

Sphingosine-1-phosphate stimulates aldosterone secretion through a mechanism involving the PI3K/PKB and MEK/ERK 1/2 pathways

Leyre Brizuela, Miriam Rábano, Patricia Gangoití, Natalia Narbona, José María Macarulla, Miguel Trueba, and Antonio Gómez-Muñoz¹

Department of Biochemistry and Molecular Biology, Faculty of Science and Technology, University of the Basque Country, 48080 Bilbao, Spain

Abstract We reported recently that sphingosine-1-phosphate (S1P) is a novel regulator of aldosterone secretion in zona glomerulosa cells of adrenal glands and that phospholipase D (PLD) is implicated in this process. We now show that S1P causes the phosphorylation of protein kinase B (PKB) and extracellularly regulated kinases 1/2 (ERK 1/2), which is an indication of their activation, in these cells. These effects are probably mediated through the interaction of S1P with the Gi protein-coupled receptors S1P1/3, as pretreatment with pertussis toxin or with the S1P1/3 antagonist VPC 23019 completely abolished the phosphorylation of these kinases. Inhibitors of phosphatidylinositol 3-kinase (PI3K) or mitogen-activated protein kinase kinase (MEK) blocked S1P-stimulated aldosterone secretion. This inhibition was only partial when the cells were incubated independently with inhibitors of each pathway. However, aldosterone output was completely blocked when the cells were pretreated with LY 294002 and PD 98059 simultaneously. These inhibitors also blocked PLD activation, which indicates that this enzyme is downstream of PI3K and MEK in this system. We propose a working model for S1P in which stimulation of the PI3K/PKB and MEK/ERK pathways leads to the stimulation of PLD and aldosterone secretion.—Brizuela, L., M. Rábano, P. Gangoití, N. Narbona, J. M. Macarulla, M. Trueba, and A. Gómez-Muñoz. Sphingosine-1-phosphate stimulates aldosterone secretion through a mechanism involving the PI3K/PKB and MEK/ERK 1/2 pathways. *J. Lipid Res.* 2007. 48: 2264–2274.

Supplementary key words adrenal gland • ceramide • mitogen-activated protein kinase • glomerulosa cells • mineralocorticoids • phospholipase D • protein kinase B • sphingolipid • steroids • phosphatidylinositol 3-kinase • mitogen-activated protein kinase kinase/extracellularly regulated kinase

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid that regulates critical biological processes, including cell proliferation and survival, cell differentiation,

inhibition of tumor cell invasion, migration, cytoskeletal rearrangement, cell contraction, immune responses, and angiogenesis (1–12). S1P can be formed intracellularly and act as a second messenger. However, many of its effects are elicited through interaction with specific Gi protein-coupled receptors (S1P1–S1P5) that are ubiquitously expressed in cells and can regulate numerous downstream signals. This dual effect of S1P was previously described by Spiegel and Milstien (11, 12).

We demonstrated recently that S1P is a novel stimulator of aldosterone secretion, which is a key hormone for hemodynamic stability in humans. This action involved prior activation of the phospholipase D (PLD)/lipid phosphate phosphatase pathway, Ca²⁺ influx from the extracellular milieu, and phosphorylation of the protein kinase C (PKC) isoforms α and δ (1).

S1P has been shown to stimulate the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB) and mitogen-activated kinase kinase (MEK)/extracellularly regulated kinase (ERK) pathways in different cell types. Both of these pathways are essential for many of the biological functions that are regulated by S1P (6, 11, 12). In particular, PI3K and ERK have been implicated in the stimulation of steroidogenesis by different agonists (3, 13–18), and we found that S1P is also a potent stimulator of cortisol secretion (19).

The aim of this work was to evaluate whether the stimulation of aldosterone secretion requires the intervention of signaling mechanisms other than the activation of the PLD/lipid phosphate phosphatase pathway that we described previously (1). Here, we demonstrate for the first

Abbreviations: ERK, extracellularly regulated kinase; MEK, mitogen-activated kinase kinase; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKC, protein kinase C; PLD, phospholipase D; PMA, 4 β -phorbol 12-myristate 13-acetate; PTX, pertussis toxin; S1P, sphingosine-1-phosphate; ZG, zona glomerulosa.

¹To whom correspondence should be addressed.

e-mail: antonio.gomez@ehu.es

Manuscript received 14 December 2006 and in revised form 22 June 2007.

Published, JLR Papers in Press, July 3, 2007.

DOI 10.1194/jlr.M700291-JLR200

time that activation of the PI3K/PKB and MEK/ERK pathways is essential for the stimulation of aldosterone secretion by SIP in isolated cells from the zona glomerulosa (ZG) of adrenal glands.

MATERIALS AND METHODS

Materials

Aldosterone, BSA (fraction V), DMEM, DNase I, HEPES, LY 294002, pertussis toxin (PTX), PD 98059, wortmannin, and 22-*R*-hydroxycholesterol were from Sigma (St. Louis, MO). Collagenases A and P were from Roche Diagnostics (Barcelona, Spain). [³H]myristate and [³H]aldosterone were supplied by American Radiolabeled Chemicals (St. Louis, MO). SIP, phosphatidylethanol, VPC 23019, and VPC 24191 were from Avanti Polar Lipids (Alabaster, AL). 4β-Phorbol 12-myristate 13-acetate (PMA) and UO 126 were from Calbiochem-Novabiochem (San Diego, CA). Antibodies to phosphoprotein kinase B (p-PKB; Ser 473), phospho-ERK 1/2 (Thr 202/Tyr 204), PKB, ERK 1/2, and goat anti-rabbit IgG horseradish peroxidase secondary antibody were from Cell Signaling Technology (Beverly, MA). Antibodies to phosphoprotein kinase Cα (p-PKC-α; Ser 657) and p-PKC-δ (Ser 643) were supplied by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Fluo-4-AM was from Invitrogen (Barcelona, Spain). Other chemicals were of the highest grade available.

Cell preparation and culture

Bovine adrenal glomerulosa cells were isolated and cultured as described previously (20). Briefly, glomerulosa cell slices were prepared from 1 year old steers obtained from a local slaughterhouse. The slices of adrenal gland were digested four times for 30 min with a mixture of collagenase A (0.18 U/mg), collagenase P (4.04 U/mg), and DNase I (0.4 mg/ml). Cells were

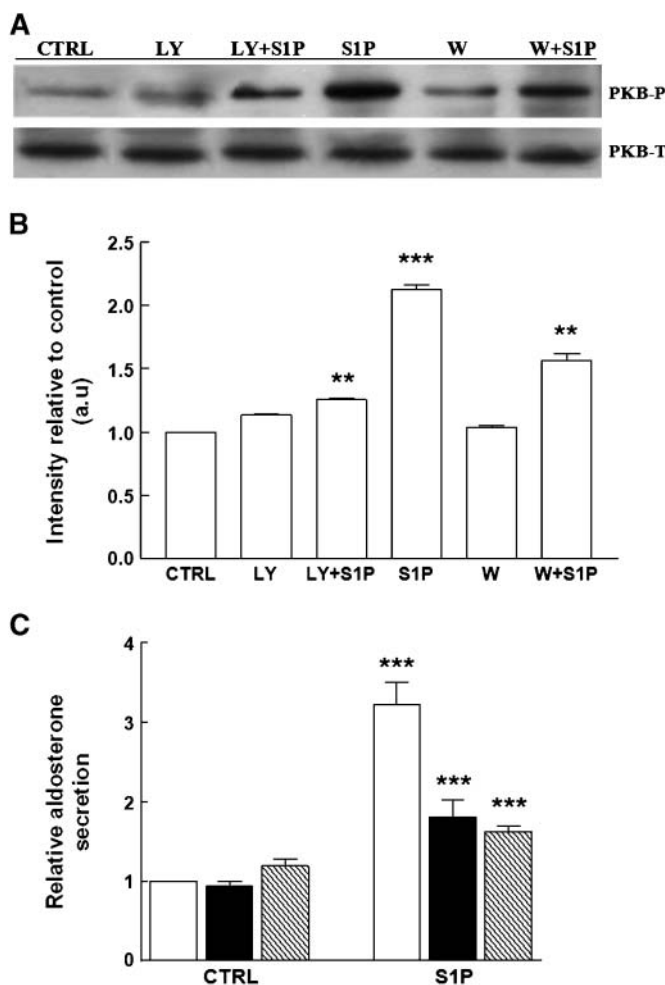


Fig. 1. Involvement of the phosphatidylinositol 3-kinase/protein kinase B (PI3K/PKB) pathway in sphingosine-1-phosphate (SIP)-stimulated aldosterone secretion. A: Zona glomerulosa (ZG) cells were seeded at 3.5×10^6 cells/100 mm dish and preincubated in DMEM without BSA or serum for 3 h. They were then preincubated with 50 μ M LY 294002 (LY) or 100 nM wortmannin (W) for 15 min, as indicated. SIP (5 μ M) was then added to the cells for 5 min. Phosphorylation of PKB was examined by immunoblotting using a specific antibody to phospho-PKB (PKB-P; phospho-Ser 473). Equal loading of protein was monitored using a specific antibody to total PKB (PKB-T). CTRL, control. Similar results were obtained in each of three replicate experiments. B: Results of scanning densitometry of the exposed film. Data are expressed as arbitrary units (a.u.) of intensity relative to the control values and are means \pm SEM of triplicate scans of a single gel. ** $P < 0.01$, *** $P < 0.001$ for LY+SIP or W+SIP versus SIP, as indicated. Similar results were obtained in each of two replicate experiments. C: Cells were preincubated with vehicle (open bars), 50 μ M LY 294002 (closed bars), or 100 nM wortmannin (hatched bars) for 15 min. SIP (5 μ M) was then added, and incubation continued for another 2 h. Aldosterone secretion was determined as described in Materials and Methods. Results are expressed as fold stimulation relative to control values and are means \pm SEM of four independent experiments performed in triplicate. *** $P < 0.001$ for LY+SIP or W+SIP versus SIP, as indicated.

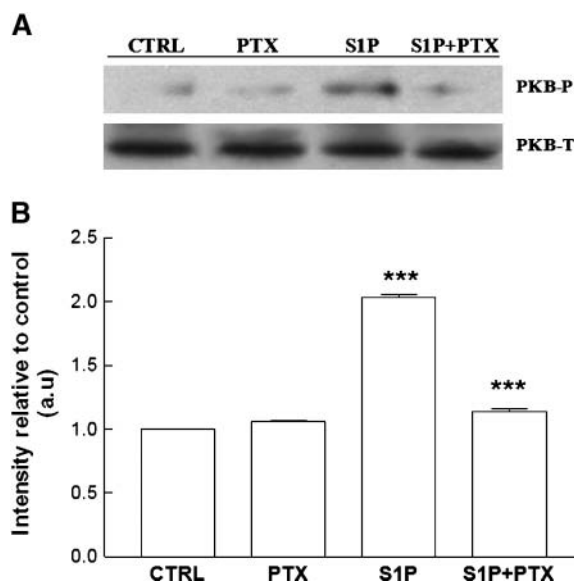


Fig. 2. Pertussis toxin (PTX) inhibits SIP-induced PKB phosphorylation. A: ZG cells were seeded at 3.5×10^6 cells/100 mm dish and preincubated with 1 μ g/ml PTX for 16 h, as indicated. The cells were then washed and incubated in DMEM without BSA or serum for 3 h in the presence of PTX (1 μ g/ml). SIP (5 μ M) was then added to the cells for 5 min. Phosphorylation of PKB was examined by immunoblotting using a specific antibody to PKB (PKB-P; anti-phospho-Ser 473). Equal loading of protein was monitored using a specific antibody to total PKB (PKB-T). Similar results were obtained in each of three replicate experiments. CTRL, control. B: Results of scanning densitometry of the exposed film. Data are expressed as arbitrary units (a.u.) of intensity relative to the control values and are means \pm SEM of triplicate scans of a single gel. *** $P < 0.001$ for SIP5 (μ M) versus SIP+PTX.

then dispersed from collagenase-digested slices by mechanical agitation. Freshly isolated cells were seeded in 35 mm culture dishes (6.5×10^5 cells per dish) or on 12-well plates ($2.5\text{--}3 \times 10^5$ cells per well). They were then cultured overnight in DMEM

containing 10% (v/v) FBS, L-glutamine (20 mM), gentamicin (50 mg/ml), NaHCO_3 (7.5%, w/v), and amphotericin B (10 mM). After 24 h, the medium was replaced by fresh DMEM, and cells were incubated further for 2 days in a gassed, humidified incubator (5% CO_2 at 37°C) before use in experiments. This time was chosen because steroid output from cells of the adrenal glands increases to a maximum by 48–72 h (21).

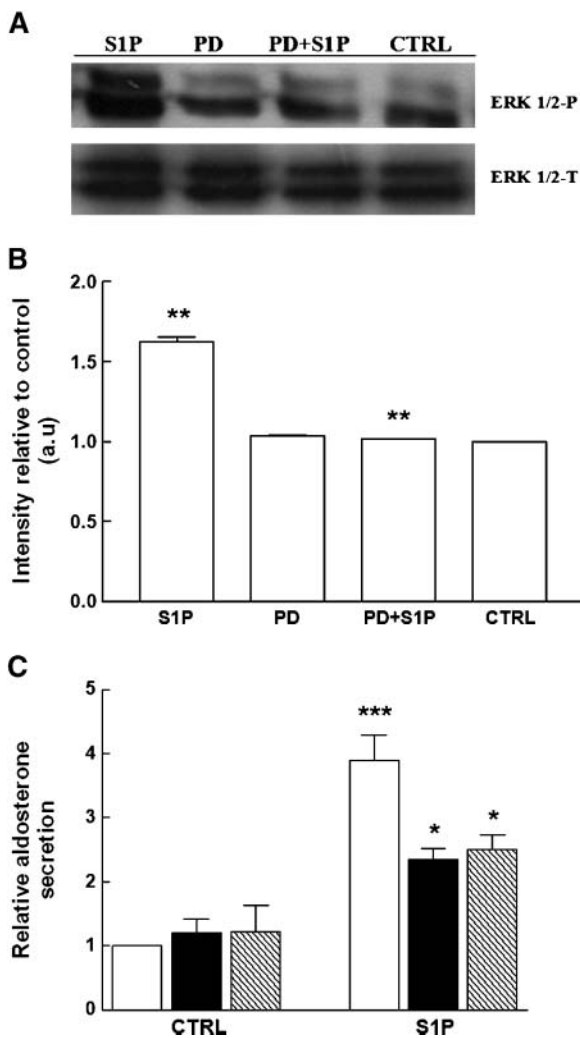


Fig. 3. Involvement of the mitogen-activated protein kinase/extracellularly regulated kinases 1/2 (MEK/ERK 1/2) pathway in SIP-stimulated aldosterone secretion. **A:** ZG cells were seeded at 3.5×10^6 cells/100 mm dish and preincubated in DMEM without BSA or serum for 3 h. They were then preincubated with 10 μM PD 98059 (PD) for 15 min, as indicated. S1P (5 μM) was then added to the cells for 5 min. Phosphorylation of ERK 1/2 was examined by immunoblotting using specific antibodies to ERK 1/2 (ERK 1/2-P; phospho-Thr 202/Tyr 204). Equal loading of protein was monitored using a specific antibody to total ERK (ERK 1/2-T). Similar results were obtained in each of three replicate experiments. CTRL, control. **B:** Results of scanning densitometry of the exposed film. Data are expressed as arbitrary units (a.u.) of intensity relative to the control values and are means \pm SEM of triplicate scans of a single gel. ** $P < 0.01$ for S1P versus S1P+PD. Similar results were obtained in each of two replicate experiments. **C:** Cells were preincubated with vehicle (open bars), 10 μM PD 98059 (closed bars), or 1 μM UO 126 (hatched bars) for 15 min. S1P (5 μM) was then added, and incubation continued for another 2 h. Aldosterone secretion was determined as described in Materials and Methods. Results are expressed as fold stimulation relative to the control values and are means \pm SEM of four independent experiments performed in triplicate. * $P < 0.05$, *** $P < 0.001$ for S1P+PD 98059 or S1P+UO 126 versus S1P.

Determination of aldosterone secretion

After incubation for 3 days in DMEM supplemented with 10% FBS, the ZG cells were washed twice with DMEM containing 0.2% BSA. Agonists were then added, and cells were incubated further for 2 h in the presence of 0.2% BSA. Quantification of aldosterone was performed by RIA, as described previously (20), using a specific monoclonal antibody against aldosterone and (1,2,6,7- ^3H)aldosterone as tracer. The antibody used had 0.06% cross-reactivity with other steroids, and the lowest detectable levels of the RIA were 3 pmol/ml for aldosterone. Analysis of the data was performed using a computer program designed specifically for this purpose.

Assay of PLD

PLD was determined in intact ZG cells by measuring the production of [^3H]phosphatidylethanol, which is the product of its transphosphatidylation activity, as described (22). Briefly, the cells were incubated for 3 h with 1 μCi [^3H]myristate/ml to label cell phosphatidylcholine. The radioactive medium was then removed, and cells were washed three times with nonradioactive

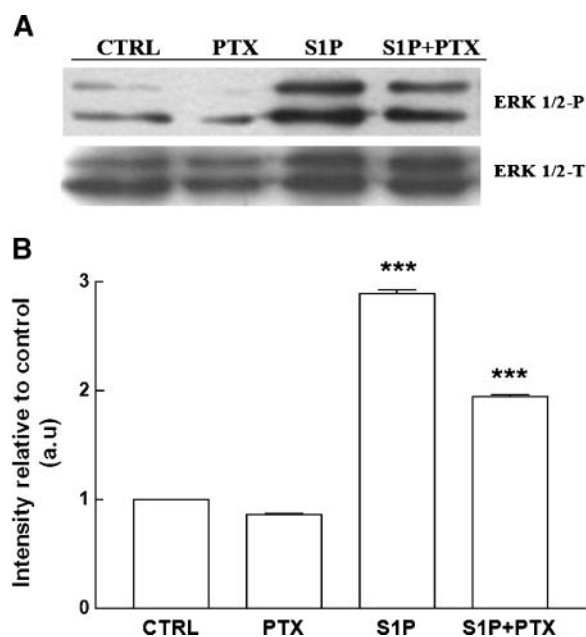


Fig. 4. PTX inhibits SIP-induced ERK 1/2 phosphorylation. **A:** ZG cells were seeded at 3.5×10^6 cells/100 mm dish and treated with 1 $\mu\text{g/ml}$ PTX as described for Fig. 2. S1P (5 μM) was added to the cells for 5 min. Phosphorylation of ERK 1/2 was examined by immunoblotting using specific antibodies to ERK 1/2 (ERK 1/2-P; phospho-Thr 202/Tyr 204). Equal loading of protein was monitored using a specific antibody to total ERK (ERK 1/2-T). Similar results were obtained in each of three replicate experiments. CTRL, control. **B:** Results of scanning densitometry of the exposed film. Data are expressed as arbitrary units (a.u.) of intensity relative to control values and are means \pm SEM of triplicate scans of a single gel. *** $P < 0.001$ for S1P versus S1P+PTX. Similar results were obtained in each of two replicate experiments.

DMEM containing 0.2% BSA. Ethanol, at a final concentration of 1%, was added at 5 min before the addition of agonists, and the cells were incubated further for 30 min because this is the optimal time to recover the maximal formation of phosphatidylethanol. This concentration of ethanol is commonly used for the determination of PLD in cells in culture and did not cause any toxic effect to these cells. Lipids were extracted as described by Bligh and Dyer (23), except that 2 M KCl in 0.2 M HCl was added to the extraction mixture instead of water for the separation of the aqueous and organic phases. Chloroform phases were then vacuum-dried in an automatic SpeedVac concentrator (Savant AS290) and resuspended in 50 μ l of chloroform.

Lipids were separated by TLC using 20 \times 20 cm Silica Gel 60-coated aluminum plates. The TLC plates were developed with chloroform-methanol-acetic acid (9:1:1, v/v/v), and the positions of lipids were identified after staining with iodine vapor by comparison with authentic standards. The silica gel-containing radioactive lipids were quantitated by liquid scintillation counting after scraping the spots off the plates.

Determination of intracellular Ca^{2+} levels

Intracellular Ca^{2+} levels were measured by flow cytometry as described (24). ZG cells were loaded with 2 mM Fluo-4-AM for

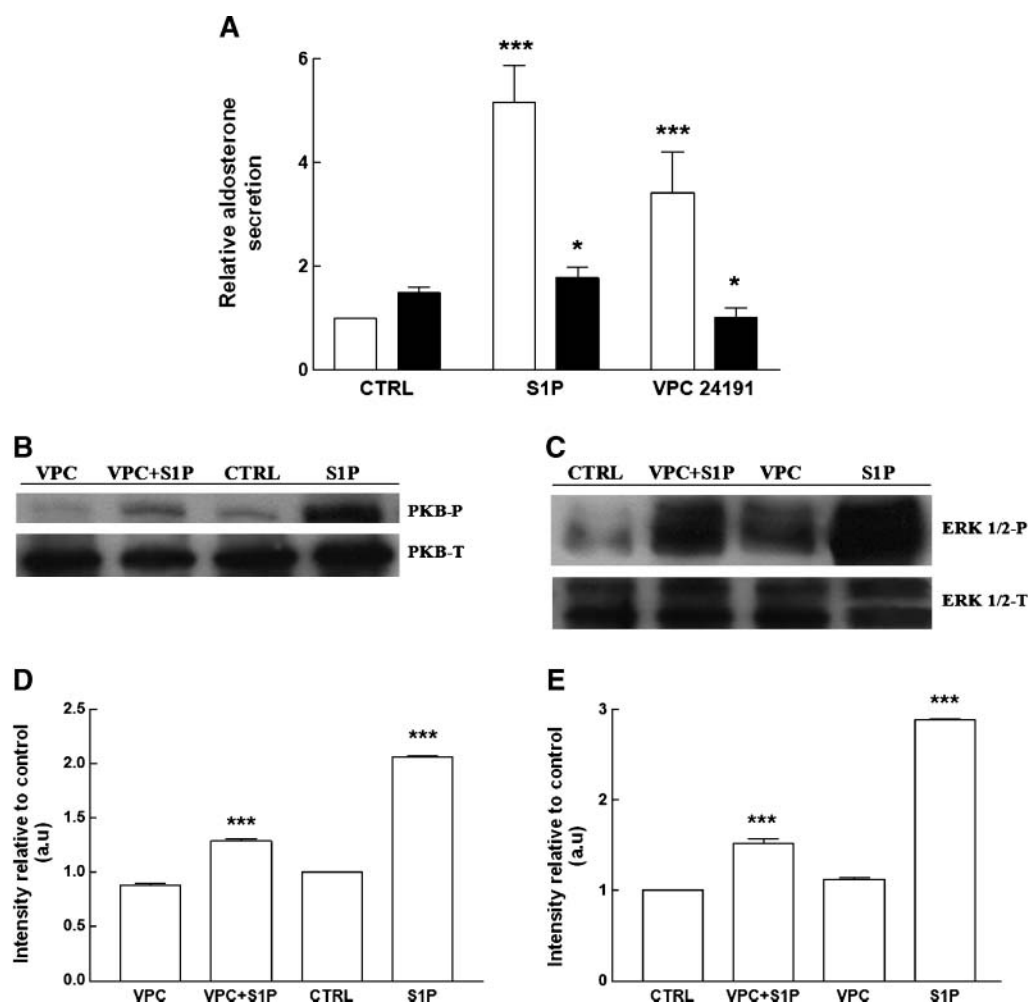


Fig. 5. The S1P1/3 receptor antagonist VPC 23019 blocks S1P-stimulated aldosterone secretion and PKB and ERK 1/2 phosphorylation. **A:** Cells were preincubated with vehicle (open bars) or 2 μ M VPC 23019 (closed bars) for 15 min. S1P (5 μ M) or the S1P1/3 receptor agonist VPC 24191 (2 μ M) was then added, and incubations were continued for another 2 h. Aldosterone secretion was determined as described in Materials and Methods. Values are means \pm SEM of three independent experiments performed in triplicate. * $P < 0.05$, *** $P < 0.001$ for S1P versus S1P+VPC 23019 or for VPC 24191+VPC 23019, as indicated. CTRL, control. **B, C:** ZG cells were seeded at 3.5×10^6 cells/100 mm dish and preincubated in DMEM without BSA or serum for 3 h. They were then preincubated with 2 μ M VPC 23019 (VPC) for 15 min, as indicated. S1P (5 μ M) was then added to the cells for 5 min. Phosphorylation of PKB and ERK 1/2 was examined by immunoblotting using specific antibodies to PKB (PKB-P; anti-phospho-Ser 473) and ERK 1/2 (ERK 1/2-P; phospho-Thr 202/Tyr 204), respectively. Equal loading of protein was monitored using a specific antibody to total PKB (PKB-T) or ERK (ERK 1/2-T). Similar results were obtained in each of three replicate experiments. **D, E:** Results of scanning densitometry of the exposed film. Data are expressed as arbitrary units (a.u.) of intensity relative to control values and are means \pm SEM of triplicate scans of a single gel in each case. *** $P < 0.001$ for S1P versus S1P+VPC 23019 or for VPC 24191 versus VPC 24191+VPC 23019, respectively. Similar results were obtained in each of two replicate experiments.

45 min at room temperature. The cells were then washed twice with PBS containing 0.2% BSA at pH 7.4. After the second wash, the cells were incubated for 10 min at room temperature. Propidium iodide stain was then added at a final concentration of 1 mg/ml for 10 min on ice in the dark. Flow cytometry analyses were performed on a FACScalibur flow cytometer (Becton Dickinson) illuminated at 530 nm (530/30 nm dichroic bandpass filter), and fluorescence was measured at 585 nm (585/42 nm bandpass filter). The Ca^{2+} response was measured as the change in green fluorescence intensity of the cells as a function of time. For each sample, a 20 s baseline monitoring was performed with the flow cytometer. To determine the background autofluorescence of the cells, one sample was recorded without the addition of agonist after baseline monitoring. Analyses were performed with an air-cooled 488 nm argon-ion laser and CellQuest software (Becton Dickinson). Forward scatter and side scatter were used to exclude cell debris from analyses.

Western blotting

ZG cells were harvested and lysed in ice-cold homogenization buffer as described previously (12). Aliquots of protein (50–75 mg) from each sample were loaded and separated by SDS-PAGE using 12% separating gels. Proteins were transferred onto nitrocellulose membranes and blocked for 1 h with 5% skim milk in TBS containing 0.1% Tween 20 and then incubated overnight with the primary antibody in the same medium at 4°C. After three washes with TBS and 0.1% Tween 20, membranes were incubated with rabbit peroxidase-conjugated secondary antibody at 1:4,000 dilution for 1 h. Bands were visualized using an enhanced chemiluminescence assay kit, Super-signal West Femto (Pierce Biotechnology, Inc.). The protein bands were identified by comparison with known molecular weight markers.

Statistical analysis

Unless stated otherwise, results are expressed as means \pm SEM of the indicated number of experiments performed in triplicate. The statistical significance of differences between means of control and experimental conditions was assessed by ANOVA. Values of $P < 0.05$ were considered significant.

RESULTS

The PI3K/PKB pathway is implicated in the stimulation of aldosterone secretion by S1P in glomerulosa cells

As mentioned above, S1P stimulated aldosterone secretion in glomerulosa cells of bovine adrenal glands (1). This action was accompanied by the activation of PLD, and inhibition of this enzyme led to a sharp decrease in aldosterone secretion. We now show that treatment of these cells with S1P causes the phosphorylation of PKB, which is an indication of its activation. PKB phosphorylation was potently inhibited by the PI3K inhibitors LY 294002 and wortmannin, suggesting that PKB is downstream of PI3K in this system (Fig. 1A, B). To evaluate whether activation of the PI3K/PKB pathway was involved in the stimulation of aldosterone secretion by S1P, aldosterone output was determined in the presence of LY 294002 or wortmannin. Figure 1C shows that both of these inhibitors were able to decrease S1P-stimulated aldosterone secretion significantly. Therefore, it can be

concluded that PI3K/PKB is an important pathway for the regulation of aldosterone levels by S1P.

It is well known that many of the biologic effects of S1P are mediated through interaction with specific receptors that are coupled to G_i proteins and are sensitive to PTX. To examine whether activation of the PI3K/PKB pathway was mediated through a receptor of this kind, the cells were preincubated with 1 μ g/ml PTX for 16 h. This treatment completely abolished S1P-stimulated PKB phosphorylation (Fig. 2), suggesting that activation of this pathway by S1P is dependent upon receptor interaction. This finding is consistent with our previous observation that S1P-stimulated aldosterone secretion is inhibited by PTX in these cells (1).

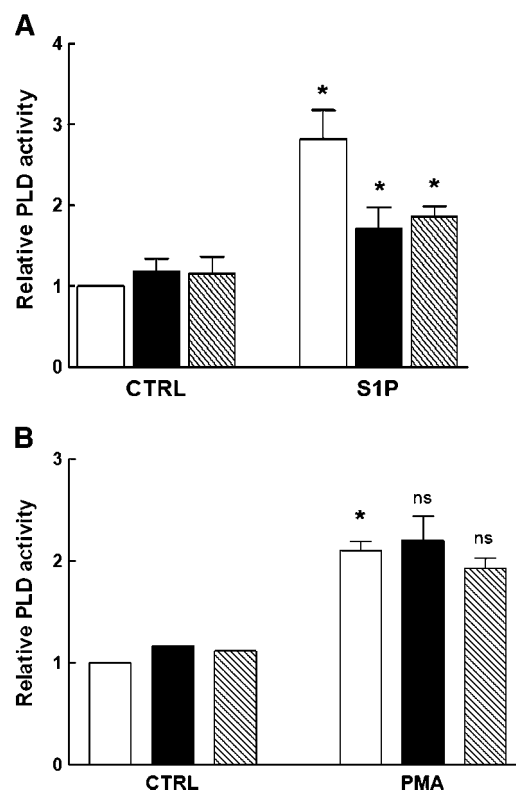


Fig. 6. Involvement of PI3K/PKB in phospholipase D (PLD) activation by S1P. **A:** Cells were preincubated with vehicle (open bars), 50 μ M LY 294002 (closed bars), or 100 nM wortmannin (hatched bars) for 15 min. Ethanol (1%) was added at 5 min before stimulation with S1P (5 μ M) or vehicle, and incubations were continued for another 30 min. PLD activity was determined by measuring the accumulation of [3 H]phosphatidylethanol as described in Materials and Methods. Results were calculated as percentage of the radioactivity present in [3 H]phosphatidylethanol compared with that in total lipids and are expressed as fold stimulation relative to control (CTRL) incubations. Values are means \pm SEM of four independent experiments performed in triplicate. * $P < 0.05$ for S1P versus control or for S1P+LY 294002 and S1P+wortmannin versus S1P. **B:** Cells were preincubated and treated as in A, except that 4 β -phorbol 12-myristate 13-acetate (PMA; 1 μ M) was used instead of S1P. Values are means \pm SEM of three independent experiments performed in triplicate. * $P < 0.05$ for PMA versus control; differences between PMA+LY 294002 or PMA+wortmannin versus PMA were not statistically significant (ns).

The MEK/ERK 1/2 pathway is implicated in SIP-stimulated aldosterone secretion in glomerulosa cells

A variety of reports showed that SIP stimulated the MEK/ERK 1/2 pathway in different cell types (3, 18). Also, it has been demonstrated that aldosterone secretion is regulated by receptors that are coupled to G proteins and by activation of tyrosine kinase receptors, leading to the activation of mitogen-activated protein kinases, including ERK 1/2 (17). Therefore, we examined whether the MEK/ERK 1/2 pathway might also be involved in the stimulation of aldosterone secretion by SIP. First, we observed that SIP stimulated the phosphorylation of ERK 1/2 in the glomerulosa cells (Fig. 3A, B). ERK phosphorylation was substantially decreased by the MEK inhibitor PD 98059 (10 μ M). The latter, along with the inhibitor UO 126 (1 μ M), which also blocks MEK activity, potently reduced SIP-stimulated aldosterone secretion (Fig. 3C), suggesting that the MEK/ERK 1/2 pathway is important in this process.

We next examined whether activation of the MEK/ERK 1/2 pathway by SIP was mediated through a receptor

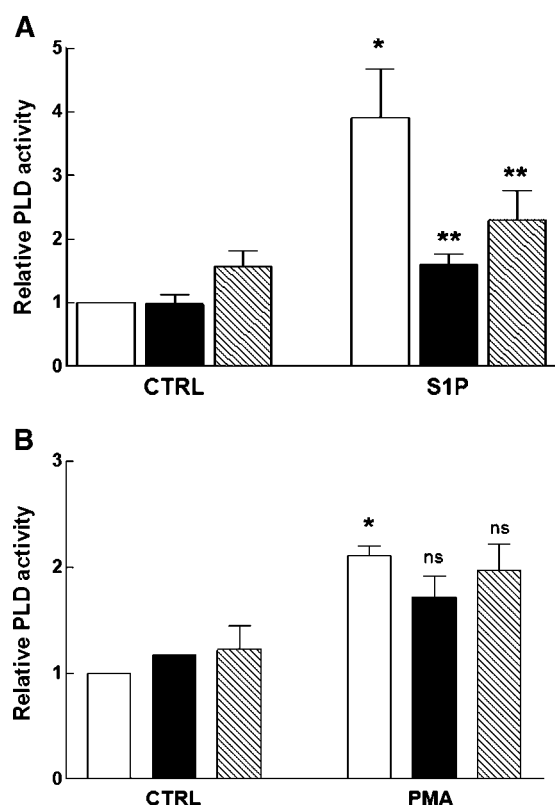


Fig. 7. Involvement of the MEK/ERK pathway in PLD activation by SIP. A: Cells were preincubated with vehicle (open bars), 10 μ M PD 98059 (closed bars), or 1 μ M UO 126 (hatched bars) for 15 min. Ethanol (1%) was added at 5 min before stimulation with SIP (5 μ M) or vehicle, and incubations were continued for another 30 min. PLD activity and results were determined and expressed as described for Fig. 6. * $P < 0.05$ for SIP versus control (CTRL); ** $P < 0.01$ for SIP+PD 98059 or SIP+UO 126. B: Cells were preincubated and treated as in A, except that PMA (1 μ M) was used instead of SIP. Results were determined and expressed as described for Fig. 6. * $P < 0.05$ for PMA versus control; differences between PMA+PD 98059 or PMA+UO 126 versus PMA were not statistically significant (ns).

coupled to a Gi protein. To test this notion, the cells were treated with PTX in the same conditions as in Fig. 2. This treatment caused a significant decrease in SIP-stimulated ERK 1/2 phosphorylation (Fig. 4), suggesting that activation of this pathway by SIP is at least partially dependent on receptor interaction. This finding is also in agreement with our previous observation that SIP-stimulated aldosterone secretion is inhibited by PTX in these cells (1). There are five receptors to which SIP can bind specifically. These receptors are named SIP1–SIP5 and belong to the endothelial differentiation gene family [reviewed by Taha, Mullen, and Obeid (25)]. SIP1 is ubiquitously expressed and is coupled exclusively to PTX-sensitive Gi proteins to stimulate ERK and PI3K. The SIP3 receptor is evolutionarily closely related to SIP1 and is coupled to Gi, Gq, or

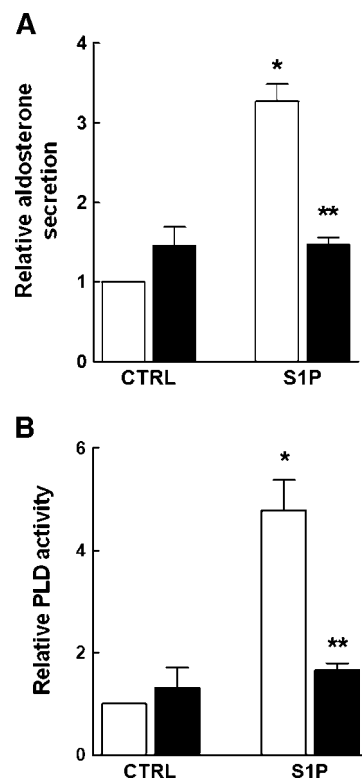


Fig. 8. SIP-stimulated aldosterone secretion and PLD activation are completely inhibited by simultaneous treatment with LY 294002 and PD 98059. A: Cells were preincubated with vehicle (open bars) or with 50 μ M LY 294002 + 10 μ M PD 98059 (closed bars) for 15 min in DMEM supplemented with 0.2% BSA. SIP (5 μ M) was then added, and incubations were continued for another 2 h. Aldosterone secretion was determined as described in Materials and Methods. Results are expressed as fold stimulation relative to incubations with vehicle and are means \pm SEM of three independent experiments performed in triplicate. * $P < 0.05$ for SIP versus control (CTRL); ** $P < 0.01$ for LY 294002+PD 98059+SIP versus SIP. B: Cells were treated and labeled as described for Fig. 6. They were then preincubated in the absence (open bars) or presence (closed bars) of 50 μ M LY 294002 + 10 μ M PD 98059 for 15 min in BSA-free DMEM. SIP (5 μ M) was then added, and incubations were continued for another 30 min in the presence of 1% ethanol. Results were determined and expressed as described for Fig. 6. * $P < 0.05$ for SIP versus control; ** $P < 0.01$ for LY 294002+PD 98059+SIP versus SIP.

G12/13, with activation of PI3K and ERK occurring via the coupling of the receptor to Gi proteins (25). Preincubation of the ZG cells with the S1P1/3 receptor antagonist VPC 23019 completely blocked S1P-stimulated aldosterone secretion (Fig. 5A), suggesting that these receptors are involved in this action. Aldosterone secretion was also enhanced by the S1P agonist VPC 24191, although to a lesser extent than by S1P, and this was also inhibited by the S1P receptor antagonist VPC 23019 (Fig. 5A).

The PI3K/PKB and MEK/ERK 1/2 pathways are implicated in the activation of PLD by S1P in glomerulosa cells

We previously demonstrated that S1P stimulates PLD activity in glomerulosa cells and that this enzyme is impli-

cated in the regulation of aldosterone secretion (1). There are also reports suggesting that PI3K activation is associated with the stimulation of PLD in some cell types (13, 26). Therefore, we hypothesized that PLD is downstream of PI3K in the cascade of events leading to aldosterone secretion. To test this possibility, PLD activity was measured in the presence of the selective PI3K inhibitors LY 294002 and wortmannin. Treatment of ZG cells with these inhibitors significantly reduced S1P-induced PLD activation (Fig. 6A) ($P < 0.05$) at similar concentrations

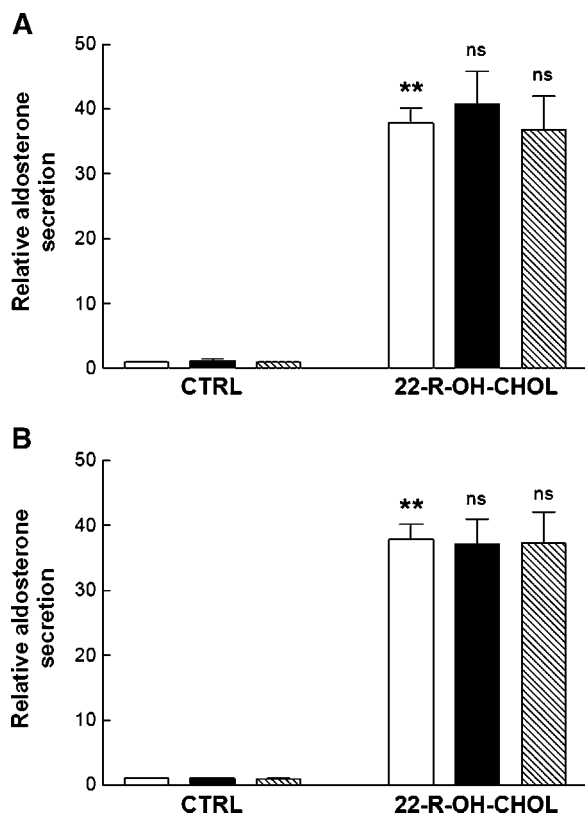


Fig. 9. PI3K/PKB and MEK/ERK 1/2 inhibitors do not block 22-*R*-hydroxycholesterol-stimulated aldosterone secretion. **A:** Cells were preincubated with vehicle (open bars), 50 μ M LY 294002 (closed bars), or 100 nM wortmannin (hatched bars) for 15 min. 22-*R*-Hydroxycholesterol (22-*R*-OH-CHOL; 10 μ M) was then added, and incubation was continued for another 2 h. Aldosterone secretion was determined as described in Materials and Methods. Values are means \pm SEM of three independent experiments performed in triplicate. CTRL, control. **B:** Cells were preincubated with vehicle (open bars), 10 μ M PD 98059 (closed bars), or 1 μ M UO 126 (hatched bars) for 15 min. 22-*R*-Hydroxycholesterol (10 μ M) was then added, and incubation was continued for another 2 h. Results were determined and expressed as in A. ** $P < 0.01$ for 22-*R*-hydroxycholesterol versus control; differences between 22-*R*-hydroxycholesterol+LY 290042, 22-*R*-hydroxycholesterol+wortmannin, 22-*R*-hydroxycholesterol+PD 98059, or 22-*R*-hydroxycholesterol+UO 126 versus 22-*R*-hydroxycholesterol were not statistically significant (ns).

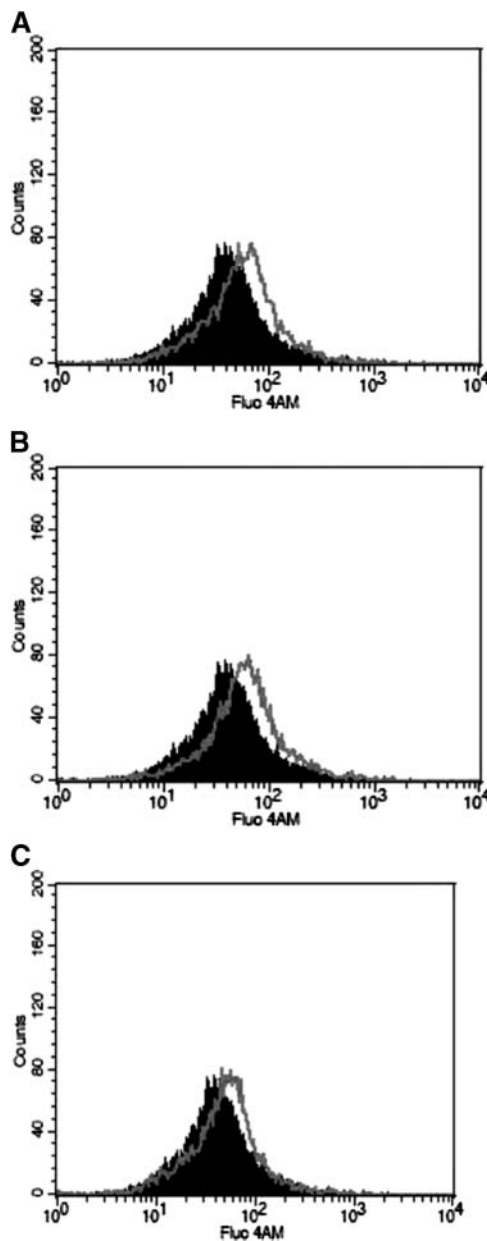


Fig. 10. The PI3K inhibitor LY 294002 and the MEK inhibitor PD 98059 do not alter S1P-stimulated calcium entry in ZG cells. Intracellular Ca^{2+} levels were analyzed by flow cytometry using Fluo-4-AM after stimulation of the cells with 5 μ M S1P (gray line) for 1 min in the absence of agonists (dark area in A) or in the presence of 50 μ M LY 294002 (dark area in B) or 10 μ M PD 98059 (dark area in C). Results are from one experiment and were confirmed in two additional experiments.

that were effective at inhibiting SIP-stimulated aldosterone secretion (Fig. 1B). To rule out a possible nonspecific effect of LY 294002 or wortmannin on PLD activity, the cells were preincubated with the same inhibitors and then stimulated with PMA (1 μ M), a PLD activator that is independent of PI3K. Figure 6B shows that these inhibitors did not block PMA-induced PLD stimulation, suggesting that inhibition of SIP-stimulated PLD activity by LY 294002 or wortmannin is attributable to their effects on PI3K.

As for PI3K, we examined the possible implication of ERK 1/2 in the stimulation of PLD by SIP. This was tested using the selective inhibitors of MEK PD 98059 and UO 126. Figure 7A shows that both of these inhibitors were able to decrease the activation of PLD by SIP at the same concentrations used to block SIP-stimulated aldosterone secretion (Fig. 3C). To eliminate a possible nonspecific effect of PD 98059 or UO 126 on PLD, the cells were pre-treated with these inhibitors and then stimulated with PMA (1 μ M) to activate PLD. Neither of these inhibitors was able to alter PMA-induced PLD activation (Fig. 7B). Together, these results suggest that the MEK/ERK 1/2 pathway is also important for the stimulation of PLD by

SIP in glomerulosa cells. Treatment of these cells with inhibitors of PI3K and MEK simultaneously led to the complete inhibition of SIP-stimulated aldosterone secretion (Fig. 8A) and PLD activation (Fig. 8B), suggesting that both of these pathways are essential for the regulation of the two processes.

A possible nonspecific effect of the PI3K and MEK inhibitors on aldosterone secretion was ruled out by using 22-*R*-hydroxycholesterol, a hydrophobic cholesterol analog that can bypass the usual signaling pathways necessary for steroid secretion (27). We found that 22-*R*-hydroxycholesterol-stimulated aldosterone secretion was not altered significantly by any of these inhibitors (Fig. 9A, B). In a previous report, we showed that SIP-stimulated aldosterone secretion required calcium influx and phosphorylation of the PKC isoforms α and δ leading to PLD activation (1). Therefore, we sought for possible cross-talk of these pathways with PKB or ERK. Figure 10 shows that SIP-stimulated calcium entry was not altered by inhibitors of PI3K/PKB or MEK/ERK. Likewise, phosphorylation of PKC- α and PKC- δ was not altered significantly by any of the PI3K or MEK inhibitors (Figs. 11, 12, respectively). These results suggest a lack of interaction between PI3K/

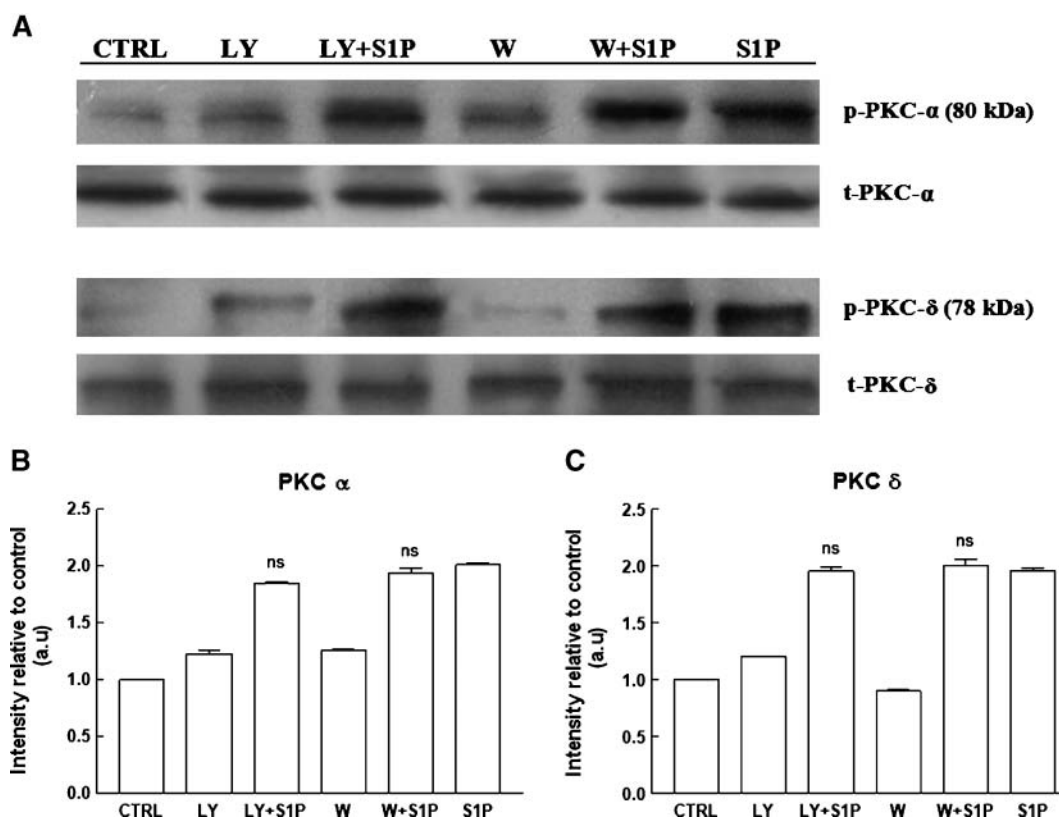


Fig. 11. Activation of protein kinase C- α (PKC- α) and PKC- δ is not affected by inhibitors of PI3K. A: ZG cells were seeded at 3.5×10^6 cells/100 mm dish and preincubated in DMEM without BSA or serum for 3 h. They were then preincubated with 50 μ M LY 294002 (LY) or 100 nM wortmannin (W) for 15 min, as indicated. SIP (5 μ M) was then added to the cells for 5 min. Phosphorylation of PKC- α and PKC- δ was examined by immunoblotting using specific antibodies to PKC- α (p-PKC- α ; anti-phospho-Ser 657) and PKC- δ (p-PKC- δ ; anti-phospho-Ser 643), respectively. Equal loading of protein was monitored using a specific antibody to total PKC- α (t-PKC- α) or total PKC- δ (t-PKC- δ). Similar results were obtained in each of two replicate experiments. CTRL, control. B, C: Results of scanning densitometry of the exposed film. Data are expressed as arbitrary units (a.u.) of intensity relative to control values and are means \pm SEM of triplicate scans of a single gel. Differences between SIP+LY 290042 or SIP+wortmannin versus SIP were not statistically significant (ns).

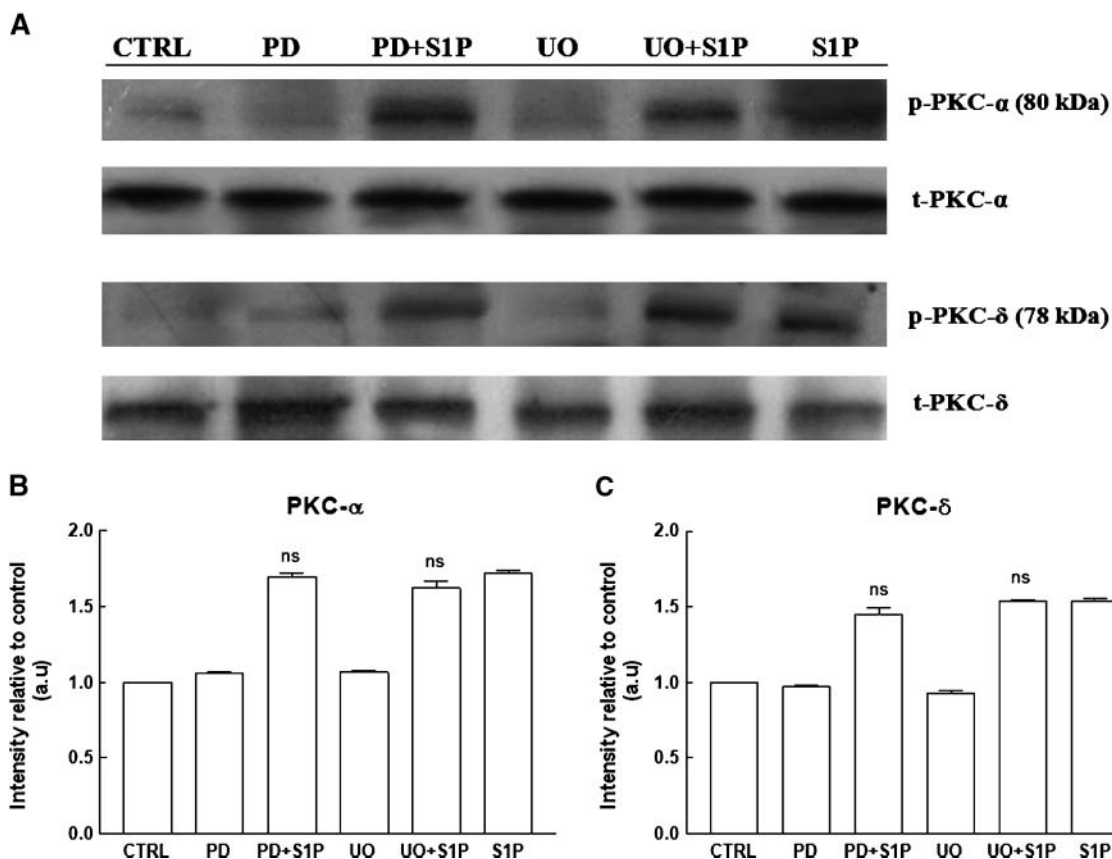


Fig. 12. Involvement of MEK/ERK 1/2 in PKC- α and PKC- δ activation mediated by S1P in ZG cells. **A:** ZG cells were seeded at 3.5×10^6 cells/100 mm dish and preincubated in DMEM without BSA or serum for 3 h. They were then preincubated with 10 μ M PD 98059 (PD) or 1 μ M UO 126 (UO) for 15 min, as indicated. S1P (5 μ M) was then added to the cells for 5 min. Phosphorylation of PKC- α and PKC- δ was examined by immunoblotting using specific antibodies to PKC- α (p-PKC- α ; anti-phospho-Ser 657) and PKC- δ (p-PKC- δ ; anti-phospho-Ser 643), respectively. Equal loading of protein was monitored using a specific antibody to total PKC- α (t-PKC- α) or total PKC- δ (t-PKC- δ). Similar results were obtained in each of two replicate experiments. CTRL, control. **B, C:** Results of scanning densitometry of the exposed film. Data are expressed as arbitrary units (a.u.) of intensity relative to the control value and are means \pm SEM of triplicate scans of a single gel. Differences between S1P+PD 98059 or S1P+UO 126 versus S1P were not statistically significant (ns).

PKB and MEK/ERK with PKC- α , PKC- δ , or calcium levels to stimulate PLD and aldosterone secretion.

DISCUSSION

Previously, we demonstrated that S1P stimulates aldosterone secretion in ZG cells to a similar extent to that of angiotensin II or K^+ ions (1), which are classical regulators of the secretion of this hormone. This observation suggested that S1P might also be a major regulator of aldosterone secretion. Also in that report, we found that part of the mechanism whereby S1P exerted this action involved prior stimulation of PLD activity, calcium influx, and phosphorylation of the PKC isoforms α and δ (1). We have now demonstrated that PI3K/PKB and MEK/ERK 1/2 are major signaling pathways involved in the stimulation of aldosterone secretion by S1P. Interestingly, these two pathways have been shown previously to be involved in secretory processes, including the secretion of histamine from basophiles (28), insulin secretion from β cells of the endocrine pancreas (29), and the production

of catecholamines by chromaffin cells of rat adrenal glands (30, 31). In addition, the MEK/ERK 1/2 pathway is implicated in the release of prostaglandin E_2 after stimulation of A549 lung adenocarcinoma cells with interleukin- 1β (32). We also found that the activation of PLD by S1P, but not PMA-stimulated PLD activity, was blocked by selective inhibitors of MEK and PI3K, suggesting that PLD is downstream of these kinases in the cascade of events leading to aldosterone secretion. In addition, these inhibitors did not affect the stimulation of PKC- α and PKC- δ or calcium influx by S1P, suggesting that these pathways act independently of PKB and ERK to activate PLD.

These observations are consistent with previous work showing that PI3K is implicated in the stimulation of PLD activity by formyl-methionyl-leucil-phenylalanine in HL60 cells (13, 33, 34) or HEK293T cells (35) and in the activation of PLD-1 by chemoattractants in neutrophils (36). However, Zheng and Bollag (37) demonstrated that in human adrenocortical carcinoma cells, the PI3K inhibitors enhanced angiotensin II-stimulated PLD activity. The reasons for this discrepancy are unknown at present, but it is possible that the regulation of these pathways is distinct

in different species (i.e., human versus bovine) or that they are altered in neoplastic cells compared with normal cells. Furthermore, it was shown that 8-bromo-cAMP stimulated PLD activity after activation of ERK 1/2 in primary cells of rat endometrium (38), supporting the hypothesis that PLD is downstream of ERK in that system. The mechanism whereby SIP activates PI3K and ERK 1/2 most likely involves an interaction of SIP with its G_i protein-coupled receptors, because preincubation of cells with PTX before treatment with SIP abolished the activation of these two pathways. PTX also blocked PLD activation and the stimulation of aldosterone secretion by SIP (1), consistent with our observation that PLD is downstream of PI3K and ERK to regulate this process. The SIP1/3-specific receptor antagonist VPC 23019 inhibited the activation of PKB and ERK and aldosterone secretion by SIP, suggesting that SIP1/3 are the receptors involved in these processes. Also, we recently demonstrated that PLD is implicated in the stimulation of cortisol secretion by SIP (19) and by angiotensin II (39), suggesting that this enzyme is a key factor in the regulation of steroidogenesis.

The fact that SIP can stimulate aldosterone secretion is intriguing. Although SIP is as potent as angiotensin II or K^+ in stimulating aldosterone secretion (1), it is unclear whether this is a physiological or a pathological effect of SIP. In this context, SIP and aldosterone have both been associated with cardiovascular diseases. SIP, by stimulating the proliferation of fibroblasts (40) and by enhancing the survival of inflammatory cells such as macrophages (41), is likely to play a role in the excessive fibroproliferative and

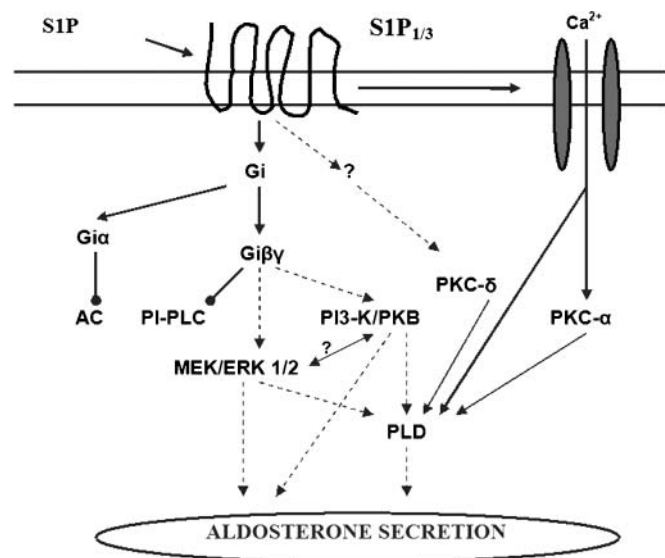


Fig. 13. Working model of SIP action to stimulate aldosterone secretion in ZG cells. SIP binds to a G_i protein-coupled receptor (possibly SIP1/3). This causes stimulation of the PI3K/PKB and MEK/ERK pathways, leading to PLD activation. Also, SIP causes calcium influx and activation of the PKC isoforms α and δ , which are upstream of PLD (1). All of these actions contribute to the stimulation of aldosterone secretion by SIP in ZG cells. AC, adenylyl cyclase; PI-PLC, phosphatidylinositol-specific phospholipase C.

inflammatory response to vascular injury that characterizes the progression of atherosclerosis. The potential role of aldosterone in promoting vascular lesions is highlighted by the Randomized Aldactone Evaluation Study (42), which showed that in patients with severe heart failure undergoing optimal medical therapy, addition of the mineralocorticoid receptor antagonist spironolactone reduced cardiac morbidity and mortality. Also, aldosterone has been shown to mediate some of the proatherogenic effects of angiotensin II (18), and rats treated with this mineralocorticoid exhibited increased arterial NADPH oxidase activity (43). The latter enzyme is responsible for the production of superoxide anions by monocytes/macrophages, an action that contributes to oxidant stress in inflammatory sites (44). NADPH oxidase is also required for monocyte-mediated LDL oxidation and alteration of basic cell functions such as adhesion and proliferation, all of which contribute to atherosclerotic lesion formation (45). Although there are no *in vivo* studies in this work, it could be speculated that aldosterone might mediate at least some of the proatherogenic effects of SIP. However, there are great differences between findings with cells in culture, as in the present work, and the assumption that these will be recapitulated in whole animals. Whether or not SIP and aldosterone could in fact contribute to atherogenesis will require the establishment of specific animal models to help clarify this question.

In conclusion, the present study demonstrates that SIP stimulates the PI3K/PKB and MEK/ERK 1/2 signaling pathways through interaction with the G_i protein-coupled receptors SIP1/3 in primary cultures of ZG cells. Blockade of these pathways using PD 98059 and UO 126 to inhibit MEK, or LY 290042 and wortmannin to inhibit PI3K, abrogated SIP-stimulated aldosterone secretion, suggesting that the two pathways are major mechanisms by which SIP regulates this process. Our working model of the mechanisms involved in the stimulation of aldosterone secretion by SIP is shown in **Fig. 13**.

The authors are grateful to the veterinarians of the Slaughterhouse Gurokela, located at Zorroza-Bilbao (Spain), for kindly providing fresh adrenal glands. This study was supported by Grant BFU2006-13689/BFI from the Ministerio de Educacion y Ciencia (Madrid, Spain) and Grant 9/UPV 00042.310-15852/2004 from the Universidad del País Vasco (Bilbao, Basque Country, Spain). L.B. and M.R. are fellows of the Departamento de Educación, Universidades e Investigación, Gobierno Vasco (Basque Country, Spain).

REFERENCES

- Brizuela, L., M. Rábano, A. Peña, P. Gangoiti, J. M. Macarulla, M. Trueba, and A. Gómez-Muñoz. 2006. Sphingosine 1-phosphate: a novel stimulator of aldosterone secretion. *J. Lipid Res.* **47**: 1238–1249.
- Brownlee, C. 2001. Intracellular signaling: sphingosine-1-phosphate branches out. *Curr. Biol.* **11**: R535–R538.
- Pyne, S., and N. J. Pyne. 2002. Sphingosine-1-phosphate signalling and termination of lipid phosphate receptors. *Biochim. Biophys. Acta.* **1582**: 121–131.

4. Hla, T. 2003. Signaling and biological actions of sphingosine 1-phosphate. *Pharmacol. Res.* **47**: 401–407.
5. Hla, T. 2004. Physiological and pathological actions of sphingosine 1-phosphate. *Semin. Cell. Dev. Biol.* **15**: 513–520.
6. Payne, S. G., S. Milstien, S. E. Barbour, and S. Spiegel. 2004. Modulation of adaptive immune responses by sphingosine 1-phosphate. *Semin. Cell. Dev. Biol.* **15**: 521–527.
7. Pettus, B. J., C. E. Chalfant, and Y. A. Hannun. 2004. Sphingolipids in inflammation: roles and implications. *Curr. Mol. Med.* **4**: 415–418.
8. Saba, J. D., and T. Hla. 2004. Point-counterpoint of sphingosine 1-phosphate metabolism. *Circ. Res.* **94**: 724–734.
9. Stunff, H. L., S. Milstien, and S. Spiegel. 2004. Generation and metabolism of bioactive sphingosine-1-phosphate. *J. Cell. Biochem.* **92**: 882–899.
10. Damirin, A., H. Tomura, M. Komachi, M. Tobo, K. Sato, C. Mogi, H. Nochi, K. Tamoto, and F. Okajima. 2005. Sphingosine 1-phosphate receptors mediate the lipid-induced cAMP accumulation through cyclooxygenase-2/prostaglandin I₂ pathway in human coronary artery smooth muscle cells. *Mol. Pharmacol.* **67**: 1177–1185.
11. Spiegel, S., and S. Milstien. 2003. Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat. Rev. Mol. Cell Biol.* **4**: 397–407.
12. Spiegel, S., and S. Milstien. 2002. Sphingosine 1-phosphate, a key cell signaling molecule. *J. Biol. Chem.* **277**: 25851–25854.
13. Nakamura, M., S. Nakashima, Y. Katagiri, and Y. Nozawa. 1997. Effect of wortmannin and 2-(L-morpholinyl)-8-phenyl-leucyl-phenylalanine-induced phospholipase D activation in differentiated HL60 cells. *Biochem. Pharmacol.* **53**: 1926–1936.
14. Meroni, S. B., M. F. Riera, E. H. Pellizzari, and S. B. Cigorraga. 2002. Regulation of rat Sertoli cell function by FSH: possible role of phosphatidylinositol 3-kinase/protein kinase B pathway. *J. Endocrinol.* **174**: 195–204.
15. Banno, Y., Y. Takuwa, M. Yamada, N. Takuwa, K. Ohguchi, A. Hara, and Y. Nozawa. 2003. Involvement of phospholipase D in insulin-like growth factor-I-induced activation of extracellular signal-regulated kinase, but not phosphoinositide 3-kinase or Akt, in Chinese hamster ovary cells. *Biochem. J.* **369**: 363–368.
16. Spät, A. 2004. Glomerulosa cell—a unique sensor of extracellular K⁺ concentration. *Mol. Cell. Endocrinol.* **217**: 23–26.
17. Shah, B. H., A. J. Baukal, F. B. Shah, and K. J. Catt. 2005. Mechanisms of extracellularly regulated kinases 1/2 activation in adrenal glomerulosa cells by lysophosphatidic acid and epidermal growth factor. *Mol. Endocrinol.* **19**: 2535–2538.
18. Zondag, G. C., F. R. Postma, I. V. Etten, I. Verlan, and W. H. Moolenaar. 1998. Sphingosine 1-phosphate signalling through the G-protein-coupled receptor EDG-1. *Biochem. J.* **330**: 605–609.
19. Rábano, M., A. Peña, L. Brizuela, A. Marino, J. M. Macarulla, M. Trueba, and A. Gómez-Muñoz. 2003. Sphingosine-1-phosphate stimulates cortisol secretion. *FEBS Lett.* **535**: 101–105.
20. Williams, B. C., E. R. T. Lightly, A. R. Ross, I. M. Bird, and S. W. Walker. 1989. Characterization of the steroidogenic responsiveness and ultrastructure of purified zona fasciculata/reticularis cells from bovine adrenal cortex before and after primary culture. *J. Endocrinol.* **121**: 317–324.
21. Jung, E., S. Betancourt-Calle, R. Mann-Blakeney, T. Foushee, M. C. Isles, and W. G. Bollag. 1998. Sustained phospholipase D activation in response to angiotensin II but no carbachol in bovine adrenal glomerulosa cells. *Biochem. J.* **330**: 445–451.
22. Martin, A., A. Gomez-Muñoz, D. W. Waggoner, J. C. Stone, and D. N. Brindley. 1993. Decreased activities of phosphatidate phosphohydrolase and phospholipase D in ras and tyrosine kinase (fps) transformed fibroblasts. *J. Biol. Chem.* **268**: 23924–23932.
23. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**: 911–916.
24. Uekert, J. E., G. Neve von-Caron, A. P. Bos, and P. F. Steeg. 1997. Flow cytometric analysis of *Lactobacillus plantarum* to monitor lag times, cell division and injury. *Lett. Appl. Microbiol.* **25**: 295–299.
25. Taha, T. A., T. D. Mullen, and L. M. Obeid. 2006. A house divided: ceramide, sphingosine, and sphingosine-1-phosphate in programmed cell death. *Biochim. Biophys. Acta.* **1758**: 2027–2036.
26. Vlahos, C. J., W. F. Matter, K. Y. Hui, and R. F. Brown. 1994. A specific inhibitor of PI-3K, 2-(4-morpholinyl)-8-phenyl-4H-1 benzopyran-4-one (LY 294002). *J. Biol. Chem.* **269**: 5241–5248.
27. Betancourt-Calle, S., R. A. Calle, C. M. Isles, S. White, H. Rasmussen, and W. B. Bollag. 2001. Differential effects of agonists of aldosterone secretion in steroidogenic acute regulatory phosphorylation. *Mol. Cell. Endocrinol.* **173**: 87–94.
28. Yano, H., S. Nakanishi, and K. Kimura. 1993. Inhibition of histamine secretion by wortmannin through the blockade of PI-3K in RBL-2H3 cells. *J. Biol. Chem.* **268**: 25846–25856.
29. Bernal-Mizrachi, E., S. Fatrai, J. D. Johnson, M. Ohsugi, K. Otani, Z. Han, K. S. Polonsky, and M. A. Permutt. 2004. Defective insulin secretion and increased susceptibility to experimental diabetes are induced by reduced Akt activity in pancreatic islet β cells. *J. Clin. Invest.* **114**: 928–937.
30. Warashina, A. 2001. Mechanism by which wortmannin and LY 294002 inhibit catecholamine secretion in the rat adrenal medullary cells. *Cell Calcium.* **29**: 239–247.
31. Chasserot-Golaz, S., P. Hubert, and D. Thierse. 1998. Possible involvement of PI3-K in regulated exocytosis: studies in chromaffin cells with the inhibitor LY 294002. *J. Neurochem.* **70**: 2347–2356.
32. Newton, R., L. Cambridge, L. A. Hart, D. A. Stevens, M. A. Lindsay, and P. J. Barnes. 2000. The MAP kinase inhibitors, PD098059, UO126 and SB203580, inhibit IL-1 β -dependent PGE₂ release via mechanistically distinct processes. *Br. J. Pharmacol.* **130**: 1353–1361.
33. Bureleout, C., N. Thibault, S. Levasseur, S. Simard, P. H. Naccache, and S. G. Bourgoin. 2004. Prostaglandin E₂ inhibits PLD pathway stimulated by formyl-methionyl-leucyl-phenylalanine in human neutrophils. Involvement of EP₂ receptors and PI3-K γ . *Mol. Pharmacol.* **66**: 293–301.
34. Exton, J. H. 1999. Regulation of phospholipase D. *Biochim. Biophys. Acta.* **1439**: 121–133.
35. Paruch, S., J. E. Benna, B. Djerdjouri, S. Marullo, and A. Périanin. 2006. A role of p44/42 mitogen-activated protein kinases in formyl-peptide receptor-mediated phospholipase D activity and oxidant production. *FASEB J.* **20**: 142–144.
36. Reinhold, S. L., S. M. Prescott, G. A. Zimmerman, and T. M. McIntyre. 1990. Activation of human neutrophil PLD by three separable mechanisms. *FASEB J.* **4**: 208–214.
37. Zheng, X., and W. B. Bollag. 2003. Angiotensin II induces transient phospholipase D activity in the H295R glomerulosa cell model. *Mol. Cell. Endocrinol.* **206**: 113–122.
38. Yoon, M. S., J. B. Koo, J. H. Hwang, K. S. Lee, and J. S. Hana. 2005. Activation of phospholipase D by 8-Br-cAMP occurs through novel pathway involving Src, Ras, and ERK in human endometrial stromal cells. *FEBS Lett.* **579**: 5635–5642.
39. Rábano, M., A. Peña, L. Brizuela, J. M. Macarulla, A. Gómez-Muñoz, and M. Trueba. 2004. Angiotensin-II-stimulated cortisol secretion is mediated by phospholipase D. *Mol. Cell. Endocrinol.* **222**: 9–20.
40. Gómez-Muñoz, A., D. W. Waggoner, L. O'Brien, and D. N. Brindley. 1995. Interaction of ceramides, sphingosine, and sphingosine 1-phosphate in regulating DNA synthesis and phospholipase D activity. *J. Biol. Chem.* **270**: 26318–26325.
41. Gómez-Muñoz, A., J. Kong, B. Salh, and U. Steinbrecher. 2003. Sphingosine 1-phosphate inhibits sphingomyelinase and blocks apoptosis in macrophages. *FEBS Lett.* **539**: 56–60.
42. Pitt, B., F. Zannad, W. J. Remme, R. Cody, A. Castaigne, A. Perez, J. Palensky, and J. Wittes. 1999. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study investigators. *N. Engl. J. Med.* **341**: 709–717.
43. Sun, Y., J. Zhang, L. Lu, S. S. Chen, M. T. Quinn, and K. T. Weber. 2002. Aldosterone-induced inflammation in the rat heart: role of oxidative stress. *Am. J. Pathol.* **161**: 1773–1781.
44. Keidar, S., M. Kaplan, E. Pavlotzky, R. Coleman, T. Hayek, S. Hamoud, and M. Aviram. 2004. Aldosterone administration to mice stimulates macrophage NADPH oxidase and increases atherosclerosis development. *Circulation.* **109**: 2213–2220.
45. Cathcart, M. 2004. Regulation of superoxide anion production by NADPH oxidase in monocytes/macrophages. *Arterioscler. Thromb. Vasc. Biol.* **24**: 23–28.