Protein kinase Cε (PKCe) Promotes Synaptogenesis through Membrane Accumulation of the Postsynaptic Density Protein PSD-95*

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Protein kinase Cε (PKCe) promotes synaptic maturation and synaptogenesis via activation of synaptic growth factors such as BDNF, NGF, and IGF. However, many of the detailed mechanisms by which PKCe induces synaptogenesis are not fully understood. Accumulation of PSD-95 to the postsynaptic density (PSD) is known to lead to synaptic maturation and strengthening of excitatory synapses. Here we investigated the relationship between PKCe and PSD-95. We show that the PKCe activators dicyclopropanated linoleic acid methyl ester and bryostatin 1 induce phosphorylation of PSD-95 at the serine 295 residue, increase the levels of PSD-95, and enhance its membrane localization. Elimination of the serine 295 residue in PSD-95 abolished PKCe-induced membrane accumulation. Knockdown of either PKCe or JNK1 prevented PKCe activator-mediated membrane accumulation of PSD-95. PKCe directly phosphorylated PSD-95 and JNK1 in vitro. Inhibiting PKCe, JNK, or calcium/calmodulin-dependent kinase II activity prevented the effects of PKCe activators on PSD-95 phosphorylation. Increase in membrane accumulation of PKCe and phosphorylated PSD-95 (p-PSD-95S295) coincided with an increased number of synapses and increased amplitudes of excitatory post-synaptic potentials (EPSPs) in adult rat hippocampal slices. Knockdown of either PKCe or JNK1 also reduced the synthesis of PSD-95 and AMPA receptors GluA4 and GluA1 by PKCe.

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1 This article contains supplemental Fig. 1.
2 The abbreviations used are: CaMKII, calcium/calmodulin-dependent kinase II; DCPLA-ME, dicyclopropanated linoleic acid methyl ester; ANOVA, analysis of variance; PSD, postsynaptic density; r-, recombinant; BisI, bisindolylmaleimide I; EPSP, excitatory synaptic potential; KD, knockdown; OE, overexpression.

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and stabilization (30–32) and, thus, synapse number. Phosphorylation of the serine 295 residue of PSD-95 enhances the synaptic accumulation of PSD-95 and its ability to recruit surface α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and potentiate excitatory postsynaptic currents (33).

In the present study we examined the role of PKCε signaling and PKCε activators in PSD-95 regulation and induction of synaptogenesis in cultured neurons and CA1 hippocampal slices. We report that PKCε activation induces membrane translocation and phosphorylation of PSD-95 at the serine 295 residue, coinciding with an increased number of synapses. Our data suggest that an important mechanism by which PKCε induces synaptogenesis is by increasing the phosphorylation of PSD-95 at the postsynaptic site and by regulating the expression of synaptophysin at the presynaptic site.

Results

PKC Activation Prevents Degradation of Primary Human Neurons—PKCε is present in high concentration in central neuronal tissues and has been implicated in broad spectrum neuronal functions. To determine the effect of PKCε activation on survival and maintenance, primary human neurons were treated for 40 days with two different PKC activators (bryostatin 1 and DCPLA-ME, which are relatively specific for PKCε) (13, 34–36). Culture media and activators were changed every 3 days. Cells were imaged from three independent wells every 5 days, and neurite-positive cells were counted from 508-μm² field images. Cells treated with either DCPLA-ME (100 nM) or bryostatin 1 (0.27 nM) showed an improved survival with increased neuritic branching (Fig. 1A). Untreated cells showed degeneration and 50% cell loss by 36 days, whereas the treated cells remained healthy for at least 40 days (Fig. 1B). The number of viable neurite-positive cells was also significantly higher at 40 days (F(3, 6) = 705.4; ANOVA, p < 0.0001) in the activator-treated cells than untreated cells (bryostatin 1, 369.7 ± 12.2; DCPLA-ME, 334.7 ± 1.8; untreated 109.7 ± 6.4).

Prolonged PKCε Activation Prevents Loss of Synaptic Proteins—We quantified the mRNA expression of PKCε, PSD-95, and synaptophysin at 40 days in untreated and PKCε activator-treated neurons. At 40 days the mRNA levels of PKCε (F(3,8) = 18.3; p = 0.0006) and PSD-95 (F(3,8) = 44.6; p < 0.0001) were significantly higher in the PKCε activator-treated cells compared with 40 day control cells (Fig. 1C and D). Synaptophysin mRNA showed no significant change in between treated and untreated groups (Fig. 1E). We also quantified the protein expression of phosphorylated PSD-95 (p-PSD-95S295), PSD-95, and synaptophysin at 40 days in untreated and PKCε activator-treated neurons by immunoblot (Fig. 1F). Expression levels of PKCε (F(3,8) = 16.60; p < 0.001), p-PSD-95S295 (F(3,8) = 66.83; p < 0.0001), PSD-95 (F(3,8) = 21.22; p < 0.001) and synaptophysin were significantly higher in the 40-day PKCε activator-treated cells compared with 40-day control cells (Fig. 1, G–J). Moreover, protein expression levels of PKCε, PSD-95, and synaptophysin showed a marked decrease in 40-day untreated cells compared with 1-day cells, even after correction for total protein, whereas PKCε activation prevented the time-dependent loss. This indicates an essential role of PKCε in maintenance of synapses and preserving normal levels of both PSD-95 and synaptophysin.

Bryostatin 1 and DCPLA-ME Specifically Activate PKCε—We then investigated whether this phenomenon is specific to PKCε or whether other PKC isoforms are involved. PKC translocation to the plasma membrane generally has been considered the hallmark of activation and frequently has been used as a surrogate measure of PKC isoform activation in cells (37). Expression levels of PKCa, PKCε, and PKCδ in the soluble (cytosol) and particulate (membrane) were measured by immunoblot at 1, 4, and 24 h after either bryostatin 1 (0.27 nM) or DCPLA-ME (100 nM) treatment (Fig. 2, A and C). Both DCPLA-ME and bryostatin 1 increased membrane translocation of PKCε but not PKCa or PKCδ (Fig. 2, B and D), confirming that both the compounds activate PKCε but not PKCa or PKCδ.

PKCε Activation Induces Membrane Translocation of Phosphorylated PSD-95 (Serine 295)—Phosphorylation of PSD-95 on serine 295 is known to promote localization of PSD-95 in the postsynaptic density (PSD), strengthening the excitatory synapse (33). To determine whether time-dependent PKCε activation has an effect on localization and expression of p-PSD-95S295, we measured the expression of p-PSD-95S295 in the soluble and particulate fractions of the primary human neurons at 1, 4, and 24 h post PKCε activator treatment (Fig. 2, E and F). PKCε activation increased the level of p-PSD-95S295 in the particulate fraction of both bryostatin 1 (F(3,8) = 4.9; ANOVA, p = 0.03) and DCPLA-ME-treated cells (F(3,8) = 11.7; ANOVA, p = 0.003) (Fig. 2F). The total PSD-95 expression in whole cell lysate from primary human neurons was unchanged among different groups (Fig. 2E). At 4 h p-PSD-95S295 levels were significantly higher in bryostatin 1 (156.4 ± 14.9%; p = 0.004) and DCPLA-ME (160.1 ± 9.5%; p = 0.003)-treated neurons compared with untreated neurons. In adult rat hippocampal slices PKCε activation increased p-PSD-95S295 expression at 1 and 4 h (bryostatin 1, F(3,8) = 4.95; ANOVA p = 0.031; DCPLA-ME: F(3,8) = 4.34; ANOVA p = 0.043) (Fig. 2G and H). Negligible amounts of p-PSD-95S295 were detected in the soluble fraction. These results show that the increase in membrane localization of p-PSD-95S295 corresponded with the kinetics of PKCε activation at 1 and 4 h.

PKCε-mediated Phosphorylation of PSD-95 at Serine 295 Is Essential for Its Membrane Association—Purified recombinant human PSD-95 (r-PSD-95) protein was readily phosphorylated by activated recombinant PKCε (r-PKCε) in vitro, and the PKC inhibitor bisindolylmaleimide I (Go 6850) (BisI, 100 nM) blocked the reaction (Fig. 3A). Both bryostatin 1 and DCPLA-ME increased the amount of r-PSD-95S295 in vitro compared with unactivated PKC alone (bryostatin 1 + r-PKCε + r-PSD-95, 200.3 ± 5.06%; p = 0.004; DCPLA-ME + r-PKCε + r-PSD-95, 194.6 ± 12.95%; p = 0.032; r-PKCε + r-PSD-95 control, 146.9 ± 7.06%) (Fig. 3B). The PKC inhibitor BisI blocked the phosphorylation. These results show that PKCε can phosphorylate PSD-95 at serine 295 in vitro.

Next we tested if the serine 295 residue in PSD-95 is essential for its membrane translocation. We created two separate clones, one containing the wild type human PSD-95 and the other containing mutant-PSD-95S295K in which the serine res-
idue at 295 (AGT) was changed to lysine (AAA). Both the clones were transfected and expressed in HEK-293 cells, and their expression was measured by immunoblot. Both transfected cell lines showed PSD-95 immunoreactivity against a PSD-95 antibody raised against the N-terminal region of PSD-95. The anti-p-PSD-95S295 antibody showed positive bands only with the wild type PSD-95-transfected cell lysate. Untransfected HEK-293 cells showed no PSD-95 expression (Fig. 3C). The wild PSD-95 and PSD-95S295K-expressing HEK-293 cells were then treated with bryostatin 1 and DCPLA-ME for 4 h in presence or absence of PKCe translocation inhibitor (EAVALKPT; 5 μM) and fractionated into cytosol and membrane fractions. Only small amounts of p-PSD-95 were found in soluble fractions (Fig. 3D). As expected, bryostatin 1 and DCPLA-ME significantly increased membrane translocation of PKCe in both PSD-95- and PSD-95S295K-expressing cells (in wild type cells, bryostatin 1 = +54.4 ± 4.9%, p = 0.0004; DCPLA-ME = +19.1 ± 4.2%, p = 0.01; F(4,10) = 28.8, ANOVA, p < 0.0001) (Fig. 3E). PKCe activators also increased translocation of wild type PSD-95 (bryostatin 1, +29.9 ± 2.3%, p = 0.0002; DCPLA-ME, +20.5 ± 2.5%, p = 0.001; F(4,10) = 35, ANOVA, p <

**FIGURE 1. PKCe activation prevents degeneration of human primary neurons.** Primary human neurons were treated with either DCPLA-ME (100 nM) or bryostatin 1 (0.27 nM) for 40 days. Fresh drug was added every third day with 50% media change. A, image of 40-day-old untreated (Control) and bryostatin 1- and DCPLA-ME-treated neurons. B, number of neurite-positive cells counted from three 20× fields (508 μm²) over time. DCPLA-ME and bryostatin 1 treatment stabilized cellular viability for at least 40 days. Viability of untreated cells declined after 20 days. C–F, PKCe, PSD-95 and synaptophysin mRNA levels in 40-day-old cells compared with 1-day neurons. F, immunoblot analysis of PKCe, p-PSD-95S295, PSD-95, and synaptophysin in 40-day-old neurons compared with 1-day neurons. M, mass markers. G–J, immunostaining of p-PSD-95 S295, PSD-95, and synaptophysin calculated from immunoblots. Staining is significantly higher in DCPLA-ME- and bryostatin 1-treated cells. Data are represented as the mean ± S.E. of three independent experiments (Student’s t test, *, p < 0.05; **, p < 0.005).
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Involves JNK1 and CaMKII—Previously it has been reported that accumulation of PSD-95 in the PSD is increased by synaptic activity and by a Rac1-JNK1 signaling pathway (33). PKCe is involved in JNK activation in macrophages (38, 39), and CaMKII inhibitors inhibit PKC-mediated signaling in hippocampal neurons (40). Thus we investigated the involvement of PKCe, JNK, and CaMKII in PSD-95 translocation in primary human neurons. Cells were pretreated for 30 min with BisI (Go 6850) (100 nM, PKC inhibitor), SP600125 (20 μM, JNK inhibitor), or KN-93 (10 μM, CaMKII inhibitor) and then treated with PKCe activators for 4 h. Cells were fractioned into cytosolic and membrane fractions, and the membrane fractions were analyzed for the expression of p-PSD-95. The inhibitors alone reduced the expression of membrane-bound p-PSD-95 (F(4,10) = 23.04; ANOVA, p < 0.0001) (Fig. 4, A and C). DCPLA-ME treatment increased membrane localization of p-PSD-95 (147.3 ± 2.8%; F(3, 8) = 39.2; ANOVA, p < 0.0001). PSD-95 phosphorylation was prevented by blocking PKC activation using bisindolylmaleimide 1 (Fig. 4, B and D), confirming the involvement of PKCe in localization of p-PSD-95 in the membrane. The JNK inhibitor SP600125 and the CaMKII inhibitor K-93 also prevented PKCe-mediated phosphorylation and translocation of PSD-95 (Fig. 4, B and D).

PKCe phosphorylated PSD-95 in vitro, incorporating 1.46 ± 0.05 mol of [32P]ATP/mol of PSD-95. Western blotting with p-PSD-95-specific antibody confirmed that this included the Ser-295 site (Fig. 3, A and B). PKC and JNK inhibitors fully inhibited the PKCe-mediated PSD-95 phosphorylation, whereas a CaMKII inhibitor partially prevented PSD-95 phosphorylation (Fig. 4E). PKCe also phosphorylated JNK1 in vitro, incorporating 1.02 ± 0.04 mol of [32P]ATP per mol of JNK1; BisI prevented JNK1 phosphorylation (Fig. 4F). PKCe is also reported to phosphorylate CaMKII in vitro (41); we also found an increase in phosphorylation of CaMKII by PKCe (Fig. 4G). Because both JNK and CaMKII inhibitors prevented PSD-95 phosphorylation by PKCe (Fig. 4E), we considered the possibility that the JNK inhibitor might not be specific. Therefore, we performed a siRNA knockdown of PKCe and JNK in human neurons. PKCe or JNK knockdown caused a 50% reduction in their respective protein expression (Fig. 4H). DCPLA-ME failed to induce the membrane accumulation of p-PSD-95 in PKCe and JNK knockdown human neurons (F(3,12) = 24.6; ANOVA, p < 0.001) (Fig. 4, I and J). These results confirm that PKCe is required for membrane translocation of p-PSD-95 and that JNK and CaMKII are intermediates in the pathway (Fig. 4K).
Next we investigated if PKC/H9280-mediated phosphorylation of PSD-95 at serine 295 leads to synaptogenesis. Because 4-h PKC/H9280 activator treatment produced the highest p-PSD-95S295 level, we quantified the number of synapses from within 100 μm² of 30–35 CA1 regions from untreated, bryostatin 1, and DCPLA-ME-treated slices using electron microscopy (3 different slices in each group) (Fig. 5A). Both bryostatin 1 and DCPLA-ME increased the number of synapses at 4 h compared with only vehicle-treated control (8.97 ± 0.63, p = 0.002, n = 35; 6.97 ± 0.50, p = 0.04, n = 30; 5.77 ± 0.50; n = 35 CA1 areas, respectively) (Fig. 5B). Presynaptic vesicle density was measured in a series of three-dimensional stacked images from 6–10 presynaptic boutons from three different hippocampal slices. Bryostatin 1 treatment increased presynaptic vesicle density at 4 h (93.23 ± 4.1, p < 0.001, n = 30 presynaptic boutons) in comparison to control (71.33 ± 4.45, n = 22 presynaptic boutons).

Next we investigated the effect of bryostatin 1 on basal synaptic transmission of hippocampal CA1 pyramidal neurons to determine whether the new synapses are functional. Field potential recordings were measured from rat hippocampal

**FIGURE 3. PKCe-mediated phosphorylation of PSD-95 at serine 295 is essential for its membrane accumulation.** A, immunoblot representing p-PSD-95S295 and PSD-95 expression after incubation of the different combinations of recombinant PKCe, PSD-95, PKCe activators, and PKCe inhibitors (inh) mentioned above at 37 °C for 10 min in vitro. B, bryostatin 1 (Bry, 0.27 nM) and DCPLA-ME (100 nM) induced the phosphorylation of PSD-95 at serine 295 position. C, expression of p-PSD-95S295 and PSD-95 and β-actin in HEK-293 cells transfected with empty vector, wild type human PSD-95, and mutant PSD-95S295K. D, expression of PSD-95, p-PSD-95S295, PKCe, and β-actin in the soluble (S) and particulate (P) fraction of wild-PSD-95- and PSD-95S295K-transfected HEK-293 cells treated with bryostatin 1 and DCPLA-ME for 4 h in the presence or absence of EAVSLKPT (5 μM). S, soluble fractions; P, membrane fractions. Percentage of total protein in the membrane; PKCe (E), PSD-95 (F) and p-PSD-95S295 (G). Data are represented as the mean ± S.E. of three independent experiments (Student’s t test. *, p < 0.05; **, p < 0.005; ***, p < 0.005).
slices. An input-output curve was calculated with stimulus intensity versus the slope of excitatory synaptic potentials (EPSPs) elicited in response to increasing intensity of stimulation to the Schaffer collateral. The mean EPSP slope increased with stronger intensity of stimulus. Slices preincubated with bryostatin 1 for 1 h exhibited greater EPSP slope without any change in fiber volley amplitude. This was abolished with 30-min pretreatment with the PKC inhibitor bisindolylmaleimide I (BisI, 100 nM) (Fig. 5, C and D). Bryostatin 1 increased the area under the curve, which represents the overall basal synaptic transmission, and a PKC inhibitor prevented the increase (bryostatin1, 0.71 ± 0.08, p = 0.03; Bis1 + bryostatin 1, 0.49 ± 0.07; untreated (ethanol only), 0.51 ± 0.06) (Fig. 5E). Treatment of slices for 4 h with bryostatin (12 slices, 3 rats) dramatically increased the EPSP slope compared with the ethanol-treated slices (6 slices, 3 rats) (Fig. 5, F and G). The smaller response in the 4-h untreated slices compared with 1-h untreated slices may be attributed to the vehicle (ethanol) added to the slices. EPSPs in hippocampal slices are reduced by a smaller percentage after ethanol treatment (42). Thus, the prolonged treatment of slices with ethanol for 4 h may have slightly reduced the EPSP slope in these groups. Bryostatin increased the area under the curve by nearly 2-fold (p < 0.0001, Fig. 5H). These results suggest that bryostatin 1 treatment facili-
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Our results indicate that increased phosphorylation of PSD-95 by PKCe leads to an increase in synapse number with increased synaptic activity. Together these data demonstrate that the new synapses are functional.

PKCe Knockdown Reduces the Expression of PSD-95 and Synaptophysin—PKCe is known to perform important functions both in presynaptic (14) and postsynaptic sites. To investigate whether PKCe is essential for the expression of synaptic proteins, we measured the effect of PKCe knockdown (PKCe KD) and PKCe overexpression (PKCe OE) on the expression of postsynaptic PSD-95 and presynaptic synaptophysin. Knockdown of PKCe was achieved by transfecting the neurons with a mixture of siRNA containing a pool of three to five siRNAs. PKCe siRNA effectively reduced PKCe expression both at the mRNA and protein levels by 2- and 3.4-fold (Fig. 6, A and I) after 72 h. Scrambled siRNA did not cause any change in PKCe expression (Fig. 6, F and G). PKCe overexpression in the neurons was obtained by transfecting pCMV6-ENTRY vector containing human PKCe cDNA. Transfected neurons showed a 7.4-fold increase in PKCe mRNA level (Fig. 6A) and a 3.6-fold increase in PKCe protein level (Fig. 6, H and I). Overexpressing PKCe by 7-fold increased the level of synaptophysin mRNA by 59.3 ± 1.3% and also increased the level of PSD-95 by 71.6 ± 3.8%. Knockdown of PKCe had opposite effects (Fig. 6, B and C). PKCe overexpression or knockdown did not alter SNAP-25 and syntaxin-1 mRNA levels (Fig. 6, D and E). Loss of PKCe expression reduced the protein levels of PSD-95 by 30% (0.71 ± 0.07; p = 0.043 (Fig. 6K) and synaptophysin by 44% (0.56 ± 0.08; p = 0.021) (Fig. 6I) compared with controls transfected with scram-

FIGURE 5. PKCe activation induces synaptogenesis. Adult rat hippocampal organotypic slices were treated with ethanol (C), bryostatin 1 (0.27 nM), or DCPLA-ME (100 nM) for 1 and 4 h, A, electron micrographs of the stratum radiatum in the hippocampal CA1 area (100 μm² CA1 area at ×5000) treated with bryostatin 1 and DCPLA-ME for 4 h. 30–35 CA1 areas each from three different hippocampal slices were analyzed. Dendritic spines showing synapse are highlighted in yellow. B, PKCe activation increased the synapse number at 4 h (F = 9.05; ANOVA p < 0.0005) in bryostatin 1 (8.97 ± 0.63, p < 0.005, n = 34 CA1 area) and DCPLA-ME (6.97 ± 0.5; p < 0.05, n = 30 CA1 area)-treated slices compared with control (5.77 ± 0.50). Typical traces of EPSPs evoked at a stimulus intensity of 200 μA from bryostatin 1-treated hippocampal slices after 1 h (C) and 4 h (D). The input-output response, reflecting basal synaptic transmission, increased after treatment with bryostatin 1 (0.27 nM) after 1 h (D) and 4 h (G). Areas under the curves (AUC) were calculated to compare the basal levels of synaptic transmission. Bryostatin 1 increased the EPSP slope significantly at 1 h and 4 h (E and H). Data are represented as the mean ± S.E. of three independent experiments (Student’s t test, *, p < 0.05; **, p < 0.005; ***, p < 0.0005).
bled siRNA. PKCε OE produced a 50% increase in synaptophysin (1.51 ± 0.1 versus 1.0 ± 0.1 in control; *p* = 0.015) (Fig. 6A) and a 30% increase in PSD-95 expression (1.31 ± 0.08 versus 1.0 ± 0.07 in control; *p* = 0.024) compared with vector-only transfected cells (Fig. 6D).

**Knockdown of PKCε Reduces Synaptogenesis**—To further establish the role of PKCε in synaptogenesis and its underlying role in expression of PSD-95 and synaptophysin we used confocal microscopy to measure the effect of PKCε knockdown on the localization of PSD-95 and colocalization of PSD-95 and synaptophysin. Punctate colocalization (clusters of proximal pre- and post-synaptic markers on neurites) of PSD-95 and synaptophysin is widely accepted as an indicator of synapses (43, 44). Primary human neurons were treated with bryostatin 1 or DCPLA-ME alone or after PKCε KD for 10 days. PSD-95 clusters and colocalized PSD-95 and synaptophysin (as recognized by staining grains along a 40-μm length of neurite, *n* = 10) were counted in 4 independent experiments (Fig. 7A). In normal cells, PKCε activation by bryostatin 1 and DCPLA-ME significantly induced PSD-95 clustering in the neurites compared with untreated controls (*p* < 0.05) (Fig. 7B). The number of synapses was also significantly higher in cells treated with bryostatin 1 and DCPLA-ME than in untreated neurons at 10 days (Fig. 7C). The increase in the number of synapses was independent of the neuron density. We found no change in neuron density (measured by NeuN staining) after 10 days of PKCε activator treatment (supplemental Fig. 1). In PKCε KD cells, immunofluorescence staining of human neurons showed a loss of synaptic networks, and bryostatin 1 and DCPLA-ME had no effect. PKCε KD prevented the effect of PKCε activators, and more importantly, reduced the basal level of PSD-95 clusters and synapses by 50%. (Fig. 7, A–C).
We also quantified the expression levels of PKCe, p-PSD-95S295, PSD-95, and synaptophysin by immunoblot after 10 days of PKCe-siRNA transfection (Fig. 7D). PKCe KD cells expressed significantly lower amounts of PSD-95 (F(5,12) = 19.24; ANOVA p < 0.0001) (Fig. 7, E and F) and synaptophysin (F(5,12) = 12.79; ANOVA p = 0.0002) (Fig. 7G). Bryostatin 1 and DCPLA-ME failed to induce PSD-95 and synaptophysin expression in PKCe KD neurons. Bryostatin 1, but not DCPLA-ME, produced a 40% decrease in PKCe protein staining (Fig. 7D). No loss in PKCe mRNA was found in bryostatin 1-treated neurons (data not shown). Down-regulation of PKCe after activation by bryostatin 1 is a well documented phenomenon (45, 46).
We further confirmed the effect of long term PKCe activation on synaptogenesis using rat hippocampal brain slices. Slices were treated with bryostatin 1 and DCPLA-ME for 10 days. The serum-free culture medium was changed every 3 days with fresh additions of activators. Synapse number in each case was quantified using electron microscopy (Fig. 8A). Bryostatin 1 (7.97 ± 0.68, p = 0.013, n = 29 CA1 areas) and DCPLA-ME (8.71 ± 0.78, p = 0.001, n = 24 CA1 areas) treatment increased the number of synapses in hippocampal slices compared with vehicle-only treated slices (4.5 ± 0.45; n = 24 CA1 area) (Fig. 8B). Presynaptic vesicle density was also significantly higher in the bryostatin 1 (59.6 ± 6.4, p < 0.05, n = 19 presynaptic boutons)- and DCPLA-ME (60.4 ± 5.1, p = 0.04, n = 19 presynaptic boutons)-treated slices than vehicle-treated controls (48.4 ± 4.3, n = 20 presynaptic boutons) (Fig. 8, C and D). Together, these findings confirm that PKCe is essential for bryostatin 1- and DCPLA-ME-mediated increase in PSD-95 and synaptophsin expression leading to increased synaptogenesis at 10 days.

**Discussion**

The outgrowth of neurites and formation of synapses depends on interactions among a number of regulatory proteins. These interactions are required for synaptic structure rearrangement, spinogenesis, and synaptogenesis. PKCe is one of the key regulators of synaptogenesis (3, 24), and PKCe activators promote the maturation of dendritic spines (9, 47). PSD-95 is a scaffold protein that also plays an important role in formation of excitatory synapses (48, 49).

Here we showed that PKCe activation induces translocation and phosphorylation of PSD-95 at the serine 295 residue leading to PSD-95 accumulation at the postsynaptic density. Our findings showed that PKCe activation not only increased the survival of neurons but also preserved the neuronal structure. Untreated cells showed gradual degeneration over 25 days, suggesting that PKCe activation is beneficial for both maturation and survival of neurons, confirming a previous report by Hama et al. (14). We have shown that short term acute changes in PKCe activity induce structural and biochemical changes in post-synaptic density scaffolding protein PSD-95 as well as increased synaptic activity. Synaptic activity is important for neuronal survival. Synaptic activity induces expression of survival genes and suppresses pro-death genes (50). Therefore, the increased survival of neurons treated with PKCe activators may be due to the increased connectivity induced in the early stages; however, other factors such as elevated neurotrophins may also play a role. PKCe induces BDNF (10, 19), and elevated expression and release of BDNF is associated with elevated synaptic activity, which contributes to neuroprotection (51, 52).

PKCe activation and membrane translocation occur both presynaptically (14, 53) and postsynaptically (8) where it phosphorylates PKCe.
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phorylates important substrate proteins required for synaptic facilitation and synaptogenesis. We found that p-PSD-95S295 accumulation increased in the membrane of PKCe-activated neurons and followed the same time course as PKCe activation at 1 h and 4 h. The serine 295 residue was essential for the PKCe-mediated membrane accumulation of PSD-95. In vitro, PKCe phosphorylated both PSD-95 and JNK1. The JNK1 inhibitor also prevented PKCe activation-mediated increase in p-PSD-95S295, confirming previous findings showing that serine 295 phosphorylation of PSD-95 is regulated by Rac1-JNK1 and PP1/PP2A signaling (33, 54). PKCe is involved in JNK activation; PKD, a downstream effector of PKC, also regulates JNK (38, 39, 55). Knockdown of either PKCe or JNK1 inhibited the PKCe activator-mediated p-PSD-95S295 accumulation in the membrane, thus confirming that PKCe and JNK act collectively in regulating PSD-95. Although it has been reported that synaptic localization of PSD-95 is regulated by JNK signaling and not by CaMKII (33, 56), our data demonstrate a role of both JNK1 and CaMKII. This is possible as PKC activation induces a simultaneous translocation of CaMKII to synapses (21), and CaMKII activation is needed for PSD-95-induced synaptic strengthening (22). CaMKII is a downstream target of PKCe in many pathways, including the events responsible for the induction of neuroplastic changes associated with hyperalgesic priming (57). In this study we found that both PKCe and CaMKII inhibitors prevented the PKCe-mediated membrane association of p-PSD-95S295. These results suggest that PKCe and CaMKII are downstream to PKCe in events responsible for phosphorylation and membrane accumulation of PSD-95.

We also demonstrated that PKCe activation increases the levels of PSD-95 and the number of synapses. In adult hippocampal slices, bryostatin 1 increased basal synaptic activity. Our results indicate an important link between PKCe activation and the membrane localization of PSD-95, specifically enriching the membrane with the p-PSD-95S295 form, which is known to strengthen the excitatory synapses (33). PSD-95 also regulates membrane insertion of AMPA receptor and dendritic spine morphology during synaptic plasticity (22, 30–32).

Overexpression of PSD-95 converts silent synapses to functional synapses (58), whereas synaptophysin may be required for increased presynaptic vesicle density, thereby facilitating neurotransmitter release (59). We found that overexpressing PKCe in primary human neurons induces the mRNA and protein levels of PSD-95 and synaptophysin, whereas knockdown of PKCe reduces PSD-95 and synaptophysin mRNA and protein levels. Our results indicate that PKCe regulates the gene expression of PSD-95 and synaptophysin. PKCe may play a critical role in synapse maintenance by regulating the synthesis of PSD-95 and synaptophysin (18). PKCe is known to drive the mitogenic response and DNA synthesis (60) via the Raf-MEK-ERK cascade and regulates transcription of essential genes through JNK/AP1, NF-κB, and JAK/STAT cascades (61, 62). PSD-95 is a critical transcriptional target of NF-κB, which is known to induce excitatory synapse formation and regulate dendritic spine formation and morphology in murine hippocampal neurons (63). Synaptophysin mRNA expression is induced by the BDNF-cFos pathway (64). NF-κB and synaptophysin have a common regulator in BDNF (65). PKCe up-regulates BDNF expression (19–20, 66).

In conclusion, PKCe has two specific roles in synaptogenesis; at the postsynaptic site it regulates PSD-95, either directly or through JNK1 and CaMKII, and at the presynaptic site it induces the expression of synaptophysin. Repeated treatment with PKCe activators induces synthesis of PKCe, PSD-95, and synaptophysin, resulting in an increased number of synapses. PKCe knockdown inhibits the synthesis of PSD-95 and synaptophysin leading to a reduced number of synapses. Besides the PKC-JNK1/CaMKII-PSD-95 pathway, PKCe can also induce synaptogenesis through the HuD-BDNF pathway. PKCe stabilizes HuD, which increases the stability and rate of translocation of target mRNAs. HuD increases as a result of PKCe activation after learning (67) and stabilizes the mRNA for BDNF, nerve growth factor (NGF), and neurotrophin-3 (NT-3) (19). PKCe activation induces the synthesis of BDNF (10, 20, 47), and BDNF induces transport of PSD-95 to the dendrites (68), which is required for maintenance of mature spines (69). Deficits of PKCe function could also contribute to the synapse loss in Alzheimer disease (15), whereas the therapeutic elimination of such deficits may offer a strategy for the treatment of synaptic loss in Alzheimer disease and other synaptic disorders.

Experimental Procedures

Materials—Bryostatin 1 was purchased from Biomol International (Farmingdale, NY). DCPLA-ME was synthesized in our laboratory following the method described earlier (34, 70) and shown to be specific for PKCe. Primary antibodies (rabbit polyclonal anti-PKCε (sc-214), rabbit polyclonal anti-PKCα (sc-208), rabbit polyclonal anti-PKCβ (sc-213), mouse monoclonal anti-synaptophysin (sc-17750), and mouse monoclonal anti-β-actin (sc-47778)) were obtained from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA). Rabbit polyclonal anti-synaptophysin (TA300431) and rabbit polyclonal anti-phospho-PSD-95 (serine 295) (TA303850) were obtained from Origene (Rockville, MD), and rabbit polyclonal anti-PSD-95 (sc#450) and rabbit polyclonal anti-JNK1/2 (#9258) were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Chicken polyclonal anti-NeuN (ab134014) was obtained from Abcam (Cambridge, MA). All secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). The anti-chicken Cy5 conjugated antibody was purchased from Abcam. Bisindolylmaleimide I (Go 6850) and PKCe translocation inhibitor (EAVSLKPT) were obtained from Santa Cruz Biotechnology, and SP600125 and KN-93 were obtained from Cell Signaling Technology.

Cell Culture—Human primary neurons (hippocampal neurons, catalogue #1540, ScienCell Research Laboratories, Carlsbad, CA) were plated on poly-L-lysine-coated plates and were maintained in neuronal medium (ScienCell) supplemented with the neuronal growth supplement (NGS, ScienCell). For maintenance of neurons half of the media was changed every 3 days. Fresh activators were added with every media change. Human HEK-293 cells were obtained from ATCC, Manassas, VA. Cells were maintained in Eagle's minimum essential medium and 10% fetal bovine serum.
Organotypic Slice Culture—Organotypic hippocampal slices were prepared mainly according to the method described by Stoppini et al. (71) with slight modifications (72). Rats were sacrificed and immediately decapitated under sterile conditions. Brains were rapidly removed and placed into a chilled dissection medium composed of Hibernate A (BrainBits, Springfield, IL), 2% B27 supplement, 2 mM L-glutamine by GlutaMax and antibiotic-antimycotics (all from Invitrogen). The hippocampi were dissected out in fresh chilled dissection medium. Isolated hippocampi were washed in new chilled dissection medium and placed on a wet 3-mm paper on the Teflon stage of a manual tissue slice chopper (Vibratome Co., Saint Louis, MO) for coronal sectioning at 300 μm. Each slice with intact pyramidal and granular layers was transferred to one membrane insert (Millipore, Bedford, MA) in 12-well plates containing Neurobasal A, 20% horse serum, 2 mM L-glutamine, and antibiotics-antimycotics for 4 days. For long term maintenance slices were cultured in serum-free medium consisting of Neurobasal A with 2% B27, 2 mM L-glutamine, and antibiotic-antimycotics. Slices were incubated in a humidified 5% CO2 atmosphere at 37 °C. The entire medium was replaced with fresh medium at day 1. After that, half the medium was removed and replaced with fresh medium twice a week.

Cell Lysis and Western Blotting Analysis—Cells were harvested in homogenizing buffer containing 10 mM Tris-Cl (pH 7.4), 1 mM phenylmethylsulfonyl fluoride), 1 mM EGTA, 1 mM EDTA, 50 mM NaF, and 20 μM leupeptin and lysed by sonication. The homogenate was centrifuged at 100,000 × g for 15 min at 4 °C to obtain the cytosolic fraction (soluble) and membrane (particulate). The pellet was resuspended in the homogenizing buffer by sonication. For whole cell protein isolation from primary neurons the homogenizing buffer contained 1% Triton X-100. Protein concentration was measured using the Coomassie Plus (Bradford) Protein Assay kit (Pierce). After quantification, 20 μg of protein from each sample was subjected to SDS-PAGE analysis in a 4–20% gradient Tris-glycine polyacrylamide gel (Invitrogen). The separated protein was transferred to a nitrocellulose membrane. The membrane was finally washed 3× then blocked with BSA and incubated with primary antibody overnight at 4 °C. All the primary antibodies were used at a 1:1000 dilution except rabbit polyclonal anti-p-PSD-95S295 (1:10,000) and rabbit polyclonal anti-synaptophysin (1:1000). After incubation, it was washed 3× with Tris-buffered saline-Tween 20 and further incubated with alkaline phosphatase-conjugated secondary antibody at 1:10,000 dilution for 45 min. The membrane was finally washed 3× with Tris-buffered saline-Tween 20 and developed using the 1-step NBT-BCIP (Sigma) was incubated for 15 min at 37 °C in the presence of 10 ng of JNK1 or 100 ng of PSD-95 or 100 ng of CaMKII, 4.89 mM CaCl2, 1.2 μg/μl phosphatidyl-l-serine, 0.18 μg/μl 1,2-dioctanoyl-sn-glycerol, 10 mM MgCl2, 20 mM HEPES (pH 7.4), 0.8 mM EDTA, 4% glycerol, 8 μg/ml aprotinin, 8 μg/ml leupeptin, 0.9 μCi of [γ-32P]ATP. [32P]Phosphoprotein formation was measured by adsorption onto phosphocellulose as described previously (70).

Knockdown and Overexpression—Human PSD-95 was cloned into pCDNA3.1 plasmid (GenScript, Piscataway, NJ). Mutant PSD-95 mutated at serine 295 residue was also cloned into pCDNA3.1 plasmid and was obtained from GenScript. PKCe knockdown was done using PKCe-siRNA constructs purchased from Santa Cruz Biotechnology. JNK knockdown was done using SAPK/JNK-siRNA from Cell Signaling Technology. Overexpression of PKCe was obtained by transfecting pCMV6-ENTRY vector containing human PKCe cDNA (Origene). Transfection was done using Lipofectamine 3000 (Invitrogen). Medium was changed after 6 h of Lipofectamine treatment. Protein expression was measured after 72 h of transfection.

Electrophysiology—Rats were housed under 12-h light/12-h dark cycles and had free access to food and water. They were anesthetized with intra-peritoneal injection of sodium pentobarbital (50 mg/kg body weight). After recording, the hippocampi were removed and transferred to ACSF at room temperature for 1 h until recording (ACSF: 124 mM NaCl, 3 mM KCl, 1.2 mM MgSO4, 2.1 mM CaCl2, 1.4 mM NaH2PO4, 26 mM NaHCO3, and 20 mM dextrose, saturated with 95% O2 and 5% CO2, which maintains the pH at 7.4). Slices were treated with ethanol or bryostatin 1 for 1 h or 4 h. All recordings were made at room temperature. For synaptic stimulation and field EPSP recordings, pyramidal neurons in the CA1 field were identified with an Olympus BX50WI microscope. Field potential recordings were determined to measure synaptic function. A bipolar stimulating electrode (100-μm separation, FHC, Bowdoinham, ME) was placed in the hippocampal Schaffer collateral pathway to elicit EPSPs in CA1 stratum radiatum, EPSPs were recorded through patch pipettes (2–5 megohms, 1.5 mm outer diameter, 0.86 mm inner diameter, P87 Brown-Flaming Puller, Sutter Instruments) filled with ACSF. All parameters including pulse duration, width, and frequency were computer-controlled. Constant-current pulse intensity was controlled by a stimulus isolation unit. Basal synaptic transmission, represented by input-output responses, was determined by the slopes of stabilized EPSP to different stimulus intensities. The strength of EPSPs was assessed by measuring the slopes (initial 20–80%) of the EPSPs rising phase.

PKC Assay—To measure PKC activity, 100 ng of recombinant PKCe (Sigma) was incubated for 15 min at 37 °C in the presence of 100 ng of JNK1 or 100 ng of PSD-95 or 100 ng of CaMKII, 4.89 mM CaCl2, 1.2 μg/μl phosphatidyl-l-serine, 0.18 μg/μl 1,2-dioctanoyl-sn-glycerol, 10 mM MgCl2, 20 mM HEPES (pH 7.4), 0.8 mM EDTA, 4% glycerol, 8 μg/ml aprotinin, 8 μg/ml leupeptin, 0.9 μCi of [γ-32P]ATP. [32P]Phosphoprotein formation was measured by adsorption onto phosphocellulose as described previously (70).

Quantitative Real-time-PCR—Quantitative real-time-PCR was done following the method described earlier (13). Total RNA (500 ng) was reverse-transcribed using oligo(dT) and Superscript III (Invitrogen) at 50 °C for 1 h. The cDNA products were analyzed using a LightCycler 480 II (Roche Applied Science) PCR machine and LightCycler 480 SYBR Green 1 master mix following the manufacturer’s protocol. Primers for PKCe (forward primer, AGCCTCGTTCACCGGTCTATGC; reverse primer, GCATGACCTTTCTGGACCA), PKCe-95 (forward primer, TCACACTTGACAGTGACACCGA; reverse primer, GCATCGTGTCTGAGACCA), knockdown (forward primer, TGGCCTTTGGTAAGGGTGCAC; reverse primer, TCACCTCTGGTCTGTTGGGAC), SNAP-25 (forward primer, CCTCGTATGTCGACAACTGTGTG; reverse primer, GGTTCATGCCTTCTGACAG), Syntaxin-1 (forward primer, TGGAGAACAGCATCCGTGAGCT; reverse primer, GGTTCATGCCTTCTGCATCCAG).
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primer, CCTCTCCACATAGTCTACCGC; GAPDH (forward primer, GTCTCTCTCTGACTCTCAACAGGC; reverse primer, ACCACCTGGTCTGGTAGGCAA) were purchased from Origene.

Electron Microscopy—Electron microscopy of slices were done following methods described earlier (9). Hippocampi were sectioned with a vibratome at 100 μm. Hippocampi were fixed in 1% OsO4. Electron micrographs (100 μm2 CA1 area at × 5000) were made of Epon-embedded hippocampal sections with a JEOL 200CX electron microscope. These sections were 90 nm thick and had been previously stained with uranyl acetate and lead citrate. During quantification, electron micrographs were digitally zoomed up to ×20,000 magnification. Spines were defined as structures that formed synapses with axon boutons and did not contain mitochondria. Presynaptic vesicle density was measured from within the presynaptic spines of diameter >600 nm. Increased numbers of presynaptic vesicles in axon boutons were measured as an increase in the frequency of axon boutons with presynaptic vesicles that occupied >50% of the cross-section space not occupied by other organelles.

Immunofluorescence and Confocal Microscopy—Cells were grown in four-chambered slides (Nunc) at low density. For immunofluorescence staining the cells were washed with PBS (pH 7.4) and fixed with 4% paraformaldehyde for 4 min. After fixation, cells were blocked and permeabilized with 5% horse serum and 0.3% Triton X-100 in 1× PBS for 30 min. Cells were washed 3× with 1× PBS and incubated with primary antibodies (rabbit polyclonal anti-PSD-95, mouse monoclonal anti-synaptophysin, and chicken polyclonal anti-NeuN) for 1 h at 1:100 dilution. After the incubation slides were again washed 3× in 1× PBS and incubated with the FITC anti-rabbit IgG, rhodamine anti-mouse IgG, and Cy5 anti-chicken IgY for 1 h at 1:400 dilution. Cells were further washed and mounted in Pro Long Gold antifade mounting solution (Invitrogen). Stained cells were viewed under the LSM 710 Meta confocal microscope (Zeiss) at 350-, 667-nm emission for DAPI, FITC, rhodamine, and Cy5, respectively. Six individual fields at 40 or 63× oil lens magnification were analyzed for the mean fluorescence intensity in each channel. Punctate colocalization was done following the methods described earlier (43, 44).

Statistical Analysis—All experiments were performed at least three times. Data are represented as the mean ± S.E. All data were analyzed by one-way ANOVA and Newman-Keuls multiple comparison post test. Significantly different paired groups were further analyzed by two-tailed Student’s t test using GraphPad Prism 6.1 software (La Jolla, CA). p values < 0.05 were considered statistically significant.

Author Contributions—A.S., T. J. N., and D. L. A. designed the study and wrote the paper. A. S. performed and analyzed all the biochemical and immunofluorescence experiments. J. H. performed and analyzed all the electron microscopy data. D. W. performed and analyzed the electrophysiology experiments.

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