulation of the transepithelial fluxes of water and solutes (*57*).

Another point disclosed in our work is the interplay between the two signaling molecules S1P and PtdIns(4)P and their respective kinases. As pointed out above, Mg^{2+} is not required for BLM-SPK activity whereas it is an activator of PI-4K (Fig. 1A). Moreover, after the addition of Mg^{2+}, the decrease in S1P levels with time is the mirror image of PtdIns(4)P synthesis (Fig. 4A) and the addition of ≈300 nM PtdIns(4)P obtained from the same renal membranes prior to the addition of ATP strongly inhibits (by 75%) the initial rate of S1P formation in the absence of Mg^{2+} (Fig. 6A). These findings show that it is likely that the activation of the PI-4K pathway reduces tissue S1P levels by inhibiting BLM-SPK activity. Although the membrane-bound, type 2, lipid phosphohydrolases are Mg^{2+}-independent (*24*, *30*, *58*), it is also possible that a different Mg^{2+}-dependent degradation mechanism could be activated under the experimental conditions described for Fig. 4A.

Conversely, an opposite effect of S1P on the synthesis of PtdIns(4)P was found. The addition of exogenous Sph leads to a strong activation of PI-4K (Fig. 1A, lane 2), as in the case of a membrane fraction from rabbit platelets (*59*). Furthermore, the addition of ≈400 nM S1P formed in and extracted from the renal membranes promotes the stimulation of PI-4K to a similar extent as that observed in Fig. 1A, lane 2, in contrast to a report by Hashizume *et al*. (*59*), who did not observe an activation of PI-4K by S1P. Presumably, kidney epithelial cells, but not circulating cells, such as platelets, can recognize S1P as a modulator of PI-4K activity. The same three-fold increase in PtdIns(4)P formation found with either 0.1 mM Sph or 0.4 μM S1P (Fig. 6, compare main panel and inset) supports the view that the latter is more effective for the activation of PI-4K and, consequently, that the activation of SPK is an earlier event in modulating the PI-4K cascade. This conclusion is confirmed by the data also presented in the inset to Fig. 6B, showing that the addition of 0.4 μM sphingosine has no effect on PtdIns(4)P formation (compare empty and filled bars). Interestingly, the addition of Sph during the formation of S1P also reduces the formation of phosphatidic acid (PA) (Fig. 1A, lane 2). This is evidence that S1P formed in BLM also participates in another regulatory loop involving glycerolipids, as recently demonstrated in a human embryonic kidney cell line (*60*).

The mutual influence of PI-4K and SPK on one another described in this manuscript is consistent with crosstalk involving sphingolipids and glycerolipids in the basolateral aspect of renal proximal tubule cells. It may be, therefore, that synchronized control mechanisms that simultaneously regulate PtdIns(4)P and S1P levels exist in the plasma membranes of kidney cells, and that these mechanisms are relevant in physiological events such as transepithelial fluxes. This view is supported by the observation that PtdIns(4)P is a potent activator of the ATP-dependent Ca^{2+} pumping mechanism present in different membranes (*42*, *61–63*).

The interaction of sphingolipids with PKCs triggered by hormones and autacoids (*64*), which play an important role in the modulation of renal ion pumps such as the $(Ca^{2+}+Mg^{2+})$ATPase (*41*), the $(Na^{+}+K^{+})$ATPase (*57*, *65*), and the ouabain-insensitive Na^{+}-ATPase (*66*) in proximal tubules is also well known. Therefore, the interaction between the SPK and PI-4K pathways described in this work may represent an important physiological mechanism by which different processes involved in ionic homeostasis are switched on or off.

The authors would like to thank Dr. Alan Leyva for recommending needed changes in the English; and Ms. Ivone Diniz Barbosa (Bolsista AT, FAPERJ, Brasil) and Ms. Monica Cristina S. Vieira (Bolsista AT / PROFIX, CNPq, Brasil) for technical assistance. This work was supported by CNPq, PRONEX, FINEP, FAPERJ, FUJB/UFRJ and PADCT (Brazil).

REFERENCES

1. Zhang, H., Desai, N.N., Olivera, A., Seki, T., Brooker, G., and Spiegel, S. (1991) Sphingosine-1-phosphate, a novel lipid, involved in cellular proliferation. *J. Cell Biol.* **114**, 155–167
2. Kolesnick, R. and Golde, D.W. (1994) The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling. *Cell* **77**, 325–328
3. Spiegel, S. and Merrill Jr., A.H. (1996) Sphingolipid metabolism and cell growth regulation. *FASEB J.* **10**, 1388–1397
4. Meyer zu Heringdorf, D., Van Koppen, C.J., and Jakobs, K.H. (1997) Molecular diversity of sphingolipid signalling. *FEBS Lett.* **410**, 34–38
5. Pyne, S. and Pyne, N.J. (2000) Sphingosine 1-phosphate signalling in mammalian cells. *Biochem. J.* **349**, 385–402
6. Stoffel, W., LeKim, D., and Sticht, G. (1969) Distribution and properties of dihydrosphingosine-1-phosphate aldolase (sphinganine-1-phosphate alkanal-lyase). *Hoppe-Seyller's Z. Physiol. Chem.* **350**, 1233–1241
7. Louie, D.D., Kisic, A., and Schroefer Jr., G.J., (1976) Sphingolipid base metabolism. Partial purification and properties of sphinganine kinase of brain. *J. Biol. Chem.* **251**, 4557–4563
8. Gosh, T.K., Bian, J., and Gill, D.L. (1990) Intracellular calcium release mediated by sphingosine derivatives generated in cells. *Science* **248**, 1653–1656
9. Mattie, M., Brooker, G., and Spiegel, S. (1994) Sphingosine-1-phosphate, a putative second messenger, mobilizes calcium from internal stores via an inositol trisphosphate-independent pathway. *J. Biol. Chem.* **269**, 3181–3188
10. Goodemote, K.A., Mattie, M.E., Berger, A., and Spiegel, S. (1995) Involvement of a pertussis toxin-sensitive G protein in the mitogenic signalling pathway of sphingosine-1 phosphate. *J. Biol. Chem.* **270**, 10272–10277
11. Berger, A., Bittman, R., Schmidt, R.R., and Spiegel, S. (1996) Structural requirements of sphingosylphosphocholine and sphingosine-1-phosphate for stimulation of activator protein-1 activity. *Mol. Pharmacol.* **50**, 451–457
12. Cuvillier, O., Pirianov, G., Kleuser, B., Vcanek, P.G., Coso, O.A., Gutkind, S., and Spiegel, S. (1996) Suppression of ceramide-mediated programmed cell death by sphingosine-1 phosphate. *Nature* **381**, 800–803
13. Goetzl, E.J. and An, S. (1998) Diversity of cellular receptors and functions for the lysophospholipid growth factors lysophosphatidic acid and sphingosine 1-phosphate. *FASEB J.* **12**, 1589–1598
14. Sadahira, Y., Ruan, F., Hakomori, S., and Igarashi, Y. (1992) Sphingosine 1-phosphate, a specific endogenous signalling molecule controlling cell motility and tumor cell invasiveness. *Proc. Natl Acad. Sci. USA* **89**, 9686–9690
15. Bornfeldt, K.E., Graves, L.M., Raines, E.W., Igarashi, Y., Wayman, G., Yamamura, S., Yatomi, Y., Sidhu, J.S., Krebs, E.G., and Hakomori, S.I. (1995) Sphingosine-1-phosphate inhibits PDGF-induced chemotaxis signal transduction. *J. Cell Biol.* **130**, 193–206
16. Postma, F.R., Jalink, K., Hengeveld, T., and Moolenaar, W.H. (1996) Sphingosine-1-phosphate rapidly induces Rho-dependent neurite retraction: action through a specific cell surface receptor. *EMBO J.* **15**, 2388–2392
17. Ancellin, N., Colmont, C., Su, J., Li, Q., Mittereder, N., Chae, S.-S., Stefansson, S., Liau, G., and Hla, T. (2002) Extracellular export of sphingosine kinase-1 enzyme. *J. Biol. Chem.* **277**, 6667–6675
18. Banno, Y., Kato, M., Hara, A., and Nozawa, Y. (1998) Evidence for the presence of multiple forms of Sph kinase in human platelets. *Biochem. J.* **335**, 301–304
19. Gijsbers, S., Van der Hoeven, G., and Van Veldhoven, P.P. (2001) Subcellular study of sphingoid base phosphorylation in rat tissues: evidence for multiple sphingosine kinases. *Biochim. Biophys. Acta* **1532**, 37–50
20. Olivera, A. and Spiegel, S. (1993) Sphingosine-1-phosphate as second messenger in cell proliferation induced by PDGF and FCS mitogens. *Nature* **365**, 557–560
21. Saba, J.D., Nara, F., Bielawska, A., Garrett, S., and Hannun, Y.A. (1997) The *BST1* gene of *Saccharomyces cerevisiae* is the sphingosine-1-phosphate lyase. *J. Biol. Chem.* **272**, 26087–26090
22. Mandala, S.M., Thornton, R., Tu, Z., Kurtz, M.B., Nickels, J., Broach, J., Menzeleev, R., and Spiegel, S. (1998) Sphingoid base 1-phosphate phosphatase: a key regulator of sphingolipid metabolism and stress response. *Proc. Natl Acad. Sci. USA* **95**, 150–155
23. Van Veldhoven, P.P., Gijsbers, S., Mannaerts, G.P., Vermeesch, J.R., and Brys, V. (2000) Human sphingosine-1-phosphate lyase: cDNA cloning, functional expression studies and mapping to chromosome 10q22(1). *Biochim. Biophys. Acta* **1487**, 128–134
24. Le Stunff, H., Peterson, C., Liu, H., Milstien, S., and Spiegel, S. (2002) Sphingosine-1-phosphate and lipid phosphohydrolases. *Biochim. Biophys. Acta* **1582**, 8–17
25. Hla, T. and Maciag, T. (1990) An abundant transcript induced in differentiating human endothelial cells encodes a polypeptide with structural similarities to G protein-coupled receptors. *J. Biol. Chem.* **265**, 9308–9313
26. Hla, T., Lee, M.-J., Ancellin, N., Liu, C.H., Thangada, S., Thompson, B.D., and Kluk, M. (1999) Sphingosine-1-phosphate: extracellular mediator or intracellular second messenger? *Biochem. Pharmacol.* **58**, 201–207
27. An, S., Zheng, Y., and Bleu, T. (2000) Sphingosine-1 phosphate-induced cell proliferation, survival and related events mediated by G protein-coupled receptors Edg3 and Edg5. *J. Biol. Chem.* **275**, 288–296
28. Liu, Y., Wada, R., Yamashita, T., Mi, Y., Deng, C.-X., Hobson, J.P., Rosenfeldt, H.M., Nava, V.E., Chae, S.-S., Lee, M.-J., Liu, C.H., Hla, T., Spiegel, S., and Proia, R.L. (2000) Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. *J. Clin. Invest.* **106**, 951–961
29. Yamazaki, Y., Kon, J., Sato, K., Tomura, H., Sato, M., Yoneya, T., Okazaki, H., Okajima, F., and Ohta, H. (2000) Edg6 as a putative sphingosine-1 phosphate receptor coupling to Ca^{2+} signalling pathway. *Biochem. Biophys. Res. Commun.* **268**, 583–589
30. Pyne, S. and Pyne, N.J. (2002) Sphingosine 1-phosphate signalling and termination. *Biochim. Biophys. Acta* **1582**, 121–131
31. Spiegel, S. and Milstien, S. (2000) Sphingosine-1-phosphate: signaling inside and out. *FEBS Lett.* **476**, 55–57
32. Divecha, N. and Irvine, R.F. (1995) Phospholipid signaling. *Cell* **80**, 269–278
33. Fruman, D.A., Meyers, R.E., and Cantley, L.C. (1998) Phosphoinositide kinases. *Annu. Rev. Biochem.* **67**, 481–507
34. Gehrmann, T. and Heilmeyer Jr., L.M. (1998) Phosphatidylinositol 4-kinases. *Eur. J. Biochem.* **253**, 357–370
35. Toker, A. and Cantley, L.C. (1997) Signalling through the lipid products of phosphoinositide-3-OH kinase. *Nature* **387**, 673–676
36. Olivera, A., Kohama, T., Tu, Z., Milstein, S., and Spiegel, S. (1998) Purification and characterization of a rat kidney sphingosine kinase. *J. Biol. Chem.* **273**, 12576–12583
37. Maia, J.C.C., Gomes, S.L., and Juliani, M.H. (1983) Preparation of (gamma-^{32}P) and (alpha-^{32}P)-nucleoside triphosphates with high specific activity in *Genes of Parasites: a Laboratory Manual* (Morel, C.M., ed.) pp. 146–167, Editora Fundação Oswaldo Cruz, Rio de Janeiro, Brazil
38. Boumendil-Podevin, E.F. and Podevin, R.A. (1983) Isolation of basolateral and brush-border membranes from the rabbit kid-

ney cortex. Vesicle integrity and membrane sidedness of the basolateral fraction. *Biochim. Biophys. Acta* **735**, 86–94
39. Kato, M. and Kako, K.J. (1987) Orientation of vesicles isolated from baso-lateral membranes of renal cortex. *Mol. Cell. Biochem.* **78**, 9–16
40. Vieyra, A., Nachbin, L., de Dios-Abad, E., Goldfeld, M., Meyer-Fernandes, J.R., and de Moraes, L. (1986) Comparison between calcium transport and adenosine triphosphatase activity in membrane vesicles derived from rabbit kidney proximal tubules. *J. Biol. Chem.* **261**, 4247–4255
41. Coka-Guevara, S., Markus, R., Caruso-Neves, C., Lopes, A.G., and Vieyra, A. (1999) Adenosine inhibits the renal plasma-membrane (Ca^{2+}+Mg^{2+})ATPase through a pathway sensitive to cholera toxin and sphingosine. *Eur. J. Biochem.* **263**, 71–78
42. Guilherme, A., Reis-Silva, C., Moraes-Albuquerque, J.H., Oliveira, M.M., and Vieyra, A. (1998) A novel phosphorylated lipid counteracts activation of the renal plasma membrane (Ca^{2+}+Mg^{2+})ATPase by endogenous phosphatidylinositol-4 phosphate. *Biosci. Reps.* **18**, 79–89
43. Horwitz, J. and Perlman, R.L. (1987) Phospholipid metabolism in PC12 pheochromocytoma cells. *Methods Enzymol.* **141**, 169–175
44. Oliveira, M.M., Alves, M.F., and Brigante, A.A. (1990) Differences in phospholipid composition of autotrophic and heterotrophic *Euglena gracilis*. *Braz. J. Med. Biol. Res.* **23**, 1237–1241
45. Serunian, L.A., Auger, K.R., and Cantley, L.C. (1991) identification and quantification of phosphoinositides produced in response to platelet-derived growth factor stimulation. *Methods Enzymol.* **198**, 78–87
46. Todorov, A.G., Einicker-Lamas, M., De Castro, S.L., Oliveira, M.M., and Guilherme, A. (2000) Activation of host cell phosphatidylinositol-3-kinases by *Trypanosoma cruzi* infection. *J. Biol. Chem.* **275**, 32182–32186
47. Nakagawa, Y. and Waku, K. (1986) Improved procedure for the separation of the molecular species of dimethylphosphatidate by high-performance liquid chromatography. *J. Chromatogr.* **381**, 225–231
48. Pike, L.J. (1992) Phosphatidylinositol 4-kinases and the role of phosphoinositides in cellular regulation. *Endocr. Rev.* **13**, 692–706
49. Balla, T. (1998) Phosphatidylinositol 4-kinases. *Biochim. Biophys. Acta* **1436**, 69–85
50. Tolan, D., Conway, A.-M., Steele, L., Pyne, S., and Pyne, N.J. (1996) The identification of DL-*threo* dihydrosphingosine and sphingosine as novel inhibitors of extracellular signal-regulated kinase signalling in airway smooth muscle. *Br. J. Pharmacol.* **119**, 185–186
51. Melendez, A.J., Carlos-Dias, E., Gosink, M., Allen, J.M., and Takacs, L. (2000) Human sphingosine kinase: molecular cloning, functional characterization and tissue distribution. *Gene* **251**, 19–26
52. Buehrer, B.M. and Bell, R.M. (1992) Inhibition of sphingosine kinase *in vitro* and in platelets. Implications for signal transducing pathways. *J. Biol. Chem.* **267**, 3154–3159
53. Pitson, S.M., D'Andrea, R.J., Vandeleur, L., Moretti, P.A.B., Xia, P., Gamble, J.R., Vadas, M.A., and Wattemberg, B.W. (2000) Human sphingosine kinase: purification, molecular cloning and characterization of the native and recombinant enzymes. *Biochem. J.* **350**, 429–441
54. Kosk-Kosicka, D., Bzdega, T., and Wawrzynow, A. (1989) Fluorescence energy transfer studies of purified erythrocyte Ca^{2+}-ATPase. Ca^{2+}-regulated activation by oligomerization. *J. Biol. Chem.* **264**, 19495–19499
55. Coelho-Sampaio, T., Ferreira, S.T., Benaim, G., and Vieyra, A. (1991) Dissociation of purified erythrocyte Ca^{2+}-ATPase by hydrostatic pressure. *J. Biol. Chem.* **266**, 22266–22272
56. Meyer zu Heringdorf, D., Lass, H., Kuchar, I., Lipinski, M., Alemany, R., Rümenapp, U., and Jakobs, K.H. (2001) Stimulation of intracellular sphingosine-1-phosphate production through G-protein-coupled sphingosine-1-phosphate receptors. *Eur. J. Pharmacol.* **414**, 145–154
57. Aperia, A., Holtbäch, U., Syrén, M.-L., Svenson, L.-B., Fryckstedt, J., and Greengard, P. (1994) Activation/deactivation of renal Na^+, K^+-ATPase: a final common pathway for regulation of natriuresis. *FASEB J.* **8**, 436–439
58. Mandala, S.M. (2001) Sphingosine-1-phosphate phosphatases. *Prostaglandins* **64**, 143–156
59. Hashizume, T., Nakao, M., and Sato, T. (1996) Sphingosine enhances phosphatidylinositol 4-kinase activity in rabbit platelets. *J. Biochem.* **120**, 61–65
60. Alderton, F., Darroch, P., Sambi, B., McKie, A., Ahmed, I.S., Pyne, N., and Pyne, S. (2001) G-protein-coupled receptor stimulation of the p42/p44 mitogen-activated protein kinase pathway is attenuated by lipid phosphate phosphatases **1**, 1a, and 2 in human embryonic kidney 293 cells. *J. Biol. Chem.* **276**, 13452–13460
61. Missiaen, L., Raeymaekers, L., Wuytack, F., Vrolis, M., de Smedt, H., and Casteels, R. (1989) Phospholipid-protein interactions of the plasma-membrane Ca^{2+}-transporting ATPases. Evidence for a tissue-dependent functional difference. *Biochem. J.* **263**, 687–694
62. Lee, A.G., Dalton, K.A., Duggleby, R.C., East, J.M., and Starling, A.P. (1995) Lipid structure and Ca^{2+}-ATPase function. *Biosci. Rep.* **15**, 289–298
63. Starling, A.P., East, J.M., and Lee, A.G. (1995) Phosphatidylinositol-4 phosphate increases the rate of dephosphorylation of the phosphorylated Ca^{2+}-ATPase. *J. Biol. Chem.* **270**, 14467–14470
64. Smith, E.R., Merrill Jr., A.H., Obeid, L.M., and Hannun, Y.A. (2000) Effects of sphingosine and other sphingolipids on protein kinase. *Methods Enzymol.* **312**, 361–373
65. Feschenko, M.S. and Sweadner, K.J. (2000) Interaction of protein kinase C and cAMP-dependent pathways in the phosphorylation of the Na^++K^+-ATPase. *J. Biol. Chem.* **275**, 34693–34700
66. Rangel, L.B.A., Malaquias, A.T., Lara, L.S., Silva, I.V., De Souza, A.M., Lopes, A.G. and Caruso-Neves, C. (2001) Protein kinase C-induced phosphorylation modulates the Na^+-ATPase activity from proximal tubules. *Biochim. Biophys. Acta* **1512**, 90–97