0026-895X/05/6706-2162–2172$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 67:2162–2172, 2005

Vol. 67, No. 6
10470/3035781
Printed in U.S.A.

Somatostatin Increases Phospholipase D Activity and Phosphatidylinositol 4,5-bisphosphate Synthesis in Clonal β Cells HIT-T15

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Received December 19, 2004; accepted March 22, 2005

ABSTRACT

In the presence of arginine vasopressin (AVP), somatostatin increases $[Ca^{2+}]_i$, leading to a transient increase in insulin release from clonal β cells HIT-T15 via $G_{i/o}$ and phospholipase C (PLC) pathway (Cheng et al., 2002a). The present study was to elucidate the mechanisms underlying somatostatin-induced $[Ca^{2+}]_i$ increase in the presence of AVP. We found that the effect of somatostatin was mediated by βγ subunits but not by the α subunit of $G_{i/o}$. Because somatostatin alone failed to increase $[Ca^{2+}]_i$, we hypothesized that somatostatin increases phosphatidylinositol 4,5-bisphosphate (PIP_2) synthesis, providing extra substrate for preactivated PLC-β to generate inositol 1,4,5-trisphosphate (IP_3). Somatostatin alone did not increase IP_3 levels, but AVP + somatostatin did. Somatostatin increased PIP_2 levels but decreased phosphatidylinositol 4-phosphate levels. We further hypothesized that PLD mediates somatostatin-induced changes in PIP_2 levels. Both the phospholipase D (PLD) inhibitors and antibody versus PLD1 antagonized AVP-somatostatin-induced increases in $[Ca^{2+}]_i$. PLD inhibitor also antagonized somatostatin-induced increase in PIP_2 levels. In addition, somatostatin increased PLD activity. These results suggest that activation of somatostatin receptors that are coupled to the βγ dimer of $G_{i/o}$ led to PLD1 activation, thus promoting the synthesis of phosphatidic acid. Phosphatidic acid activates PIP-5 kinase, which evokes an increase in PIP_2 synthesis. The PIP_2 generated by somatostatin administration increases substrate for preactivated phospholipase C-β, which hydrolyzes PIP_2 to form IP_3, leading to an increase in $[Ca^{2+}]_i$. The regulation of PIP_2 synthesis by $G_{i/o}$-coupled receptors via PLD activation represents a novel signaling mechanism for somatostatin and a novel concept in the cross-talk between G_q- and $G_{i/o}$-coupled receptors in β cells.

Signaling via the large family of G protein-coupled receptors (GPCRs) can lead to many cellular responses, ranging from regulation of hormone release to stimulation of gene transcription. Cross-talk between two GPCRs can lead to paradoxical cellular responses. Signaling between G_q-coupled receptors and $G_{i/o}$-coupled receptors produces a synergistic increase in $[Ca^{2+}]_i$ in numerous cell types (Muller and Lohse, 1995; Quitterer and Lohse, 1999; Yeo et al., 2001). PLC is known to hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP_2) into the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 triggers Ca^{2+} release from the endoplasmic reticulum, whereas DAG activates protein kinase C (Berridge et al., 1984; Nishizuka, 1984). In general, activation of G_q-coupled receptors increases $[Ca^{2+}]_i$ via the PLC pathway, whereas activation of $G_{i/o}$-coupled receptors inhibits adenylyl cyclase (Patel et al., 1994; Rhee, 2001). However, when these receptors are stimulated concurrently, they produce a synergistic increase in $[Ca^{2+}]_i$ via enhancement of PLC-β activity (Muller and Lohse, 1995; Quitterer and Lohse, 1999; Yeo et al., 2001). This enhancement by $G_{i/o}$-coupled receptors is mediated by $G_{i/o}$-βγ dimer (Selbie et al., 1997; Quitterer and Lohse, 1999; Chan et al., 2000).

We reported recently that in the presence of AVP, somatostatin increased $[Ca^{2+}]_i$ and evoked a paradoxical, transient insulin

H.C. and J.A.G. contributed equally to the present work.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.104.010470.

ABBREVIATIONS: GPCR, G protein-coupled receptor; PLC, phospholipase C; PLD, phospholipase D; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; ER, endoplasmic reticulum; AVP, arginine vasopressin; U-73122, 1-[6-[[17β-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione; AM, acetoxymethyl ester; ct-βARK, C terminus of the β-adrenergic receptor kinase; KRB, Krebs-Ringer-bicarbonate buffer; RFU, relative fluorescent unit; PIP, phosphatidylinositol 4-phosphate; PA, phosphatidic acid; PC, phosphatidylcholine; PTX, pertussis toxin; zLYCK, carbobenzyloxy-leucine-tyrosine-chloromethylketone; HEK, human embryonic kidney; PVDF, polyvinylidene difluoride.

release from HIT-T15 cells (Cheng et al., 2002a). Pretreatment with somatostatin for 100 s does not enhance AVP-induced increase in $[Ca^{2+}]_i$ (Cheng et al., 2002a). Others have found similar results using somatostatin as a $G_{i/o}$-coupled receptor agonist. For example, in SH-SY5Y cells, somatostatin increases $[Ca^{2+}]_i$ after pretreatment with carbachol, a cholinergic agonist, which signals via G_q (Connor et al., 1997). In another β cell line, RINm5F, cyclopentyladenosine, a $G_{i/o}$-coupled receptor agonist, does not increase $[Ca^{2+}]_i$ when administered alone but increases $[Ca^{2+}]_i$ after pretreatment with carbachol (Biden and Browne, 1993). The cross-talk effects of somatostatin with AVP in HIT-T15 cells are mediated by $G_{i/o}$, the PLC pathway, and Ca^{2+} release from endoplasmic reticulum (Cheng et al., 2002a). The increase in $[Ca^{2+}]_i$ by somatostatin is attributable to a cross-talk between G_q and $G_{i/o}$ (Cheng et al., 2002a). To date, most of the studies examining cross-talk signals between G_q- and $G_{i/o}$-coupled receptors have been performed through quantification of inositol phosphates and/or $[Ca^{2+}]_i$ (Selbie et al., 1997; Chan et al., 2000; Yeo et al., 2001). In addition, antibodies versus PLC-β isozymes (Murthy et al., 1996) or PLC inhibitors such as U-73122 (Cheng et al., 2002a) have also been used to investigate the cross-talk mechanism of $G_{i/o}$-coupled somatostatin receptors. The results from some of these studies suggest that the $\beta\gamma$ dimer of $G_{i/o}$ directly activates PLC-β (Murthy et al., 1996; Chan et al., 2000). However, in our previous study, treatment with somatostatin alone, even at a high concentration of 1 μM, failed to increase $[Ca^{2+}]_i$ or insulin release (Cheng et al., 2002a). We therefore hypothesized that somatostatin increases PIP_2 synthesis in β cells, thereby increasing the substrate for preactivated PLC-β by AVP. In addition, we hypothesized that somatostatin activates PLD to increase the synthesis of phosphatidic acid, which in turn activates PIP-5 kinase to increase PIP_2 synthesis.

Our present findings indicate that somatostatin alone cannot increase IP_3 production. In addition, somatostatin-induced increase in IP_3 and $[Ca^{2+}]_i$ in the presence of AVP is mediated through the G$\beta\gamma$-dimer of $G_{i/o}$, an increase in PLD activity, and a subsequent increase in PIP_2 synthesis.

Materials and Methods

Materials. All reagents were purchased from Sigma Chemical (St. Louis, MO), except that fura-2 acetoxymethyl ester (fura-2AM) was from Molecular Probes (Eugene, OR), and rabbit polyclonal antibodies versus $G\alpha_{i1}/G\alpha_{i2}$ and $G\alpha_{i3}/G\alpha_o$, and Gβ were from BIOMOL Research Laboratories (Plymouth Meeting, PA), myo-[2-^{3}H]inositol and [γ-^{32}P]ATP were from PerkinElmer Life and Analytical Sciences (Boston, MA).

Cell Culture and Transfection. HIT-T15 cells were maintained in RPMI 1640 medium with 10% FBS and aerated with 5% CO_2/95% air at 37°C. All experiments were performed using cells from passages 80 to 90. For overexpression of the C terminus of the β-adrenergic receptor kinase (ct-βARK), HIT-T15 cells were transfected with pcDNAIIIB T8βark plasmid (donated by Dr. Silvio Gutkind, National Institutes of Health), using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

Measurement of $[Ca^{2+}]_i$ in Single Cells. $[Ca^{2+}]_i$ was measured as described previously (ZhuGe and Hsu, 1995). Cells were loaded with 2 μM fura-2AM in Krebs-Ringer-bicarbonate buffer (KRB) for 30 min at 37°C. Measurement was performed in custom-made 35-mm culture dishes or a 20 × 20-mm perifusion chamber on the stage of an inverted fluorescence microscope (Carl Zeiss). Fluorescence images were obtained (λ_{ex} = 340 and 380 nm; λ_{em} = 510 ± 20 nm), and background was subtracted and divided on a pixel-by-pixel basis to generate spatially resolved maps of the $[Ca^{2+}]_i$. The emitted signals were digitalized, recorded and processed using Attofluor Digital Fluorescence Imaging System (Atto Instruments, Rockville, MD). The $[Ca^{2+}]_i$ was calculated according to a method published previously (Grynkiewicz et al., 1985). Calibration was performed according the procedure provided by Attofluor, using fura-2 penta K^+ as a standard.

Measurement of $[Ca^{2+}]_i$ in Transfected Cells. T8βARK-transfected HIT-T15 cells were seeded into black-walled, clear-based, poly-D-lysine–coated 96-well plates (Costar; Corning, Acton, MA) at a density of 10^5 cells per well in RPMI 1640 medium, supplemented as described above, and cultured overnight. The cells were then incubated with the FLIPR Calcium 3 assay kit (Molecular Devices, Sunnyvale, CA) at 37°C for 60 min. After incubation, the plates were inserted into a fluorometric imaging plate reader (FLEXstationII; Molecular Devices, Sunnyvale, CA), and the fluorescence at λ_{ex} = 488 nm, and λ_{em} = 515 to 535 nm was used to monitor changes in $[Ca^{2+}]_i$. Data were expressed as percentage increase of relative fluorescent units (RFU) divided by baseline RFU using Softmax Pro.

Microinjection Protocol. Single cells were grown for 2 days on glass coverslips of custom-made 35-mm culture dishes. Thereafter, cells were loaded with fura-2AM and mounted on the stage of an inverted microscope. For microinjection of antibodies, two cells from each dish were injected with intracellular buffer and rabbit antibodies, respectively, using a disposable glass pipette (VWR Scientific, West Chester, PA) held by a Narishige MW-3 micromanipulator. Pipettes were made by a PE-2 Micropipette puller (Narishige Scientific Instrument, Tokyo, Japan). All antibodies were diluted at 1:100 with intracellular buffer solution containing 27 mM K_2HPO_4, 8 mM NaH_2PO_4, and 26 mM KH_2PO_4, pH 7.3. Injection pressure was controlled by a pressure injection system (Picospritzer II; General Valve, Fairfield, NJ). A 30-min incubation period was allowed between the antibody injection and $[Ca^{2+}]_i$ measurement. For microinjection of PIP_2, one cell from each dish was injected with Na^+ salt of PIP_2, which had been dissolved and further diluted in intracellular buffer at the respective PIP_2 concentration or buffer alone as the control. Microinjection of PIP_2, PIP, and phosphatidic acid (PA) was performed at 100 s after the perfusion with AVP into the culture dish. At the end of each experiment, the cell was depolarized with 10 mM KCl to test membrane integrity.

Determination of PIP and PIP_2. PIP and PIP_2 levels were measured using thin-layer chromatography as described previously (Norris and Majerus, 1994). HIT-T15 cells were labeled with 200 μCi/ml of [γ-^{32}P]ATP in phosphate-free KRB for 60 min and washed twice with centrifugation at 300*g* for 2 min. For experiments, cells were resuspended at a density of 15 × 10^6 cells/ml/treatment. The reactions were terminated by addition of 1 ml of ice-cold 1 N HCl. Phospholipids were separated by a chloroform/methanol (1:1) mixture. The lower phase was dried under a stream of nitrogen, resuspended in 200 to 500 μl of chloroform/methanol (1:1) mixture, and spotted on silica gel plates. PIP and PIP_2 were identified by comigration with unlabeled standards, which were visualized by iodine staining and radiograph. One-centimeter blocks of the corresponding lanes for the samples were subsequently scraped, and radioactivity was quantified by liquid scintillation counting.

Determination of IP_3. Measurement of inositol phosphates followed modified procedures from a published report (Hoque et al., 2001). Cells were labeled with 20 μCi/ml of myo-[2-^{3}H]inositol at 37°C for 90 min, by which time the incorporation of ^{3}H into inositol lipids had reached a plateau (Hoque et al., 2001).

Cells were washed twice in phosphate-free KRB and centrifuged at 300*g* for 2 min. For experiments, cells were resuspended at a density of 20 × 10^6 cells/ml/treatment. The reactions were terminated by addition of 0.5 ml of ice-cold 10% trichloroacetic acid, and samples were centrifuged at 3,000*g* for 20 min at 4°C. The supernatants were passed through a 200 to 400 mesh Dowex AG1-X 8 in formate form column (Bio-Rad Laboratories, Hercules, CA). Inositol phosphates were eluted by stepwise addition of 0.2, 0.5, and 1 M ammonium formate, which yielded inositol 1-monophosphate, inositol 1,4-bisphosphate, and IP_3, respectively.

Radioactivity associated with IP_3 from each sample was quantified by liquid scintillation counting.

Determination of PLD Activity. PLD assay was performed using a method described previously (O'Launaigh et al., 2002). In brief, HIT-T15 cells were grown in 24-well plates overnight in RPMI 1640 medium with 10% FBS. The medium was then discarded, and 500 μl of 3 μCi/ml of [^{3}H]myristic acid was added to each well for 60 min. Cells were then washed twice with KRB, and 400 μl of KRB 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 treatment had been administered. Phosphatidylcholine (PC) and phosphatidylbutanol were identified by migration with unlabeled standards, which were visualized by iodine staining and radiograph. One-centimeter blocks of the corresponding lanes for the samples were subsequently scraped and radioactivity was quantified by liquid scintillation counting.

Intracellular Delivery of Antibodies versus PLD. This technique was performed using BioPORTER protein delivery reagent (Gene Therapy Systems, San Diego, CA). Undiluted PLD antibodies (a gift from Dr. Sylvain Bourgoin of the University of Laval, Canada) were delivered into HIT-T15 cells following manufacturer's protocol. In brief, HIT-T15 cells were plated on a glass coverslip of custom-made 35-mm culture dish. Antibody versus PLD (2 μg) (or normal rabbit plasma as control) was mixed with 1.5 μl of the protein delivery reagent and 150 μl of FBS-free RPMI medium and added to each well. The cells were incubated at 37°C for 4 h. The culture medium was then removed and replaced by KRB. Thereafter, cells were loaded with fura-2AM and determination of single cell $[Ca^{2+}]_i$ was performed as mentioned above.

Western Blot Analysis of PLD. PLD protein levels were determined by Western blot analysis. Ten micrograms of whole cell protein of HIT-T15 was separated by reduced SDS-PAGE (10%). Protein was transferred to PVDF membranes in transfer buffer (35 mM Tris, 190 mM glycine, and 20% methanol). The PVDF membranes were blocked with 5% nonfat dry milk in PBS for 1 h at room temperature. The primary PLD antibodies were diluted 1:30 in wash buffer (0.01% Tween 20 in PBS) and incubated with the PVDF 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, Rockford, IL) was diluted 1:2000 in wash buffer and incubated with the polyvinylidene difluoride membranes for 1 h at room temperature. The blots were then washed and developed using diaminobenzidine.

Data Analyses. All values are presented as mean ± 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

Mediation by G$\beta\gamma$ of Somatostatin-Induced Increase in $[Ca^{2+}]_i$ in the Presence of AVP. The $G_{i/o}$-$\beta\gamma$ dimer has been demonstrated to be the essential subunit of the G pro-

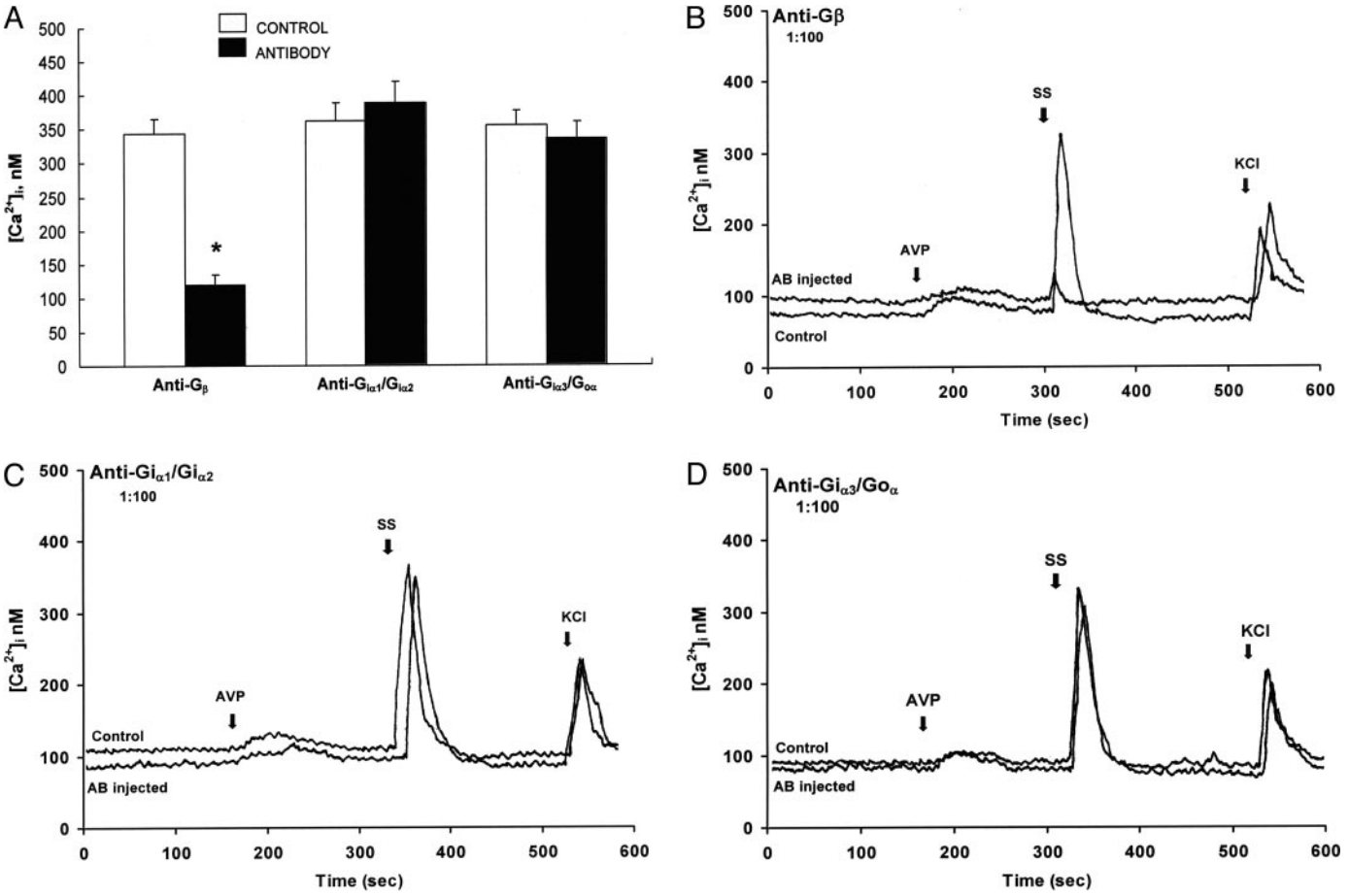


Fig. 1. Effect of antibodies versus $G_{i/o}$ subunits on somatostatin (SS)-induced increase in $[Ca^{2+}]_i$ in the presence of AVP in HIT-T15 cells. A, antibodies versus Gβ, Gα_{i1}/Gα_{i2}, and Gα_{i3}/Gα_o were diluted 1:100 and microinjected into single cells followed by a 30-min incubation period and Ca^{2+} image analysis. Somatostatin (100 nM) was given 100 s after administration of AVP (1 nM). Baseline $[Ca^{2+}]_i$ were approximately 100 nM. Values are the mean ± S.E.; $n = 10$ cells/treatment from three independent cell preparations. *, $p < 0.05$ compared with somatostatin controls. B, representative calcium trace of microinjected antibodies versus Gβ, in the presence of AVP, inhibited SS induced increase in $[Ca^{2+}]_i$. C and D, representative calcium traces of microinjected antibodies versus Gα_{i1}/Gα_{i2} and Gα_{i3}/Gα_o, respectively, in the presence of AVP, did not inhibit SS-induced increase in $[Ca^{2+}]_i$.

tein that elicits this synergistic increase in $[Ca^{2+}]_i$ in the presence of G_q activation (Selbie et al., 1997; Quitterer and Lohse, 1999; Chan et al., 2000). To determine whether our cell system is also dependent on $G_{i/o}$-βγ dimer, we microinjected antibodies versus $G_{i/o}$ subunits into single cells in the presence of 1 nM AVP and then treated the cells with somatostatin (100 nM) to induce an increase in $[Ca^{2+}]_i$. Antibody (1:100) versus Gβ reduced the response to somatostatin by 89% ($p < 0.05$). In contrast, antibodies versus $G\alpha_{i1}/G\alpha_{i2}$ or $G\alpha_{i3}/G\alpha_o$ (1:100) did not significantly change the response to somatostatin (Fig. 1). To further demonstrate the involvement of $G_{i/o}$-βγ dimer, ct-βARK was expressed in HIT-T15 cells. Expressing ct-βARK has been shown to sequester Gβγ, thereby inhibiting its ability to stimulate downstream mediators (Inglese et al., 1992; Koch et al., 1994). Protein expression of the myc-tagged ct-βARK was determined by immunocytochemistry using anti-myc monoclonal antibody; 80 to 90% of HIT-T15 cells expressed the myc epitope (data not shown). When ct-βARK was expressed in HIT-T15 cells, it dramatically reduced somatostatin (100 nM)-induced increase in $[Ca^{2+}]_i$ in the presence of AVP (1 nM). In mock-transfected HIT-T15 cells, somatostatin in the presence of AVP caused a 81 ± 14% increase in RFU/baseline RFU, whereas cells that expressed ct-βARK elicited only a 14 ± 9% increase in RFU/baseline RFU when stimulated by AVP-somatostatin. All cells responded to 10 mM KCl by increasing $[Ca^{2+}]_i$ (data not shown). These results suggested that $G_{i/o}$-βγ dimer is essential for the somatostatin-induced increase in $[Ca^{2+}]_i$ in the presence of AVP.

Somatostatin- and AVP-Induced Increase in IP_3 Levels. Somatostatin alone was unable to elicit any increase in $[Ca^{2+}]_i$ in HIT-T15 cells (Cheng et al., 2002a). We then hypothesized that somatostatin does not directly activate PLC-β. We first determined the time course in which somatostatin + AVP produced the highest IP_3 levels. In the presence of AVP (1 nM), somatostatin (100 nM) increased IP_3 levels after 10 to 12 s of administration (Fig. 2A, $p < 0.05$). We then used this time frame to compare IP_3 levels among four treatment groups, terminating all reactions at 12 s after somatostatin administration. Somatostatin (100 nM) alone failed to increase IP_3 levels. AVP (1 nM) alone induced a small but significant increase in IP_3 levels (Fig. 2B). In cells pretreated with AVP (1 nM) for 100 s, followed by somatostatin (100 nM), IP_3 levels were the highest among four treatment groups. These results suggested that somatostatin alone cannot activate PLC-β in HIT-T15 cells, because it did not increase IP_3 levels. AVP (1 nM) alone increased IP_3 levels in these cells, and concurrent administration of AVP and somatostatin caused a synergistic increase in IP_3 levels.

Somatostatin-Induced Increase in PIP_2 and Decrease in PIP Levels. Because somatostatin alone was unable to increase IP_3 levels in HIT-T15 cells, we hypothesized that somatostatin increases PIP_2 synthesis, which in turn provides more substrate for preactivated PLC-β by AVP. If this hypothesis is correct, injection of PIP_2 into single cells in the presence of 1 nM AVP should increase $[Ca^{2+}]_i$ in a similar manner to somatostatin administration. In the control group, microinjection of intracellular buffer into single cells did not increase $[Ca^{2+}]_i$ in the presence of AVP (Fig. 3). In the presence of AVP, microinjection of PIP_2 (5–50 amol) increased $[Ca^{2+}]_i$ in a concentration-dependent manner. In the absence of AVP, PIP_2 at the highest concentration studied (50 amol) failed to increase $[Ca^{2+}]_i$ (Fig. 3C). In addition, in the presence of AVP (1 nM), microinjection of PIP (50 amol) was unable to increase $[Ca^{2+}]_i$ (data not shown). These results are consistent with our hypothesis that somatostatin increases PIP_2 levels, providing more substrate for preactivated PLC-β to synthesize IP_3 and increase $[Ca^{2+}]_i$. Again, all cells responded to 10 mM KCl by increasing $[Ca^{2+}]_i$ (data not shown).

We determined whether AVP-somatostatin increased PIP_2 levels and the time course of this increase. In the presence of 1 nM AVP, 100 nM somatostatin increased PIP_2 at 8 s after somatostatin administration ($p < 0.05$) (Fig. 4A). In addition, there was a decrease in PIP levels, the precursor for PIP_2, at 8 s after somatostatin administration ($p < 0.05$). We then used this time frame to compare PIP_2/PIP levels among four treatment groups.

We determined whether somatostatin increases PIP_2 and decreases PIP levels in the presence and absence of AVP. Somatostatin (100 nM) increased PIP_2 and decreased PIP levels in the presence and absence of 1 nM AVP ($p < 0.05$), compared with basal controls (Fig. 4, B and C). AVP alone failed to alter PIP_2 or PIP levels. Because somatostatin with or without AVP decreased PIP levels but increased PIP_2 levels, these findings suggest that somatostatin increased the synthesis of PIP_2 from PIP or decreased the breakdown of PIP_2 to PIP.

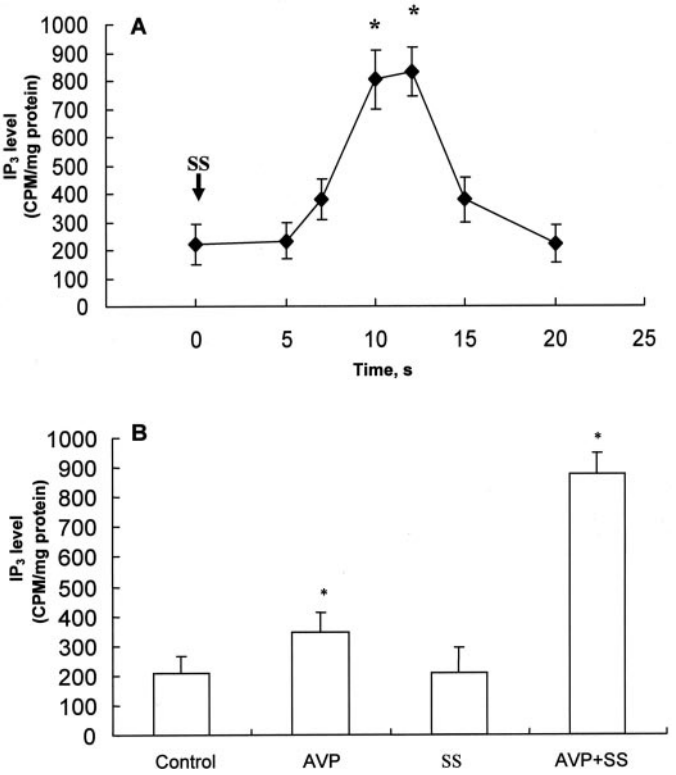
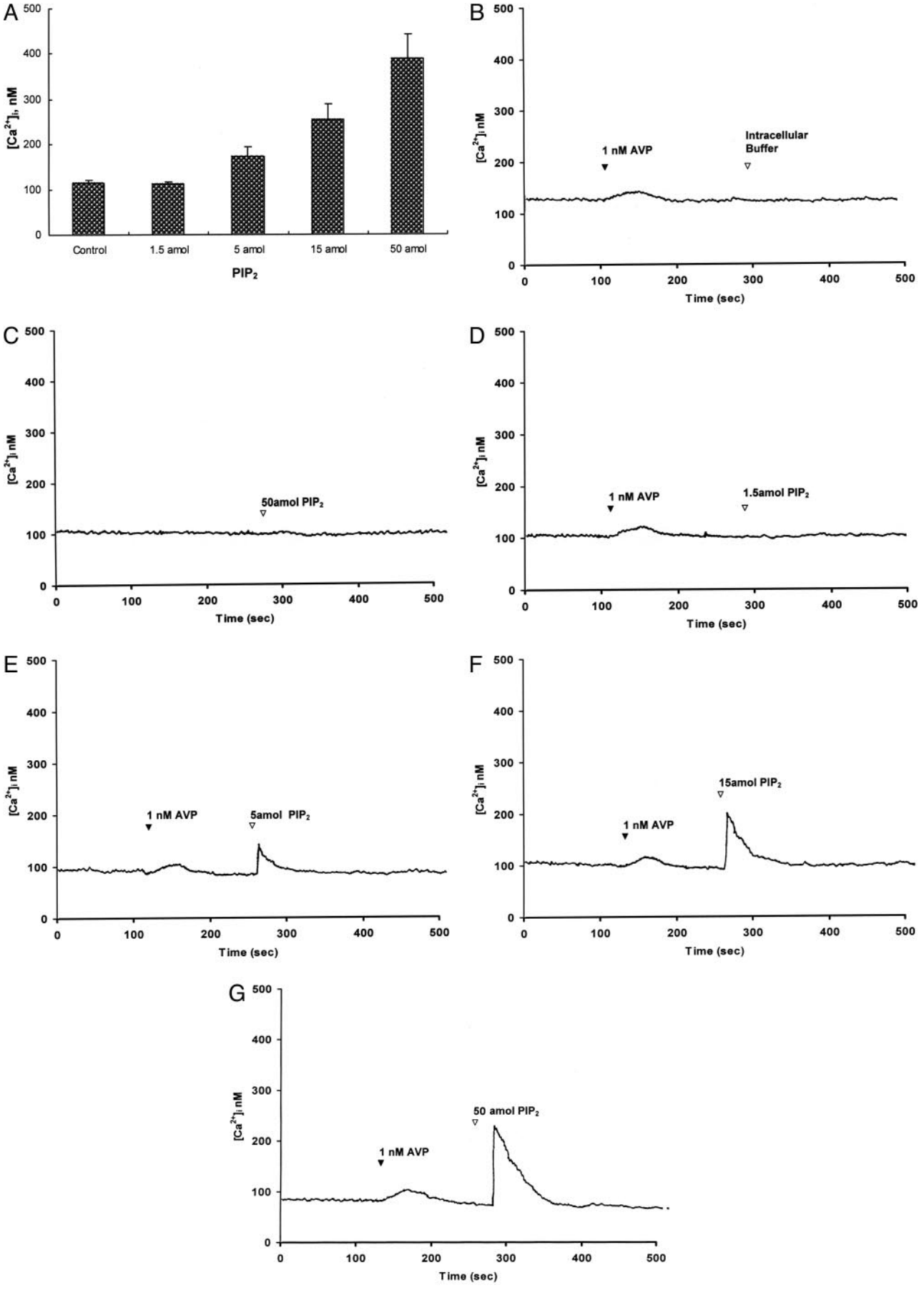


Fig. 2. Somatostatin (SS)-induced increase in IP_3 levels in the presence and absence of AVP in HIT-T15 cells. A, time course of AVP-somatostatin induced increase in IP_3 levels, as determined by ion-exchange chromatography. Somatostatin (100 nM) was given 100 s after AVP (1 nM) and experiments terminated at the respective time; *, $p < 0.05$ compared with 0 s ($n = 3$). B, determination of IP_3 levels among four treatment groups. Somatostatin (100 nM) was applied 100 s after AVP (1 nM); experiment was terminated at 12 s of somatostatin treatment; *, $p < 0.05$ compared with basal controls. Values are the mean ± S.E. ($n = 4$ independent cell preparations).



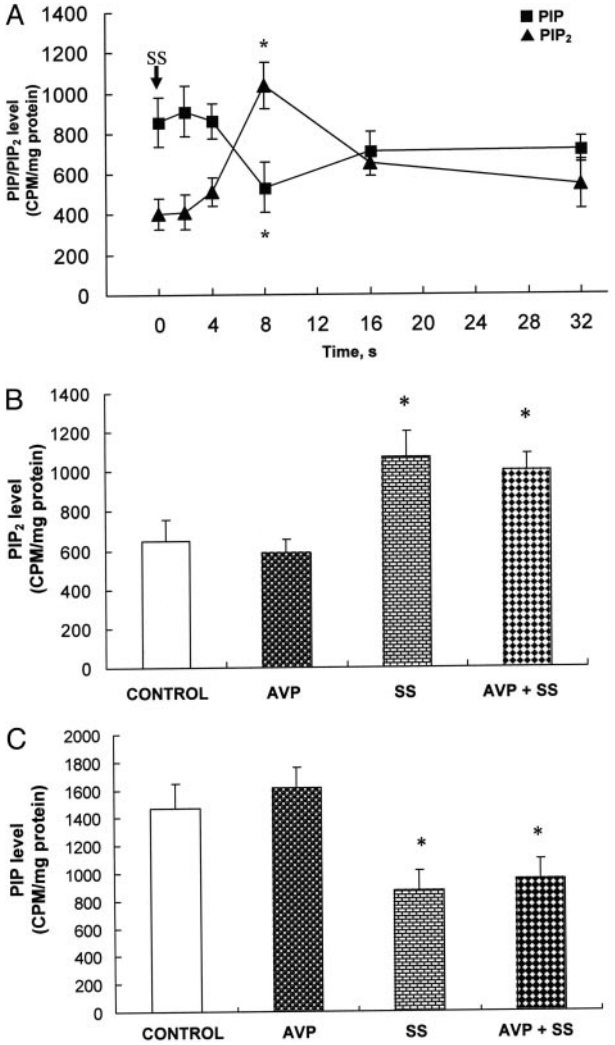


Fig. 4. Somatostatin (SS)-induced changes in PIP and PIP_2 levels in HIT-T15 cells. PIP and PIP_2 levels were determined by thin-layer chromatography. Values are the mean ± S.E. A, time course for changes in PIP and PIP_2 levels by somatostatin in the presence of AVP. Somatostatin (100 nM) was administered 100 s after AVP (1 nM); the experiment was terminated at the indicated time; $n = 3$ independent cell preparations. *, $p < 0.05$ compared with 0 s. B and C, somatostatin-induced changes in PIP and PIP_2 levels in the presence and absence of AVP. PIP_2 (B) and PIP (C) levels were determined after exposure to different treatments. Somatostatin (100 nM) was applied 100 s after AVP (1 nM) and experiments terminated at 8 s after somatostatin administration; $n = 4$ independent cell preparations. *, $p < 0.05$ compared with basal controls.

Somatostatin-Induced Increase in PLD Activity. PIP_2 synthesis is catalyzed predominantly by PIP 5-kinase (Hawkins et al., 1992), which is activated by several input signals, including PLD (Jenkins et al., 1994). PLD converts PC into PA, which is known to activate PIP 5-kinase. We hypothesized that somatostatin stimulates PLD to increase PA formation, thereby activating PIP 5-kinase to increase PIP_2 synthesis. Somatostatin (100 nM) increased PLD activity by 2-fold over the control group ($p < 0.05$; Fig. 5A). In

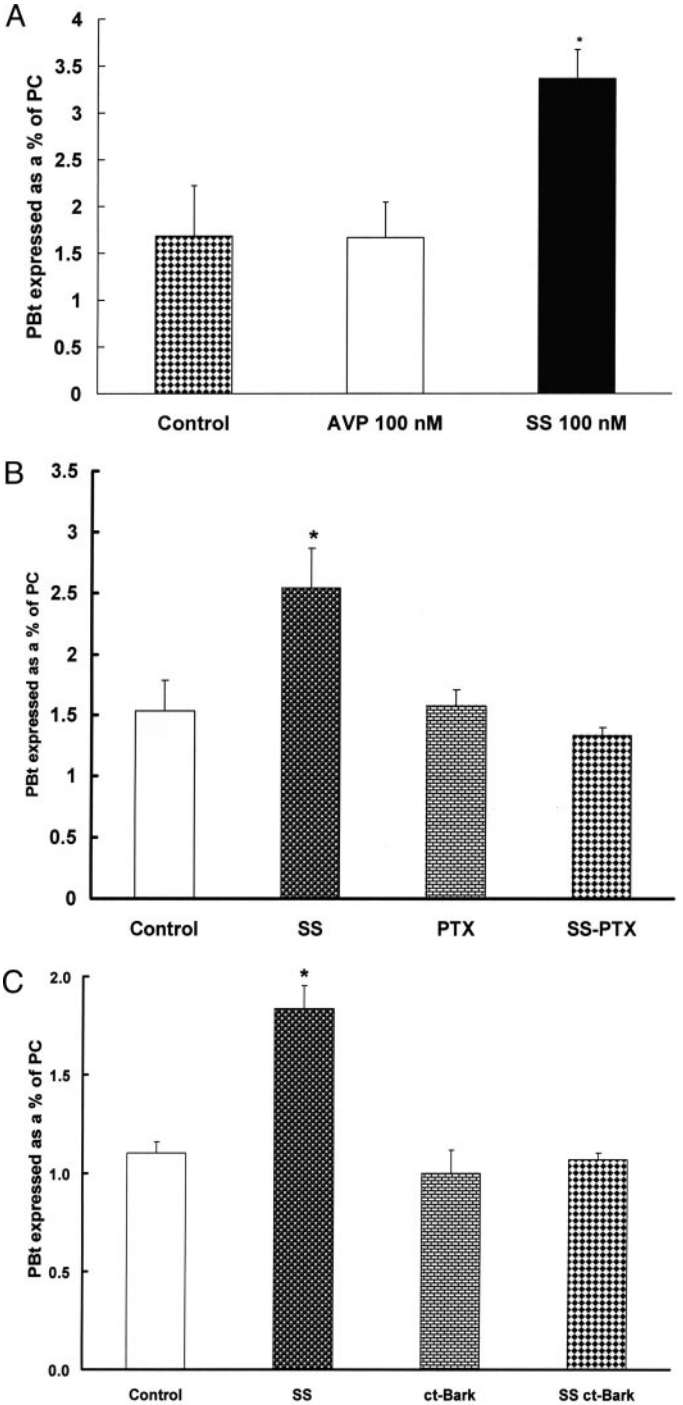


Fig. 5. Somatostatin (SS)-induced increase in PLD activity in HIT-T15 cells. Experiments were terminated 30 s after somatostatin or AVP had been administered. A, effect of somatostatin and AVP. B, effect of PTX. HIT-T15 cells were pretreated overnight with PTX (100 ng/ml) before treatments. C, effect of ct-βARK expression on somatostatin-induced increase on PLD activity. HIT-T15 cells were transfected 24 h before PLD assay. Values are the mean ± S.E. ($n = 4$ independent cell preparations). *, $p < 0.05$ compared with controls. PBt, phosphatidylbutanol; PC, phosphatidylcholine.

Fig. 3. Effect of PIP_2 microinjection on $[Ca^{2+}]_i$ in the presence of AVP in HIT-T15 cells. A, administration of PIP_2 (1.5–50 amol) into single cells increased $[Ca^{2+}]_i$ in a dose-dependent manner after 100 s of AVP (1 nM). Values are the mean ± S.E.; $n = 3$ independent cell preparations. B, representative calcium trace of HIT-T15 cells microinjected with intracellular buffer after the addition AVP did not increase $[Ca^{2+}]_i$. C, representative calcium trace of HIT-T15 cells microinjected with PIP_2 alone at 50 amol did not increase $[Ca^{2+}]_i$. D, Representative calcium trace of HIT-T15 cells microinjected with 1.5 amol of PIP_2 after the addition of AVP did not increase $[Ca^{2+}]_i$. E–G, representative calcium traces of microinjected PIP_2 (5, 15, and 50 amol) did increase $[Ca^{2+}]_i$, while in the presence of AVP, in a dose-dependent manner.

contrast, 100 nM AVP did not increase PLD activity (Fig. 5A). Pretreatment of HIT-T15 cells with pertussis toxin (PTX, 100 ng/ml) for 14 to 18 h abolished somatostatin (100 nM)-induced activation of PLD ($p < 0.05$; Fig. 5B), suggesting a $G_{i/o}$-coupled receptor-mediated response. HIT-T15 cells that expressed ct-βARK domain was able to abolish somatostatin (100 nM)-induced activation of PLD ($p < 0.05$, Fig. 5C). To demonstrate that activation of PLD can lead to an increase in PIP_2 levels, which can be used by PLC as a substrate to promote IP_3 production and a subsequent increase in $[Ca^{2+}]_i$, we microinjected PA into HIT-T15 cells. Microinjection of PA (10 amol) in the absence of AVP (1 nM) produced no changes in $[Ca^{2+}]_I$; however, in the presence of AVP, it increased $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$: PA, 2.1 ± 0.8 nM; AVP + PA, 39.1 ± 8.8 nM, $n = 10$ cells, $p < 0.05$). To determine whether somatostatin's ability to increase PLD activity has an impact on PIP_2 levels and its subsequent increase in $[Ca^{2+}]_i$, we used 1-butanol, a PLD inhibitor, to determine its effects on somatostatin-induced increase in PIP_2 levels. Pretreatment of cells with 1-butanol (0.5%) abolished somatostatin-induced increase in PIP_2 levels ($p < 0.05$. Figure 6), but pretreatment with 2-butanol (0.5%), an inactive constitutional isomer of 1-butanol, did not (Fig. 6). To further demonstrate the involvement of PLD in AVP + somatostatin-

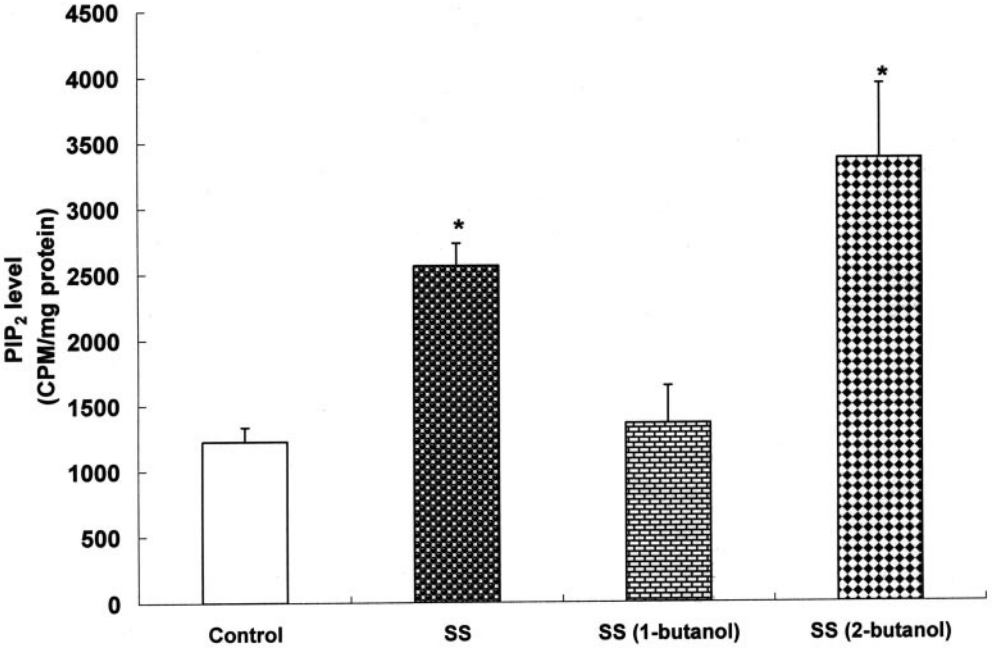


Fig. 6. Effect of 1-butanol and 2-butanol on somatostatin (SS)-induced increase in PIP_2 levels. HIT-T15 cells were pretreated with 0.5% 1-butanol or 2-butanol 5 min before somatostatin treatment. Experiments were terminated at 8 s after somatostatin administration. Values are the mean ± S.E.; $n = 4$ independent cell preparations. *, $p < 0.05$ compared with basal controls.

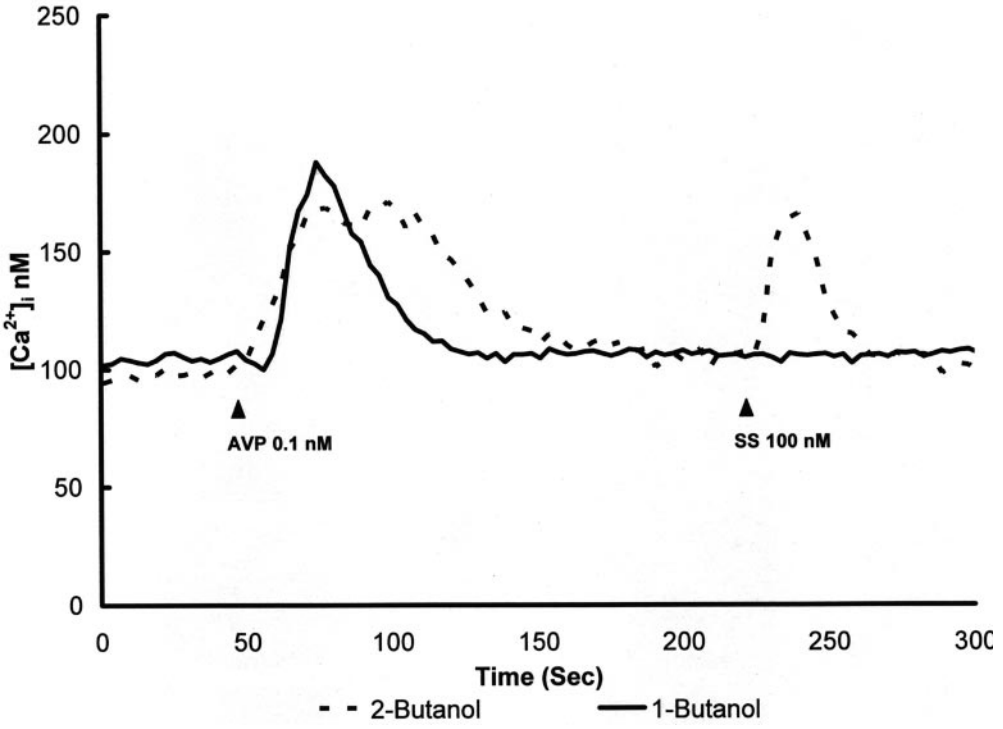


Fig. 7. Effect of 1-butanol and 2-butanol on somatostatin (SS)-induced (100 nM) increase in $[Ca^{2+}]_i$ in the presence of AVP (1 nM). HIT-T15 cells were pretreated with 1-butanol or 2-butanol (0.5%) 5 min before treatments. Cells treated with 1-butanol or 2-butanol were exposed to AVP (1 nM) for 150 s before the addition of somatostatin. Each line depicts the mean from 8 to 14 cells. The lines are representative of four independent experiments.

induced increase in $[Ca^{2+}]_i$, cells were pretreated with PLD inhibitor 1-butanol. 1-Butanol (0.5%) abolished AVP + somatostatin-induced increase in $[Ca^{2+}]_i$, but 2-butanol (0.5%), did not (Fig. 7). Pretreatment with another PLD inhibitor, carbobenzyloxy-leucine-tyrosine-chloromethylketone (zLYCK, 10 μM) (Kessels et al., 1991) also abolished AVP + somatostatin-induced increase in $[Ca^{2+}]_i$ (Fig. 8). A high concentration of AVP (100 nM) caused an increase in both the control and zLYCK-pretreated cells (Fig. 8), suggesting that zLYCK does not inhibit G_q-PLC signaling pathway. To further classify PLD involvement in this pathway, we used antibodies versus PLD1 and PLD2, respectively, to determine which PLD isoform was responsible for the paradoxical increase in $[Ca^{2+}]_i$ caused by AVP-somatostatin. Only PLD1 was detected in HIT-T15 cells, using Western blot analysis (Fig. 9, inset). Antibody versus PLD1 inhibited somatostatin-induced $[Ca^{2+}]_i$ increase in the presence of AVP, whereas antibody versus PLD2 did not (Fig. 9). These results suggested that PLD1 mediates somatostatin-induced increases in PIP_2.

Discussion

We reported previously that in clonal β cells HIT-T15, somatostatin increased $[Ca^{2+}]_i$ and transiently stimulated insulin release in the presence of AVP (Cheng et al., 2002a). These effects of somatostatin in HIT-T15 cell are caused by activation of sstr2 (Cheng et al., 2002b) and are attributable to cross-talk between $G_{i/o}$ and G_q (Cheng et al., 2002a). Cross-talk between G_q- and $G_{i/o}$-coupled receptors have been reported in other systems. For example, activation of $G_{i/o}$-coupled adenosine A_1-receptors enhances the stimulation of PLC-β by G_q-coupled receptors such as α_1-adrenergic, bradykinin, histamine H_1, and muscarinic receptors (Selbie and

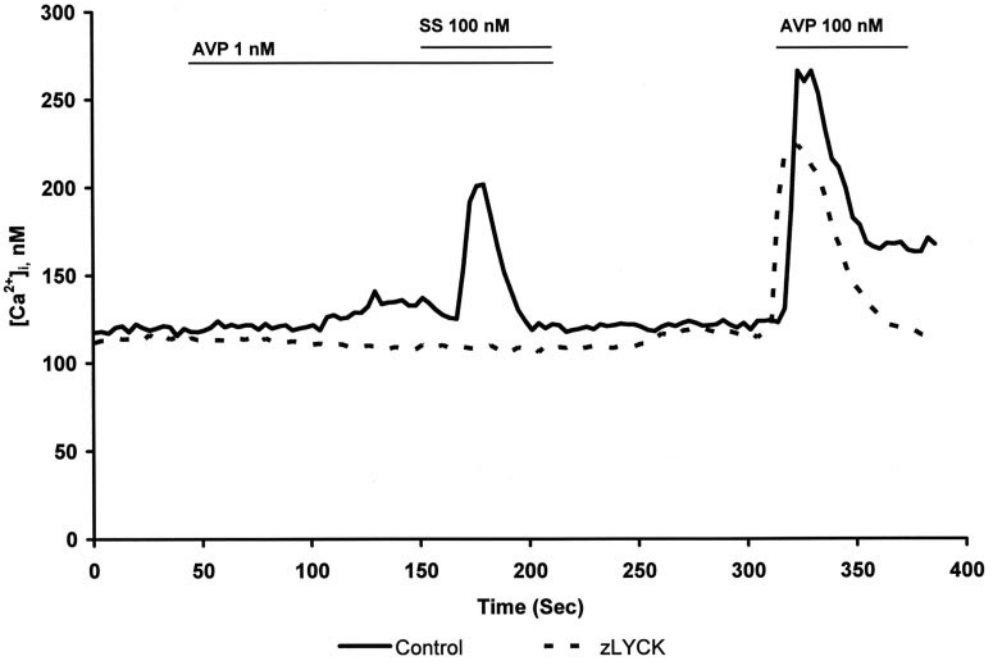


Fig. 8. Effect of zLYCK on somatostatin (SS)-induced (100 nM) increase in $[Ca^{2+}]_i$ in the presence of AVP (1 nM). HIT-T15 cells were pretreated with zLYCK (10 μM) 60 min before treatments. Control and zLYCK-treated cells were exposed to AVP (1 nM) for 120 s before the addition of somatostatin. After the 60-s coexposure to AVP (1 nM) and somatostatin, cells were perfused with KRB for 120 s before the addition of 100 nM AVP. Each line depicts the mean from five to eight cells. The lines are representative of four independent experiments.

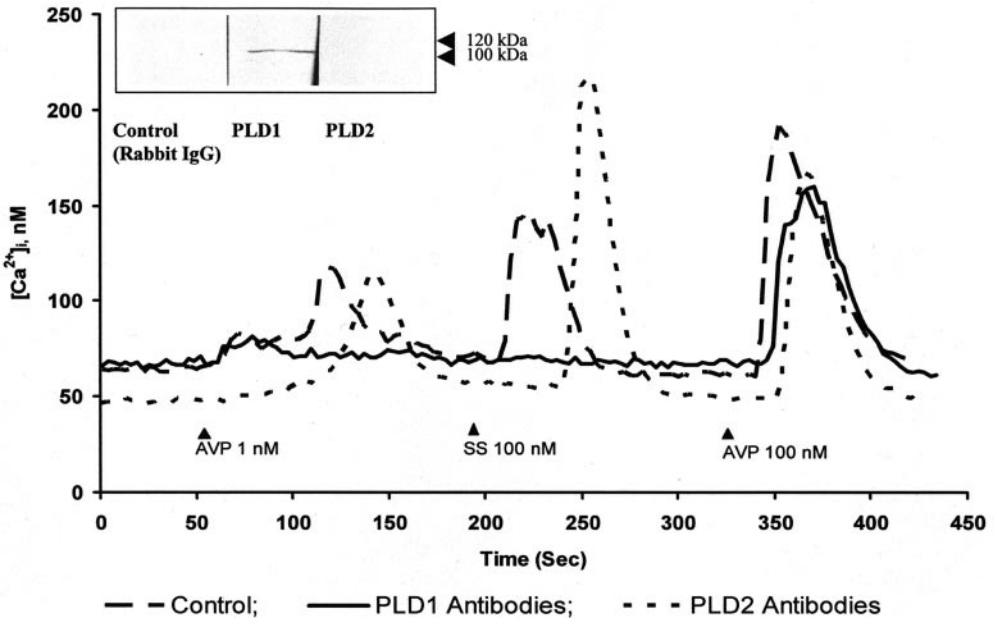


Fig. 9. PLD immunoblot and effect of PLD antibodies on somatostatin (SS)-induced increase in $[Ca^{2+}]_i$ in the presence of AVP. Inset, 10 μg of the whole cell protein was run on SDS-PAGE, transferred to the PVDF membrane, and blotted with PLD antibodies. For $[Ca^{2+}]_i$ determination, HIT-T15 cells were pretreated with PLD antibodies using the BioPORTER protein delivery system 4 h before Fura-2AM loading. Cells were exposed to AVP (1 nM) for 120 s before the addition of somatostatin (100 nM). AVP (100 nM) was added 120 s after somatostatin treatment. Each line depicts the mean from five to eight cells. The lines are representative of four independent experiments.

Hill, 1998). For such cross-talk, activation of $G_{i/o}$ alone usually has no effect, but it enhances G_q-mediated increases in PLC-β activity, particularly when G_q is activated before $G_{i/o}$ (Muller and Lohse, 1995; Connor et al., 1997; Quitterer and Lohse, 1999; Yeo et al., 2001). Activation of $G_{i/o}$-coupled adenosine A_1-receptors, α_2-adrenoceptors in COS cells (Quitterer and Lohse, 1999) or δ- or κ-opioid receptors in SH-SY5Y cells (Yeo et al., 2001) enhances inositol phosphate formation generated by activation of G_q-coupled receptors. In Chinese hamster ovary cells, neuropeptide Y, a $G_{i/o}$-coupled receptor agonist, enhances inositol phosphate formation generated by ATP, a G_q-coupled receptor agonist (Selbie et al., 1997). Somatostatin also increases $[Ca^{2+}]_i$ after activation of the G_q-coupled muscarinic receptors in SH-SY5Y cells (Connor et al., 1997).

So far, studies on the cross-talk between G_q and $G_{i/o}$ indicate that G$\beta\gamma$ of $G_{i/o}$ is responsible for the enhancement of G_q-generated signals (Selbie et al., 1997; Quitterer and Lohse, 1999; Chan et al., 2000). We demonstrated that antibody versus Gβ nearly abolished somatostatin-induced increase in $[Ca^{2+}]_i$, whereas antibodies versus Gα_{i1}/Gα_{i2} and Gα_{i3}/Gα_o failed to do so. In addition, overexpressing ct-βARK, which binds avidly to G$\beta\gamma$ (Inglese et al., 1992; Koch et al., 1994), greatly reduced somatostatin-induced increases in $[Ca^{2+}]_i$. Our findings are consistent with what is found in the literature (Selbie et al., 1997; Quitterer and Lohse, 1999; Chan et al., 2000) and further suggest that in β cells, the increase in $[Ca^{2+}]_i$ by somatostatin is mediated through G$\beta\gamma$.

Several studies have shown that G$\beta\gamma$ of $G_{i/o}$ can activate a number of enzymes, including PLC-β (Blake et al., 2001), PLA_2 (Kim et al., 1989), mitogen-activated protein kinase (Koch et al., 1994), Raf-1 (Pumiglia et al., 1995), βARK (Goldman et al., 1997), phosphatidylinositol 3-kinase (Lopez-Ilasaca et al., 1998), and adenylyl cyclase (Myung and Garrison, 2000). In intestinal smooth muscle cells, somatostatin alone activates PLC-β_3, thereby increasing IP_3 levels, $[Ca^{2+}]_I$, and contractions through the G$\beta\gamma$ of $G_{i/o}$ (Murthy et al., 1996).

Although others have suggested that enhancement of G_q signals by $G_{i/o}$ occurs through activation of PLC-β (Chan et al., 2000) or interaction with a step after PLC activation (Yeo et al., 2001), none of them has attributed the effect of $G_{i/o}$ to a step before PLC activation, except that Schmidt et al. (1996 and 1998) suggested that $G_{i/o}$ mediates an increase in PIP_2 levels. In the present study with HIT-T15 cells, after somatostatin treatment, PIP levels decreased but PIP_2 levels increased, suggesting that somatostatin may increase PIP_2 synthesis. Thus, we have suggested for the first time that somatostatin can increase PIP_2 synthesis, which in turn provides extra substrate for preactivated PLC-β by AVP to generate high levels of IP_3. Without a preactivated PLC-β, somatostatin failed to increase IP_3 levels (Fig. 3) and $[Ca^{2+}]_i$ (Cheng et al., 2002a). However, we cannot rule out the possibility that the increase in PIP_2 and decrease in PIP levels might have been caused by a decrease in phosphatase activity. In addition, because the IP_3 assay that we used in the present study could not differentiate inositol 1,3,4-trisphosphate from 1,4,5-trisphosphate, some of the IP_3 observed in the present study could be attributable to inositol 1,3,4-trisphosphate. Nevertheless, our results are consistent with the findings using cyclopentyladenosine, an adenosine A_1-receptor agonist, in RINm5F cells, another β cell line (Biden and Browne, 1993). In RINm5F cells, activation of adenosine A_1-receptors alone fails to increase IP_3 levels and $[Ca^{2+}]_i$ but increases them after activation of M_3 receptors (Biden and Browne, 1993). Our findings are further supported by those of the administration of PIP_2 into the cells, in which PIP_2 alone failed to increase $[Ca^{2+}]_i$, but PIP_2 in the presence of a small concentration of AVP (1 nM) increased $[Ca^{2+}]_i$. Because PIP_2 and PIP failed to increase $[Ca^{2+}]_i$ in the absence of AVP (1 nM), we believe that these phospholipids are unable to elicit any $[Ca^{2+}]_i$ response in HIT-T15 cells. The failure of microinjected PIP (50 amol) to induce changes in $[Ca^{2+}]_i$ in the presence of AVP (1 nM) demonstrated that the microinjection of PIP_2 in the presence of AVP, which caused an increase in $[Ca^{2+}]_i$, was caused by the use of PIP_2 as a substrate for low-grade PLC activation and was not an artifact of the system. In HEK 293 cells with stable expression of M_3 receptors, carbachol increases PIP_2 levels for at least 30 min (Schmidt et al., 1996). The M_3 receptor-mediated increase in PIP_2 levels is attributable to G_i-protein coupling, because this effect was inhibited by PTX pretreatment. In HEK 293 cells, activation of plasma membrane purinergic receptors and lysophosphatidic acid receptors, respectively, also increases PIP_2 levels for $\geq$40 min (Schmidt et al., 1998). These findings are somewhat different from those of ours, because somatostatin-induced increase in PIP_2 levels in HIT-T15 cells lasted $<$ 12 s, but the effect of $G_{i/o}$-coupled receptor agonists in HEK 293 cells lasted $\geq$40 min. It is not clear why these two systems are so much different in terms of the duration of the agonist-induced PIP_2 levels. However, in the

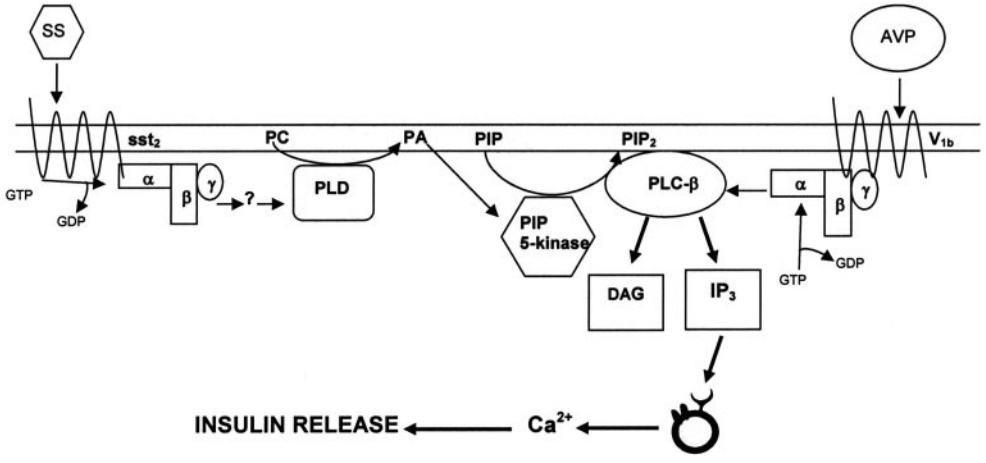


Fig. 10. Summary of the cross-talk between AVP receptor (V_{1b}) and somatostatin receptor (sstr2) in stimulation of insulin release from β cells. Activation of the $G_{i/o}$-coupled receptor by somatostatin increases PIP_2 synthesis from PIP through G$\beta\gamma$. G$\beta\gamma$ activates PLD to increase the synthesis of phosphatidic acid (PA) from phosphatidylcholine (PC). PA activates PIP 5-kinase to increase synthesis of PIP_2, providing extra substrate for preactivated PLC-β by AVP. These increases in DAG and IP_3 levels and $[Ca^{2+}]_i$ lead to insulin release. ER, endoplasmic reticulum.

study with HEK 293 cells, for preactivation of PLC, Schmidt et al. (1998) used G_q-coupled receptor agonists, which markedly lowered PIP_2 levels. Upon removal of the agonists, the PIP_2 levels increased again, which were above the initial control levels (K. H. Jakobs, personal communication).

In the present study, we demonstrated for the first time that somatostatin can increase PLD activity. This effect of somatostatin was PTX-sensitive, and was blocked by expression of ct-βARK, suggesting that Gi/o-βγ dimer mediates this effect of somatostatin. We further hypothesized that somatostatin-induced increase in PLD activity mediates the increase in PIP_2 formation, because PLD is the enzyme that catalyzes the formation of PA, which may in turn activate PIP 5-kinase to increase PIP_2 synthesis (Hawkins et al., 1992). In the present study, we demonstrated that microinjection of PA increased $[Ca^{2+}]_i$, which has been used as an indicator for IP_3 increase. In addition, the PLD inhibitors 1-butanol and zLYCK and antibody versus PLD1 all blocked somatostatin-induced $[Ca^{2+}]_i$ increase in the presence of AVP. 1-Butanol further abolished somatostatin-induced PIP_2 increase. Therefore, the present findings strongly support the notion that somatostatin increased PA synthesis, which in turn activated PIP-5 kinase, the enzyme catalyzing the formation of PIP_2.

In this system, somatostatin was able to increase PIP_2 levels, providing extra substrate for PLC-β. On the other hand, PIP_2 can regulate a wide range of cellular processes, including exocytosis (Cremona and De Camilli, 2001), clathrin-mediated endocytosis (Gillooly and Stenmark, 2001), actin rearrangement (Tolias et al., 2000), vesicle docking (Brown et al., 2001), opening of G-protein-gated inwardly rectifying K^+ channels (Zhang et al., 1999), K_{ATP} channels (Baukrowitz et al., 1998), and membrane ruffling and trafficking (Honda et al., 1999). Because of the diverse role of PIP_2 in cellular processes, the effect of somatostatin-induced increase in PLD activity and its subsequent increase in PIP_2 levels on normal physiology of β cells is uncertain.

In the present study, when AVP was needed to cause a low-grade PLC activation, we used 1 nM AVP in most of the experiments. This concentration of AVP usually caused an increase in $[Ca^{2+}]_i$ of 0 to 50 nM, which was consistent with our previous study (Cheng et al., 2002a). However, in the experiment in which butanols were studied (Fig. 7), AVP at 1 nM increased $[Ca^{2+}]_i$ by ~90 nM. In this particular experiment, probably as a result of different FBS used in cell culture, the cells became more responsive to AVP than usual. As a result of the hyper-response to AVP, somatostatin-induced $[Ca^{2+}]_i$ increase was attenuated. This phenomenon was also seen in our previous study (Cheng et al., 2002a).

In summary, we have demonstrated a novel signaling mechanism for somatostatin. The activation of somatostatin receptors, which are coupled to $G_{i/o}$, leads to an increase in PIP_2 synthesis through Gβγ activation of PLD. The PIP_2 generated by somatostatin administration provides extra substrate for preactivated PLC-β, which hydrolyzes PIP_2, thereby increasing IP_3 levels, $[Ca^{2+}]_i$, and a transient release of insulin from HIT-T15 cells (Fig. 10). This is the first report regarding somatostatin-induced increase in PLD activity and PIP_2 synthesis by activation of $G_{i/o}$-coupled receptors.

Acknowledgments

We greatly appreciate the generous donation of pcDNAIIIB T8βARK plasmid by Dr. Silvio Gutkind of National Institute of Dental and Craniofacial Research, National Institutes of Health, and antibodies versus PLD1 and PLD 2 by Dr. Sylvain Bourgoin of the University of Laval, Canada. We would also like to acknowledge Dr. Guangxing Bai for contribution to Western blot analysis.

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