Sphingosine 1-phosphate: a novel stimulator of aldosterone secretion

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Abstract Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid capable of regulating critical physiological and pathological functions. Here, we report for the first time that S1P stimulates aldosterone secretion in cells of the zona glomerulosa of the adrenal gland. Regulation of aldosterone secretion is important because this hormone controls electrolyte and fluid balance and is implicated in cardiovascular homeostasis. S1P-stimulated aldosterone secretion was dependent upon the protein kinase C (PKC) isoforms α and δ and extracellular Ca^{2+}, and it was inhibited by pertussis toxin (PTX). S1P activated phospholipase D (PLD) through a PTX-sensitive mechanism, also involving PKC α and δ and extracellular Ca^{2+}. Primary alcohols, which attenuate the formation of phosphatidic acid (the product of PLD), and cell-permeable ceramides, which inhibit PLD activity, blocked S1P-stimulated aldosterone secretion. Furthermore, propranolol, chlorpromazine, and sphingosine, which are potent inhibitors of phosphatidate phosphohydrolase (PAP) (the enzyme that produces diacylglycerol from phosphatidate), also blocked aldosterone secretion. These data suggest that the PLD/PAP pathway plays a crucial role in the regulation of aldosterone secretion by S1P and that Gi protein-coupled receptors, extracellular Ca^{2+}, and the PKC isoforms α and δ are all important components in the cascade of events controlling this process.—Brizuela, L., M. Rábano, A. Peña, P. Gangoiti, J. M. Macarulla, M. Trueba, and A. Gómez-Muñoz. **Sphingosine 1-phosphate: a novel stimulator of aldosterone secretion. *J. Lipid Res.* 2006.** 47: **1238–1249.**

Supplementary key words adrenal gland • atherosclerosis • ceramides • phospholipase D • protein kinase C • sphingolipids

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid that regulates critical biological processes, including cell proliferation, apoptosis, cell differentiation, migration, tumor cell invasion, and angiogenesis (1–6). Although S1P can be produced intracellularly and act as a second messenger, it can be released into the blood stream upon activation of a variety of cell types, including platelets, mast cells, fibroblasts, monocytes, glioma cells, or melanoma cells (1, 2, 7, 8). Also, S1P is stored in serum, where it is bound to albumin. Many of the effects of S1P are elicited through interaction with specific Gi protein-coupled receptors, termed $S1P_{1\text{-}5B}$, which are ubiquitously expressed in cells, and can regulate numerous downstream signals (1, 2). For example, the effects of S1P on cytoskeletal rearrangement and cell motility are mediated by the small GTPases Rac and Rho, whereas stimulation of cell proliferation or cell survival involves mitogen-activated protein kinases, transcription factors such as AP-1, Ca^{2+} mobilization, or phospholipase D (PLD) activation (2, 9). We demonstrated recently that S1P blocks apoptosis in bone marrow-derived macrophages through a mechanism involving the inhibition of acid sphingomyelinase (6). Also, it was reported (10) that sphingosine kinase, the enzyme that catalyzes the formation of S1P from sphingosine, is involved in signaling pathways of proinflammatory cytokines, such as tumor necrosis factor-α. In particular, S1P is implicated in the tumor necrosis factor-α-induced expression of E-selectin and vascular cell adhesion molecule (10) and can regulate the expression of intercellular adhesion molecule-1 (11) and the tumor necrosis factor-α-induced expression of monocyte chemoattractant protein-1.

Aldosterone regulates electrolyte and fluid balance and subsequent blood pressure homeostasis, and more recently it was defined as a key cardiovascular hormone (12). In addition, aldosterone plays an important role in the pathophysiology of heart failure, and it was suggested to mediate at least some of the proatherogenic effects of angiotensin II (13–15). A number of factors have been shown to stimulate or inhibit aldosterone production, including adrenaline, vasoactive intestinal polypeptide, serotonin, ouabain, atrial natriuretic peptide, dopamine, and heparin. However, the principal regulators of aldosterone synthesis and secretion are angiotensin II, potassium ions, and

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Abbreviations: ACTH, adrenocorticotropic hormone; C_2-ceramide, *N*-acetylsphingosine; C_6-ceramide, *N*-hexanoylsphingosine; PA, phosphatidate; PAP, phosphatidate phosphohydrolase; PKC, protein kinase C; PLD, phospholipase D; PMA, 4β-phorbol 12-myristate 13-acetate; PTX, pertussis toxin; S1P, sphingosine-1-phosphate; ZG, zona glomerulosa.

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adrenocorticotropic hormone (ACTH) (12). Here, we demonstrate that S1P is a novel stimulator of aldosterone secretion in cells of the zona glomerulosa (ZG) of the adrenal gland, and we establish the mechanism whereby this effect is brought about.

MATERIALS AND METHODS

Materials

Aldosterone, angiotensin II, BSA (fraction V), DMEM, chlorpromazine, EGTA, HEPES, pertussis toxin (PTX), lysophosphatidate, lysophosphatidylcholine, L-propranolol, dequalinium analog, C14-linked, and rottlerin were from Sigma (St. Louis, MO). Collagenases A and P were from Roche Diagnostics (Barcelona, Spain). [^{3}H]Myristate and [^{3}H]aldosterone were supplied by American Radiolabeled Chemicals (St. Louis, MO). Sphingosine, S1P, *N*-acetylsphingosine (C_2-ceramide), *N*-hexanoylsphingosine (C_6-ceramide), and phosphatidylethanol were from Avanti Polar Lipids (Alabaster, AL). Ro-32-0432, 4β-phorbol 12-myristate 13-acetate (PMA), and hispidine were from Calbiochem-Novabiochem (San Diego, CA). ACTH was supplied by Novartis Pharma S.A (Rueil-Malmaison, France). Antibodies to phosphoprotein kinase C (PKC-α; Ser 657), phospho-PKC-δ (Ser 643), and β-actin were supplied by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Goat anti-rabbit IgG horseradish peroxidase secondary antibody was from Cell Signaling Technology (Beverly, MA). 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 (16). Briefly, glomerulosa cell slices were prepared from 1 year old steers obtained from a local slaughterhouse, and the cells were 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 (2.5×10^5 cells per well). They were then cultured overnight in DMEM containing 10% (v/v) fetal bovine serum, L-glutamine (20 mM), gentamicin (50 μg/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 gland increases to a maximum by 48–72 h (17).

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 (17) using a specific monoclonal antibody against aldosterone and [1,2,6,7-^{3}H]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 specifically designed for this purpose.

Assay of PLD

PLD was determined in intact ZG cells by measuring the production of [^{3}H]phosphatidylethanol, which is the product of its transphosphatidylation activity, as described (18). Briefly, the cells were incubated for 3 h with 1 μCi [^{3}H]myristate/ml to label cell phosphatidylcholine. The radioactive medium was then removed, and cells were washed three times with nonradioactive DMEM containing 0.2% BSA. Ethanol, at a final concentration of 1%, was added 5 min before the addition of agonists, and the cells were incubated further for the indicated times. This concentration of ethanol provided maximal formation of phosphatidylethanol with no toxicity for the cells. Lipids were extracted as described by Bligh and Dyer (19), 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 μl of chloroform. Lipids were separated by TLC using 20 × 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 after scraping the spots off the plates by liquid scintillation counting.

Assay of phosphatidylinositol-dependent phospholipase C

Phosphatidylinositol-dependent phospholipase C was determined as described previously (20). Briefly, ZG cells were incubated for 24 h at 37°C in DMEM supplemented with 10% FBS containing 1 μCi/ml *myo*-[1-2-^{3}H]inositol to label cell phosphoinositides. The cells were then washed twice in the absence of radioactive inositol and incubated in BSA-free medium with 10 mM LiCl for 2.5 h. Reactions were stopped with 0.5 ml of 5% $HClO_4$ and 100 μl of BSA (20 mg/ml). The [^{3}H]inositol phosphates were separated from [^{3}H]inositol by retention on columns of ion-exchange resin (Dowex AG1-X8; 100–200 mesh), as described (21). The radioactive samples eluted off the column were quantified by liquid scintillation counting.

cAMP determination

The levels of cAMP were determined essentially as described (22). Briefly, ZG cells were incubated for 72 h at 37°C in DMEM supplemented with 10% FBS. The cells were then washed twice and incubated in BSA-free medium supplemented with 1 mM isobutylmethylxanthine for 15 min before any addition. Agonists were added as required, and the reactions were stopped with 0.5 ml of 10% trichloroacetic acid after 20 min. The cells were then left on ice for ~1 h to allow cell disruption. The lysates were centrifuged at 10,000 *g* for 5 min. The supernatants were then collected and washed four times with diethylether saturated with water. cAMP levels were determined by radioimmunoassay using a commercial kit, Cyclic AMP (^{3}H) Assay System Code TRK 432 (Amersham Biosciences UK Ltd.).

Western blotting

ZG cells were harvested and lysed in ice-cold homogenization buffer as described previously (1). Aliquots of protein (50–75 μg) from each sample were loaded and separated by SDS-PAGE using 12% separating gels. Proteins were transferred onto nitrocellulose paper 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/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, Supersignal West Femto (Pierce Biotechnology,

Inc.).The protein bands were identified by comparison with known molecular weight markers.

Measurement of intracellular Ca^{2+} levels by flow cytometry

Intracellular Ca^{2+} levels were determined essentially as described (23). ZG cells were loaded with 2 μM Fluo-4-AM for 45 min at room temperature. The cells were then washed twice with phosphate-buffered saline containing 0.2% BSA at pH 7.4 to remove extracellular dye. After the second wash, the cells were incubated for 10 min at room temperature. To determine the percentage of dead cells after loading with Fluo-4-AM, propidium iodide staining was performed at a final concentration of 1 μg/ml for 10 min on ice in the dark. Flow cytometry analyses were done on a FACScalibur flow cytometer (Becton Dickinson) at 530 nm (530/30 nm dichroic bandpass filter), and propidium iodide 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 (23).

Statistical analysis

Unless stated otherwise, results are expressed as means ± SEM of the indicated number of experiments performed in triplicate. The statistical significance of the difference between means of control and experimental conditions was assessed with Student's paired *t*-test. Values of $P < 0.05$ were considered significant.

RESULTS

S1P stimulates aldosterone secretion in ZG cells

It is well established that the secretion of aldosterone is mainly regulated by the renin-angiotensin system, K^+ ions, and ACTH. Here, we report on the existence of a novel regulator of aldosterone secretion: S1P. Like these agonists (24, 25), S1P stimulated aldosterone secretion in a time- and concentration-dependent manner at concentrations that are achievable in vivo. Maximal stimulation was attained after 2 h of incubation of ZG cells (**Fig. 1**, upper panel) with 5 μM S1P (Fig. 1, lower panel). This action was selective for S1P, as other structurally related sphingolipids or glycerolipids, including sphingosine, ceramide, lysophosphatidic acid, and lysophosphatidylcholine, were much less effective or failed to stimulate the secretion of this hormone (Fig. 1).

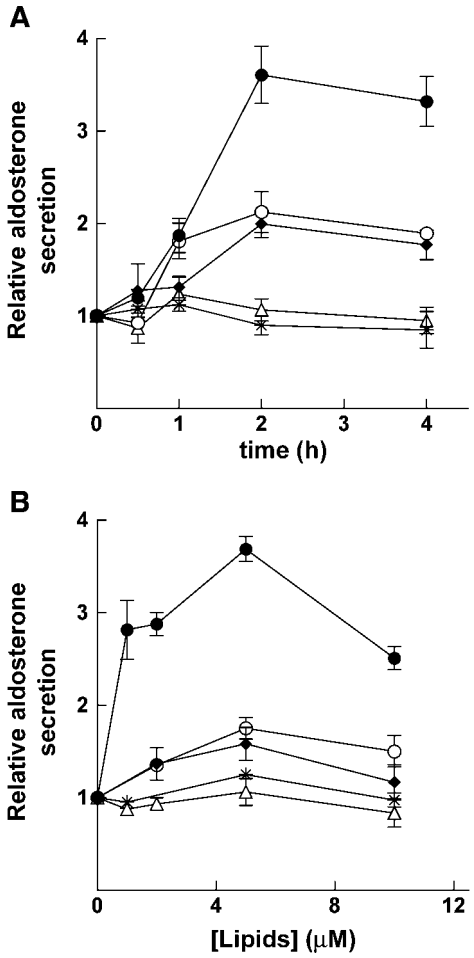

Fig. 1. Sphingosine-1-phosphate (S1P) stimulates aldosterone secretion in zona glomerulosa (ZG) cells. Upper panel: ZG cells were incubated for various times with 5 μM S1P (closed circles), lysophosphatidylcholine (open circles), lysophosphatidate (diamonds), *N*-acetylsphingosine (C_2-ceramide; stars), or sphingosine (triangles). For experimental details, see Materials and Methods. Results are expressed as fold stimulation relative to incubations with vehicle, and they are means ± SEM of four independent experiments performed in triplicate. Lower panel: ZG cells were incubated for 2 h with increasing concentrations of S1P (closed circles), lysophosphatidylcholine (open circles), lysophosphatidate (diamonds), C_2-ceramide (stars), or sphingosine (triangles). Results are expressed as fold stimulation relative to incubations with vehicle, and they are means ± SEM of three independent experiments performed in triplicate, except for the value at 5 μM S1P, which is the mean ± SEM of 18 experiments. The basal value of aldosterone secretion was 0.5 ± 0.02 ng/mg protein (mean ± SEM; n = 24).

S1P activates PLD in ZG cells

Given the relevance of the observations described above, experiments were aimed at elucidating the mechanism whereby S1P controls the secretion of aldosterone. As mentioned previously, S1P can stimulate the activity of key enzymes that regulate important signal transduction processes. Two major pathways that are affected by S1P are the cAMP/protein kinase A pathway and the PLD/phosphatidate phosphohydrolase (PAP) pathway. Therefore, we first examined whether S1P could affect the concentration of cAMP in these cells. S1P did not cause any significant change in the levels of cAMP and did not modify the ACTH-induced production of this nucleotide, which indicates that the stimulation of aldosterone secretion by S1P is independent of adenylyl cyclase activation [basal value of cAMP concentration was 2.93 ± 0.88 pmol/10^6 cells, and this was increased up to 6.84 ± 1.35 pmol/10^6 cells (mean ± SEM of four independent experiments performed in triplicate) by 10 nM ACTH].

We next evaluated whether S1P might be able to stimulate the PLD/PAP pathway. In particular, activation of PLD is important because this enzyme is implicated in the regulation of vital cellular processes, such as cell proliferation and survival, cytoskeleton rearrangement, and the secretory pathway (26–29). The activity of PLD was determined by measuring the accumulation of [^{3}H]phosphatidylethanol using [^{3}H]myristic acid to label cellular lipids, as indicated in Materials and Methods. [^{3}H]myristic acid is preferentially incorporated into phosphatidylcholine (30–33), which is the major substrate for PLD in mammalian tissues (30, 34). As shown in **Fig. 2**, S1P activated PLD in a time- and concentration-dependent manner, reaching maximal values at 30 min of incubation of the cells with 5 μM S1P. Angiotensin II, a major stimulator of aldosterone secretion, can also activate PLD in these cells (35, 36). However, the mechanisms of action of angiotensin II and S1P on aldosterone secretion (**Fig. 3**, upper panel) and PLD activation (Fig. 3, lower panel) are likely to be independent, as their effects are approximately additive. Also, the combined effects of S1P and ACTH, or S1P and K^+ ions, on the stimulation of aldosterone secretion were additive (**Fig. 4**, upper and lower panel, respectively), suggesting that the mechanisms of action of ACTH and K^+ differ from that of S1P. These data also indicate that the extent of activation of aldosterone secretion by S1P is approximately half that of ACTH and similar to that of

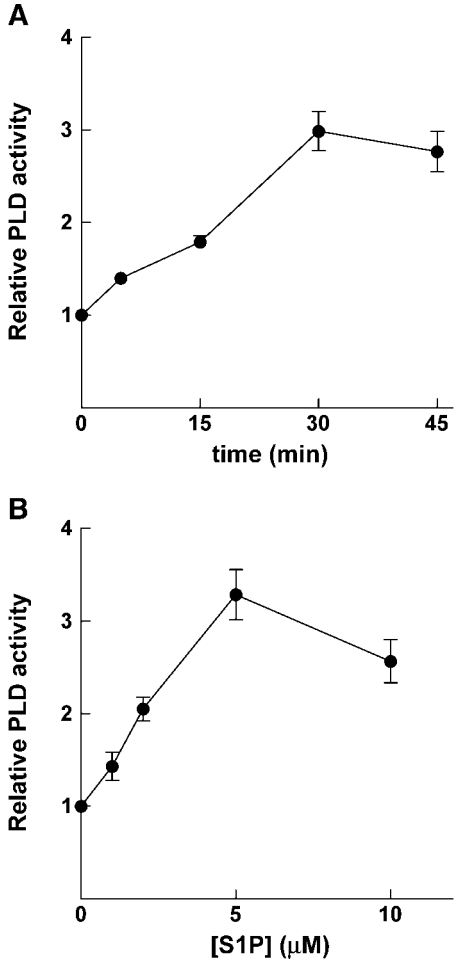

Fig. 2. S1P stimulates phospholipase D (PLD) activity in ZG cells. ZG cells were labeled with 1 μCi/ml [^{3}H]myristate in DMEM containing 0.2% BSA for 3 h. The cells were then washed three times with this same medium, but in the absence of label, and incubated further in BSA-free DMEM for 2.5 h. Cells were stimulated with 5 μM S1P for various times (upper panel) or with increasing concentrations of S1P for 30 min, as indicated (lower panel), in the presence of 1% ethanol. [^{3}H]phosphatidylethanol formation was determined as indicated in Materials and Methods. Results were calculated as a percentage of the radioactivity present in [^{3}H]phosphatidylethanol compared with that in total lipids and are expressed as fold stimulation relative to incubations with vehicle. Values are means ± SEM of four independent experiments performed in triplicate, except for the value at 5 μM S1P, which is the mean ± SEM of 15 experiments.

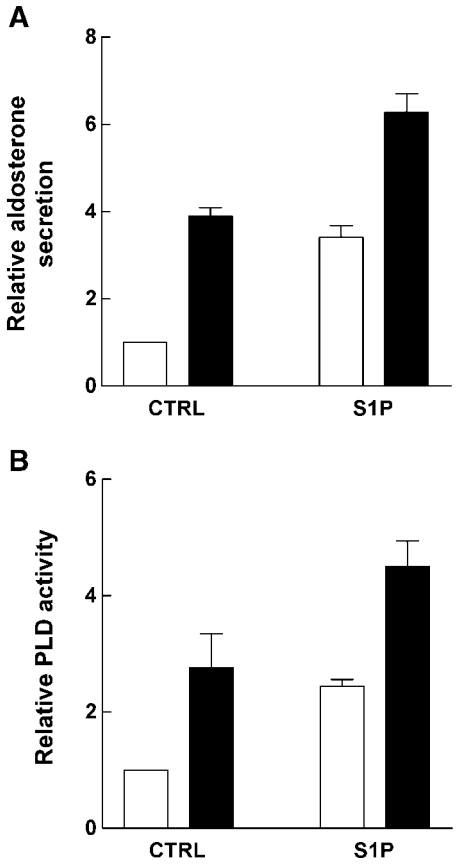

Fig. 3. Effect of S1P and angiotensin II on aldosterone secretion and PLD activation in ZG cells. Cells were incubated for 2 h with S1P (5 μM) in the presence (closed bars) or absence (open bars) of angiotensin II (10 nM). Upper panel: Aldosterone secretion was determined as described in Materials and Methods. Results are calculated as described for Fig. 1 and are expressed as means ± SEM of three independent experiments performed in triplicate. Lower panel: For determination of PLD activity, ethanol (1%) was added 5 min before cell stimulation, and incubations were continued further for 30 min. The enzyme activity was determined by measuring the accumulation of [^{3}H]phosphatidylethanol as described in Materials and Methods. Results were calculated as a 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 ± SEM of five independent experiments performed in triplicate.

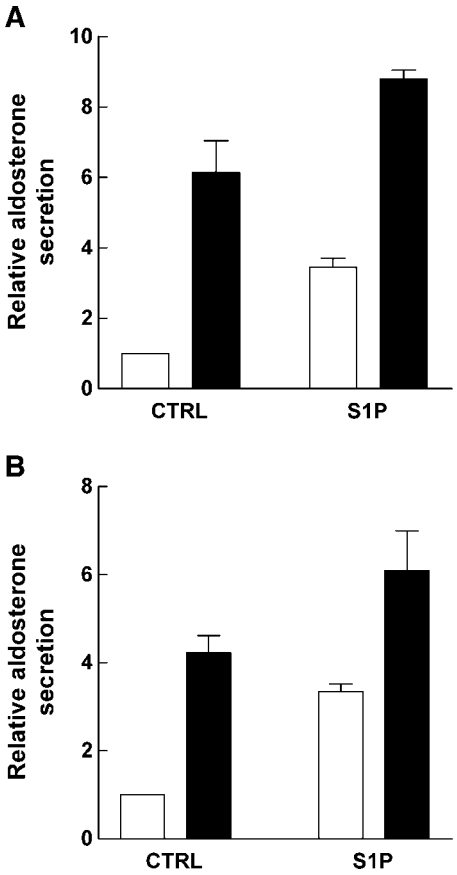

Fig. 4. Effect of S1P on adrenocorticotropic hormone (ACTH)- or K^+-stimulated aldosterone secretion. ZG cells were incubated for 2 h with S1P (5 μM) in the presence (closed bars) or absence (open bars) of ACTH (1 nM; upper panel) or K^+ ions (16 mM; lower panel). Aldosterone secretion was determined as described in Materials and Methods. Results are calculated as described for Fig. 1 and are expressed as means ± SEM of three independent experiments performed in triplicate. CTRL, control.

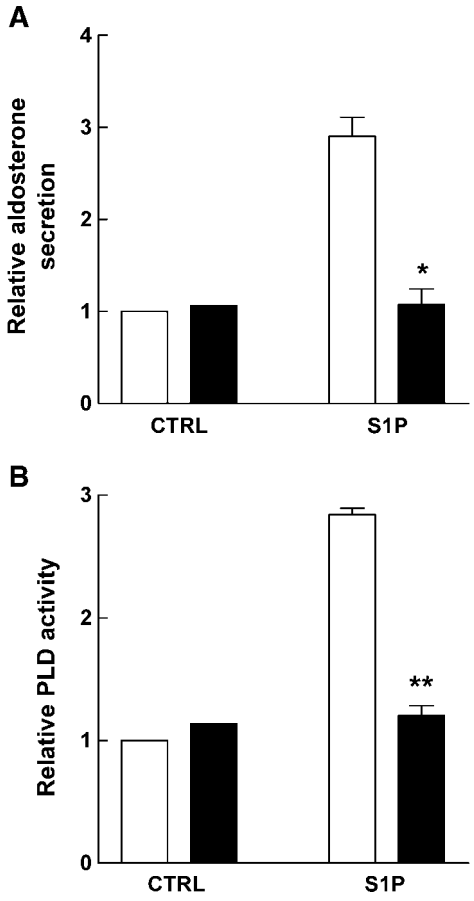

Fig. 5. Effect of pertussis toxin (PTX) on aldosterone secretion and PLD activation by S1P in ZG cells. Upper panel: Cells were preincubated in the absence (open bars) or presence (closed bars) of 1 μg/ml PTX for 16 h. S1P (5 μM) or vehicle [control (CTRL)] was then added, as indicated, and the incubations were continued further for 2 h. Aldosterone secretion was determined as described in Materials and Methods. Results are calculated and expressed as described for Fig. 1 and are means ± SEM of three independent experiments performed in triplicate. Lower panel: Cells were preincubated in the absence (open bars) or presence (closed bars) of 1 μg/ml PTX for 16 h. S1P (5 μM) or vehicle [control (CTRL)] was then added, as indicated. Ethanol (1%) was added 5 min before stimulation with S1P, and incubations continued further for 30 min. PLD activity was determined by measuring the accumulation of [^{3}H]phosphatidylethanol as described in Materials and Methods. Results were calculated as a percentage of the radioactivity present in [^{3}H]phosphatidylethanol compared with that in total lipids and are expressed as fold stimulation relative to control incubations. Values are means ± SEM of three independent experiments performed in triplicate. ** $P < 0.01$; * $P < 0.05$.

angiotensin II or K^+, suggesting that S1P could be also considered a major regulator of this process.

S1P-stimulated aldosterone secretion and PLD activation are inhibited by PTX

Although S1P is rapidly taken up by cells and can act intracellularly, many of its biological effects are caused by binding to specific receptors that are coupled to PTX-sensitive Gi proteins (1, 2). We observed that the stimulation of aldosterone secretion by S1P was completely blocked by PTX ($P < 0.05$), suggesting that this is a process mediated by this type of receptor (**Fig. 5**, upper panel). Like stimulation of aldosterone secretion, and in agreement with previous work in other cell types (32, 37), PLD activation was also inhibited by PTX ($P < 0.01$) (Fig. 5, lower panel).

Roles of PKC and Ca^{2+} ions in aldosterone secretion and PLD activation by S1P

It was reported previously that steroid secretion, including aldosterone, can be regulated by PKC and Ca^{2+} independently of cAMP (21, 32, 33, 38). Therefore, we investigated the possible involvement of these factors in the stimulation of aldosterone secretion by S1P. The involvement of PKC was first studied by preincubating the cells with PMA (2 μM) for 48 h, as this treatment causes downregulation of PKC in these cells (39). Under these conditions, the cells lost their sensitivity to stimulation by PMA [aldosterone output was decreased from 2.01 ± 0.08-fold to 0.86 ± 0.12-fold (mean ± SEM of three independent experiments performed in triplicate; $P < 0.05$)],

and the effect of S1P was almost completely abolished (**Fig. 6**, upper panel). Similar results were obtained by preincubating the cells for 30 min with the PKC inhibitor Ro-32-0432 (5 μM) before S1P addition (data not shown). These results indicate that PKC activity is involved in S1P-stimulated aldosterone secretion in ZG cells. To identify the specific PKC isoforms that are involved in this action of S1P, experiments were carried out in the presence of selective inhibitors of PMA-downregulatable PKC isoforms. We found that dequalinium and rottlerin, which are selective inhibitors of PKC-α and PKC-δ, respectively, partially inhibited S1P-stimulated aldosterone secretion when added alone to the cells in culture and that this effect was almost completely inhibited by the combined action of the two inhibitors (Fig. 6, lower panel). By contrast, hispidine, a specific inhibitor of PKC-β, had no effect (Fig. 6, lower panel). These results suggest that the PKC isoforms that are involved in the stimulation of aldosterone secretion by S1P are PKC-α and PKC-δ. To support this hypothesis, we performed Western blot analyses using specific antibodies to the phosphorylated forms of these PKC isoforms, as phosphorylation of PKC-α and PKC-δ is an indication of their activation (40–42). We found that S1P caused the phosphorylation of both PKC isoforms and that dequalinium and rottlerin abolished PKC-α and PKC-δ phosphorylation, respectively (**Fig. 7**).

We then tested whether Ca^{2+} was necessary for the stimulation of aldosterone secretion by S1P. Addition of the extracellular Ca^{2+} chelator EGTA to the culture medium substantially decreased this effect of S1P, suggesting that extracellular Ca^{2+} ions are essential in this process (**Fig. 8**, upper panel). Although S1P can also increase cytosolic Ca^{2+} from internal stores by both inositol trisphosphate-dependent and -independent mechanisms, it did not stimulate phosphatidylinositol-specific phospholipase C (data not shown), and the secretion of aldosterone was not inhibited further by the combined pretreatment of cells with EGTA and the intracellular Ca^{2+} chelator BAPTA-AM (Fig. 8, upper panel). The efficiency of BAPTA-AM to chelate intracellular Ca^{2+} was previously tested using the calcium ionophore A23187 to allow Ca^{2+} entry to the cells (32). Failure of S1P to mobilize intracellular Ca^{2+} in ZG cells was further confirmed by flow cytometry through direct measurement of intracellular Ca^{2+} using Fluo-4-AM as dye (23) (see Materials and

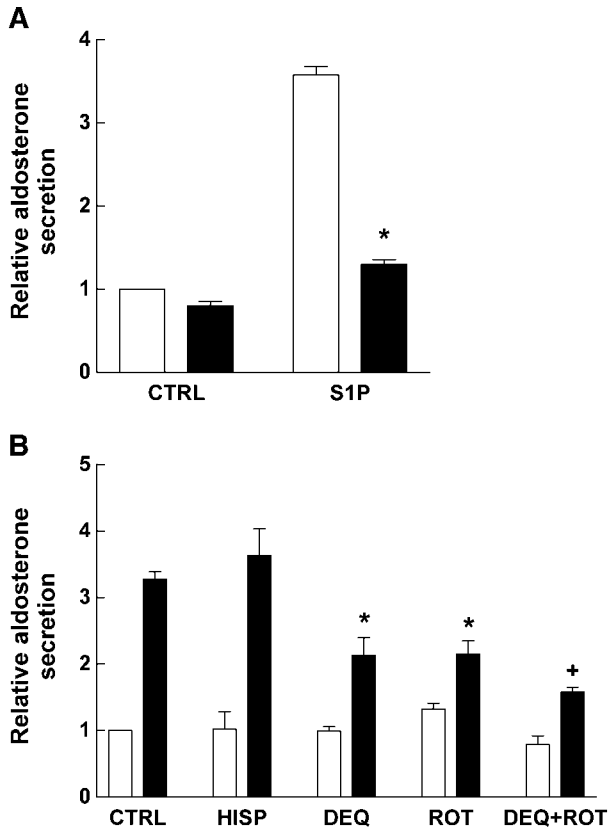

Fig. 6. Involvement of protein kinase C (PKC) in S1P-stimulated aldosterone secretion in ZG cells. Upper panel: Cells were preincubated with vehicle (open bars) or 2 μM 4β-phorbol 12-myristate 13-acetate (PMA; closed bars) for 48 h. S1P (5 μM) was then added, and incubation continued further for 2 h. Aldosterone secretion was determined as described in Materials and Methods. Results are expressed as described for Fig. 1 and are means ± SEM of three independent experiments performed in triplicate. Lower panel: Cells were preincubated for 30 min with vehicle (CTRL), 2 μM hispidine (HISP), 0.5 μM dequalinium (DEQ), 5 μM rottlerin (ROT), or 0.5 μM dequalinium + 5 μM rottlerin (DEQ+ROT), as indicated (hispidine, dequalinium, and rottlerin selectively inhibit PKC-β, PKC-α, and PKC-δ, respectively). The cells were then stimulated with vehicle (open bars) or 5 μM S1P (closed bars) and incubated further for 2 h. Results are calculated and expressed as described for Fig. 1 and are means ± SEM of four independent experiments performed in triplicate. * $P < 0.05$, DEQ or ROT versus CTRL; $^{+}$ $P < 0.05$, DEQ+ROT versus DEQ or ROT.

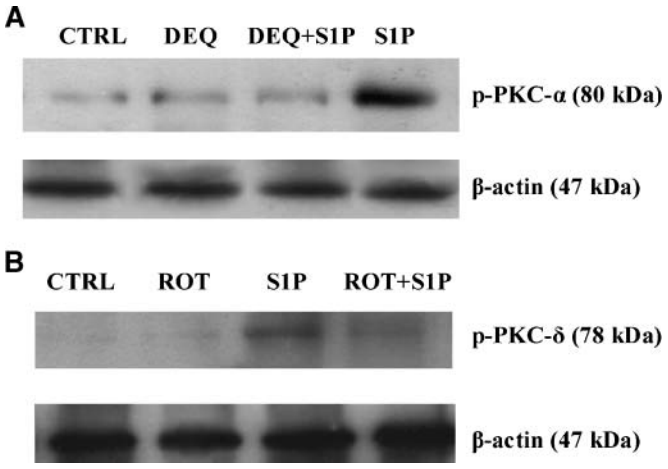

Fig. 7. S1P induces the phosphorylation of PKC-α and PKC-δ. ZG cells were seeded at 3.5×10^6 cells/100 mm dish and preincubated in DMEM without BSA and serum for 3 h. They were then preincubated with 0.5 μM dequalinium (DEQ) (A) or 5 μM rottlerin (ROT) (B) for 30 min, as indicated. S1P (5 μM) was then added to the cells for 5 min. Phosphorylation of the different PKC isoforms was examined by immunoblotting using specific antibodies to PKC-α (phospho-Ser 657) or PKC-δ (phospho-Ser 643). Equal loading of protein was monitored using a specific antibody to β-actin. Similar results were obtained in each of two replicate experiments. CTRL, control.

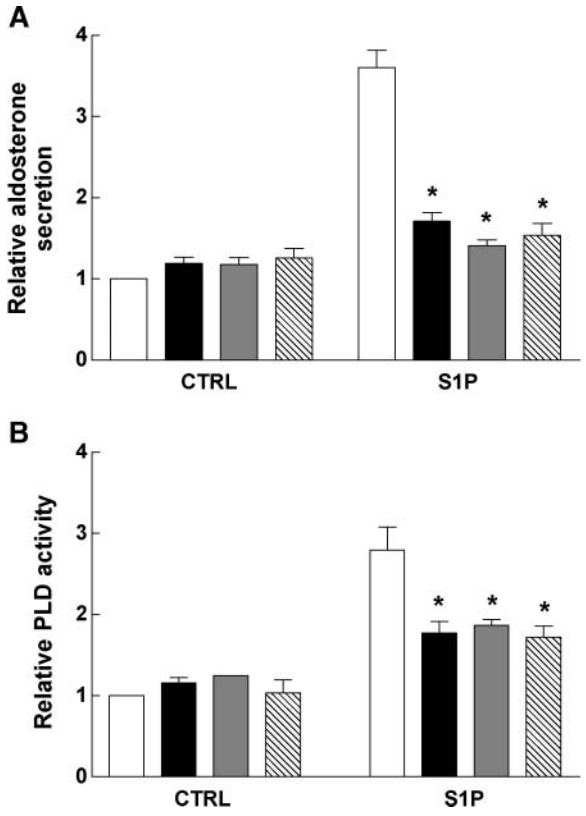

Fig. 8. Involvement of extracellular Ca^{2+} in S1P-induced aldosterone secretion and PLD activation. Upper panel: Cells were treated as described for Fig. 1 and preincubated for 30 min with 5 mM EGTA (closed bars), 5 μM BAPTA-AM (gray bars), 5 mM EGTA + 5 μM BAPTA-AM (hatched bars), or vehicle (open bars). They were then stimulated with vehicle (CTRL) or S1P (5 μM) as indicated and incubated further for 2 h. Aldosterone secretion was determined as described in Materials and Methods. Results are expressed as described for Fig. 1 and are means ± SEM of three independent experiments. Lower panel: Cells were treated and labeled as described for Fig. 2 and preincubated for 30 min with 5 mM EGTA (closed bars), 5 μM BAPTA-AM (gray bars), 5 mM EGTA + 5 μM BAPTA-AM (hatched bars), or vehicle (open bars), as indicated. Ethanol (1%) was added 5 min before stimulation with vehicle or S1P (5 μM), and incubations were continued further for 30 min. PLD activity was determined by measuring the accumulation of [^{3}H]phosphatidylethanol as described in Materials and Methods. Results were calculated and expressed as described for Fig. 2 and are means ± SEM of three independent experiments. * $P < 0.05$.

Methods for details). Intracellular Ca^{2+} levels did not change upon stimulation of the cells with 5 μM S1P for 1–10 min in the presence of EGTA (data not shown).

As shown in the upper panel of Fig. 8, extracellular Ca^{2+} is important for the regulation of aldosterone secretion by S1P, so we tested whether this cation was also required for the activation of PLD by S1P. Although PLD is not affected by physiological concentrations of Ca^{2+} in vitro, there is evidence suggesting that Ca^{2+} regulates PLD activity in intact cells (32–34, 37, 43–45). In fact, Ca^{2+}ionophores increase the activity of PLD in different cell types, including Sf 9 cells that were transfected with this enzyme (34, 46). In agreement with these observations, we found that the calcium ionophore A23187 (1 μM) stimulated PLD by ~2.76 ± 0.32-fold (mean ± SEM of three independent experiments performed in triplicate) in ZG cells and that this effect was almost completely abolished by EGTA [PLD activity was decreased to 1.35 ± 0.15-fold (mean ± SEM of three independent experiments performed in triplicate) by 5 mM EGTA]. Likewise, EGTA significantly decreased ($P < 0.05$) the stimulation of PLD by S1P, suggesting a requirement for extracellular Ca^{2+} in this process (Fig. 8, lower panel). As for aldosterone secretion, S1P-stimulated PLD was not inhibited further by the combined pretreatment of cells with EGTA and the intracellular Ca^{2+} chelator BAPTA-AM, which is consistent with the lack of effect of S1P on intracellular Ca^{2+} mobilization. It can be concluded that extracellular Ca^{2+} ions are involved in the regulation of both aldosterone secretion and PLD activation by S1P in ZG cells.

PKC is an important regulator of PLD, and this enzyme activity has been implicated in the regulation of steroid secretion (32, 33, 35, 36). Therefore, we hypothesized that PKC might play an important role in the regulation of PLD by S1P in ZG cells. As for aldosterone secretion, the involvement of PKC in PLD activation was first investigated by treating the cells with PMA (2 μM) for 48 h to downregulate this enzyme activity. Under these conditions, the cells lost their sensitivity to rapid stimulation of PLD by PMA ($P < 0.05$) and the effect of S1P was completely abolished ($P < 0.01$) (**Fig. 9**, upper panel). Moreover, PMA did not enhance PLD activation by S1P significantly, suggesting that both of these agonists function through similar mechanisms to activate PLD (data not shown). As for aldosterone secretion, the involvement of the PKC-α and PKC-δ isoforms in PLD activation was assessed using the selective inhibitors dequalinium and rottlerin, respectively. These inhibitors partially blocked ($P < 0.05$) PLD activation by S1P when added to the cells independently; however, PLD was completely inhibited by the combined actions of the two inhibitors (Fig. 9, lower panel).

S1P-stimulated aldosterone secretion is mediated by PLD

These results, together with our previous observations (32, 33) and those of others (35, 36), led us to hypothesize that PLD might be involved in the stimulation of aldosterone secretion by S1P. This possibility was assessed by examining aldosterone output in the presence of primary alcohols, which can reduce the levels of the PLD product, phosphatidic acid (PA). We found that optimal concentrations of primary alcohols that are used for the determination of PLD activity, 1% ethanol and 0.3% 1-butanol (3, 30, 31, 47), significantly decreased ($P < 0.05$) S1P-stimulated aldosterone secretion (**Fig. 10**). To rule out any possible nonspecific effect of the alcohols on aldosterone secretion, in some experiments the cells were stimulated in the presence of 2-butanol, as secondary alcohols are not substrates for PLD (48). Unlike 1-butanol, 0.3% 2-butanol did not alter S1P-stimulated aldosterone secretion significantly (Fig. 10), suggesting that the inhibitory effects of the primary alcohols were caused by decreasing the levels of PLD-derived PA. A possible nonspecific effect of alco-

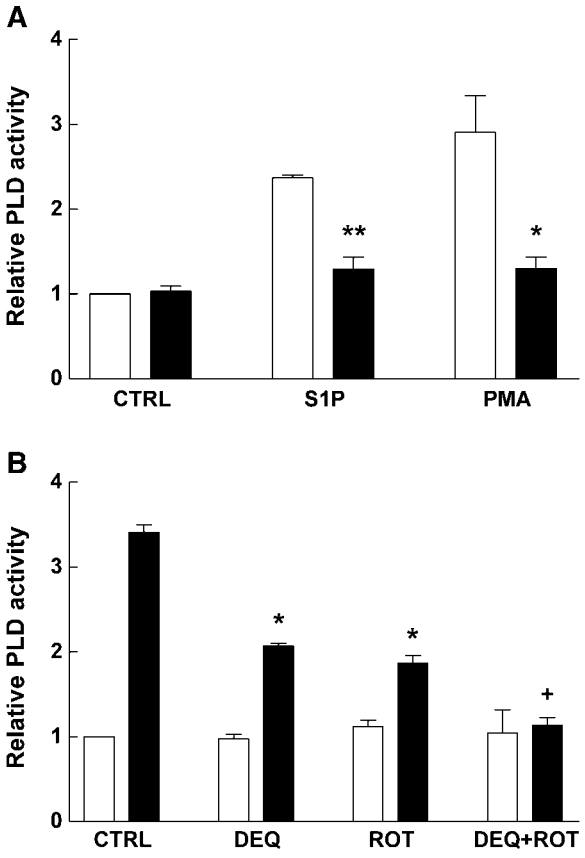

Fig. 9. Involvement of PKC in PLD activation by S1P in ZG cells. Upper panel: Cells were preincubated with vehicle (open bars) or 2 μM PMA for 48 h (closed bars) to downregulate PKC. Ethanol (1%) was added 5 min before stimulation with S1P (5 μM), PMA (2 μM), or vehicle, and incubations were continued further for 30 min. PLD activity was determined by measuring the accumulation of [^{3}H]phosphatidylethanol as described in Materials and Methods. Results were calculated as a 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 ± SEM of three independent experiments performed in triplicate. Lower panel: Cells were preincubated for 30 min with vehicle (CTRL), 2 μM hispidine (HISP), 0.5 μM dequalinium (DEQ), 5 μM rottlerin (ROT), or 0.5 μM dequalinium + 5 μM rottlerin (DEQ+ROT), as indicated. Ethanol (1%) was added 5 min before stimulation with 5 μM S1P or vehicle, and incubations were continued further for 30 min. Results were calculated and expressed as for the upper panel and are means ± SEM of three independent experiments performed in triplicate. ** $P < 0.01$; * $P < 0.05$; $^+$ $P < 0.05$, DEQ+ROT versus DEQ or ROT.

hols on aldosterone secretion was previously ruled out using 22-*R*-hydroxycholesterol, a hydrophobic cholesterol analog that can bypass the usual signaling pathways necessary for steroid secretion (49). In this work, we also observed that 22-*R*-hydroxycholesterol-stimulated aldosterone secretion was not altered by either 1% ethanol or 0.3% 1-butanol [22-*R*-hydroxycholesterol stimulated aldosterone secretion by ~10.81 ± 2.80-fold (mean ± SEM of three independent experiments performed in triplicate) under the conditions used in these experiments].

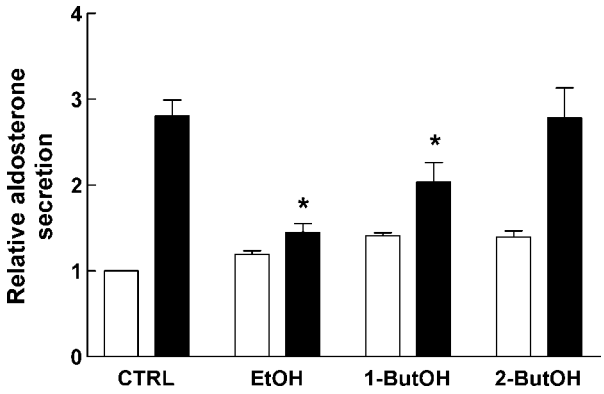

Fig. 10. Primary alcohols inhibit S1P-stimulated aldosterone secretion in ZG cells. Cells were treated as described for Fig. 1 and preincubated for 5 min with 1% ethanol (EtOH), 0.3% 1-butanol (1-ButOH), or 0.3% 2-butanol (2-ButOH), as indicated. They were then stimulated with vehicle (open bars) or 5 μM S1P (closed bars) and incubated further for 2 h. Aldosterone secretion was determined as described in Materials and Methods. Results are expressed as fold stimulation relative to control (CTRL) incubations and are means ± SEM of three independent experiments performed in triplicate. * $P < 0.05$.

The involvement of PLD in S1P-induced aldosterone secretion was further evaluated by stimulating the cells in the presence of short-chain cell-permeable ceramides, which are potent inhibitors of PLD (5, 50) but do not affect phosphatidylinositol-dependent phospholipase C activity (29, 32). As expected, C_6-ceramide completely blocked ($P < 0.05$) the stimulation of PLD by S1P (**Fig. 11**, upper panel). Likewise, the stimulation of aldosterone secretion by S1P was completely abolished ($P < 0.01$) by C_6-ceramide (Fig. 11, lower panel). The latter experiments included relatively high concentrations of ceramide (100 μM), because aldosterone secretion is determined in the presence of a high concentration of BSA (0.2%), which binds ceramide and makes it less available to cells (51). Nonetheless, C_6-ceramide at 10 μM substantially decreased aldosterone secretion in the presence of 0.2% BSA (Fig. 11, lower panel). The inhibitory effect of ceramide on aldosterone secretion may not be entirely attributable to the inhibition of PLD, as ceramides can also inhibit enzyme activities that are implicated in steroidogenesis. In fact, it was reported previously that C_6-ceramide caused a significant decrease in testosterone biosynthesis induced by 22-*R*-hydroxycholesterol (52), and we have observed that C_2-ceramide decreases the stimulation of cortisol secretion induced by 22-*R*-hydroxycholesterol in zona fasciculata cells of bovine adrenal glands (32). Likewise, C_2-ceramide decreased 22-*R*-hydroxycholesterol (10 μM)-stimulated aldosterone secretion from 10.81 ± 2.80-fold to 7.08 ± 1.63-fold (mean ± SEM; n = 3). In addition, we observed that S1P-stimulated aldosterone secretion was inhibited by the amphiphilic amines propranolol, chlorpromazine, and sphingosine (**Fig. 12**), which are all potent inhibitors of PAP (53, 54), the enzyme that produces diacylglycerol from PA. Together, these data suggest that the stimulation of aldosterone se-

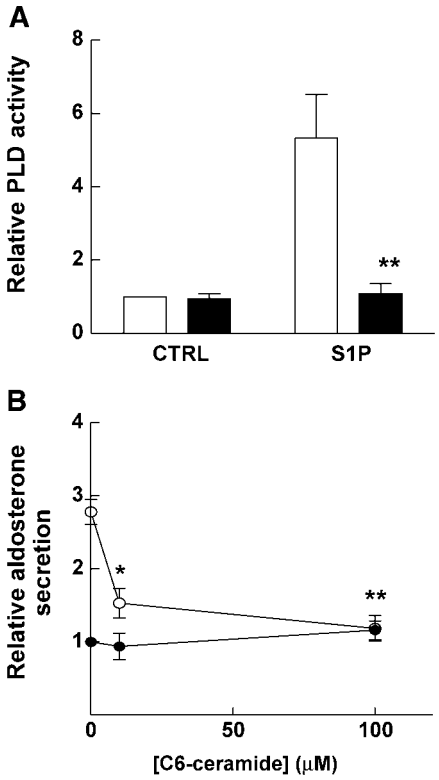

Fig. 11. Inhibition of S1P-stimulated PLD and aldosterone secretion by *N*-hexanoylsphingosine (C_6-ceramide) in ZG cells. Upper panel: Cells were treated and labeled as described for Fig. 3. They were then preincubated in the absence (open bars) or presence (closed bars) of 10 μM C_6-ceramide for 2 h in BSA-free DMEM. S1P (5 μM) was added, and incubations were continued further for 30 min in the presence of 1% ethanol. PLD activity was determined by measuring the accumulation of [^{3}H]phosphatidylethanol as described in Materials and Methods. Results were calculated as a percentage of the radioactivity present in [^{3}H]phosphatidylethanol compared with that in total lipids and expressed as fold stimulation relative to control (CTRL) incubations. Values are means ± SEM of four independent experiments performed in triplicate. Lower panel: Cells were preincubated with vehicle (open circles) or with the indicated concentrations of C_6-ceramide (closed circles) for 2 h in DMEM supplemented with 0.2% BSA. S1P (5 μM) was then added, and incubations were continued further for 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 ± SEM of four independent experiments performed in triplicate. ** $P < 0.01$.

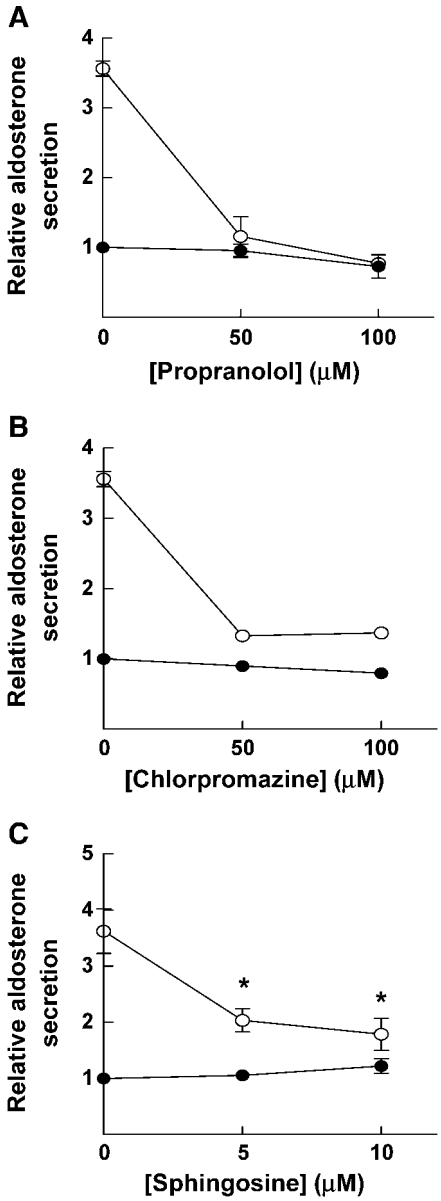

Fig. 12. Effect of propranolol, chlorpromazine, and sphingosine on S1P-stimulated aldosterone secretion in ZG cells. Upper panel: Cells were treated as described for Fig. 1 and preincubated for 15 min with the indicated concentrations of propranolol. They were then stimulated with 5 μM S1P (open circles) or vehicle (closed circles) and incubated further for 2 h. Aldosterone secretion was determined as described in Materials and Methods. Results are calculated as described for Fig. 1 and expressed as means ± range of two independent experiments performed in triplicate. Middle panel: The cells were preincubated for 15 min with the indicated concentrations of chlorpromazine and then stimulated with 5 μM S1P (open circles) or vehicle (closed circles) for 2 h. Results are calculated and expressed as for the upper panel. Lower panel: The cells were preincubated for 15 min with the indicated concentrations of sphingosine and then stimulated with 5 μM S1P (open circles) or vehicle (closed circles) for 2 h. Results are expressed as fold stimulation relative to incubations with vehicle and are means ± SEM of four independent experiments performed in triplicate. * $P < 0.05$.

cretion by S1P likely involves the concerted action of PLD and PAP activities.

DISCUSSION

S1P plays an essential role in the regulation of pathophysiological processes, including cell death, proliferation, differentiation, angiogenesis, cancer, inflammation, and cardiovascular disease, including atherosclerosis (1, 5, 6, 29, 55–57). However, the mechanisms whereby S1P exerts its actions are not well understood. As S1P binds to and activates multiple Gi protein-coupled receptors, the

beneficial and harmful actions of S1P are critically dependent on the expression profiles of their target receptor subtypes and their coupling to different signal transduction pathways in the target cells. In this study, we present strong evidence for a novel action of S1P: stimulation of aldosterone secretion, an effect that is completely inhibited by PTX, suggesting that it is a process mediated by Gi protein-coupled receptors. Secretion of aldosterone is critical for the transport of Na^+, K^+, and water in the organism; therefore, this novel action of S1P places it as a central metabolite for controlling fluid and electrolyte balance and blood pressure homeostasis. Also, like S1P (56, 57), aldosterone has been implicated in atherogenesis (15, 58) and associated with vascular injury (58–60).

There are several pathways involved in the regulation of steroid secretion. In particular, we demonstrated recently that S1P stimulated the secretion of cortisol by a mechanism involving PLD (32) and that this enzyme is also implicated in the angiotensin II-stimulated secretion of this hormone (33). Interestingly, PLD has also been associated with the stimulation of aldosterone secretion by angiotensin II (35). In this work, we show that S1P stimulates both aldosterone secretion and PLD activity through a mechanism that is dependent upon the presence of extracellular Ca^{2+} and PKC activation. The downregulation of PKC by prolonged incubation with phorbol esters such as PMA is widely used to implicate PKC in the regulation of cell function. However, this experimental approach only provides information on generic PKC activity. This is also the case for many inhibitors of PKC, including those of the Ro family, which are not specific and block the activity of different PKC isoforms. Here, we show that S1P causes the phosphorylation of PKC-α and PKC-δ, and that selective inhibitors of these two PKC isozymes block both aldosterone secretion and PLD activation. These observations are consistent with previous work showing that S1P stimulated PLD activity through the activation of PKC-α in C2C12 myoblasts (61) and through the activation of PKC-δ in human airway epithelial cells (62) and A549 lung adenocarcinoma cells (63).

Two isoforms of PLD (PLD-1 and PLD-2) have been identified and cloned in mammalian cells [reviewed by Exton (27, 34)]. Both of these isozymes are expressed in cells of the ZG (64) and also in the zona fasciculata of bovine adrenal gland (33). Although PLD-1 and PLD-2 are differentially regulated in vitro, there are no clear differences in whether the two isoforms are regulated by distinct mechanisms in vivo. In fact, we (31) and others (27) have shown that both PLD-1 and PLD-2 are regulated by Ca^{2+} ions and PKC in intact cells. Therefore, it is possible that the two PLD isoforms may be involved in the regulation of aldosterone secretion by S1P.

In addition, we have observed that blockade of PAP, the enzyme that produces diacylglycerol from PA (the product of PLD), results in the inhibition of S1P-stimulated aldosterone secretion, thereby supporting the notion that the PLD/PAP pathway regulates this process. There are no specific inhibitors of PAP; however, amphiphilic amines, including chlorpromazine, propranolol, and sphingosine, block this enzyme activity very potently both in intact cells and in cell-free systems (53, 54). The involvement of the PLD/PAP pathway in the stimulation of aldosterone secretion by S1P was further assessed by examining aldosterone output in the presence of primary alcohols, which can reduce the levels of PA by forming phosphatidylalcohols via transphosphatidylation, a reaction that is uniquely catalyzed by PLD. We found that optimal concentrations of ethanol or 1-butanol (3, 30, 31, 33) significantly reduced S1P-stimulated aldosterone secretion. By contrast, secondary alcohols, which are not substrates for PLD (48), did not affect aldosterone secretion. Furthermore, and in agreement with our previous observations (5, 50), the cell-permeable C_6-ceramide inhibited PLD activity in these cells, and this treatment also blocked S1P-stimulated aldosterone secretion. These observations support the hypothesis of an involvement of PLD in the stimulation of aldosterone secretion by S1P. Nonetheless, although ceramide blocks PLD, this may not be the only reason for the inhibition of aldosterone secretion by this sphingolipid, as ceramides can also affect the activities or the expression of enzymes that participate in steroid synthesis (52, 65).

It can be concluded that S1P is a novel regulator of aldosterone secretion, which is crucial for hemodynamic stability. The data presented here suggest that stimulation of aldosterone secretion by S1P involves the PLD/PAP pathway and that Gi proteins, extracellular Ca^{2+}, and the PKC isoforms α and δ are all important components of the signaling pathways controlling this process.

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