Insulin regulates soluble amyloid precursor protein release via phosphatidyl inositol 3 kinase-dependent pathway

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ABSTRACT **Several lines of biochemical evidence correlate the presence of energy metabolic defects with the functional alterations associated with brain aging and with the pathogenesis of neurodegenerative disorders such as Alzheimer's disease. Within this context we tested the ability of insulin to regulate the amyloid precursor protein (APP) processing in SH-SY5Y neuroblastoma cells. Our findings show that insulin promotes APP metabolism by a glucose-independent mechanism. We demonstrate a novel intracellular pathway that increases the rate of secretion of soluble APP through the activity of phosphatidyl-inositol 3 kinase (PI3-K). This pathway, downstream of insulin receptor tyrosine kinase activity, does not involve either the activation of protein kinase C or the mitogen-activated protein kinase (MAP-K) pathway. Because of the physiological role of PI3-K in the translocation of glucose transporter-containing vesicles, we speculate that PI3-K involvement in APP metabolism may act at the level of vesicular trafficking.—Solano, D. C., Sironi, M., Bonfini, C., Solerte, S. B., Govoni, S., Racchi, M. Insulin regulates soluble amyloid precursor protein release via phosphatidyl inositol 3 kinase-dependent pathway. *FASEB J.* 14, 1015–1022 (2000)**

Key Words: signal transduction · PI3 kinase · Alzheimer's disease · α secretase

THE AMYLOID PLAQUES found in the brains of patients with Alzheimer's disease (AD) are mainly composed of β-amyloid (Aβ), a peptide derived from a larger molecule that is known, in its several isoforms, as the amyloid precursor protein (APP). APP is normally cleaved within its extracellular domain by a protease known as 'α-secretase', and the processing releases a large, soluble APP fragment (sAPPα) into the extracellular space (1). Because cleavage occurs within the Aβ domain, α secretase processing does not generate Aβ (2, 3). A number of alternative metabolic pathways have also been described that have the potential to generate intact Aβ fragments (for review, see refs 4, 5). Because sAPPα and Aβ seem to be formed by two mutually exclusive mechanisms, stimulation of secretory processing of APP might prevent the formation of Aβ and its accumulation into amyloid plaques. Although other evidence indicates that in some cell types the release of sAPPα and Aβ can proceed independently of each other (6), the ability of sAPPα to protect neurons against cytotoxic insults (reviewed in ref 7) provides an additional basis for the hypothesis that the sustained release of sAPPα is important for neuronal viability.

Several intracellular signaling cascades are involved in the regulation of APP processing (4, 5). Protein kinase C (PKC) is involved in the pathway that leads to the nonamyloidogenic processing of APP; in addition, it has been suggested that other PKC-independent, Ca^{2+}-dependent processes are also involved in the activation of the α secretase pathway (8, 9). Neurotransmitters, hormones, or cytokines, as well as other neuroactive compounds that activate PKC and other transduction signals, increase secretion of sAPPα via the nonamyloidogenic pathway (4, 5). Many plasma membrane receptors regulate cellular processes through protein tyrosine kinase. Ligands of receptors with intrinsic tyrosine kinase activity, including nerve growth factor (NGF) and epidermal growth factor (EGF), also increase sAPPα secretion (5). In this context, we investigated the role of insulin in the modulation of the secretory processing of APP.

The study of the effect of insulin on APP processing is also suggested by several other considerations detailed below. Several lines of biochemical evidence suggest a link between glucose utilization and energy metabolic defects to the functional alterations associated with brain aging and with the pathogenesis of

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neurodegenerative disorders such as AD. Disturbed brain energy metabolism is a prominent feature of AD brains (10–13). In fact, impaired glucose metabolism has been observed in parietal, temporal, and frontal cortex of AD patients (14, 15). Inhibition of energy metabolism can alter APP processing and induce amyloidogenic products (16, 17). Markers of altered energy metabolism in AD patients also seem to be present peripherally (reviewed in ref 18) and include alteration of glucose metabolism, glutamine oxidation, and Ca^{2+} homeostasis in fibroblasts (19, 20) as well as decreased cytochrome *c* oxidase activity in platelets (21, 22) and fibroblasts (23). Furthermore, fibroblasts from AD patients are more sensitive to glucose deprivation and energy depletion compared to control cells (23, 24). Thus the depletion of metabolic energy, tightly linked to glucose, may significantly alter the metabolism of a key protein in AD.

We therefore tested the ability of insulin to regulate APP processing in SH-SY5Y neuroblastoma, a cellular model that expresses insulin receptors endogenously. Our findings suggest that insulin modulates APP secretion by a tyrosine kinase-dependent and glucose-independent mechanism. We also show that within the insulin signaling pathway, the activity of phosphatidyl inositol 3 kinase (PI3-K) is necessary for insulin-mediated sAPPα release.

EXPERIMENTAL PROCEDURES

Materials

All culture media, supplements, and fetal calf serum (FCS) were obtained from Life Technologies, Inc. (Paisley, Scotland, U.K.). Electrophoresis reagents were obtained from Bio-Rad (Hercules, Calif.). Recombinant human insulin (Humalog) was from Eli Lilly Corp. (Indianapolis, Ind.). All other reagents were of the highest grade available and were purchased from Sigma Chemical Co. (St. Louis, Mo.). PMA, GF109203X, genistein, PD98059, rapamycin, wortmannin, and LY294002 were dissolved in dimethyl sulfoxide and stored at −20°C. Stocks were diluted in serum-free medium prior to experiments.

Cell culture and experimental treatments

SH-SY5Y neuroblastoma cells were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% FCS, penicillin/streptomycin, nonessential amino acids, and sodium pyruvate (1 mM) at 37°C in 5% CO_2/95% air. For the experiments, 4×10^6 cells were seeded on 60 mm dishes and cultured for 48 h. The cells were exposed to serum-free MEM for 24 h, then experimental treatments were performed in serum-free MEM with incubation for 2 h at 37°C. For the experiments without glucose or with 2-deoxy-D-glucose (2DG), cells were incubated for 2 h at 37°C in MEM without glucose and pyruvate with the addition of glucose or 2DG, as indicated in Results and in the figure legends.

Harvesting the cells and preparation of conditioned medium

Proteins in conditioned medium were quantitatively precipitated by the deoxycholate/trichloroacetic acid procedure as described previously (25). Cell monolayers were washed twice with ice-cold phosphate-buffered saline and lysed on the tissue culture dish by addition of ice-cold lysis buffer (50 mM Tris/HCl, pH=7.5, 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100). An aliquot of the cell lysate was used for protein analysis with the Bio-Rad Bradford kit for protein quantification.

Immunodetection of sAPPα

Normalization of protein loading on each blot was obtained by loading a volume of sample of conditioned medium standardized to the protein concentration in the total cell lysate. Proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%) and transferred onto PVDF membrane (DuPont NEN, Boston, Mass.). The membrane was blocked for 1 h with 10% non-fat dry milk in Tris-buffered saline containing 1% Tween 20. Membranes were immunoblotted with the antibodies 22C11 (Boehringer Mannheim, Mannheim, Germany) or 6E10 (Senetek, St. Louis Mo.). The detection was carried out by incubation with alkaline phosphatase-conjugated goat anti-mouse immunoclobulin G (IgG) (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) for 1 h. The blots were then washed extensively and sAPPα was visualized using an enhanced chemiluminescent method (DuPont NEN, Boston, Mass.).

Quantitative densitometry and statistics

Quantitative analysis of Western blots was performed by calculating the relative density of the immunoreactive bands after acquisition of the blot image with a Nikon CCD video camera module and analysis with the Image 1.47 program (Wayne Rasband, NIH, Research service Branch, NIMH, Bethesda, Md.). Statistical analyses were done by one-way ANOVA, followed by a two-tailed student's *t* test or multiple comparison test where appropriate; a value of $P<0.05$ was considered significant.

RESULTS

Insulin stimulates sAPPα release

Treatment of SH-SY5Y cells for 2 h with increasing concentrations of human recombinant insulin (Humalog) resulted in a concentration-dependent increase in sAPPα release into the conditioned media of the cells. The maximal effect was obtained at the concentration of 1 μM insulin (**Fig. 1*A***), which resulted in an ~2.5-fold increase of sAPPα over the basal level (Fig. 1*B*). Western blots show that sAPPα released into the conditioned medium migrates as a doublet with an apparent molecular mass of 100–120 kDa (Fig. 1*A*). This protein pattern is consistent with previous reports using the same cellular model (26, 27) and likely reflects the expression of the major isoforms of APP (M. Racchi, unpublished data). Since the same immunoreactive band was detected

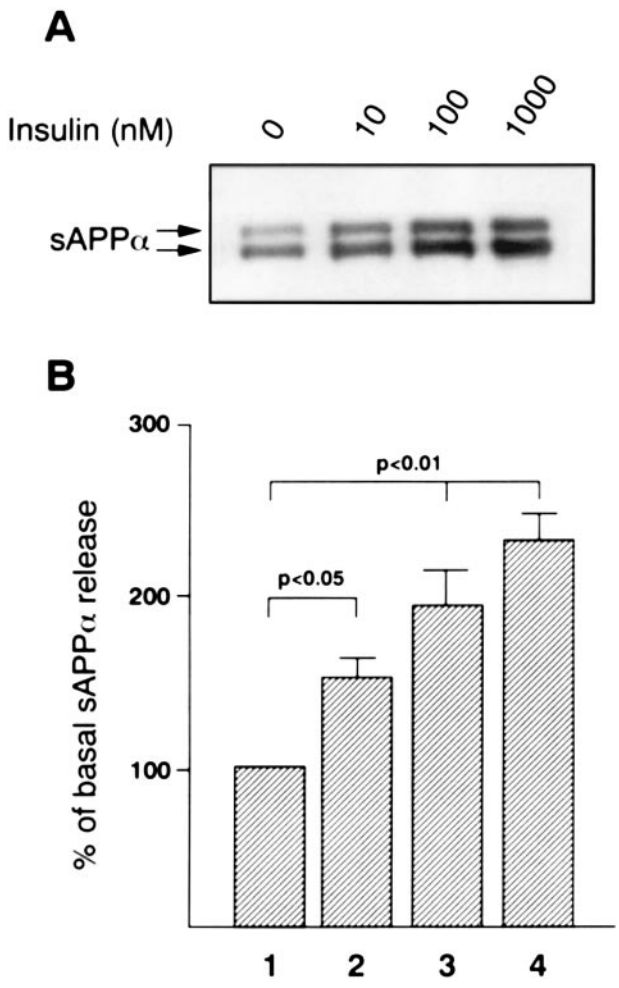

Figure 1. Release of sAPPα is stimulated by insulin. *A)* SH-SY5Y cells expressing insulin receptors endogenously were deprived of serum for 24 h. The next day cells were incubated for 2 h with serum-free MEM alone (lane 1) or increasing concentrations of insulin (10 nM, 100 nM, and 1 μM) (lanes 2, 3, and 4 respectively) in serum-free MEM. Proteins released into the conditioned media were collected and subjected to Western blot analysis for sAPPα. *B)* Densitometric analysis of Western blots expressed as percent of basal release and as mean ± SE of three independent experiments.

by the antibodies 22C11 and 6E10 and the latter antibody recognizes epitopes in the first 16 amino acids of Aβ (which also constitutes the carboxyl terminus of APP cleaved by α-secretase), the identified bands can be assumed to be authentic sAPPα.

Insulin-induced sAPPα release is independent of glucose

The effect of insulin on sAPPα release is independent of the presence of glucose. As previously shown in COS cells and fibroblasts from AD patients (17, 24), basal sAPPα release is decreased in conditions of glucose deprivation (~30% below control basal levels) and even more so in the presence of 2DG (~50%). Treatment of SH-SY5Y cells with 1 μM insulin in medium without glucose showed an increase of sAPPα release relative to basal levels, similar to that obtained in glucose-containing (50 mM) medium (**Fig. 2**). The presence of 2-DG similarly did not block the relative ability of insulin to stimulate sAPPα release (Fig. 2).

Tyrosine kinase activity is necessary for insulin-induced sAPPα secretion

Cells were treated for 2 h with 1 μM insulin in the presence or absence of 20 μM genistein. Western blot analysis showed that genistein completely

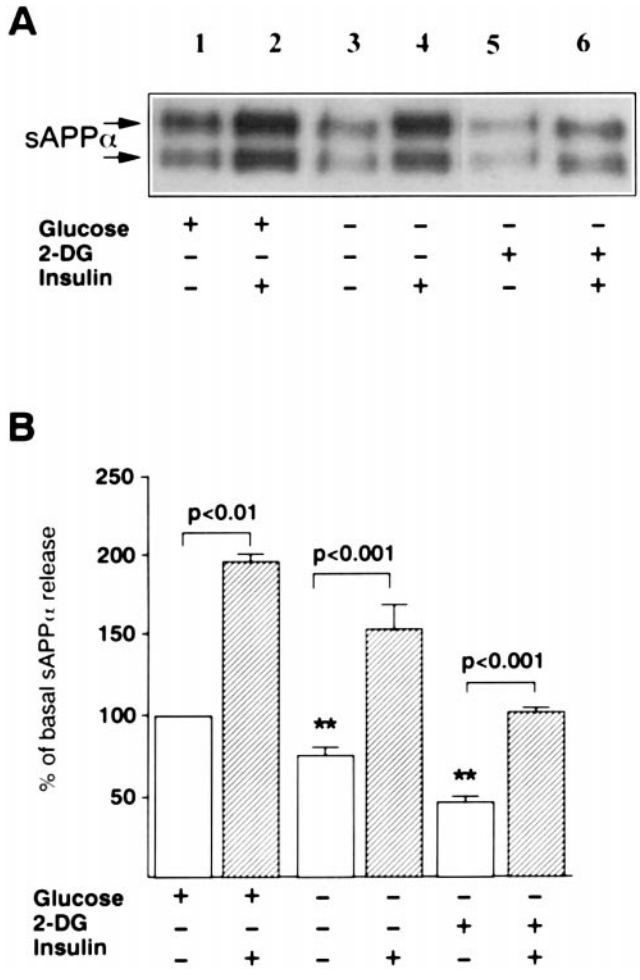

Figure 2. Insulin-induced sAPPα release is not dependent on glucose. *A)* SH-SY5Y cells pretreated as in Fig. 1 were then treated for 2 h with 50 mM glucose in serum-free MEM in the absence (lane 1) or presence of 1 μM insulin (lane 2). A second set of cells was treated for 2 h in serum-free MEM without glucose in the absence (lane 3) or presence of 1 μM insulin (lane 4). Finally, a third set of cells was treated for 2 h without glucose in serum-free MEM, but with the addition of 50 mM 2DG (lane 5) or 1 μM insulin (lane 6). Proteins released into the conditioned media were collected, precipitated, and subjected to Western blot analysis. *B)* Results of densitometric analysis are expressed as percent of basal release and as a mean ± SE of three independent experiments.

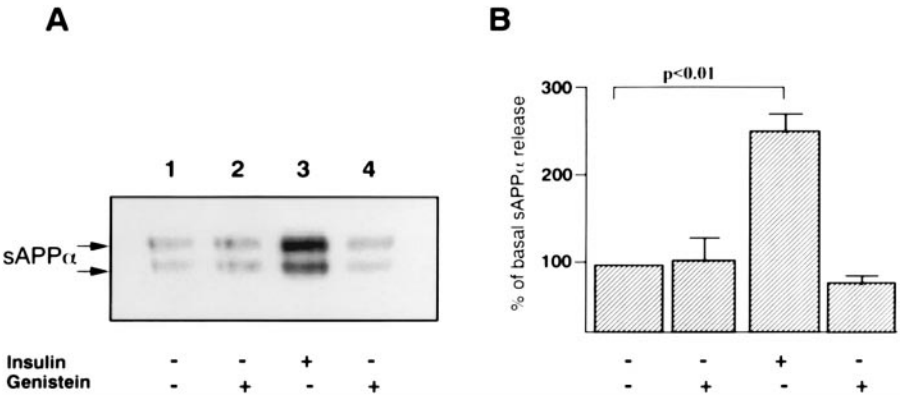

Figure 3. Genistein inhibits insulin-mediated sAPPα release. *A)* SH-SY5Y cells as in Fig. 1 were treated for 2 h at 37°C with serum-free MEM (lane 1), with 20 μM genistein (lane 2), with insulin 1 μM (lane 3), or with 1 μM insulin plus 20 μM genistein (lane 4). Proteins released into the conditioned media were collected and subjected to Western blot analysis. *B)* Densitometric analysis of Western blots expressed as percent of basal release and as means ± SE of three independent experiments.

blocked the effect of insulin, returning the level of sAPPα release to basal levels, whereas the constitutive sAPPα release of SH-SY5Y cells was not affected by the inhibitor (**Fig. 3**).

Insulin-induced sAPPα release is independent from PKC activation

We tested the possibility that insulin regulation of the release of sAPPα was secondary to activation of PKC by using the selective PKC inhibitor GF-109203X (28). The cells were incubated for 2 h with 200 nM PdBu in the presence or absence of 2.5 μM GF-109203X. Treatment of SH-SY5Y cells with PdBu elicited an increase of sAPPα release of approximately twofold over basal levels. As expected, simultaneous treatment with GF-109203X abolished the response to phorbol ester (**Fig. 4**). As previously shown, 1 μM insulin increased the release of sAPPα whereas addition of GF-109203X did not inhibit the effect of insulin.

Insulin-induced sAPPα release is independent of MAP kinase activation

Activation of tyrosine kinase receptors can activate MAP Kinase through a Ras/Raf/MAP-K kinase (ERK) pathway. Cells were treated with 1 μM insulin as indicated before, but with the simultaneous addition of 30 μM PD98059, a MEK inhibitor. This concentration was chosen on the basis of previously published data (29–31). As shown in **Fig. 5**, inhibition of MEK by PD-98059 did not block the secretory effect of insulin, suggesting that the MAP-K pathway was not involved. Treatment of the cells with PD98059 in the absence of insulin did not inhibit constitutive release of sAPPα (Fig. 5).

Insulin-mediated sAPPα release is dependent on PI3-K activation

Insulin receptor phosphorylation results in the activation of PI3-K. **Figure 6** shows that treatment of the cells with 1 μM insulin and 10 μM wortmannin, a specific inhibitor of PI3-K (32), blocked the insulin-induced increase of sAPPα release. To confirm the result, we also used the compound LY-294002, another selective inhibitor of PI3-K (33). We observed

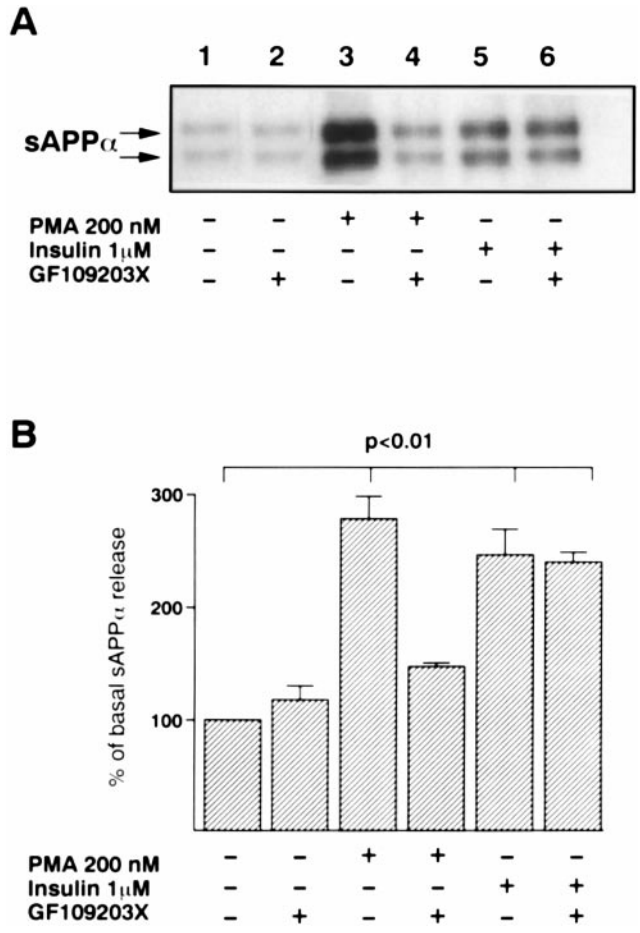

Figure 4. The effect of insulin on APP metabolism is not dependent on PKC activity. *A)* SH-SY5Y cells preincubated overnight with serum-free MEM were then treated for 2 h at 37°C as follows: with serum-free MEM (lanes 1); with 2.5 μM GF109203X (lanes 2); with 200 nM PMA (lanes 3); with 200 nM PMA and 2.5 μM GF109203X (lanes 4); with 1 μM insulin (lanes 5); or with 1 μM insulin and 2.5 μM GF109203X (lanes 6). Proteins released into the conditioned media were collected, precipitated, and subjected to Western blot analysis. *B)* Results are expressed as percent of basal release and as means ± SE of three independent experiments.

that 10 μM LY-294002 blocked the release of sAPPα induced by insulin (Fig. 6). Neither wortmannin nor LY-294002 was able to affect constitutive release of sAPPα (data not shown).

Insulin-mediated sAPPα release is independent from p70^{s6K} activity

PI3-K and subsequent activation of the PKB/PDK system are involved in the activation of p70^{S6K}. There is evidence that the phosphorylation of this kinase plays a role in the regulation of protein synthesis (34, 35). We tested whether this branch of the insulin signaling pathway was involved in the regulation of sAPPα release. Cells were incubated with 1 μM insulin and in the absence or presence of 20 ng/ml rapamycin, a macrolide that inhibits the activation pathway of p70^{s6K} downstream of PI3-K. After treatment, Western blot and densitometric analysis demonstrated that rapamycin did not interfere with insulin-induced sAPPα release, thus ruling out the involvement of p70^{s6K} (**Fig. 7**).

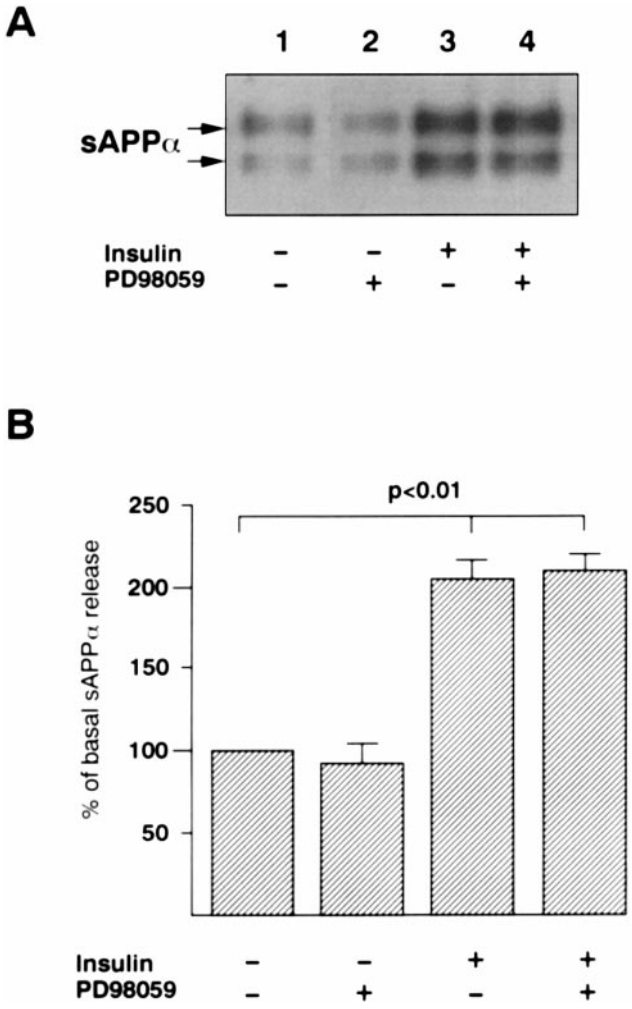

Figure 5. The effect of insulin on APP metabolism is not dependent on MAP-K activity. *A)* SH-SY5Y cells preincubated overnight with serum-free MEM were incubated at 37°C for 2 h with serum-free MEM (lane 1), with 30 μM PD98059 (lane 2), with 1 μM insulin (lane 3), or with 1 μM insulin and 30 μM PD98059 (lane 4). Proteins released into the conditioned media were collected and subjected to Western blot analysis. *B)* Densitometric analysis of Western blots expressed as percent of basal release ± SE of three independent experiments.

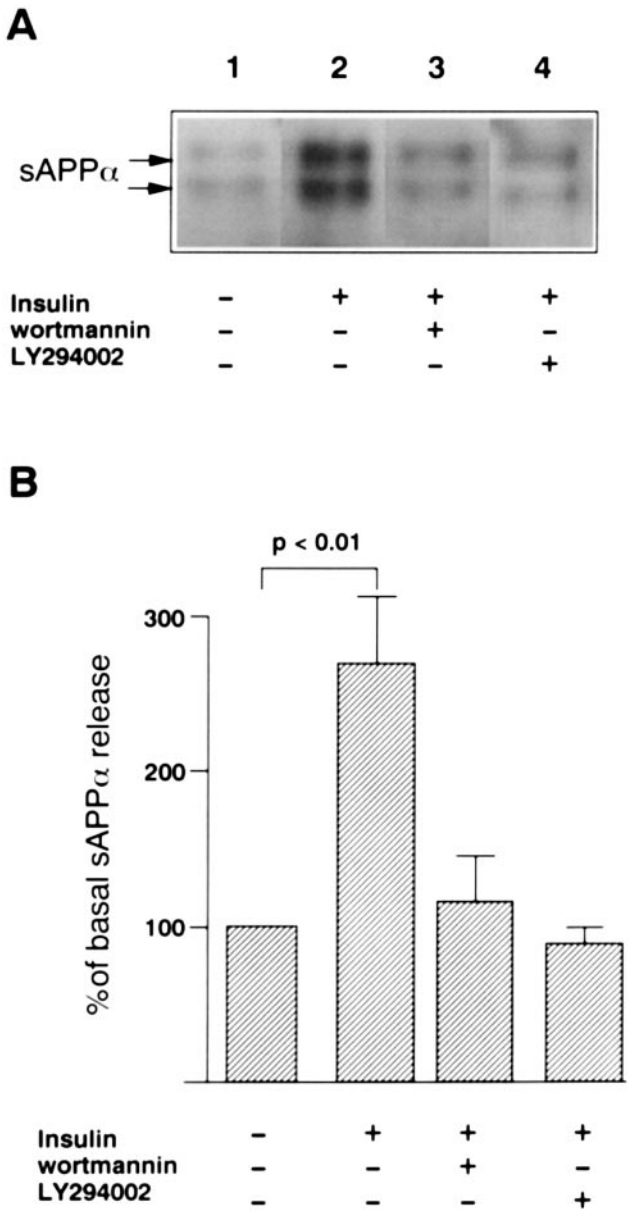

Figure 6. PI3-K activation is necessary for insulin stimulation of sAPPα release. *A)* SH-SY5Y as in Fig. 1 were incubated for 2 h at 37°C with serum-free MEM (lane 1), with 1 μM insulin (lane 2), with 1 μM insulin and 10 μM wortmannin (lane 3), or with insulin and 10 μM LY294002 (lane 4). Proteins released into the conditioned media were collected, precipitated, and subjected to Western blot analysis. *B)* Results are expressed as percent of basal release ± SE of three independents experiments

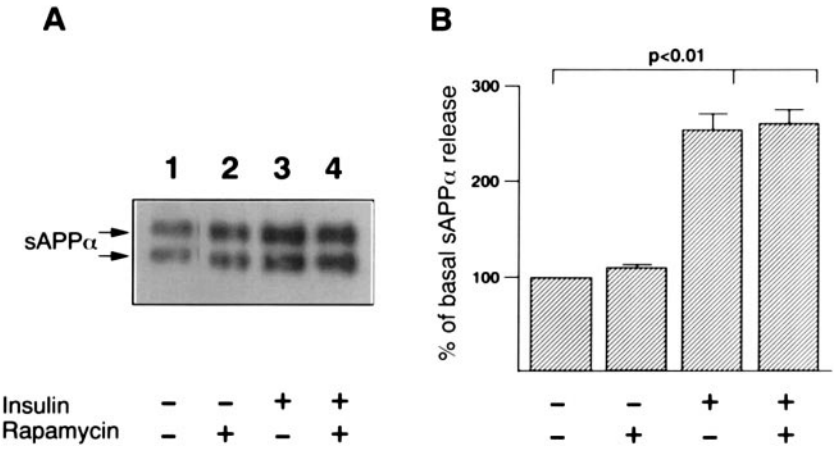

Figure 7. Insulin-mediated sAPPα release is not dependent on $p70^{s6K}$ activation. *A)* SH-SY5Y cells were preincubated overnight with serum-free MEM and the day after were incubated for 2 h at 37°C with serum-free MEM (lane 1), with 20 ng/ml rapamycin (lane 2), with 1 μM insulin (lane 3), or with 1 μM insulin and 20 ng/ml rapamycin (lanes 4). Proteins released into the conditioned media were collected and subjected to Western blot analysis. *B)* Results are expressed as percent of basal release ± SE of three independent experiments.

DISCUSSION

The data presented here show that human recombinant insulin can modulate sAPPα release from SH-SY5Y cells in a concentration- and tyrosine kinase-dependent manner.

It has been extensively shown that α secretase cleavage of APP is increased by the activation of a variety of signal transduction pathways (for review, see refs 4, 5), including tyrosine kinase receptors. Among other ligands, insulin acts through a receptor with intrinsic protein tyrosine kinase activity. Genistein has been reported to exhibit specific inhibitory activity against tyrosine kinases. As reported here, 20 μM and 40 μM genistein (the latter concentration not shown) blocked sAPPα release induced by insulin, thus showing that a tyrosine kinase dependent mechanism was necessary for the effect of insulin on APP metabolism, a mechanism similar to that described for EGF (36).

Deprivation of glucose from the culture medium in our experiments decreased the basal release of sAPPα, although insulin was still able to induce a relative increase of sAPPα secretion. Reduction of basal release by glucose deprivation clearly indicates that glucose is necessary in a key step of APP metabolism. Nevertheless, the finding that glucose deprivation did not interfere with insulin-mediated increase of sAPPα secretion suggests the possibility that insulin can act as a growth factor to induce sAPPα release through a mechanism similar to EGF and other tyrosine kinase receptor ligands.

The activation of the secretory metabolism of APP by EGF is mediated at least partially by PKC, because treatment of the EGF-stimulated cells with the specific PKC inhibitor GF-109203X decreases the response by ~35% (36). In our experiments, the simultaneous treatment of neuroblastoma cells with GF-109203X at the same concentration used in the former study completely blocked the effect of phorbol esters, but did not block the activation of sAPPα release by insulin. This result suggests that PKC is not involved in insulin-mediated release of sAPPα.

Insulin receptor kinase activity results in the tyrosine phosphorylation of substrates like the insulin receptor substrate 1 (IRS-1). Phosphorylated IRS-1 interacts with many other proteins that bind phosphotyrosine with their SH2 domains. GRB2 is one such protein, and its function involves the role of an 'adapter molecule' that links the guanine nucleotide exchange factor for $p21^{ras}$ (named mSOS) to phosphorylated IRS-1. This complex may then activate Ras and the MAP-K cascade (37). Recent reports showed that MAP-K (ERK) is involved in NGF, phorbol esters, and carbachol-stimulated sAPPα secretion; it is notable that a reduction of sAPPα secretion via inhibition of MAP-K was reported for several cell lines (29–31). Our experiments demonstrate that the effect of insulin signaling on sAPPα release was not inhibited by the addition of PD-98059, a selective MEK inhibitor ruling out the involvement of the MAP-K pathway in the effect of insulin on APP metabolism.

Phosphorylated IRS-1 can bind and activate PI3-K, and it is now suggested that such a pathway may be one of the key signaling events in the insulin effect on end point responses (38). PI3-K plays a critical role in growth factor signaling to cell growth and proliferation, differentiation, apoptosis, glycogen synthesis, protein synthesis, vesicle traffic, and glucose transporter translocation. PI3-K appears to be an upstream activator of uptake of amino acids, gene transcription, and mRNA translation. Acute cellular responses to insulin include the activation of protein synthesis (39) through a pathway involving PI3-K, PKB and $p70^{s6K}$ (40). The latter kinase is activated through a pathway sensitive to the macrolide rapamycin, which can block the pathway of activation of $p70^{s6K}$ downstream of PI3-K. We show here that the compound does not inhibit insulin-mediated sAPPα release, suggesting that the effect is not mediated by an increase in protein translation.

The activation of sAPPα release by insulin is inhibited by wortmannin and LY-294002. The former is a cell-permeable fungal metabolite that inhibits PI3-K

by covalent modification of the catalytic subunit of PI3-K. The compound LY-294002, another specific inhibitor of PI3-K, is mechanistically different from wortmannin since it directly competes for the ATP binding sites of the catalytic subunit of PI3-K. The kinase targeted to phosphoinositides is important for one of the basic physiological roles of insulin: translocation of the Glut4 glucose transporter to the cell surface to increase the rate of glucose uptake into target cells. Wortmannin blocks the rate of insulin-stimulated exocytosis of Glut4 glucose transporters, with little effect on endocytosis (41, 42). Similarly, PI3-K inhibitors block insulin-stimulated trafficking of transferrin receptors (43). Since transferrin receptors are not present on Glut4-containing vesicles, this suggests that PI3-K is involved in the regulation of a step in exocytosis that is not restricted to movement of Glut4-containing vesicles. Moreover, phosphoinositides could serve as specific membrane targets that bind proteins required for the formation of transport vesicles. For example, the targets could be the polypeptides of the adaptor complex that link clathrin to the cytoplasmic tail of certain transmembrane receptor proteins (44). Thus, all these observations lead us to the conclusion that the effect of insulin on APP metabolism may be mediated by a PI3-K-dependent modulation of vesicular trafficking. This would mean either an increase of the transport of APP into secretory compartments or trafficking of 'secretase' containing vesicles toward APP-containing membrane domains. These hypotheses are based on experimental evidence that ruled out all alternatives within insulin signaling pathway and describe a novel intracellular pathway involved in the regulation of APP metabolism.

Finally, we can speculate that the mechanism described here may also serve a function of neuroprotection from the toxic activity of Aβ itself. It has been demonstrated that insulin and insulin-like growth factors can protect neurons in culture from Aβ toxicity (45, 46). Thus, sAPPα derived from insulin-mediated metabolism of APP can also serve as a neuroprotective molecule (see ref. 7) and work synergistically with insulin in a putative system that can both prevent and offer rescue from toxic events. FJ

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Received for publication July 28, 1999.
Revised for publication December 21, 1999.