Targeting the signaling pathway of acylation stimulating protein

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Abstract **Acylation stimulating protein (ASP; C3adesArg) stimulates triglyceride synthesis (TGS) and glucose transport in preadipocytes/adipocytes through C5L2, a G-protein-coupled receptor. Here, ASP signaling is compared with insulin in 3T3-L1 cells. ASP stimulation is not $G\alpha_s$ or $G\alpha_i$ mediated (pertussis and cholera toxin insensitive), suggesting $G_{\alpha q}$ as a candidate. Phospholipase C (PLC) is required, because the Ca^{2+} chelator 1,2-bis(*o*-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid tetra(acetoxymethyl) ester and the PLC inhibitor U73122 decreased ASP stimulation of TGS by 93.1% ($P < 0.0.001$) and 86.1% ($P < 0.004$), respectively. Wortmannin and LY294002 blocked ASP effect by 69% ($P < 0.001$) and 116.1% ($P < 0.003$), respectively, supporting phosphatidylinositol 3-kinase (PI3K) involvement. ASP induced rapid, transient Akt phosphorylation (maximal, 5 min; basal, 45 min), which was blocked by Akt inhibition, resembling treatment by insulin. Downstream of PI3K, mamalian target of rapaycin (mTOR) is required for insulin but not ASP action. By contrast, both ASP and insulin activate the mitogen-activated protein kinase/extracellular signal-regulated kinase ($MAPK/ERK_{1/2}$) pathway, with rapid, pronounced increases in $ERK_{1/2}$ phosphorylation, effects partially blocked by PD98059 (64.7% and 65.9% inhibition, respectively; $P < 0.001$). Time-dependent (maximal, 30 min) transient calcium-dependent phospholipase A_2 ($cPLA_2$)$_{\text{-Ser505}}$ phosphorylation (by $MAPK/ERK_{1/2}$) was demonstrated by Western blot analysis. ASP signaling involves sequential activation of PI3K and PLC, with downstream activation of protein kinase C, Akt, $MAPK/ERK_{1/2}$, and $cPLA_2$, all of which leads to an effective and prolonged stimulation of TGS.—** Maslowska, M., H. Legakis, F. Assadi, and K. Cianflone. **Targeting the signaling pathway of acylation stimulating protein. *J. Lipid Res.* 2006.** 47: **643–652.**

Supplementary key words insulin • 3T3-L1 cells • triglyceride synthesis • phosphatidylinositol 3-kinase • phospholipase C

Obesity is one of the most common health problems of our society (1), and our biggest challenge is understanding how to limit its progress. Adipose tissue provides a long-term storage reservoir for energy surplus in the form of triglycerides (lipogenesis), which in turn can be mobilized (lipolysis) when necessary to provide energy for essential cellular processes (2). Normally, the balance between lipogenesis and lipolysis is tightly controlled by numerous hormonal components. Predominance of the lipogenic state, whether driven by increases in food intake or lack of exercise, is dependent on the activation of specific intracellular enzymatic pathways. Regrettably, the continuous augmentation of adipose tissue stores leads to obesity, which, in turn, can lead to a number of metabolic perturbations, such as diabetes, coronary artery disease, and hypertension.

In addition to being a storage organ, adipose tissue produces a variety of adipokines, some of which are closely involved in adipose tissue metabolism in an autocrine and paracrine manner (for review, see 3). Acylation Stimulating Protein (ASP) is generated by adipose tissue through the interaction of Factor B and adipsin with complement C3 and is identical to C3adesArg (4). ASP production, along with its precursor proteins Factor B, adipsin, and C3, is increased during the differentiation of human and mouse adipocytes (5, 6), a production that can be further augmented by insulin and dietary chylomicrons (7). In vivo production of ASP in the adipose environment has been elegantly demonstrated by studying arterial-venous differences across an adipose tissue bed (8, 9). Local adipose ASP production increased postprandially and correlated with plasma triglyceride (TG) clearance. This correlation of ASP with postprandial TG clearance has been demonstrated across a wide range of fasting ASP levels in men and woman (10). Moreover, the circulating levels of ASP are increased in obesity, with greater increases observed in women than in men (11). Upon weight loss, ASP levels return to normal (12–14). Studies have shown that ASP levels are also significantly higher in diabetes and cardiovascular disease (15).

ASP plays a key role in the regulation of lipid storage in that it stimulates the esterification of fatty acids onto a glycerol backbone, resulting in the augmentation of intracellular triglyceride depots in human preadipocytes, adipocytes, and skin fibroblasts (16, 17). In stimulating

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triglyceride synthesis (TGS), ASP increases the activity of diacylglycerol acyltransferase (DGAT; the final enzyme in TGS) in membrane preparations from adipocytes (18). ASP stimulates glucose transport in both adipocyte and muscle cells (19, 20) through the translocation of the glucose transporters GLUT1, GLUT4, and GLUT3 (21). Finally, as with insulin, ASP also inhibits lipolysis (22), yet the effects of ASP and insulin are additive.

Recently, C5L2, an orphan receptor, was identified as an ASP receptor (23, 24). C5L2 is a seven transmembrane Gprotein belonging to the C5a, C3a, and N-formylmethionyl-leucyl-phenylalanine (fMLP) family of receptors. In HEK-293 cells transfected with the receptor, ASP binds C5L2 with high affinity, and cells become responsive to ASP (but not insulin) for TGS and glucose transport (24). However, how ASP interacts with the receptor to generate a signal is not well understood. Our initial study on ASP signaling demonstrated the involvement of protein kinase C (PKC) in ASP-stimulated TGS (25). Even though numerous studies have reported on the postreceptor signaling targets of the insulin pathway regulating glucose transport and lipolysis, the main components of the signaling cascade(s) resulting in the stimulation of TGS are unknown.

The aim of this study was to identify the signaling pathway for ASP stimulation of TGS compared with insulin. Using murine 3T3-L1 preadipocytes as a cell model, we evaluated the involvement of the phospholipase C (PLC), PLD, PLA_2, phosphatidylinositol 3-kinase (PI3K), Akt, and mitogen-activated protein kinase/extracellular signal–regulated kinase ($MAPK/ERK_{1/2}$) pathways.

MATERIALS AND METHODS

Materials

3T3-L1 preadipocytes were obtained from the American Type Culture Collection (Manassas, VA). All tissue culture reagents, such as DMEM/F12, PBS, FBS, and trypsin, were from Gibco (Burlington, Ontario, Canada). Inhibitors used were pertussis toxin (PTX), isotetrandrine, PD098059, wortmannin, rapamycin, bisindolylmaleimide V, Akt inhibitor, 1,2-bis(*o*-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA-AM), arachidonyltrifluoromethyl ketone ($AACOCF_3$; Calbiochem, La Jolla, CA), LY294002 (Promega, Madison, WI), and U73122 (Sigma, Oakville, Ontario, Canada). Stimulators of TGS, insulin, and phorbol 13-myristate 12-acetate (PMA), were from Sigma. Oleic acid[9,10-^{3}H(N)] was from DuPont-New England Nuclear (Mississauga, Ontario, Canada). Thin-layer chromatography plates (silica gel 150A) came from Fisher (Nepean, Ontario, Canada). Organic solvents, scintillation vials, general chemicals, and tissue culture materials were from VWR (Montreal, Quebec, Canada). Scintillation fluid was from ICN (Costa Mesa, CA). Bio-Rad reagent for protein measurements was from Bio-Rad (Mississauga, Ontario, Canada).

ASP preparation

ASP was isolated and purified from human plasma as described previously (4). Each batch was verified for purity by ion-spray mass spectrometry at the McGill University Mass Spectrometry Unit. The activity of each ASP preparation was checked by its ability to stimulate TGS in 3T3-L1 preadipocytes.

Cell culture and net TGS

3T3-L1 preadipocytes, maintained at low passage number, were grown in DMEM/F12 supplemented with 10% FBS. TGS was evaluated as described in detail previously (16, 17). At 80% confluence, the cells were plated at 7,000 cells/well on 24-well plates for experiments. On the 4th day after plating (at 100% cell confluence), preadipocytes were switched to serum-free medium (DMEM/F12) for 2 h followed by incubation with various inhibitors for 30 min (U73122, *n*-butanol, wortmannin, LY294002, rapamycin, bisindolylmaleimide V, PD98059, isotetrandrine, $AACOCF_3$, or BAPTA-AM) or 4 h (PTX). Subsequently, the medium was changed to fresh serum-free DMEM/F12 supplemented with 100 μM [^{3}H]oleate/BSA (5:1 molar ratio; average specific activity, 65 dpm/pmol), appropriate inhibitors, and stimulators (ASP, insulin, or PMA). Inhibitors were reconstituted and stored (working solution) according to the manufacturer's instructions and were added at appropriate concentrations from freshly prepared working solutions diluted in PBS. TGS was measured over 4 h at 37°C in the presence of inhibitors and hormones. Appropriate vehicle controls were used in each experiment. After the incubation period, radioactive medium was removed and the cells were washed two times in ice-cold PBS. The lipids were extracted for 1 h in 1 ml of heptane-isopropanol (3:2, v/v) and then rinsed with an additional 1 ml of the same solvent mix. Lipid extracts were evaporated to dryness in a centrifuge-evaporator (Canberra-Packard Canada) and redissolved in 100 μl of chloroform-methanol (2:1, v/v), and lipids were resolved by TLC in hexane-ethyl ether-acetic acid (75:25:1, v/v/v) with reference lipids run concurrently. Separated lipids were visualized with iodine vapor, and the spots corresponding to triglyceride were scraped into scintillation vials and counted by liquid scintillation counting (Beckman). Cell proteins were solubilized in 0.1 N NaOH for 3 h and measured by the method of Bradford (26). Note that, because TGS and lipolysis are ongoing, these experiments measure net TGS. Medium oleate depletion during the 4 h did not exceed 6%. Average basal TGS was 30–50 pmol/μg cell protein, which constitutes 50–70% of the radioactive lipids, with the remainder primarily in phospholipids. Monoacylglycerol, diacylglycerol (DAG), and free fatty acids constitute <5%. On average, TGS stimulation with ASP was 168.65 ± 24.17% (range, 132.7 ± 8.5% to 209.4 ± 17.0%), that with insulin was 233.71 ± 50.81% (range, 185.9 ± 15.1% to 308.4 ± 54.8%), and that with PMA was 167.23 ± 29.4% (range, 139.6 ± 10.7% to 198.2 ± 17.2%), where basal was always set as 100%.

Cell treatment, lysis, and Western blot analysis

Cells were grown to confluence on 60 mm dishes, preincubated in serum-free medium for 2 h, and then stimulated with ASP or insulin in fresh serum-free medium for 0, 5, 15, 30, 45, and 60 min. The medium was quickly removed, and 500 μl of ice-cold lysing buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 1 mM PMSF, 200 μM orthovanadate, 20 mM β-glycerophosphate, 100 μM 4-(2-aminoethyl) benzenesulphonyl fluoride (irreversible serine protease inhibitor), 150 nM aprotinin, 1 μM E-64, and 1 μM leupeptin, pH 7.5) was added to the plates for 10 min at 4°C with gentle shaking. Total cell lysates were collected and centrifuged to remove particulate material (14,000 *g* for 10 min at 4°C). Aliquots of the supernatant were stored at −80°C for further analysis. Proteins were measured with the Bio-Rad assay. For Western blot analysis, Laemmli sample buffer was added to the aliquots of cell lysates and the samples were boiled for 3 min. Twenty-five microliters of cell lysate was loaded per lane, and the proteins were resolved by 10% SDS-PAGE. Gels were then transferred to a polyvinylidene difluoride membrane and

were immunoblotted with the appropriate antibody to phosphorylated forms of intracellular proteins and reblotted with the antibodies directed to the nonphosphorylated proteins in question. The immobilized proteins were detected with the ECL Plus kit from Amersham Biosciences (Piscataway, NJ) using Kodak film.

Statistical analysis

The results were normalized to basal TGS in each experiment (set as 100%) and are presented as means ± SEM. Differences were analyzed by two-way ANOVA for drug treatment and hormone treatment with the Bonferoni posthoc test, with $P < 0.05$ considered significant. Western immunoblots were quantified with the ChemiImager Ready System Alpha DigiDoc Imaging system (San Leonardo, CA).

RESULTS

Immediate postreceptor events: involvement of G-proteins

PTX was used to evaluate the involvement of the $G\alpha_i$ subunit in ASP action. Confluent 3T3-L1 preadipocytes were pretreated for 4 h with 100 ng/ml PTX before stimulation with ASP or insulin. As shown in **Fig. 1A**, PTX did not affect basal TGS (which is set as 100%). ASP stimulation of TGS is unaltered by PTX treatment (181.9 ± 14.6% for ASP alone vs. 216.3 ± 10.7% for ASP + PTX, where basal is set at 100%; P = NS). Insulin also stimulates TGS in 3T3-L1 preadipocytes (27) through the insulin receptor, which belongs to the family of tyrosine kinase receptors. As expected, PTX had no inhibitory effect on the TGS stimulatory action of insulin (302.1 ± 2.1% for insulin alone vs. 346.1 ± 10.6% for insulin + PTX; P = NS).

Activation of PLC but not PLD

Our data demonstrating the rapid biphasic increases in intracellular DAG and PKC translocation by ASP (25) are suggestive of PLC and/or PLD involvement in ASP signaling (28). This was tested using specific inhibitors and Ca^{2+} chelators. The effect of U73122, a potent and widely used inhibitor of PLC (29), was tested. As shown in Fig. 1B, U73122 resulted in a slight but significant

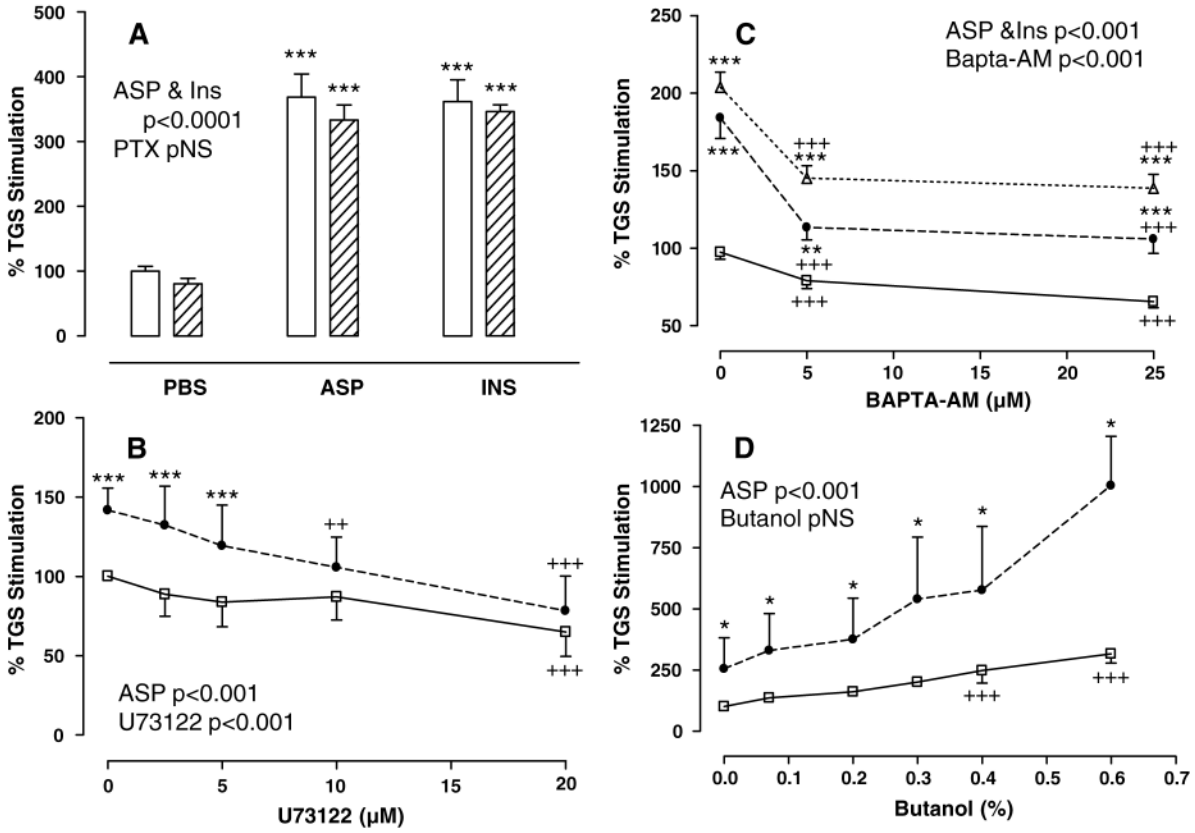

Fig. 1. Effect of pertussis toxin (PTX) and phospholipase inhibition on Acylation-Stimulating Protein (ASP)-stimulated triglyceride synthesis (TGS) in 3T3-L1 preadipocytes. Confluent cells were pretreated with serum-free medium containing 100 ng/ml PTX (A; hatched bars), 2.5–20 μM U73122, a phospholipase C (PLC) inhibitor (B), 5 and 20 μM 1,2-bis(*o*-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA-AM), a Ca^{2+} chelator (C), or 0.1–0.6% *n*-butanol, a PLD substrate substitute (D) for 30 min (with the exception of PTX, which required a 4 h pretreatment) and were then stimulated with 5 μM ASP (circles) or 100 nM insulin (Ins; triangles) for an additional 4 h. All inhibitors were present throughout the experiment. TGS was measured as picomoles of [^{3}H]oleate incorporated into TG. The results were normalized to basal TGS (open bars for A; squares for B–D) in each of the experiments performed (n = 9 values per treatment) and are presented as percentage TGS stimulation (means ± SEM), where basal (no addition and no treatment) was set as 100%. The appropriate amount of vehicle was included in the medium for each of the inhibitors tested. The data were analyzed by two-way ANOVA (results shown in graphs) followed by the Bonferoni posthoc test: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for ASP or insulin stimulation versus control; $^{++}P < 0.01$ and $^{+++}P < 0.001$ for inhibitor treatment versus no inhibitor.

decrease in basal TGS at the highest concentration only (35.2% inhibition; $P < 0.001$). The ASP stimulatory effect was gradually blocked in a concentration-dependent manner, leading to a complete loss of ASP stimulatory activity at the highest concentration tested ($P < 0.001$ at 20 μM U73122). On the other hand, insulin-stimulated TGS (308.4 ± 51.5%; $P < 0.0001$) was inhibited by only 7.1% at 20 μM U73122 (data not shown). These data suggest that although both ASP and insulin can stimulate TGS, ASP, in contrast to insulin, mediates its effects via PLC.

The Ca^{2+}-chelating agent BAPTA-AM is a cell-permeable molecule that becomes hydrolyzed and trapped inside the cell as an active chelator, thus chelating only intracellular Ca^{2+} concentration ($[Ca^{2+}]$). As shown in Fig. 1C, basal TGS decreased slightly but significantly with increasing BAPTA concentrations (32.8% inhibition at 25 μM BAPTA-AM; $P < 0.001$). ASP-stimulated TGS (184.1 ± 23%; $P < 0.001$) was inhibited significantly by 84.1% and 93.1% (5 and 25 μM, respectively; $P < 0.001$). Similarly, insulin stimulated TGS by 203.8 ± 9.6%, an effect that was attenuated by 56.6% and 62.8% with 5 and 25 μM BAPTA-AM, respectively ($P < 0.001$ compared with insulin alone).

The involvement of PLD in ASP signaling was evaluated using *n*-butanol, a primary alcohol, which serves as an artificial substrate for PLD, generating phosphatidylalcohol instead of phosphatidic acid (30). As the generated phosphatidylalcohol can no longer be converted to DAG by the enzyme phosphatidic acid phosphatase, *n*-butanol effectively prevents PLD-generated second messengers. In the absence of *n*-butanol, ASP-stimulated TGS was 265.1 ± 11.1% (where basal was set as 100%; $P < 0.0001$); however, the ASP effect was not inhibited at any *n*-butanol concentrations tested (Fig. 1D).

Participation of the PI3K/Akt pathway

The involvement of PI3K was analyzed using the two inhibitors wortmannin (cell-permeable, irreversible) and LY294002 (reversible). Both compounds inhibit the catalytic site of PI3K and have been used extensively to demonstrate the role of PI3K in insulin action on glucose transport (31, 32). As shown in **Fig. 2A**, wortmannin alone had no effect on basal TGS. The ASP stimulatory effect (258.3 ± 24.7% for ASP alone; $P < 0.0001$) was inhibited by 70% (down to 148.1 ± 6.7% for ASP + 100 nM wortmannin; $P < 0.0001$). Inhibition was evident at all concentrations tested. Wortmannin also inhibited up to 80% of the insulin-stimulated TGS (360.8 ± 20.1% for insulin alone vs. 156.1 ± 6.4% for insulin + 100 nM wortmannin; $P < 0.0001$).

Similarly, with LY294002 (Fig. 2B), ASP-stimulated TGS (187.4 ± 24.1%; $P < 0.001$) was reduced in a concentration-dependent manner down to basal TGS levels at 25 μM LY294002 ($P < 0.01$ vs. ASP alone). A similar effect was observed on insulin-stimulated TGS, although complete inhibition was already observed with 10 μM LY294002 ($P < 0.001$). A slight inhibition of basal TGS with increasing concentrations of LY294002 was negligible compared with those for ASP- and insulin-stimulated TGS.

Once activated, PI3K can stimulate the activation of 3–phosphoinositide-dependent protein kinase-1 (PDK-1) (which activates Akt), PKC, Ras, and others. Western blot analysis showed that PDK-1 phosphorylation on serine 241 (Ser241) from 0 to 60 min remains constant after ASP or insulin stimulation (data not shown). On the other hand, Akt, the immediate target of PDK-1, is rapidly phosphorylated on Ser473, an event necessary for activation. As shown in Fig. 2D, phosphorylation of Akt on Ser473 (Akt-P) by ASP is rapid, reaching its maximum between 5 and 10 min and diminishing to basal levels by 45 min. Insulin activation of Akt phosphorylation was as rapid (5 min), but Akt remained phosphorylated over the entire time course, with a slight decrease at 60 min (Fig. 2E). Furthermore, treatment of cells with the Akt inhibitor 1L-6-hydroxymethyl-*chiro*-inositol 2-[(*R*)-2-*O*-methyl-3-*O*]octadecylcarbonate (Fig. 2C) resulted in the complete elimination of ASP stimulation in a concentration-dependent manner ($P < 0.0001$). Basal TGS was only slightly affected at the highest concentration of inhibitor (23.8% inhibition at 10 μM; $P < 0.05$).

We have shown previously that PKC is implicated in ASP signaling and that phorbol 12-myristate 13-acetate (PMA), a known PKC activator, also stimulates TGS (25). PKC is regulated through phosphorylation of the newly synthesized PKC by PDK-1 (33) and activation by Ca^{2+} and lipids. 3T3-L1 preadipocytes were incubated with PMA (to stimulate TGS via PKC activation), and various inhibitors were tested. Although wortmannin and LY294002 inhibited both the ASP and insulin effects (Fig. 2A, B), the TGS stimulatory action of PMA was unaffected by LY294002 (**Table 1**; P = NS). Furthermore, PMA treatment did not induce Akt phosphorylation (data not shown), suggesting that PKC acts downstream of PI3K as a stimulator of TGS. On the other hand, intracellular $[Ca^{2+}]$ chelation with 25 μM BAPTA-AM decreased PMA stimulation of TGS from 145.5% to 105.3%, an inhibition of 88.5% (Table 1; $P < 0.005$).

Downstream effectors of PI3K/Akt

The mamalian target of rapaycin (mTOR) pathway, which involves the activation of p70 S6 kinase and 4E binding protein (gene transcription/RNA translation pathway), is a well-characterized downstream target of PI3K through Akt. We evaluated the mTOR pathway using two specific inhibitors, rapamycin (an inhibitor of mTOR) and bisindolylmaleimide V (a p70 S6 kinase inhibitor). Rapamycin (**Fig. 3A**) did not inhibit ASP TGS stimulatory activity at any concentration tested. Note that the PMA-induced TGS activity was also unaffected by mTOR inhibition (Table 1). By contrast, the insulin-mediated stimulation of TGS was significantly reduced in the presence of rapamycin, 76.8% and 84.9% at 5 and 50 nM rapamycin, respectively ($P < 0.001$). Bisindolylmaleimide V, which blocks the activation of p70 S6 kinase but not that of PKC, significantly inhibited basal TGS (Fig. 3B; $P < 0.001$). Although ASP stimulation of TGS was also reduced, the decreases paralleled those observed for basal TGS, and significant ASP stimulation remained at all concentrations. By contrast, insulin-stimulated TGS was sharply decreased, with

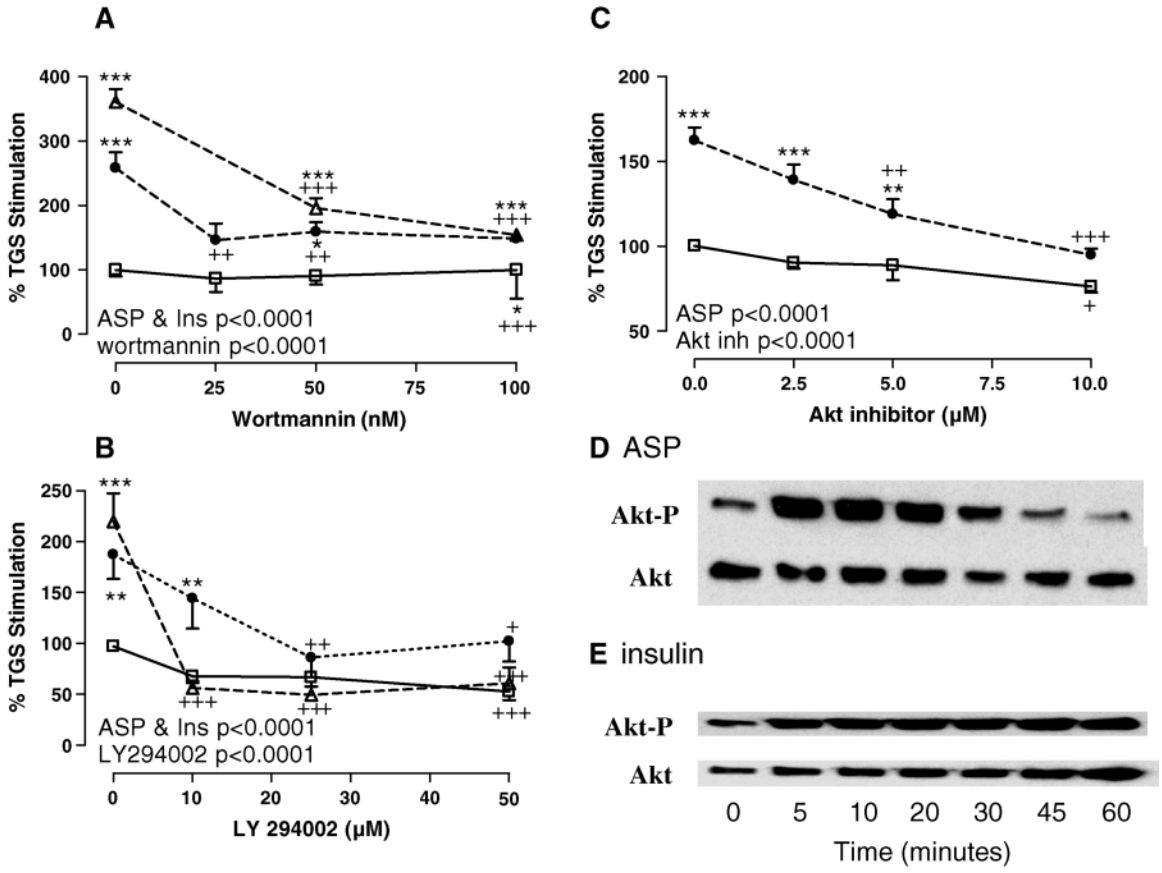

Fig. 2. Effect of phosphatidylinositol 3-kinase (PI3K) inhibition and involvement of Akt in ASP-stimulated TGS in 3T3-L1 preadipocytes. A–C: Confluent cells were pretreated with serum-free medium containing various concentrations of wortmannin (A), LY294002 (B), or Akt inhibitor (C) for 30 min and were then stimulated with 5 μM ASP (circles) or 100 nM insulin (Ins; triangles) for an additional 4 h. All inhibitors were present throughout the experiment. TGS was measured as picomoles of [^{3}H]oleate incorporated into TG. The results were normalized to basal TGS (squares) in each of the experiments performed (n = 11–17 values per point) and are presented as percentage TG stimulation (means ± SEM), where basal (no addition and no treatment) was set as 100%. The appropriate amount of vehicle was included in the medium for each of the inhibitors tested. The data were analyzed by two-way ANOVA (results shown in graphs) followed by the Bonferoni posthoc test: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for ASP or insulin stimulation versus control; $^{+}P < 0.05$, $^{++}P < 0.01$, and $^{+++}P < 0.001$ for inhibitor treatment versus no inhibitor. D, E: 3T3-L1 preadipocytes were incubated with ASP (D) and insulin (E) for 5–60 min in serum-free medium, cells were lysed, and total cell protein was subjected to Western blot analysis using antibodies to the phosphorylation site on serine 473 (Ser473) of Akt (Akt-P) and to total Akt.

no stimulation remaining at the highest concentrations tested ($P < 0.001$). In addition, Western blot analysis of glycogen synthase kinase (GSK)-3β phosphorylation at Ser9 showed no change for either ASP or insulin (data not shown), indicating that GSK-3β does not participate in the signaling pathway for TGS.

TABLE 1. Effect of inhibitors on PMA stimulation of TGS

		No Inhibitor			Inhibitor		
Inhibitor	Concentration	−PMA	+PMA	P^a	−PMA	+PMA	P^b
LY294002	50 μM	100 ± 3.6	163.9 ± 16.3	0.001	51.9 ± 7.9	134.4 ± 20.1	NS
BAPTA-AM	25 μM	100 ± 4.5	145.5 ± 19.5	0.006	65.4 ± 3.9	105.3 ± 13.0	0.05
Rapamycin	50 nM	100 ± 4.0	198.5 ± 17.2	0.0004	69.9 ± 4.4	203.1 ± 12.3	NS
Bisindolylmaleimide V	25 μM	100 ± 5.5	139.6 ± 10.7	0.02	28.9 ± 4.4	111.4 ± 11.4	NS
PD98059	25 μM	100 ± 3.5	289.3 ± 52.5	0.001	87.3 ± 9.4	160.3 ± 32.9	0.002

BAPTA-AM, 1,2-bis(*o*-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid tetra(acetoxymethyl) ester; PMA, phorbol 13-myristate 12-acetate; TGS, triglyceride synthesis. Confluent cells were pretreated with serum-free medium containing the indicated inhibitors at the indicated concentrations for 30 min and then stimulated with 20 nM PMA for an additional 4 h. All inhibitors were present throughout the experiment. TGS was measured as picomoles of [^{3}H]oleate incorporated into TG. The results were normalized to basal TGS in each of the experiments performed and are presented as percentage TGS stimulation, where basal (no addition and no treatment) was set as 100%. The appropriate amount of vehicle was included in the medium for each of the inhibitors tested. The data were analyzed using two-way ANOVA followed by the Bonferoni posthoc test.

[a] For PMA versus without PMA (no inhibitor present).

[b] For PMA = inhibitor versus PMA and no inhibitor.

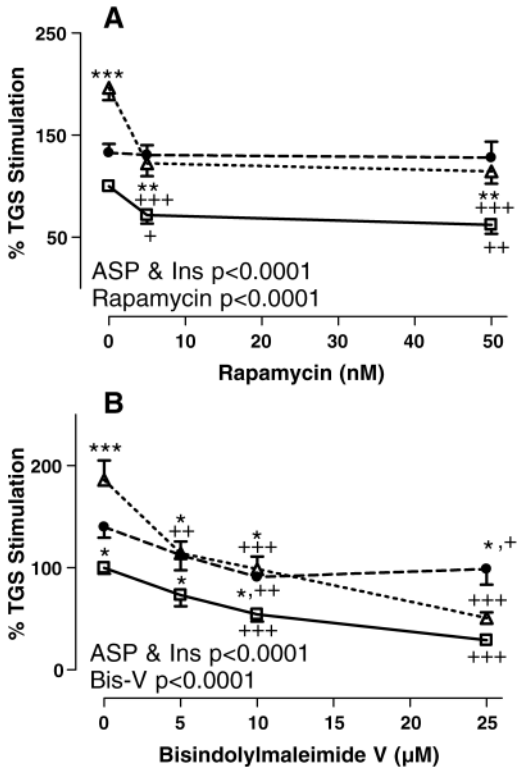

Fig. 3. Analysis of mamalian target of rapaycin (mTOR) and p70 S6 kinase involvement in ASP-stimulated TGS in 3T3-L1 preadipocytes. Confluent cells were pretreated with serum-free medium containing various concentrations of rapamycin, an mTOR inhibitor (A), or bisindolylmaleimide V (Bis-V), a p70 S6 kinase inhibitor (B), for 30 min and were then stimulated with 5 μM ASP (circles) or 100 nM insulin (Ins; triangles) for an additional 4 h. All inhibitors were present throughout the experiment. TGS was measured as picomoles of [^{3}H]oleate incorporated into TG. The results were normalized to basal TGS (squares) in each of the experiments performed (n = 9 values per point) and are presented as percentage TGS stimulation (means ± SEM), where basal (no addition and no treatment) was set as 100%. The appropriate amount of vehicle was included in the medium for each of the inhibitors tested. The data were analyzed by two-way ANOVA (results shown in graphs) followed by the Bonferoni posthoc test: $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ for ASP or insulin stimulation versus control; $^{+}P < 0.05$, $^{++}P < 0.01$, and $^{+++}P < 0.001$ for inhibitor treatment versus no inhibitor.

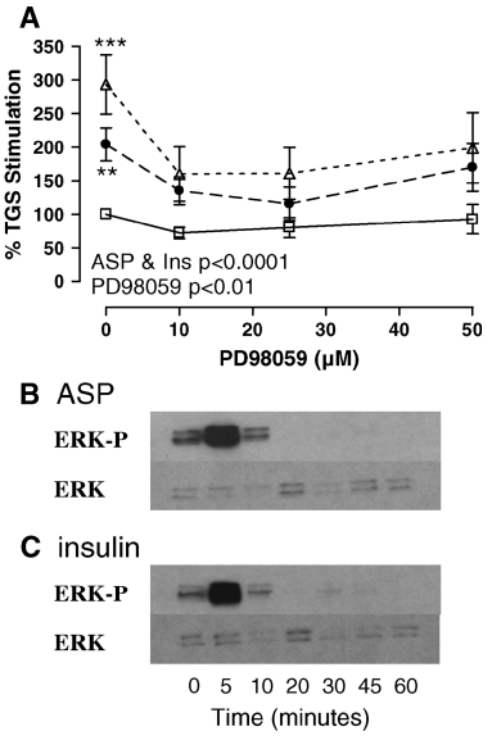

Fig. 4. Involvement of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK$_{1/2}$) pathway in ASP-stimulated TGS in 3T3-L1 preadipocytes. A: Confluent cells were pretreated with serum-free medium containing PD098059, a MAPK/ERK$_{1/2}$ inhibitor specific to mitogen-activated protein/extracellular signal-regulated kinase (MEK), for 30 min and were then stimulated with 5 μM ASP (circles) or 100 nM insulin (Ins; triangles) for an additional 4 h. All inhibitors were present throughout the experiment. TGS was measured as picomoles of [^{3}H]oleate incorporated into TG. The results were normalized to basal TGS (squares) in each of the experiments performed (n = 9 values per point) and are presented as percentage TGS stimulation (means ± SEM), where basal (no addition and no treatment) was set as 100%. The appropriate amount of vehicle was included in the medium for each of the inhibitors tested. The data were analyzed by two-way ANOVA (results shown in graph) followed by the Bonferoni posthoc test: $^{**}P < 0.01$ and $^{***}P < 0.001$ for ASP or insulin stimulation versus control. B, C: 3T3-L1 preadipocytes were incubated with ASP (B) and insulin (C) for 5–60 min in serum-free medium and lysed, and total cell protein was subjected to Western blot analysis using antibodies to the phosphorylation site on threonine 202/tyrosine 204 of ERK (ERK-P) and to total ERK.

MAPK pathway

Both PI3K and PKC activation can lead to the activation of MAPK/ERK$_{1/2}$ (cellular proliferation/differentiation pathway) (34, 35). As shown in **Fig. 4A**, using a MAPK/ERK$_{1/2}$ inhibitor, PD098059, basal TGS was unaffected, but significant inhibition of both ASP- and insulin-stimulated TGS was observed at all concentrations ($P <$ 0.001 for both ASP and insulin), with no significant stimulation remaining. Interestingly, PMA (Table 1) stimulation of TGS was also decreased (289.3 ± 57.5% for PMA alone vs. 160.3 ± 36.1% for PMA + 25 μM PD98059, 68.1% inhibition; $P < 0.002$). After ASP stimulation, ERK$_{1/2}$ was rapidly phosphorylated by 10 min (Fig. 4B), an effect also seen with insulin (Fig. 4C).

Involvement of PLA$_2$ in ASP signaling

G-protein activation as well as MAPK/ERK$_{1/2}$ activation can lead to the activation of PLA$_2$, producing arachidonic acid and lysophospholipid. Isotetrandrine, an inhibitor of G-protein-coupled PLA$_2$ (36), had no effect on the stimulatory effect of ASP (**Fig. 5A**), which remained significantly increased ($P < 0.001$). Basal TGS levels were unaffected at any concentration of isotetrandrine.

The involvement of calcium-dependent phospholipase A$_2$ (cPLA$_2$) was evaluated using the reversible inhibitor AACOCF$_3$ (37). Increasing concentrations of AACOCF$_3$ significantly attenuated ASP- and insulin-stimulated TGS (Fig. 5B). Although basal TGS was slightly inhibited, the

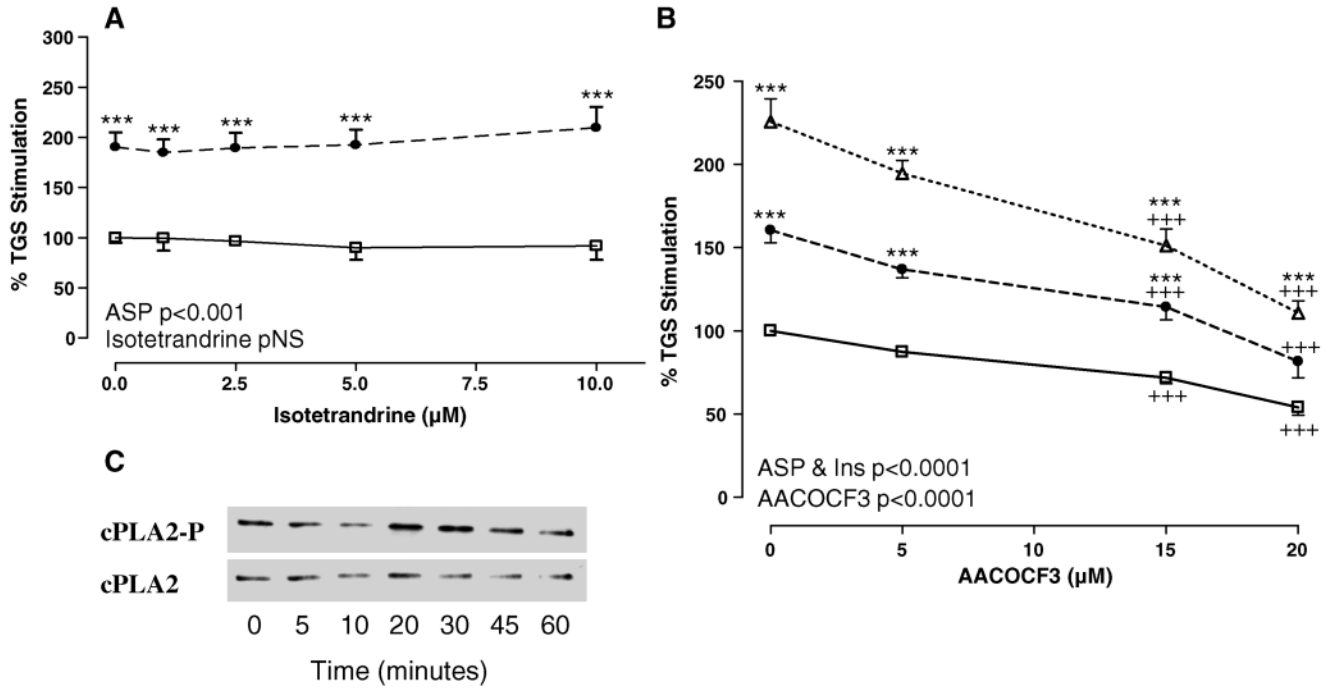

Fig. 5. Involvement of PLA_2 in ASP stimulation of TGS in 3T3-L1 preadipocytes. A, B: Confluent cells were pretreated with serum-free medium containing 1–10 μM isotetrandrine (A) or 5–20 μM arachidonyltrifluoromethyl ketone (AACOCF3; B) for 30 min, and TGS was then stimulated with 5 μM ASP (circles) or 100 nM insulin (Ins; triangles) for an additional 4 h. TGS was measured as picomoles of [^{3}H]oleate incorporated into TG. The results were normalized to basal TGS (squares) in each of the experiments performed, where basal (no addition and no treatment) was set as 100% (average of 12 values per point), and the results are presented as means ± SEM. The data were analyzed by two-way ANOVA (results shown in graphs) followed by the Bonferoni posthoc test: $^{***}P < 0.001$ for ASP or insulin stimulation versus control; $^{+++}P < 0.001$ for inhibitor treatment versus no inhibitor. C: 3T3-L1 preadipocytes were incubated with ASP for 5–60 min in serum-free medium and lysed, and total cell protein was subjected to Western blot analysis using antibodies to the phosphorylation site on Ser505 of calcium-dependent phospholipase A_2 (cPLA$_2$-P) and to total cPLA$_2$.

inhibition by $AACOCF_3$ was more pronounced on ASP- and insulin-stimulated TGS, with complete inhibition of the ASP effect and 91.5% inhibition of the insulin effect. Using Western blot analysis with anti-cPLA$_2$ to Ser505 (a site phosphorylated by MAPK/ERK$_{1/2}$ enzymes), a time-dependent phosphorylation of cPLA$_2$ was demonstrated, reaching a maximal effect at 30 min. Arachidonic acid, the product of cPLA$_2$ activity, can act as a signaling molecule directly or can be used as a precursor for the production of prostaglandins. A general cyclooxygenase inhibitor (indomethacin) was used to examine this possibility. ASP and insulin stimulated TGS by 209.4 ± 17% and 197.6 ± 18% ($P < 0.0001$), respectively, with no significant inhibition of TGS with any of the indomethacin concentrations tested (data not shown).

DISCUSSION

Based on the present study and the findings of Baldo et al. (25), a scenario for the ASP intracellular signaling pathway is proposed in **Fig. 6**. The ASP receptor, C5L2, belongs to a large family of seven transmembrane, G-protein-coupled receptors that includes C3aR, C5aR (CD88), and the fMLP receptor (38). Both the C3aR and C5aR signaling pathways are mediated through Gα_i and are PTX-sensitive (39, 40). fMLP and C5a binding to their respective receptors results in the activation of PLCβ2 by the Gβ/γ subunit (38, 41). With respect to ASP and the C5L2 receptor, we have previously demonstrated that Gα_s was not involved, nor was PKA activated by ASP in TGS (25). In this study, we have also excluded Gα_i, in agreement with recent binding data showing that C5L2 couples poorly to Gα_i (42), differentiating the ASP activation of C5L2 from that of receptors in the same family: C3a on C3aR and C5a on C5aR activation. This suggests a possible link of ASP and C5L2 via Gα_q or G$\alpha_{12/13}$. After ASP-C5L2 receptor activation, the Gα subunit dissociates from the Gβ/γ subunit, which leads to PLC and PI3K activation.

PLC activation (which in this case could occur through the Gα_q or Gβ/γ subunit) (43) results in the release of inositol 1,4,5-triphosphate and DAG. Inositol 1,4,5-triphosphate stimulates the release of intracellular Ca^{2+} from the endoplasmic reticulum, which further serves as a signaling molecule, activating a number of intracellular enzymes, including PKC, PI3K, Akt, and cPLA$_2$ (44–46). All of these enzymes are involved in the ASP pathway (as discussed below), explaining the effective inhibition by Ca^{2+} chelation of the ASP signaling pathway.

The fact that inhibition of the ASP effect was observed at low wortmannin concentrations suggests that class I (class IA and IB) PI3K is involved in ASP TGS stimulatory action

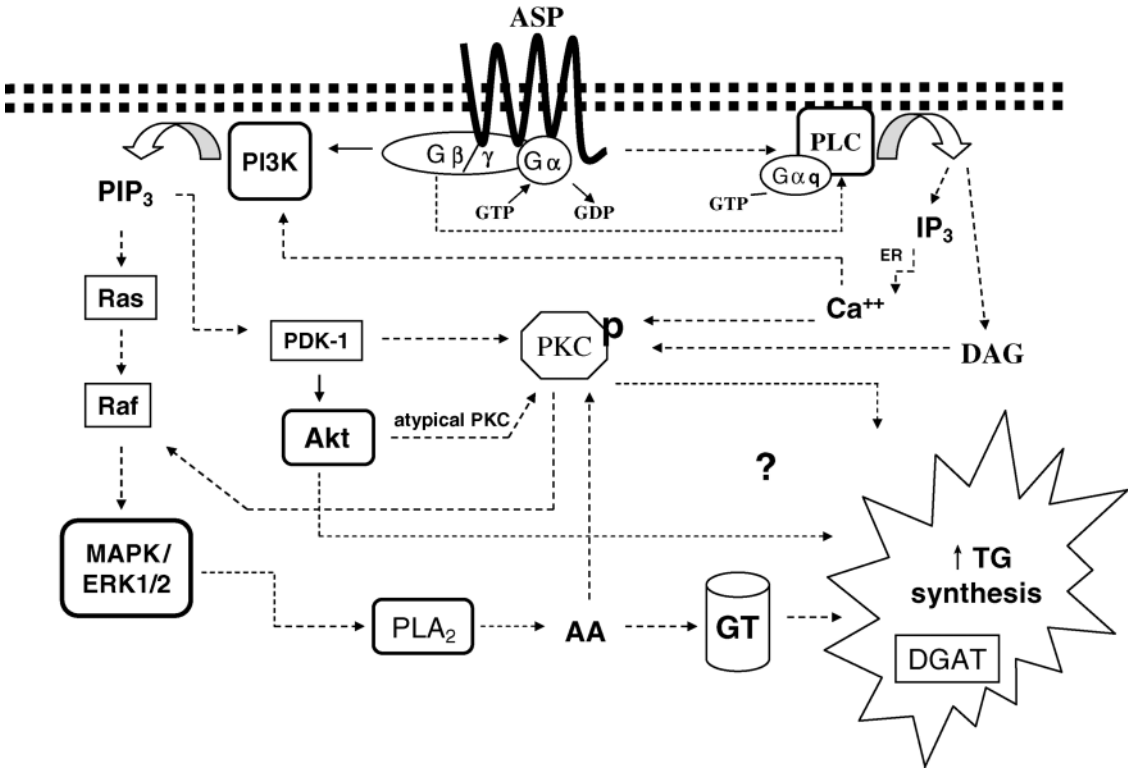

Fig. 6. Proposed intracellular signaling pathway for ASP-stimulated TGS. AA, arachidonic acid; C5L2, ASP receptor; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; ER, endoplasmic reticulum; GT, glucose transport; IP_3, inositol 1,4,5-triphosphate; PDK-1, 3-phosphoinositide-dependent protein kinase-1; PIP_2, phosphatidylinositol 4,5-biphosphate; PKC, protein kinase C.

(47). Interestingly, the class IB PI3K, p110γ, is activated via the G-protein-coupled receptors, specifically by the Gβ/γ subunit (48, 49), which leads to PDK-1 activation and, in turn, phosphorylates PKC, releasing it into the cytoplasmic compartment (33).

DAG is a known activator of conventional and novel PKC isoforms. We demonstrated previously that ASP treatment leads to rapid transient increases in DAG (10–20 min) as well as a second prolonged increase in intracellular DAG (25). The acute increase in DAG (within minutes) is associated with increased total intracellular PKC activity and PKC translocation to the membrane fraction (25). Although PLD activation by PKC (50) has been shown to produce a prolonged increase in intracellular DAG, we have ruled out the involvement of PLD in this study.

Both PKC and PI3K activation lead to $MAPK/ERK_{1/2}$ phosphorylation (34, 35). Although PI3K and PLC are proposed to be activated in parallel, activation of both appears to be necessary, because blockage of one or the other results in the inhibition of ASP-stimulated TGS. We propose that after ASP activation, the PI3K pathway as well as the PKC pathway converge with the $MAPK/ERK_{1/2}$ pathway. $cPLA_2$ is activated by increases in intracellular $[Ca^{2+}]$, enhancing membrane translocation, which stimulates MAPK phosphorylation of $cPLA_2$ (51–53). Thus, ASP signaling involves the rapid and transient phosphorylation of $ERK_{1/2}$ at 10 min, which then leads to $cPLA_2$ phosphorylation (maximal effects at 30 min). Interestingly, the results obtained here with $AACOCF_3$ and the pattern of $cPLA_2$ phosphorylation are very similar to those observed for angiotensin II, a hormone that requires PLC activation (54) and has been shown to stimulate TGS (55, 56). Intracellular arachidonic acid can then enhance PKC activity (57) as well as stimulate the translocation of appropriate glucose transporters (58) and by so doing increase glucose transport into the cell.

This study also provides important insight into the regulation of TGS by insulin. Ca^{2+}, PI3K, Akt, mTOR, and $MAPK/ERK_{1/2}$ were all shown to be implicated in the insulin stimulation of TGS. Inhibition of insulin-stimulated glucose uptake using intracellular $[Ca^{2+}]$ chelators has been reported previously in 3T3-L1 adipocytes (59–61). In contrast, this study is the first demonstration that intracellular $[Ca^{2+}]$ chelation inhibits insulin-stimulated TGS in 3T3-L1 preadipocytes. Although there are a number of similarities in the ASP and insulin signaling pathways, there are a number of differences as well. PLC is involved in ASP signaling but is not involved in insulin stimulation of TGS or, as shown previously, in glucose transport (62, 63). Furthermore, although the mTOR pathway is important for insulin-mediated TGS, it is not involved in ASP signaling. These differences in ASP and insulin implicate a divergence of signaling pathways for ASP and insulin.

Downstream of this Akt branch point, we can only speculate how PLC, PI3K, PKC, $MAPK/ERK_{1/2}$, and $cPLA_2$ activation lead to increased TGS. We have demonstrated previously that ASP increases glucose transport as well as fatty acid esterification onto the glycerol backbone

through the activation of DGAT (an enzyme that is specific to TGS) (18, 64) but not phospholipid synthesis (24). ASP effects on TGS and glucose transport appear to be independent because *i*) ASP stimulates glucose transport in the absence of TGS, and *ii*) glucose is not absolutely required for ASP stimulation of TGS, because provision of pyruvate or acetate in the medium can be used for the glycerol-3-phosphate backbone (24). However, both effects are required for maximal ASP stimulation of TGS. With respect to glucose transport, it has been demonstrated that PI3K and PKC activation lead to the translocation of glucose transporters, and ASP likely acts via this pathway (62, 65). Two DGAT genes have been cloned recently (DGAT1 and DGAT2) (64, 66), but there is little information on their regulation. Based on circumstantial evidence and similarities with other endoplasmic reticulum-associated anabolic enzymes (acyl-cholesterol-acyltransferase and glycerol-3-phosphate-acyltransferase) (66, 67), it is reasonable to assume that this enzyme might be activated through dephosphorylation. In this case, downstream of PKC, we postulate the activation of a phosphatase enzyme, leading to the activation of DGAT, although that intermediate step remains to be identified. Altogether, we have demonstrated that the ASP signaling pathway involves PI3K, PLC, PKC, MAPK/ERK$_{1/2}$, and cPLA$_2$. Together, these interact to stimulate TGS in a manner independent of that of insulin.

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