Insulin Stimulates Postsynaptic Density-95 Protein Translation via the Phosphoinositide 3-Kinase-Akt-Mammalian Target of Rapamycin Signaling Pathway*

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Insulin receptors are highly enriched at neuronal synapses, but whose function remains unclear. Here we present evidence that brief incubations of rat hippocampal slices with insulin resulted in an increased protein expression of dendritic scaffolding protein postsynaptic density-95 (PSD-95) in area CA1. This insulin-induced increase in the PSD-95 protein expression was inhibited by the tyrosine kinase inhibitor, AG1024, phosphatidylinositol 3-kinase (PI3K) inhibitors, LY294002 and wortmannin, translational inhibitors, anisomycin and rapamycin, but not by LY303511 (an inactive analogue of LY294002), and transcriptional inhibitor, actinomycin D, suggesting that insulin regulates the translation of PSD-95 by activating the receptor tyrosine kinase-PI3K-mammalian target of rapamycin (mTOR) signaling pathway. A similar insulin-induced increase in the PSD-95 protein expression was detected after stimulation of the synaptic fractions isolated from the hippocampal neurons. Furthermore, insulin treatment did not affect the PSD-95 mRNA levels. In agreement, insulin rapidly induced the phosphorylation of 3-phosphoinositide-dependent protein kinase-1 (PKD1), protein kinase B (Akt), and mTOR, effects that were prevented by the AG1024 and LY294002. We also show that insulin stimulated the phosphorylation of 4E-binding protein 1 (4E-BP1) and p70S6 kinase (p70S6K) in a mTOR-dependent manner. Finally, we demonstrate the constitutive expression of PSD-95 mRNA in the synaptic fractions isolated from hippocampal neurons. Taken together, these findings suggest that activation of the PI3K-Akt-mTOR signaling pathway is essential for the insulin-induced up-regulation of local PSD-95 protein synthesis in neuronal dendrites and indicate a new molecular mechanism that may contribute to the modulation of synaptic function by insulin in hippocampal area CA1.

Insulin and its receptor are widely dispersed throughout the brain with the highest density located in the olfactory bulb, cerebral cortex, hypothalamus, and hippocampus, where they are thought to subserve a number of functions including regulation of glucose metabolism, food intake and body weight, fertility and reproduction, learning, memory, and attention (1–4). Brain insulin receptors are present in particularly high concentrations in neurons, and in much lower levels in glia (5). Although the mRNA of insulin receptors is largely localized in neuronal somata, abundant insulin receptors are found in both cell bodies and synapses (5–7). However, very little is known about the functional significance of synaptic insulin receptors in the neurons. Recently, several studies have drawn links between insulin signaling and intracellular trafficking and plasma membrane expression of ion channels and neurotransmitter receptors at the central nervous system synapses. For example, it has been shown that insulin rapidly recruits functional GABA_A receptors to postsynaptic domains in hippocampal neurons, resulting in a long-lasting enhancement of GABA_A receptor-mediated synaptic transmission (8). In addition, we and other investigators have provided evidence that insulin can promote the internalization of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors from the synaptic membrane of neurons, which causes a long-term depression of excitatory synaptic transmission in the hippocampus and cerebellum (9–12). Moreover, insulin enhances N-methyl-D-aspartate (NMDA)¹ receptor-mediated synaptic transmission at the hippocampal CA1 synapses (13) and potentiates the activity of recombinant NMDA receptors expressed in Xenopus oocytes (14). Although these findings highlight the role of insulin signaling in modulating synaptic functions, it is not yet clear how exactly insulin contributes to these diverse actions at the molecular levels.

The postsynaptic density (PSD) is a specialization of the cytoskeleton at the synaptic junction and serves as an important organizer of the postsynaptic signal transduction machinery (15). The PSD forms a disc that consists of cytoskeletal and regulatory proteins, some of which contact the cytoplasmic domains of ion channels or neurotransmitter receptors in the postsynaptic membrane (16). One of fundamental structural proteins within the PSD is PSD-95, a 95-kDa scaffolding protein containing multiple PSD-95/Discs large/zona occludens-1 domains to anchor and associate glutamate receptors with other functional proteins in the PSD (17, 18). Although the function of the PSD-95 protein at the synapses is not yet clear, evidence from the PSD-95 mutant or expression studies has demonstrated that PSD-95 may play a modulatory role in con-

¹ The abbreviations used are: NMDA, N-methyl-D-aspartate; PSD, postsynaptic density; PSD-95, protein postsynaptic density-95; aCSF, artificial cerebrospinal fluid; 4E-BP1, 4E-binding protein 1; eIF4E, eukaryotic initiator 4E; eIF4G, eukaryotic initiator 4G; p70S6K, p70S6 kinase; IGF-1, insulin-like growth factor-1; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; mTOR, mammalian target of rapamycin; LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzo pyran-4-one; LY303511, 2-piperazinyl-8-phenyl-4H-1-benzozyperan-4-one; AG1024, 3-bromo-5-t-butyl-4-hydroxy-benzylidenemalonitrile; RT, reverse transcriptase.
control of the synaptic transmission (19), bidirectional synaptic plasticity (20, 21), maturation of excitatory synapses (19, 22), and drug addition (23). A pressing question that follows from these observations is whether and how PSD-95 protein can be elevated at synaptic sites under physiological conditions to modulate the synaptic function. A recent study has demonstrated that estrogen rapidly stimulates dendritic PSD-95 protein synthesis in NG108-15 neuroblastoma cells (24). Given that insulin receptor and its substrate are components of PSD fractions and are concentrated at the synapses in the hippocampal neurons, it is of particular interest to examine the relationship between insulin receptor activation and PSD-95 protein synthesis. Our results reveal the first evidence that brief insulin treatment substantially increases the synthesis of PSD-95 protein in local dendritic compartments via the activation of PI3K-Akt-mTOR signaling pathway. This event may provide a potentially important way to modulate synaptic transmission and plasticity in hippocampal area CA1.

**EXPERIMENTAL PROCEDURES**

**Hippocampal Slice Preparations**—All experiments were performed according to the guidelines laid down by the Institutional Animal Care and Use Committee of National Cheng Kung University. Hippocampal slices (400 μm thick) were obtained from 28- to 35-day-old young male Sprague-Dawley rats by the procedures described previously (25). In brief, animals were killed by decapitation under halothane anesthesia, and the hippocampi were removed, placed in ice-cold artificial CSF (aCSF) solution and cut with a Leica VT1000S tissue slicer (Leica, Nussloch, Germany) in 400-μm thick transverse slices. After preparation, slices were placed in a holding chamber of aCSF oxygenated with 95% O2, 5% CO2 and kept at room temperature for at least 1 h before experiments. The composition of the aCSF solution was 117 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM MgCl2, 25 mM NaHCO3, 1.2 mM NaHPO4, and 11 mM glucose at pH 7.3–7.4, and equilibrated with 95% O2-5% CO2. After gassing, the slices were then transfused with the indicated gases for 30 min before transfer onto a filter. The filtrate was then centrifuged at 1000 × g for 10 min and then was gently resuspended with same buffer at a protein concentration of 2 mg/ml.

**Western Blotting**—For each experimental group, homogenates from at least three slices were pooled. The microdissected CA1 regions were lysed in RIPA buffer solution containing 0.5% Triton X-100 (50 mM HEPES, 100 mM NaCl, and 5 mM Kac, pH 7.4) with RNase inhibitor (15 units/ml) and centrifuged at 2000 × g for 1 min. Supernatants were washed twice in 10% SDS-sodium dodecyl sulfate (SDS) solution to remove proteins of protein phosphatase and proteinase inhibitors (50 mM Tris-HCl, 100 mM NaCl, 15 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM EGTA, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM microcystin-LR, 1 μM okadaic acid, 0.5% Triton X-100, 2 mM benzamidine, 60 μg/ml aprotinin, and 60 μg/ml leupeptin) to avoid dephosphorylation and degradation of proteins, and ground with a pestle (Kontes Glassware, Vineland, NJ). Samples were sonicated and spun down at 15,000 × g at 4 °C for 10 min. The supernatant was then assayed for total protein concentration using the Bio-Rad Bradford Protein Assay Kit. Each sample was separated in 10% SDS-PAGE gels. Following the transfer on nitrocellulose membranes, blots were blocked in Tris-HCl buffer solution containing 3% bovine serum albumin and 0.1% Tween 20 for 1 h and then blotted for 2 h at room temperature with antibodies that recognize PSD-95 (1:1000; Upstate Biotechnology, Lake Placid, NY), phosphorylated PDK1 (1:1000; Cell Signaling Technologies, Beverly, MA), phosphorylated Akt (1:1000; Cell Signaling Technologies), phosphorylated mTOR (1:1000; Cell Signaling Technologies), phosphorylated p70S6K (1:1000; Cell Signaling Technologies), or phosphorylated 4E-BP1 (1:1000; Cell Signaling Technologies). It was then probed with horseradish peroxidase-conjugated secondary antibody for 1 h and developed using the ECL immuno blotting detection system. The immunoblots using phosphorylation site-specific antibodies were subsequently stripped and reprobed with the following antibodies: anti-PDK1 antibody (1:1000), anti-Akt antibody (1:1000), anti-mTOR antibody (1:1000), anti-p70S6K, or anti-4E-BP1 antibody (1:1000) that were purchased from Cell Signaling Technologies. Immunoblots were analyzed by densitometry.

**RESULTS**

**Activation of Insulin Receptors Increases the PSD-95 Protein Expression in Hippocampal Area CA1**—The initial set of experiments was designed to investigate whether the activation of insulin receptors regulates the PSD-95 protein expression. Incubation of hippocampal slices with insulin (0.1–3 μM) for 10 min resulted in dose-dependent increases in levels of PSD-95 protein as measured on Western blotting of homogenates from area CA1 (Fig. 1A). At a concentration of 0.5 μM, the level of PSD-95 protein was increased by 46.3 ± 5.4% of control 10 min after washout of insulin. Because 0.5 μM insulin could consistently increase PSD-95 protein expression, we chose this treatment protocol to identify the underlying mechanisms of this event in all subsequent experiments. In a time course analysis, PSD-95 protein levels remained elevated for at least 30 after washout of insulin (Fig. 1B). In addition, the effect of insulin on PSD-95 protein expression appears to be specific, because the levels of another PDZ-containing scaffold protein SAP97 were not altered after insulin treatment (Fig. 1C).

IGF-1 receptors are structurally very similar to insulin receptors and insulin binds to both insulin and IGF-1 receptors,
although the former is bound with about 40-fold greater affinity (27, 28). Further experiments were performed to determine whether the increases in PSD-95 protein by insulin were mediated by the activation of IGF-1 receptors. To directly test this possibility, hippocampal slices were pretreated with a monoclonal antibody directed against the external portion of IGF-1 receptors (5 μg/ml), whereas anti-IGF-1 receptor antibody completely blocked the IGF-1 (50 ng/ml for 10 min)-induced increases in PSD-95 protein expression. Group data showing the normalization of PSD-95 protein to β-actin was determined in each group of five experiments. C, representative Western blotting showing the level of PSD-95 protein taken at different times after washout of insulin (0.5 μM). Group data showing the normalization of PSD-95 protein to β-actin was determined in each group of five experiments. D, representative Western blotting showing that the insulin-induced increases in the PSD-95 protein expression was not affected by monoclonal antibody directed against the external portion of IGF-1 receptors (5 μg/ml), whereas anti-IGF-1 receptor antibody completely blocked the IGF-1 (50 ng/ml for 10 min)-induced increases in PSD-95 protein expression. Group data showing the normalization of PSD-95 protein to β-actin was determined in each group of four experiments. The number of experiments is indicated by n. *, p < 0.05 (unpaired Student’s t test) as compared with the control group.

**Insulin-induced Increases in New PSD-95 Protein Expression Are PI3K-Akt-mTOR Dependent**—The activation of insulin receptors by insulin elicits a large cascade of signal transduction events. To define the specific signaling pathway that contributes to the insulin-induced increases in the PSD-95 protein expression, the requirement for PI3K activation was first examined. Pretreatment of the hippocampal slices with the specific PI3K inhibitor, LY294002 (20 μM) (31), completely blocked insulin-induced increases in the PSD-95 protein expression (Fig. 2A). Similar results were also observed with the use of another structurally unrelated PI3K inhibitor, wortmannin (5 μM) (data not shown). In contrast, the inactive analogue of LY294002, LY303511 (20 μM), did not affect the action of insulin (Fig. 2A). None of LY294002, wortmannin, or LY303511 alone altered the basal levels of PSD-95 protein. These results support that the activation of PI3K is required for the insulin-induced increases in PSD-95 protein expression in hippocampal area CA1.

To determine whether the enhanced PSD-95 protein expression by insulin was because of an increase in the new protein synthesis, we pretreated the hippocampal slices with the protein synthesis inhibitor, anisomycin (20 μM), 60 min before insulin stimulation. As shown in Fig. 2B, anisomycin pretreatment completely blocked the insulin-induced increases in PSD-95 protein expression. To evaluate the possible contribution of mRNA synthesis to this event, we examined the effect of actinomycin D (25 μM), on the action of insulin. In contrast to the translation inhibitor, actinomycin D pretreatment for 60 min had no effect on the increases in PSD-95 protein level by insulin (Fig. 2B). We also examined whether insulin affected the levels of PSD-95 mRNA determined by quantitative real-time RT-PCR, but we observed no effect (Fig. 3), a result consistent with the suggestion that the increases in PSD-95 protein induced by insulin were derived from an activation of mRNA translation, and furthermore, that this mRNA was already present at the time of the stimulation.

The mTOR signaling pathway has been shown to play a crucial role in regulating several components of the translational machinery in mammalian cells (32), and insulin can stimulate this pathway to modulate the activity of several translation regulatory factors (33). We next asked if the increases in PSD-95 protein expression by insulin occur through
Insulin Increases PSD-95 Protein Translation

Fig. 2. Activation of the PI3K-Akt-mTOR signaling pathway is required for insulin-induced increases in new PSD-95 protein synthesis. A, after treatment with different kinase inhibitors, hippocampal slices were exposed to 0.5 μM insulin for 10 min and then the level of PSD-95 protein was determined by Western blotting. The tyrosine kinase inhibitor, AG1024 (10 μM), and PI3K inhibitor, LY294002 (20 μM), inhibited insulin-induced increases in the PSD-95 protein expression, whereas an inactive analogue of LY294002, LY303511 (20 μM), did not have any significant effect. Group data showing the normalization of PSD-95 protein to β-actin was determined in each group of five experiments. B, representative Western blotting showing that pretreatment of the hippocampal slices with translation inhibitor, anisomycin (20 μM), and mTOR inhibitor, rapamycin (200 nM), but not transcription inhibitor, actinomycin D (25 μM), blocked the insulin-induced increases in the PSD-95 protein synthesis. Group data showing the normalization of PSD-95 protein to β-actin was determined in each group of five experiments. Number of experiments is indicated by n. *p < 0.05 (unpaired Student’s t test) as compared with control group.

Fig. 3. Insulin does not affect PSD-95 mRNA levels. Equal amounts of total mRNA isolated from control and insulin (0.5 μM)-treated hippocampal slices were analyzed by real-time RT-PCR to quantitate the amount of PSD-95 mRNA. At 10 min after insulin washout, there is no significant change in PSD-95 mRNA levels (n = 5; p > 0.05; unpaired Student’s t test).

A mTOR-coupled mechanism, so the mTOR inhibitor, rapamycin, was employed. As shown in Fig. 2B, pretreatment of the hippocampal slices with rapamycin (200 nM) for 60 min completely blocked insulin-induced increases in PSD-95 protein expression. Rapamycin alone failed to alter the basal levels of PSD-95 protein. We conclude therefore that insulin stimulates the new PSD-95 protein synthesis in a rapamycin-sensitive and PI3K-dependent manner.

To further ascertain the requirement for the activation of the PI3K-Akt-mTOR signaling pathway in insulin-induced increases in PSD-95 protein expression, we used phospho-specific antibodies to measure the relative levels of phosphorylated, active forms of PDK1, Akt, and mTOR after hippocampal slices were treated with insulin. For PDK1, we used an antibody specific for phosphorylated serine 241, which is on the activation loop of PDK1 and is essential for kinase activity (34). For Akt, we used an antibody specific for phosphorylated serine 473, which is necessary for maximal activation of Akt (35). For mTOR, we used an antibody specific for phosphorylated serine 2448, which has been shown to be important in the control of mTOR activity (36, 37). As expected, stimulation of the hippocampal slices with 0.5 μM insulin for 10 min caused a marked increase in the phosphorylation of PDK1, Akt, and mTOR as measured on Western blots of homogenates from the CA1 area (Fig. 4). In addition, the insulin-induced increase in the phosphorylation of PDK1, Akt, and mTOR was transient, returning to control levels within 20–30 min after washout of insulin.

It is well documented that PDK1, Akt, and mTOR are downstream effectors of PI3K. Therefore, we determined whether LY294002 could block the insulin-induced increases in the phosphorylation of PDK1, Akt, and mTOR. We found that LY294002 (20 μM) but not LY303511 (20 μM) completely blocked the increases in PDK1, Akt, and mTOR phosphorylation induced by insulin (Fig. 5). Neither LY294002 nor LY303511 alone altered the basal levels of PDK1, Akt, and mTOR phosphorylation. Similarly, tyrosine kinase inhibitor AG1024 (10 μM) pretreatment also completely blocked the insulin-induced increases in the phosphorylation of PDK1, Akt, and mTOR phosphorylation (Fig. 5). Together, these results indicate that PI3K acted upstream of PDK1, Akt, and mTOR to initiate the insulin receptor signaling in hippocampal area CA1.

Insulin Stimulates the Phosphorylation of 4E-BP1 and p70S6K—Because activation of mTOR can contribute to translational initiation by phosphorylation of 4E-binding protein 1 (4E-BP1) that binds to and represses the function of the cap-binding translation factor eIF4E, we asked if this regulatory protein may have a role in insulin-induced increases in PSD-95 protein synthesis. We used an antibody specific for phosphorylated threonine 70, a site whose phosphorylation is required for the inactivation of 4E-BP1 and its dissociation from the eIF4E complex (38). Incubation of the hippocampal slices with 0.5 μM insulin for 10 min increased phosphorylation of 4E-BP1 (Fig. 6). Furthermore, the insulin-induced increases in the phosphorylation of 4E-BP1 were completely blocked by AG1024, LY294002, and rapamycin pretreatment, respectively. LY303511 failed to alter the increases in 4E-BP1 phosphorylation induced by insulin (data not shown).

mTOR activation may also regulate translation by direct or indirect phosphorylation of other translation-related protein factors such as p70S6K (38). We then asked if the increases in PSD-95 protein expression by insulin are p70S6K-dependent, so the phosphorylation state of p70S6K after insulin treatment was examined. We used an antibody specific for phosphorylated threonines 389 and 229, sites whose phosphorylation are vital for p70S6K activation (39). When the hippocampal slices were treated with 0.5 μM insulin for 10 min, the phosphoryla-
tion of p70S6K was significantly increased (Fig. 6). Furthermore, the insulin-induced increases in the phosphorylation of p70S6K were completely blocked by AG1024, LY294002, and rapamycin pretreatment, respectively. LY303511 failed to alter the increases in p70S6K phosphorylation induced by insulin. These results support the suggestion that activation of both 4E-BP1 and p70S6K is required for the insulin-induced increase in PSD-95 protein expression in hippocampal area CA1.

Site of Insulin-induced New PSD-95 Protein Synthesis—Although there is evidence that mRNA can be translated in the dendrites of hippocampal CA1 pyramidal cells (40), the major site of protein synthesis is the cell body. To specifically explore the presence and the possible synthesis of PSD-95 protein from the synaptic fractions, we generated synaptoneurosome, a biochemical preparation that enriches for synaptic fractions (41).

To assess the relative distribution of PSD-95 mRNA, we amplified the mRNA by RT-PCR from both the crude homogenates and synaptoneurosome fractions and compared its distribution with that of other mRNAs (Fig. 7). The end point products were separated by electrophoresis and detected by ethidium bromide staining. Because it is not a quantitative analysis, absolute amounts cannot be determined. However, this analysis did reveal that the mRNA encoding PSD-95 is present in our hippocampal CA1 synaptoneurosome preparations and that its relative distribution is similar to known dendritically targeted mRNAs encoding α-calcium-calmodulin-dependent protein kinase II. In contrast, mRNA encoding histone H1 was not detected in the synaptoneurosome fractions, providing further support that the synaptoneurosome preparations used in this study are indeed the synaptic fractions.

Having confirmed the expression of untranslated PSD-95 mRNA within synaptic compartment, we developed an in vitro assay using the synaptoneurosome fractions to assess if PSD-95 mRNA located to the synaptic compartment could be under the same translational control as we detected in intact slices. As shown in Fig. 8A, an increase in the PSD-95 protein expression was observed after insulin (0.5 μM) treatment for 10 min. Consistent with results obtained with hippocampal slices, AG1024 and LY294002 completely blocked this event, whereas LY303511 has no effect on insulin-induced increases in PSD-95 protein synthesis. In addition, pretreatment of the synaptoneurosome with protein synthesis inhibitors, anisomycin and rapamycin, but not the transcription inhibitor, actinomycin D, also prevented the increases in PSD-95 by insulin (Fig. 8B).
Therefore, these findings strongly suggest that the rapid insulin stimulation of PSD-95 new protein synthesis occurs primarily in dendrites.

**DISCUSSION**

The present study indicates a new synaptic mechanism that leads to the modulation of local PSD-95 protein expression in hippocampal area CA1. Our findings suggest a novel role for insulin in regulating the activation of dendritic translation machinery through a PI3K-Akt-mTOR signaling pathway. Insulin activates mTOR and its downstream translation regulatory molecules, 4E-BP1 and 70S6K, to stimulate the translation of the dendritic spine scaffolding protein PSD-95. We also demonstrate that insulin stimulates the translation of PSD-95 in a transcription-independent manner.

Although a decade of research has revealed the importance of PSD-95 protein in many neuronal processes (19–23), the factors or mechanisms that critically regulate the endogenous PSD-95 protein expression have not yet been defined. A recent study has shown that estrogen can stimulate a rapid increase in PSD-95 new protein synthesis in NG108-15 cells through a...
transcription-independent manner (24). Although this is the first report to address the specific mechanisms regulating the endogenous levels of PSD-95 protein, the authors did not directly determine the site of PSD-95 protein synthesis. The present study shows that insulin induces increases of PSD-95 protein expression in intact slices and biochemical synaptoneurosomal fractions in hippocampal area CA1, both of which are inhibited by translation inhibitor, strongly suggesting that a new PSD-95 protein synthesis is occurring in the synaptodendritic region. In contrast to the translation inhibitor, actinomycin D, a specific inhibitor of transcription, did not significantly affect the insulin-induced increases in the PSD-95 protein synthesis (Fig. 2B). Therefore, this event is not dependent on mRNA transcription but rather on the existing population of PSD-95 mRNA already transcribed. Consistent with this notion, we find that mRNA encoding PSD-95 protein is located to synaptic fractions in a similar proportion to that of known dendritic mRNAs encoding α-calcium-calmodulin-dependent protein kinase II (Fig. 7). Although we have been unable, however, to exclude the possibility that local protein synthesis occurs in cells other than pyramidal cells (e.g., glia, interneurons), the most likely source of translation may be dendrites of CA1 pyramidal neurons. This suggestion is supported by the observations that the levels of insulin-induced increases in PSD-95 protein are not statistically different between the intact slices and synaptoneurosome fractions.

Synapse has been shown to be an important site of specialized insulin signaling in the brain (2, 42, 43). So far, the functional significance of insulin and its receptors at the synapses have been best studied in the context of regulation of intracellular trafficking and plasma membrane expression of ion channels and neurotransmitter receptors (9–14). Recent evidence suggests that the diverse insulin actions may be coordinated through the spatially regulated expression of insulin receptor tyrosine kinase substrates and their downstream signaling transduction molecules (7). Although the identities of the insulin receptor substrates required for insulin-induced increases in PSD-95 protein synthesis are unknown, our observations suggest that the candidate proteins should initiate a signaling cascade involving PI3K, Akt, and mTOR. The finding that insulin-induced increases in PSD-95 protein synthesis is blocked by two structurally unrelated PI3K inhibitors, LY294002 and wortmannin, and mTOR inhibitor, rapamycin, supports a pathway involving PI3K and mTOR. This finding is consistent with an early report showing that activation of the PI3K-Akt-mTOR pathway by estrogen can stimulate a rapid increase in PSD-95 protein expression in NG108-15 cells (24).

How does mTOR enhance new PSD-95 protein synthesis? There are two potential mechanisms that could result in this translational regulation. First, the activation of mTOR may contribute to translation initiation by phosphorylating 4E-BP1 that binds eIF4E. The phosphorylated 4E-BP1 is replaced by eIF4G, a scaffolding translation factor that assembles several critical components of the cap-dependent translation initiation complex on the mRNA via its interaction with cap-bound eIF4E (33, 44, 45). Second, the activation of mTOR may also regulate translation via direct or indirect phosphorylation and activation of other translation-related protein factors such as p70S6K (33, 45). The activation of p70S6K then phosphorylates ribosomal protein S6, which can result in enhanced translation of 5′-oligopyrimidine tract-containing mRNAs that encode numerous components of translation machinery (33). This idea is supported by the finding that insulin treatment leads to increased phosphorylation of 4E-BP1 and p70S6K, both of which correlate with increased levels of PSD-95 protein translation and are inhibited by rapamycin (Fig. 8). Thus, activation of mTOR by insulin may enhance both cap-dependent translation (eIF4E pathway) and initiation of 5′-oligopyrimidine tract-containing mRNAs (p70S6K–ribosomal protein S6 pathway) at synapses. In addition, the presence of these molecular components of translation machinery in the dendritic regions was recently demonstrated by biochemical and immunohistochemical analyses in the synaptic fractions (46) or cultured primary hippocampal neurons in vitro (47). Although it is thought that rapamycin is a highly specific inhibitor of mTOR, the possibility that rapamycin affects other unknown kinases or molecules cannot be completely excluded.

Although our data show that 0.1–3 μM insulin can consistently stimulate new PSD-95 protein synthesis in hippocampal area CA1, it is unclear whether the endogenous insulin level could trigger this enhancing action. Because there has been no pharmacological tools currently available that can clearly distinguish insulin and IGF receptors, to our knowledge, no evidence directly identifies this issue. Under resting conditions, using insulin radioimmunoassay strategy, it has been demonstrated that the concentration of immunoreactive insulin extract from the whole hippocampal formations was at about 0.2 ng/g wet tissue (48). Because the locally insulin concentrations at the synaptic cleft may not be detectable when whole tissues are extracted, it is therefore difficult to comment on whether the concentration of insulin used in the present study is physiologically or pathophysiological relevant.

Our results reinforce the idea that mRNA is translated in synaptic locations. Whereas it is well known that dendrites can translate mRNA (49) and new protein synthesis can be induced in isolated dendrites and synaptoneurosomes after exposure of neuronal cultures to brain-derived neurotrophic factor (50) or activity blockade (51, 52), little is known about whether this event may exactly occur in situ. Our results demonstrate for the first time that mRNA encoding PSD-95 is present at the synapses and can be stimulated following activation of insulin receptors in more intact brain tissue. In addition, our results may explain, at least in part, the observations that insulin increases NMDA receptor-mediated synaptic response at hippocampal CA1 synapses (13) and NMDA receptor expression in Xenopus oocytes (14). It is conceivable that PSD-95 protein interacting with the C-terminal E(T/S)XV sequence motif of NR2 subunits through the N-terminal PDZ domains (53) may cluster and stabilize NMDA receptor expression, thereby promoting their function.

In conclusion, the data presented here suggest that the dendritic scaffolding protein PSD-95 may define a novel class of insulin signaling at the synapses of hippocampal area CA1. Thus, certain kinds of synaptic modulation induced by insulin might be mediated by mTOR-dependent, regulated local translation of PSD-95 mRNA in neuronal dendrites. Whereas the physiological significance of insulin induced the increases in the dendritic PSD-95 protein synthesis in vitro remains to be elucidated, giving that the PSD-95 protein is generally assumed as an adapter molecule to cluster ion channels and neurotransmitter receptors or organize a signaling complex at the postsynaptic membrane (18), and that considerable evidence implicates a chaperone role for PDZ-containing proteins in early events of assembly, processing, and delivery of receptor proteins (54–56), this event may therefore serve an important role in controlling synaptic strength and plasticity.

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