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Abstract

Somatostatin (SS) is a peptide hormone that inhibits insulin secretion in β -cells by activating its $G_{i/o}$ -coupled receptors. Our previous work indicated that a $\beta\gamma$ -dimer of $G_{i/o}$ coupled to SS receptors can activate phospholipase D1 (PLD1) (Cheng, H., Grodnitzky, J. A., Yibchok-anun, S., Ding, J., and Hsu, W. H. (2005) *Mol. Pharmacol.* 67, 2162–2172). The aim of the present study was to elucidate the mechanisms underlying SS-induced PLD activation. We demonstrated the presence of ADP-ribosylation factor Arf1 and Arf6 in clonal β -cells, HIT-T15. We also determined that the activation of PLD1 was mediated through Arf6. Overexpression of dominant-negative (dn) Arf6 mutant, Arf6(T27N), and suppression of mRNA levels using siRNA, both abolished SS-induced PLD activation, while overexpression of wild type Arf6 further enhanced this PLD activation. In contrast, overexpression of dn-Arf1 mutant Arf1(T31N) or dn-Arf5 mutant Arf5(T31N) failed to reduce SS-induced PLD activation. These findings suggested that Arf6, but not Arf1 or Arf5, mediates the effect of SS. We further determined the involvement of the Arf6 guanine nucleotide exchange factor (GEF) EFA6A, a GEF previously thought to be found predominantly in the brain, in the activation of PLD1 in HIT-T15 cells. Using Northern and Western blot analyses, both mRNA and protein of EFA6A were found in these cells. Overexpression of dn-EFA6A mutant, EFA6A(E242K), and suppression of mRNA levels using siRNA, both abolished SS-induced PLD activation, whereas overexpression of dn-EFA6B mutant, EFA6B(E651K), failed to reduce SS-induced PLD activation. In addition, overexpression of dn-ARNO mutant, ARNO(E156K), another GEF of Arf6, had no effect on SS-induced activation of PLD. Taken together, these results suggest that SS signals through EFA6A to activate Arf6-PLD cascade.

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Somatostatin Receptors Signal through EFA6A-ARF6 to Activate Phospholipase D in Clonal β -Cells*

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Somatostatin (SS) is a peptide hormone that inhibits insulin secretion in β -cells by activating its $G_{i/o}$ -coupled receptors. Our previous work indicated that a $\beta\gamma$ -dimer of $G_{i/o}$ coupled to SS receptors can activate phospholipase D1 (PLD1) (Cheng, H., Grodnitzky, J. A., Yibchok-anun, S., Ding, J., and Hsu, W. H. (2005) *Mol. Pharmacol.* 67, 2162–2172). The aim of the present study was to elucidate the mechanisms underlying SS-induced PLD activation. We demonstrated the presence of ADP-ribosylation factor Arf1 and Arf6 in clonal β -cells, HIT-T15. We also determined that the activation of PLD1 was mediated through Arf6. Overexpression of dominant-negative (dn) Arf6 mutant, Arf6(T27N), and suppression of mRNA levels using siRNA, both abolished SS-induced PLD activation, while overexpression of wild type Arf6 further enhanced this PLD activation. In contrast, overexpression of dn-Arf1 mutant Arf1(T31N) or dn-Arf5 mutant Arf5(T31N) failed to reduce SS-induced PLD activation. These findings suggested that Arf6, but not Arf1 or Arf5, mediates the effect of SS. We further determined the involvement of the Arf6 guanine nucleotide exchange factor (GEF) EFA6A, a GEF previously thought to be found predominantly in the brain, in the activation of PLD1 in HIT-T15 cells. Using Northern and Western blot analyses, both mRNA and protein of EFA6A were found in these cells. Overexpression of dn-EFA6A mutant, EFA6A(E242K), and suppression of mRNA levels using siRNA, both abolished SS-induced PLD activation, whereas overexpression of dn-EFA6B mutant, EFA6B(E651K), failed to reduce SS-induced PLD activation. In addition, overexpression of dn-ARNO mutant, ARNO(E156K), another GEF of Arf6, had no effect on SS-induced activation of PLD. Taken together, these results suggest that SS signals through EFA6A to activate Arf6-PLD cascade.

Somatostatin (SS)³ is a peptide hormone that is well known for its ability to inhibit the secretion of growth hormone, glu-

cagon, and insulin (1, 2). This peptide hormone is secreted from many tissues in the body including the pancreatic δ -cells (1, 2). SS secreted from δ -cells exerts an inhibitory paracrine effect on insulin secreting β -cells and is released in two sizes; a 14-amino acid peptide and its prohormone NH_2 -terminal extended form, a 28-amino acid peptide (1, 2). There are six SS receptors, all of which are G-protein coupled receptors (GPCRs) (3, 4), which couple through $G_{i/o}$. SS transduces its physiological signal through many effectors, which include adenylyl cyclase (5), inwardly rectifying K^+ channels (6), tyrosine phosphatase (7), phospholipase C (8), voltage-dependent Ca^{2+} channel (9), $Na^+ - H^+$ antiporter (10), and mitogen-activated protein kinase (11). We recently reported that SS can activate phospholipase D (PLD), causing a subsequent increase in phosphoinositol 4,5-bisphosphate (PIP_2) concentration (12). This receptor-mediated activation of PLD was attributed to the release of the $\beta\gamma$ -dimer coupled to SS-receptors and occurred in a pertussis toxin-sensitive manner. We also demonstrated that PLD1 was the only detectable isoform present in the clonal β -cells HIT-T15 and the downstream stimulatory effects of SS on PIP_2 levels and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) could be attributed to the activation of PLD1 (12).

PLD Hydrolyzes Phosphatidylcholine to phosphatidic acid. The conversion of phosphatidylcholine to phosphatidic acid regulates many cellular processes such as endocytosis, exocytosis, cell proliferation, and cell migration (13–17). There are two mammalian PLD isoforms, PLD1 and PLD2. These isoforms differ in their subcellular location and regulation. PLD1 is located in the cytosol, Golgi apparatus, nucleus, and plasma membrane, while PLD2 seems to be largely found on the plasma membrane (18). Each isoform may exist as one of two splice variants, *i.e.* PLD1a, PLD1b, PLD2a, and PLD2b (19, 20). There are many potential regulators for PLD, but only a few of them can stimulate both isoforms. For example, the membrane phospholipids, PIP_2 , and protein kinase C (PKC) stimulate both isoforms of PLD (21, 22). The family of small G-proteins, ADP-ribosylation factor (Arf), and Rho are potent stimulators of PLD1 but are unable to stimulate the endogenous forms of PLD2 (23). PLD activity can also be stimulated by the activation of various GPCRs. A plethora of GPCR agonists have been shown to stimulate PLD activity. Angiotensin II (24), bradykinin (25), carbachol (26), lysophosphatidic acid (27), gonadotropin-releasing hormone (28), vasopressin (29), endothelin (30), thyrotropin (31), and prostaglandin $F_{2\alpha}$ (32) are examples of the prevalent nature of GPCR-mediated stimulation of PLD. GPCRs can stimulate PLD through phospholipase C (PLC)-dependent signaling pathway. PLC catalyzes the conversion of PIP_2 to inositol

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³ The abbreviations used are: SS, somatostatin; Arf, ADP-ribosylation factor; dn, dominant-negative; GEF, guanine nucleotide exchange factors; GPCR, G-protein-coupled receptor; ICC, immunocytochemistry; PIP_2 , phosphoinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; FBS, fetal bovine serum; siRNA, small interfering RNA; MOPS, 4-morpholinepropanesulfonic acid; PVDF, polyvinylidene difluoride; HA, hemagglutinin.

1,4,5-trisphosphate and diacylglycerol. Inositol 1,4,5-trisphosphate mobilizes Ca^{2+} from endoplasmic reticulum stores. This increase in $[\text{Ca}^{2+}]_i$ is known to activate conventional isoforms of PKC. Diacylglycerol, the other product of PLC activation, can also stimulate PKC through its interaction with the C1 domain of the conventional and novel PKC isoforms. Both PKC isoforms are known to enhance the activity of PLD. GPCR activation of small G-proteins can be involved in stimulating PLD (6, 11). The signaling mechanisms responsible for small G-protein regulation of PLD are diverse, but two small G-proteins, Rho and Arf, have been firmly established to regulate PLD activity through GPCRs (26, 32–36). Stimulation of Rho by activation of GPCRs is attributed to the associated $\beta\gamma$ -dimer binding to the pleckstrin homology-domain of a guanine nucleotide exchange factor for Rho (37) and its direct interaction with $\text{G}\alpha_{12/13}$ -subunit (38, 39). These interactions cause GDP to dissociate from Rho, which allows Rho to bind GTP, switching it to the active form. Rho-GTP binds to the COOH terminus of PLD1. In HIT-T15 cells, activation of SS receptors stimulates PLD activity and subsequently increases PIP_2 synthesis to provide additional substrate for the inositol 1,4,5-trisphosphate formation. This leads to a synergistic increase in $[\text{Ca}^{2+}]_i$ in the presence of AVP, which activates PLC- β (12). In HIT-T15 cells, C3 exoenzyme (10 $\mu\text{g}/\text{ml}$), a Rho inhibitor isolated from *Clostridium botulinum* (40), and Ro31–8220 (10 μM), a PKC inhibitor, did not inhibit the SS-induced activation of PLD and its subsequent increase PIP_2 that leads to a synergistic increase in $[\text{Ca}^{2+}]_i$ in the presence of AVP.⁴ These findings suggest that SS-induced PLD activation is not mediated by Rho or PKC and may be mediated by another signaling protein.

In this paper, we focused on the role of Arf isoforms in the regulation of SS-induced increase in PLD activity in an insulin-secreting cell line, HIT-T15. Arfs are small G-proteins that play an important role in vesicle transport, endocytosis, and actin rearrangement (41) and insulin secretion (42). There are three classes of Arf proteins (43). Class I (Arf1–3) play an important role in Golgi vesicle transport. The role of Class II (Arf4, Arf5) in cell signaling is not fully elucidated. Class III (Arf6) is located in the plasma membrane and facilitates endocytosis pathways. Arf6 also regulates cortical actin cytoskeleton arrangement and has a profound effect on cell migration (44), wound healing (45), and phagocytosis (46).

Like all small G-proteins, Arf proteins switch from their inactive GDP-bound state to their active GTP-bound state. The intrinsic GTPase activity of Arf proteins is mainly affected by GEFs and GTPase-activating proteins. GEFs enhance the dissociation rate of GDP from Arfs, which is the rate-limiting step in Arf activation. GTPase-activating proteins increase the intrinsic GTPase activity of Arf, switching them back to their GDP-bound inactive state.

There are several well defined hypotheses for GPCR activation of Arf proteins. Evidence suggests that Arf1 and Arf6 can directly interact with the COOH terminus of GPCRs and become activated upon stimulation of the receptors (33, 47). Other studies indicate that Arf6 binds to β -arrestin and

becomes activated by ARNO, a GEF for both Arf1 and Arf6 (35). In this paper, we report that in HIT-T15 cells, SS utilizes a novel signaling pathway to regulate PLD activity. We hypothesize that in HIT-T15 cells 1) SS receptor mediates release of $\beta\gamma$ -dimer signals through Arf6 to activate PLD, and 2) Arf6 activation by SS receptors is mediated by EFA6A, a low molecular weight GEF of Arf6, thought to be primarily found in the brain (48).

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HIT-T15 cells were maintained in RPMI 1640 with 10% FBS and aerated with 5% CO_2 , 95% air at 37 °C. All experiments were performed using cells from passages 80–90. pXS plasmids expressing wild type Arf6, and dominant-negative (dn) constructs of Arf6 [Arf6(T27N)], Arf1 [Arf1(T31N)], and Arf5 [Arf5(T31N)] were generated as described previously (49).

EFA6A wild type and EFA6A(E242K), EFA6B wild type and EFA6B (E651K), and ARNO wild type and ARNO (E156K) were inserted into pFLAG-cmv6 (Sigma) with the FLAG epitope at the amino terminus (50). All plasmids were transfected into HIT-T15 cells using LipofectamineTM (Invitrogen) according to the manufacturer's protocol.

Transfection with siRNA of Arf6 and EFA6A—The siRNA sequences targeting Arf6 are GCACCGCAUUAUCAUUGACCG (sense, 5' to 3') and CGGUCAUUGAUAAUGCGGUGC (antisense, 5' to 3') (51). The siRNA sequences targeting EFA6A are AUUGGUGGUCUGGCGAGUAAUUU (sense, 5' to 3') and AUACUCGCCAGCCAAUUU (antisense 5' to 3') (52) and were manufactured by Dharmacon, Lafayette, CO. Cells were plated with 50% confluence in a T-25 flask and were transfected with siRNA on the second day of plating using LipofectamineTM and slightly modified transfection protocol. Briefly, 25 μl of 20 μM Arf6 siRNA or 40 μl of 20 μM EFA6A siRNA was diluted in 0.5 ml of RPMI 1640, and 15 μl of LipofectamineTM was diluted in another tube in 0.5 ml of RPMI 1640 with gentle mixing. The RPMI 1640 used for transfection had no FBS. After allowing them to stand for 10 min both mixtures were combined and mixed with gentle inversion. The complex mixture was incubated for 20 min and then the entire transfection mixture was added to the T-25 flask containing fresh RPMI 1640 with 5% FBS. After 6 h of incubation, the medium was replaced with regular RPMI 1640 containing 10% FBS. 48 h after transfection the cells were distributed into 12-well plates for subsequent studies. The PLD assays and protein assays were performed ≥ 72 h after transfection.

Northern Blot Analyses—Total RNA was isolated from HIT-T15 cells using TRI Reagent (Sigma), and this total RNA was used to make a HIT-T15 cDNA PCR template using the SMART RACE cDNA Amplification Kit (BD Biosciences) according to manufacturer's instructions. Primers were designed from rat EFA6A sequence (corresponding to base pairs 844–1393 of complete coding sequence) and used in a PCR reaction with the HIT-T15 cDNA template to amplify a fragment of the EFA6A encoding gene from that tissue. Amplification of the desired target was confirmed by DNA sequencing. Northern blot riboprobes were generated by subcloning this HIT-T15 EFA6A fragment into pGEM-T Easy (Promega,

⁴ J. A. Grodnitzky and W. H. Hsu, unpublished data.

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Madison, WI), confirming orientation and fidelity with DNA sequencing, then linearizing the plasmid with appropriate restriction enzymes and transcribing probes with either T7 or SP6 polymerase using MEGAscript Kit (Ambion, Austin, TX). Probes were digoxigenin-labeled by incorporating digoxigenin RNA labeling mix (Roche Applied Science) into this reaction. For Northern blot, total RNA was isolated from HIT-T15 cells and rat brain using TRI Reagent. For each sample, 1 μ g total RNA was separated on a 1% agarose MOPS/formaldehyde gel using standard protocols before being transferred to positively charged nylon membrane (Roche Applied Science). The membrane was probed before stringent washes and chemiluminescent detection with CDP-Star (Roche Applied Science).

Western Blot Analyses—Rabbit polyclonal antibodies were raised against a COOH-terminal peptide of Arf6, residues 164–175, and against a NH₂-terminal peptide of EFA6A (residues 4–18; KSPVPFLPGTSPSAD), respectively. The specificity of the antibody was assessed by Western blotting of cells expressing FLAG-tagged GEF constructs. Lysates were separated by SDS-PAGE and blots probed with antibodies to EFA6A or FLAG and visualized with secondary antibodies using Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE). Rabbit polyclonal antibodies raised against Arf1 were a generous gift of Dr. Richard Kahn of Emory University School of Medicine. Ten μ g of whole cell HIT-T15 protein was separated by reduced SDS-PAGE (10%). Protein for Arf analysis was transferred to PVDF membranes in transfer buffer (35 mM Tris, 190 mM glycine, 20% methanol), whereas protein for EFA6A analysis was transferred to nitrocellulose membrane. The PVDF and nitrocellulose membranes were blocked with 5% nonfat dry milk in phosphate-buffered saline for 1 h at room temperature. The primary Arf antibodies were diluted 1:50 in wash buffer (0.01% Tween 20 in Tris-buffered saline), whereas the primary EFA6A antibodies were diluted 1:700 in wash buffer. The diluted antibodies were incubated with the PVDF or nitrocellulose membranes for 1 h at room temperature. The blots were washed three times for 10 min each with wash buffer. The secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase, Pierce) was diluted 1:2,000 in wash buffer and incubated with the PVDF or nitrocellulose membranes for 1 h at room temperature. The blots were then washed and developed using diaminobenzidine.

Determination of PLD Activity—PLD assay was performed using a previously described method (53). Briefly, HIT-T15 cells were grown in 24-well plates overnight in RPMI 1640 medium with 10% FBS. The medium was then discarded and 500 μ l of 3 μ Ci/ml [³H]myristic acid (PerkinElmer Life Sciences) was added to each well for 60 min. Cells were then washed twice with Krebs-Ringer buffer before 400 μ l of Krebs-Ringer buffer was added to each well. Treatments were applied 15 s after the addition of 0.5% 1-butanol. The reactions were terminated 30 s after SS had been administered. Phosphatidylbutanol and phosphatidylcholine were identified by migration with unlabeled standards, visualized by iodine staining and radiographed into 1-cm blocks, and were subsequently scraped and radioactivity quantified by liquid scintillation counting.

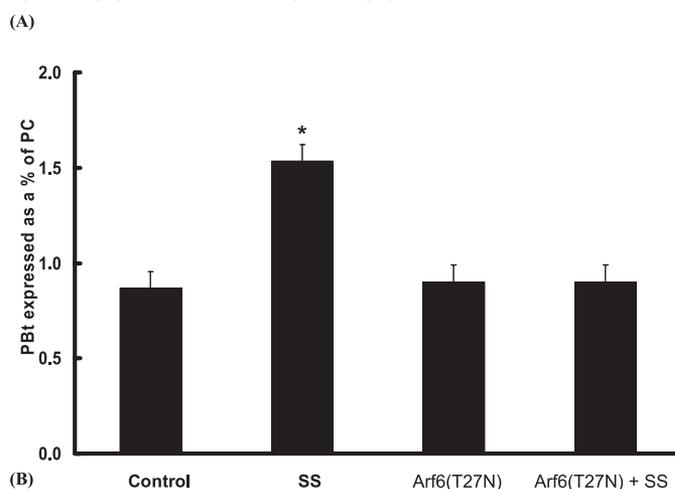
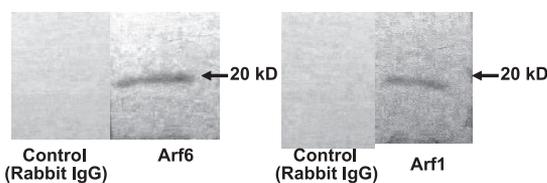


FIGURE 1. *A*, Western blot analyses of Arf1 and Arf6 in HIT-T15 cells. 10 μ g of the whole cell protein was separated using SDS-PAGE, transferred to PVDF membrane, and blotted with 1:50 Arf1 and Arf6 antibodies. *B*, effect of Arf6(T27N) expression on SS-induced increase in PLD activity. HIT-T15 cells were transfected 24 h prior to PLD assay. Cells were then pretreated with 0.5% 1-butanol 15 s prior to SS treatment. Experiments were terminated 30 s after SS had been administered. Values are the mean \pm S.E. ($n = 4$ independent cell preparations). *, $p < 0.05$, compared with the control group. PBT, phosphatidylbutanol; PC, phosphatidylcholine.

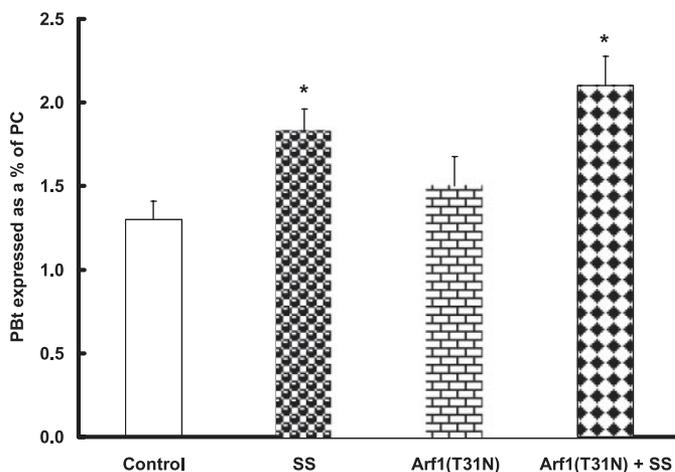


FIGURE 2. **Effect of Arf1(T31N) expression on SS-induced increase in PLD activity.** HIT-T15 cells were transfected 24 h prior to PLD assay. Treatment procedures were the same as in Fig. 1B. Values are the mean \pm S.E. ($n = 4$ independent cell preparations). *, $p < 0.05$, compared with the control group. PBT, phosphatidylbutanol; PC, phosphatidylcholine.

Data Analyses—All values are presented as mean \pm S.E. Results were analyzed using analysis of variance and individual mean comparisons were made using the Least Significant Difference test. The significance level was set at $p < 0.05$.

RESULTS

Western Blot Analysis of Arf1 and Arf6—Arf1 and Arf6 have been shown to stimulate PLD activity through GPCR signaling. To determine the particular Arf isoform that may play a role in

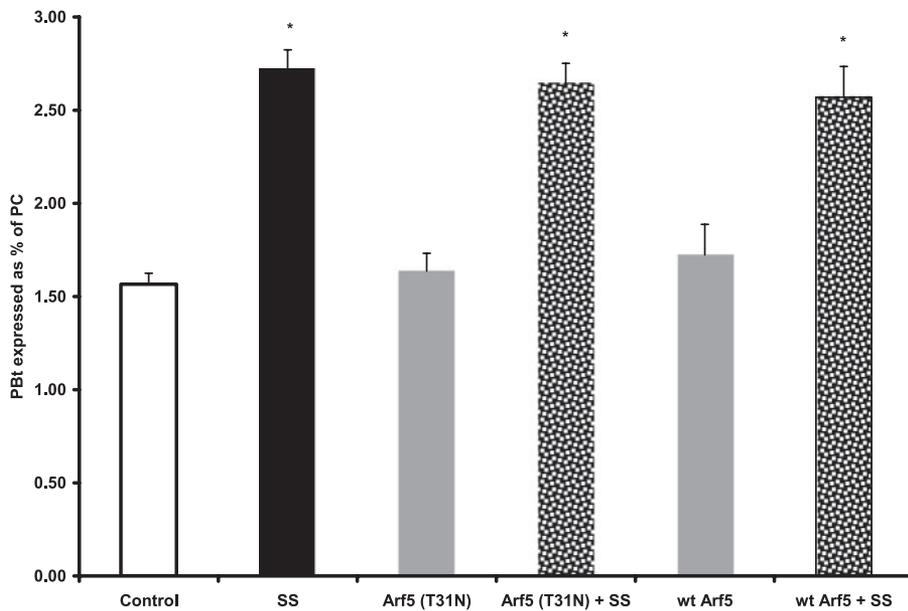
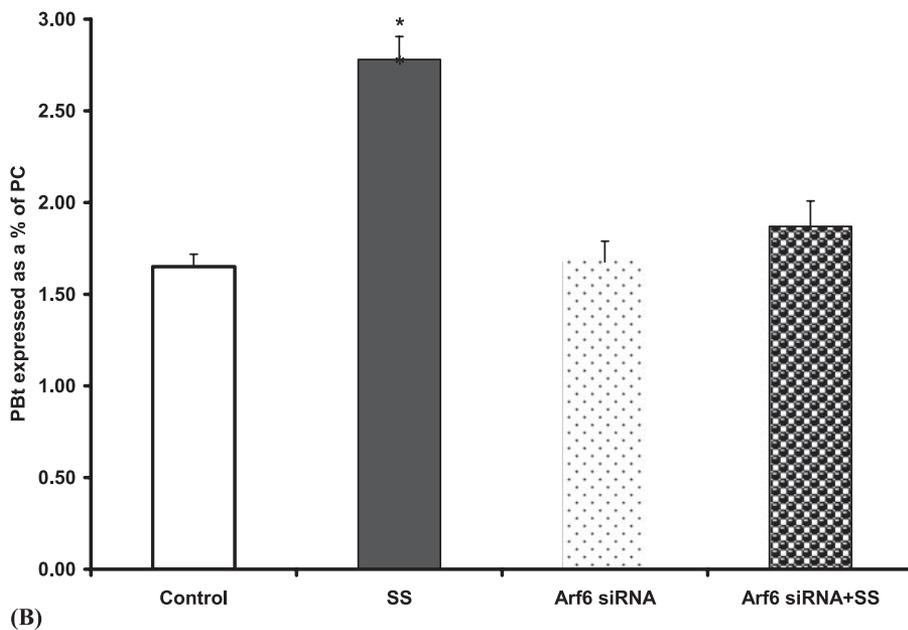


FIGURE 3. **Effect of Arf5(T31N) expression on SS-induced increase in PLD activity.** HIT-T15 cells were transfected 24 h prior to PLD assay. Treatment procedures were the same as in Fig. 1B. Values are the mean \pm S.E. ($n = 3$ independent cell preparations). *, $p < 0.05$, compared with the control group. PBt, phosphatidylbutanol; PC, phosphatidylcholine.



(A)



(B)

FIGURE 4. A, Western blot analyses of Arf6 in HIT-T15 cells transfected with siRNA. The methods were the same as Fig. 1A. B, suppression of Arf6 expression by transfecting cells with Arf6 siRNA on SS-induced increase in PLD activity. HIT-T15 cells were transfected for ≥ 72 h prior to PLD assay. Treatment procedures were the same as in Fig. 1B. Values are the mean \pm S.E. ($n = 3$ independent cell preparations). *, $p < 0.05$, compared with the control group. PBt, phosphatidylbutanol; PC, phosphatidylcholine.

this SS-receptor signaling pathway, Western blot analysis was performed to determine the Arf isoforms present in HIT-T15 cells. The results demonstrated the presence Arf1 and Arf6, with sizes of ~ 20 kDa, in HIT-T15 cells (Fig. 1A).

Arf6, but Not Arf1, Mediates SS-induced PLD Activation—Arf6 has been previously shown to stimulate PLD in other systems via a GPCR-mediated pathway (26, 33–35). To determine the involvement of Arf6 in PLD stimulation in HIT-T15 cells, we overexpressed various Arf6 mutants and analyzed their effect on SS-induced increase in PLD activity. Protein expression of the HA-tagged Arf6 mutant was determined by immunocytochemistry (ICC) using anti-HA monoclonal antibodies with 80–90% of HIT-T15 cells expressing the HA epitope (data not shown). SS (100 nM) increased PLD activity ~ 2 -fold (Fig. 1B). Overexpression of dn-Arf6 mutant Arf6(T27N) abolished SS-induced increase in PLD (Fig. 1B). Furthermore, overexpression of Arf6(T27N) did not affect base-line PLD activity (Fig. 1B). Arf1 is another well known regulator of PLD1 and has also been demonstrated to stimulate PLD activity via a GPCR-mediated pathway (47) and was present in HIT-T15 cells (Fig. 1A). To determine the involvement of Arf1 in this signaling pathway, an Arf1 dn-mutant Arf1(T31N) was overexpressed to determine its effects on SS-mediated increase in PLD activity. Protein expression of the HA-tagged Arf1 mutant was determined by ICC using anti-HA monoclonal antibodies with 80–90% of HIT-T15 cells expressing the HA epitope (data not shown). SS (100 nM) stimulation of PLD was not affected by the overexpression of Arf1(T31N) (Fig. 2). Overexpression of Arf1(T31N) had no effect on baseline PLD activity either (Fig. 2). The above data suggested that Arf6, but not Arf1, plays a significant role in SS-induced increase in PLD activity in HIT-T15 cells.

For the sake of completeness, Arf5, a class II Arf also was examined. To determine the involvement of Arf5 in this signaling pathway, an Arf5 dn-mutant Arf5(T31N) was overexpressed to determine its effects on SS-mediated increase in PLD activity. Protein expression of the HA-tagged Arf5 mutant was determined by ICC using anti-HA

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monoclonal antibodies with 80–90% of HIT-T15 cells expressing the HA epitope (data not shown). SS (100 nM) stimulation of PLD was not affected by the overexpression of Arf5(T31N) (Fig. 3). Over-expression of Arf5(T31N) had no effect on base-line PLD activity either (Fig. 3).

To confirm the effect of Arf6 on SS-induced PLD activation, we transfected cells with siRNA for Arf6. Transfection with siRNA reduced Arf6 expression to ~20% of the control level (Fig. 4A). SS-induced PLD activation was abolished in these transfected cells (Fig. 4B). These results suggest that Arf6 mediates SS-induced activation of PLD.

Overexpression of wild type Arf6 enhanced SS (100 nM)-induced increase in PLD activity but did not change baseline PLD activity (Fig. 5). These results suggested that SS stimulation of PLD activity is mediated through an Arf6-dependent pathway.

EFA6, but Not ARNO, Mediates SS-induced PLD Activation—ARNO is one of the members of the ARNO/cytohesin family of low molecular weight GEFs that are known to increase Arf activity and cause a subsequent increase in PLD activity through a GPCR-signaling pathway (34, 35, 54). To examine whether ARNO may play a role in the SS-induced increase in PLD activity, the dn-mutant of ARNO (ARNO(E156K)) was overexpressed. Protein expression of the FLAG-tagged ARNO mutant was determined by ICC using anti-FLAG monoclonal antibodies with 80–90% of HIT-T15 cells expressing the FLAG epitope (data not shown). Overexpressing ARNO(E156K) had no effect on SS-induced increase in PLD activity nor was basal activity of PLD affected (Fig. 6). These results suggested that ARNO does not play a significant role in SS-mediated activation of PLD.

The EFA6 family of GEFs acts specifically on Arf6 (55) but has not yet been reported to stimulate PLD through GPCR stimulation. To determine whether EFA6 GEFs function in this signaling pathway, HIT-T15 cells were transfected with the dn-mutant of EFA6A (EFA6A(E242K)), thought to be a neural-specific isoform (56), and the dn-EFA6B (EFA6B(B651K)), thought to be a widely expressed isoform (48). Protein expression of the FLAG-tagged EFA6A and EFA6B mutants was determined by ICC using anti-FLAG monoclonal antibodies with 80–90% of HIT-T15 cells expressing the FLAG epitope (data not shown). Overexpression of dn-EFA6A abolished SS-induced PLD activation, whereas that of dn-EFA6B failed to do so (Fig. 7). Overexpressing these EFA6 dn-mutants did not change basal PLD activity.

EFA6A Expression—To confirm that EFA6A may play a role in this SS-receptor signaling pathway, both Northern and Western blot analyses were performed to determine if this EFA6A isoform is present in HIT-T15 cells. Northern blot analyses demonstrated the presence of mRNA of EFA6A with a size of ~2.1 kb in both rat brain and HIT-T15 cells (Fig. 8A). In addition, a size of ~1.6 KB EFA6A mRNA was found in HIT-T15 cells. Western blot analyses demonstrated the presence of EFA6A with a size of ~70 kDa in both HIT-T15 cells and rat brain homogenate (Fig. 8B). In addition, a smaller EFA6A protein with a size of ~40 kDa was detected in both samples; the nature of this band is unclear, but it could be a breakdown product or an isoform of EFA6A.

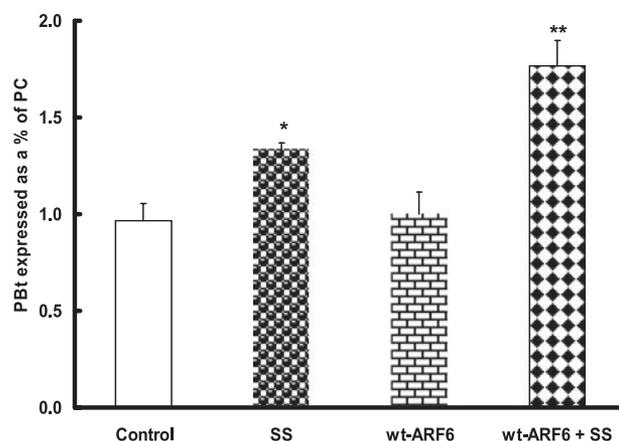


FIGURE 5. Effect of wild type (wt)-Arf6 expression on SS-induced increase on PLD activity. HIT-T15 cells were transfected 24 h prior to PLD assay. Treatment procedures were the same as in Fig. 1B. Values are the mean \pm S.E. ($n = 4$ independent cell preparations). *, $p < 0.05$, compared with the control group and **, $p < 0.05$, compared with the SS alone group. Pbt, phosphatidylbutanol; PC, phosphatidylcholine.

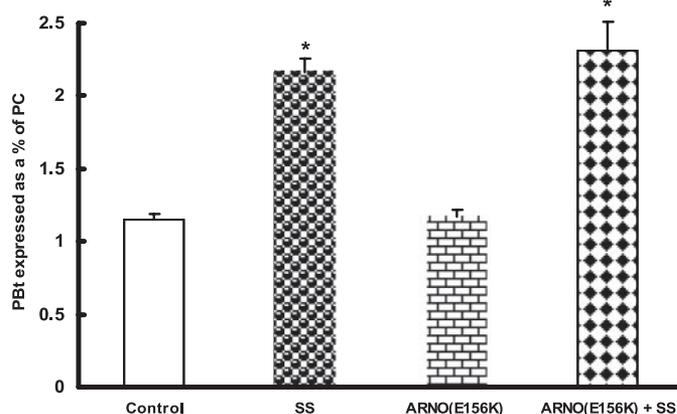


FIGURE 6. Effect of ARNO(E156K) expression on SS-induced increase on PLD activity. HIT-T15 cells were transfected 24 h prior to PLD assay. Treatment procedures were the same as in Fig. 1B. Values are the mean \pm S.E. ($n = 4$ independent cell preparations). *, $p < 0.05$, compared with the control group. Pbt, phosphatidylbutanol; PC, phosphatidylcholine.

The antibody directed against EFA6A was specific for this GEF, since it did not detect transfected, FLAG-tagged EFA6B, EFA6C, or ARNO, although these GEFs were visible with anti-FLAG antibodies (Fig. 8C).

Inhibition of EFA6A Expression on SS-induced PLD Activation—To confirm the effect of EFA6A on SS-induced PLD activation, we transfected cells with siRNA for EFA6A. Transfection with siRNA reduced EFA6A expression to ~40% of the control level (Fig. 9A). SS-induced PLD activation was abolished in these transfected cells (Fig. 9B). These results suggested that EFA6A is essential for SS-induced activation of PLD and provided further evidence for the involvement of Arf6 in SS receptor signaling pathway.

DISCUSSION

The findings of the present study strongly support the hypothesis that Arf6 mediates the SS-induced increase in PLD activity in clonal β -cells HIT-T15. Despite the fact that both Arf1 and Arf6 are present in these cells, the effects of overexpression of dn-mutant and wild type Arf6 as well as

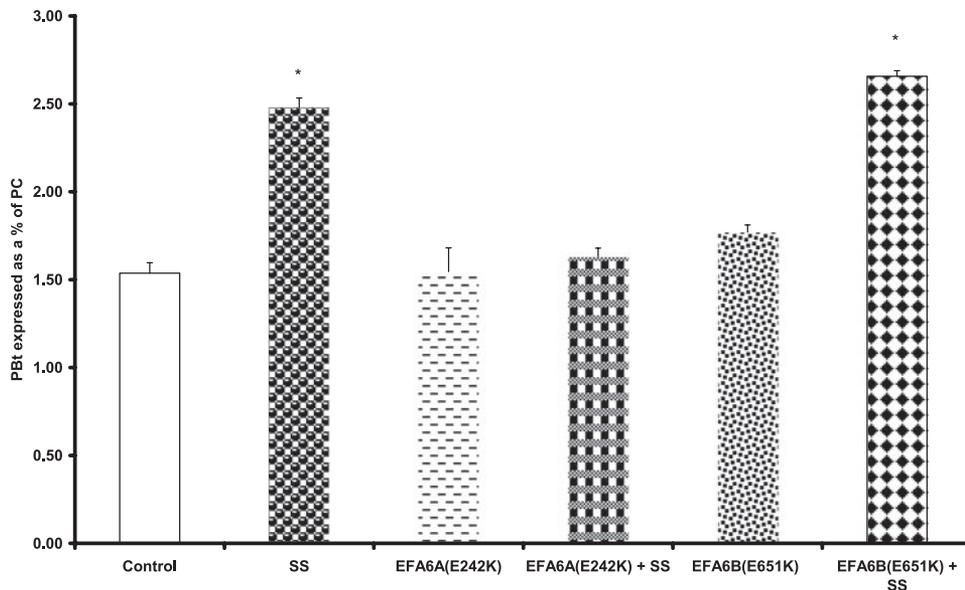


FIGURE 7. Effect of overexpression of dn-mutants of EFA6, EFA6A(E242K), and EFA6B(E651K) on SS-induced increase on PLD activity. HIT-T15 cells were transfected 24 h with EFA6A(E242K) and EFA6B(E651K), respectively, prior to PLD assay. Treatment procedures were the same as in Fig. 1B. Values are the mean \pm S.E. ($n = 4$ independent cell preparations). *, $p < 0.05$, compared with the control group. PBt, phosphatidybutanol; PC, phosphatidylcholine.

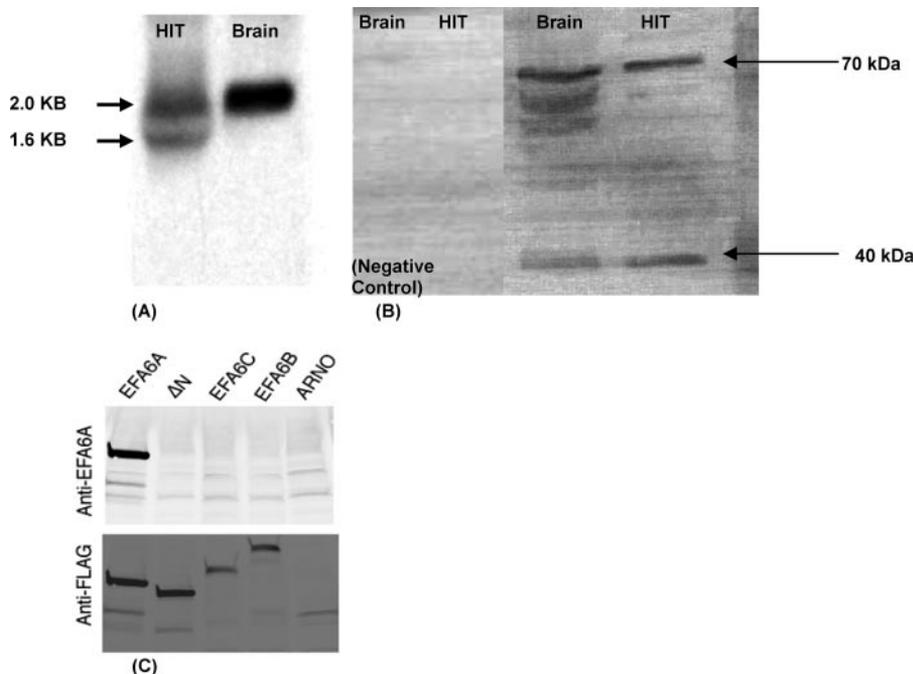


FIGURE 8. Northern (A) and Western (B) blot analyses of EFA6A in native HIT-T15 cells and rat brain. A, 1 μ g of total RNA was separated using 1% agarose gel and then transferred to positively charged nylon membrane. The membrane was probed with a fragment of the EFA6A coding gene. B, 12 μ g of the whole cell protein was separated using SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with 1:700 EFA6A antibodies. Negative controls did not have antibody versus EFA6A. C, characterization of antibody versus EFA6A. HeLa cells were transfected with FLAG-tagged constructs encoding EFA6A, an amino-terminally truncated EFA6A (Δ N), EFA6C, EFA6B, or ARNO. Lysates of cells were loaded onto SDS-PAGE and blots probed with antibodies to EFA6A (top panel) or FLAG (lower panel).

transfection with Arf6 siRNA on SS-induced PLD activation indicate that Arf6 is essential for this pathway. In addition, this effect does not seem to be an artifact of the overexpression of an Arf protein, because overexpression of dn-Arf1 or dn-Arf5 mutant did not have any effect on SS-induced PLD activation.

The EFA6 family consists of four related Arf6 GEFs (A, B, C, and D) that contain similar Sec7 and pleckstrin homology domains and are distinguished by variable amino-terminal extensions. The EFA6B isoform is widely expressed, while the EFA6A isoform is more prominent in brain (48). Nevertheless, using probes specific for EFA6A, we detected by Northern analysis a band of \sim 2.1 kb in both rat brain and HIT-T15 cells, which is consistent with the predominant 2.1 kb species reported in brain, small intestine, colon, and ovary (48). In addition, the EFA6A protein of the expected size, \sim 70 kDa, was observed with EFA6A-specific antiserum. We also detected a shorter mRNA of EFA6A of \sim 1.6 kb. Our findings are interesting, because EFA6A, and not EFA6B, is specifically involved in the SS-induced PLD activation in clonal β -cells, and this will be further explored.

The ability of the EFA6A dn-mutant, EFA6A(E242K), and siRNA for EFA6A to block SS-induced PLD activation further implicates the involvement of Arf6 and places an EFA6 family GEF, but not ARNO, in mediating SS-induced activation of PLD. Taken together, these findings strongly suggest the involvement of EFA6A-Arf6 in this signaling pathway.

We previously demonstrated that SS was able to increase PLD activity in HIT-T15 cells, leading to a subsequent increase in PIP₂ levels (12). In this paper we report a novel mechanism for PLD stimulation. In our proposed model, the $\beta\gamma$ -dimer that couples to SS receptors interacts directly or indirectly with EFA6A, which is known to increase the nucleotide exchange rate on Arf6. Arf6-GTP would activate PLD1 in HIT-T15 cells. Although our proposed model for GPCR-mediated activation of the Arf6-PLD signaling cascade is similar to other previously reported models, distinct differences between the models are apparent. In one proposed model the $\beta\gamma$ -dimer directly interacts with Arf1 and Arf4, a Class II Arf (36). In our system, Arf1 and Arf5, another Class II Arf, did not play a significant role in the SS-induced activation of PLD, indicating that the direct interaction of $\beta\gamma$ -dimer with

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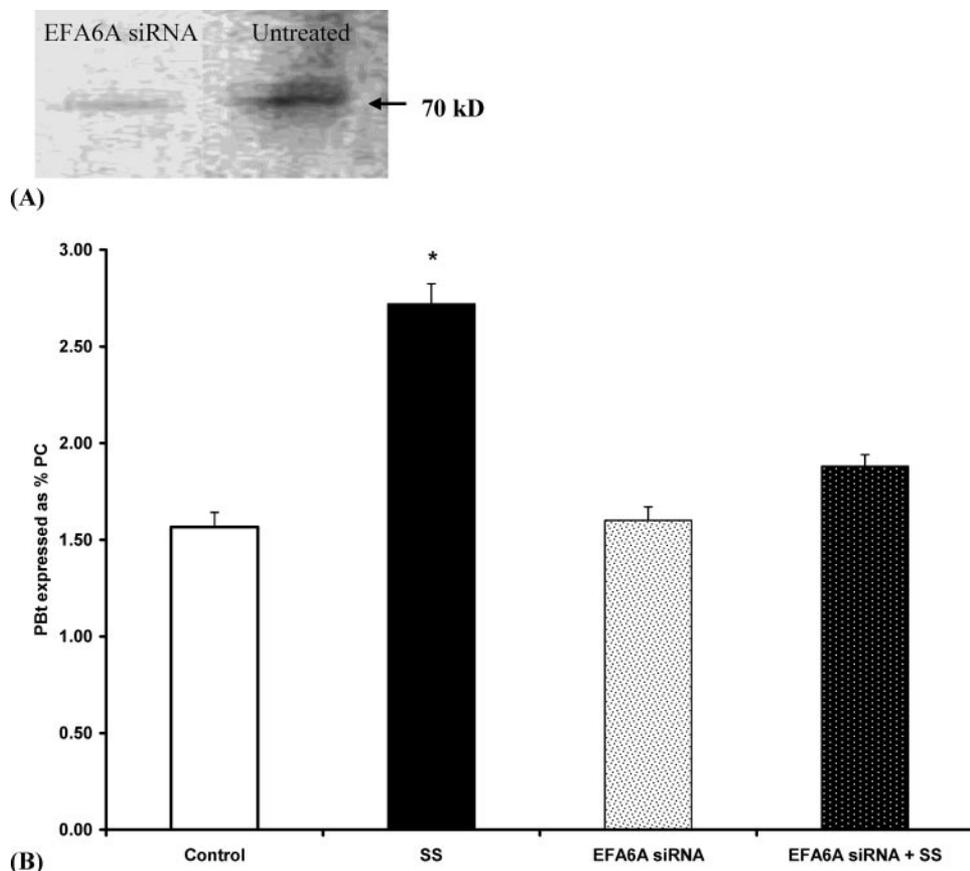


FIGURE 9. *A*, Western blot analyses of EFA6A in HIT-T15 cells transfected with siRNA for EFA6A. The methods were the same as Fig. 1*A*. *B*, suppression of EFA6A expression by transfecting cells with EFA6A siRNA on SS-induced increase in PLD activity. HIT-T15 cells were transfected for ≥ 72 h prior to PLD assay. Treatment procedures were the same as in Fig. 1*B*. Values are the mean \pm S.E. ($n = 3$ independent cell preparations). *, $p < 0.05$, compared with the control group. PBT, phosphatidylbutanol; PC, phosphatidylcholine.

Class I/II Arfs does not occur in the HIT-T15 cell system for SS-PLD signaling.

There have been several reports of GPCR activation of Arf6 occurring due to the ability of Arf6 to directly interact with activated GPCRs. This signaling cascade has been demonstrated in 5-hydroxytryptamine-2A receptors (47), gonadotropin-releasing hormone receptors (28), and M_3 -muscarinic receptors (26). These studies show the involvement of Arf1, Arf3, and Arf6 in the GPCR-mediated activation of PLD. This signaling cascade is dependent on Arf proteins interacting with the NPXXY motif located in the seventh transmembrane helix of these receptors (33, 57). NPXXY is a common motif found in most GPCRs and is found in Type 2 SS receptor, the SS receptor present in HIT-T15 cells that mediate the effect of SS in these cells (58). These studies demonstrate that the activation of a GPCR can stimulate the conversion of Arf-GDP to its active Arf-GTP-bound state; Arf-GTP can then bind to the NPXXY motif of the receptor. The direct binding model does not conflict with the results obtained from this study. The focus of this study was to determine the proteins involved in SS-induced activation of PLD and we did not address the translocation or scaffolding properties of activated Arf6 in this paper. It is possible that the $\beta\gamma$ -dimer interacts with EFA6A to switch Arf6 to the GTP bound state. Furthermore, the active Arf6 could then bind to NPXXY motif of an activated SS receptor, which may be

essential for PLD activity. Additional studies are required to determine whether a direct interaction transpires between SS receptors and Arf6-GTP.

In our proposed model, the mechanism of SS-induced activation of PLD in HIT-T15 cells is through the $\beta\gamma$ -dimer (12). We hypothesize that the $\beta\gamma$ -dimer coupling to SS receptor interacts with EFA6A, which will cause a subsequent stimulation of PLD1 in HIT-T15 cells through Arf6 activation. This study provides an exciting novel signal mechanism upon which SS can activate PLD. Another well documented model for GPCR-mediated activation of Arf6 involves the stimulation of β -adrenergic receptors (34) and luteinizing hormone receptors (35). Activation of these receptors leads to the dissociation of $\beta\gamma$ -dimer and subsequent activation of G-protein-coupled receptor kinase. G-protein-coupled receptor kinase then phosphorylates the receptors, which recruits β -arrestin to the receptor and leads to receptor desensitization (34). Upon β -arrestin binding, ARNO scaffolds with the desensitized receptor complex, thereby activating

Arf6. The two main differences between the models are the GEFs involved in Arf6 activation and the additional downstream signaling events that occur after the dissociation of $\beta\gamma$ -dimer. In our system, EFA6A controls the SS-induced activation of PLD. In the other model ARNO is responsible for Arf6 activation (34). Surprisingly, in HIT-T15 cells, EFA6A dominated this signaling cascade, and ARNO did not significantly contribute to this pathway. These results indicate that in HIT-T15 cells EFA6A, but not ARNO, is essential for the activation of Arf6. An alternative hypothesis would be that additional signaling events occur between the $\beta\gamma$ -dimer and EFA6A. Such events are likely to include the involvement of G-protein-coupled receptor kinases and β -arrestins.

Although this study provides an exciting novel signal mechanism upon which SS can activate PLD1 and cause a subsequent increase in PIP_2 , the physiological significance for SS-induced activation of Arf6 and PLD1 is not well understood. Arf6-induced increase in PIP_2 levels has been reported in another pancreatic β -cell line, MIN6, in which the ability of Arf6 to increase PIP_2 levels was essential to the slow phase of insulin secretion but had no effect on initial fast phase of insulin secretion (42). In addition, PLD1 activation has been shown to be essential for insulin secretion for both glucose and cholinergic receptor-mediated responses (61, 62). The action of PLD1 on exocytosis appears to mediate its effect through a distal step of exocytosis,

somewhere beyond vesicle recruitment and the readily releasable pool. Both Arf6 and PLD1 stimulate exocytotic processes in β -cells, so we are perplexed as to why an inhibitory hormone like SS would signal through Arf6-PLD1 pathway. On the other hand, we recently found that SS evoked its receptor endocytosis.⁵ Since activation of Arf6 (34) and PLD (59, 60, 63) can induce receptor endocytosis, we hypothesize that this novel signaling pathway mediates SS receptor endocytosis. Further work is underway to test this hypothesis.

This study describes for the first time the involvement of EFA6A in a GPCR signaling pathway and the presence of EFA6A in insulin-secreting cells. In addition, Western blot analysis using antibodies that recognize the NH₂ terminus of EFA6A detected endogenous EFA6A in HIT-T15 cells. Taken together with our findings with Northern and Western blot analyses, this is evidence for the existence of EFA6A in HIT-T15, although further studies will be required to explore the implications of this signaling pathway in insulin-secreting cells.

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⁵ N. Syed and W. H. Hsu, unpublished data.

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Somatostatin Receptors Signal through EFA6A-ARF6 to Activate Phospholipase D in Clonal β -Cells

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