Selective Association of Protein Kinase C with 14-3-3 \( \zeta \) in Neuronally Differentiated PC12 Cells

STIMULATORY AND INHIBITORY EFFECT OF 14-3-3 \( \zeta \) IN VIVO*

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The 14-3-3 protein is a family of highly conserved acidic proteins found in a wide range of eukaryotes from yeast to mammals. 14-3-3 acts as an adapter protein and interacts with signaling molecules including protein kinase C (PKC). Although 14-3-3 \( \zeta \) was originally characterized as an endogenous PKC inhibitor, it was reported to activate PKC in vitro, but the in vivo regulation of PKC by 14-3-3 is still not well understood. To examine the regulation of PKC by 14-3-3, we have established a sub-cell line, PC12-B3, that stably expresses FLAG epitope-tagged 14-3-3 \( \zeta \) isoform in PC12 cells. Here we show that PKC-\( \alpha \) and PKC-\( \epsilon \) become associated with 14-3-3 \( \zeta \) when the cells are neuronally differentiated by nerve growth factor. We found that the immunoprecipitate by anti-FLAG antibody contains constitutive and autonomous Ca\( ^{2+} \)-independent classical PKC activity. In contrast, the FLAG immunoprecipitate has no Ca\( ^{2+} \)-dependent classical PKC activity despite the fact that PKC-\( \alpha \) is present in the FLAG immunoprecipitate from differentiated PC12-B3 cells. Our results show that the association with 14-3-3 \( \zeta \) has distinct effects on classical PKC and non-classical PKC activity.

The 14-3-3 protein, a family of acidic 30-kDa proteins, is ubiquitous and highly enriched in the central nervous system (1). It is estimated that \(-1\%\) of the total cytosolic proteins expressed in the mammalian brain is 14-3-3 (2). 14-3-3 acts as an adapter protein and interacts with signaling molecules to mediate a wide variety of cellular events such as cell cycle regulation, cell growth and differentiation, anti-apoptosis, and synaptic transmission (For review, see Refs. 3–5). The 14-3-3 protein is conserved among species ranging from yeast to human and normally exists as a dimer. In the brain cytosol, seven 14-3-3 subspecies (\( \alpha \), \( \beta \), \( \gamma \), \( \delta \), \( \epsilon \), \( \eta \), and \( \zeta \)) were chromatographically separated, and of these, \( \alpha \) and \( \delta \) were found to be phosphorylated forms of \( \beta \) and \( \zeta \), respectively (6). In addition, the \( \theta \) isoform was also found in the rat brain (7) and was originally discovered in the T cell line as 14-3-3 \( \theta \) (8). Thus, at least six 14-3-3 isoforms are present in the mammalian brain.

Leonardo, a Drosophila gene homologue of the vertebrate 14-3-3 \( \zeta \), is expressed in abundance in mushroom bodies, and with low levels of expression of Leonardo show deficiency in olfactory learning (9). The Leonardo protein is also highly enriched in the presynaptic boutons in the fly neuromuscular junction (10). Significant reduction of glutamate synaptic transmission has been observed at the neuromuscular junctions of Leonardo (\( \sim \) null mutant larvae, and this effect is attributed to a presynaptic mechanism (10). Consistent with this notion, the Leonardo protein was recently shown in Drosophila neuromuscular junction to form a complex with the Ca\( ^{2+} \)-dependent K\( ^{+} \) channel, Slowpoke, at the presynaptic nerve terminals (11). Although very little is known regarding the function of vertebrate 14-3-3 \( \zeta \) in neuronal cells, several lines of evidence indicate that it plays a role in synaptic function in vertebrates as well. Immunological and biochemical studies show that mammalian 14-3-3 \( \zeta \) localizes in synapses in the rat brain (12). The 14-3-3 protein was involved in exocytosis; it stimulates Ca\( ^{2+} \)-dependent catecholamine release from digitoni-permeabilized adrenal chromaffin cells (13–15), in which 14-3-3 \( \zeta \) is one of the major isoforms (16).

One of the target molecules of 14-3-3 is protein kinase C (PKC). 14-3-3, originally discovered as an activator protein for tyrosine hydroxylase and tryptophan hydroxylase (17), was isolated as an endogenous kinase \( \zeta \) inhibitory protein (KCIP) (18). It was later found that KCIP was identical to the 14-3-3 protein (19). However, it has been reported that 14-3-3 \( \zeta \) activates rather than inhibits PKC in vitro (20, 21). Thus, although 14-3-3 was initially characterized as a PKC inhibitor, its effect on PKC is still not clear. Because PKC is critical for neuronal differentiation in PC12 cells (22–26), we have investigated whether the interaction between PKC and 14-3-3 \( \zeta \) takes place and how it affects the PKC activity in neuronal cells. For this purpose, we have established a sub-cell line of PC12 cells (PC12-B3 cells) that stably express FLAG epitope-tagged 14-3-3 \( \zeta \). Here we show the selective association of 14-3-3 \( \zeta \) with PKC-\( \alpha \) and PKC-\( \epsilon \) in NGF-differentiated PC12-B3 cells. Furthermore, we found that the 14-3-3 \( \zeta \) immunoprecipitate by anti-FLAG antibody from the NGF-differentiated cells contains Ca\( ^{2+} \)-independent non-classical PKC (nPKC) activity. This nPKC is constitutively active and does not require any addition of PKC activators. Such constitutive activation of nPKC in the 14-3-3 \( \zeta \) complex might play an important role in neuronal differentiation of PC12 cells by NGF.

**EXPERIMENTAL PROCEDURES**

14-3-3 \( \zeta \) cDNA—14-3-3 \( \zeta \) cDNA isolated from rat hippocampal cDNA library (pB5BN7, Ref. 27) and subcloned in mammalian expression vector pcDNA3 (clone pc14(12)) was used for the study. FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Lys) was tagged at the C terminus of cDNA. The vector pcDNA3 (clone pc14(12)) was used for the study. FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Lys) was tagged at the C terminus of cDNA. The vector pcDNA3 (clone pc14(12)) was used for the study. FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Lys) was tagged at the C terminus of cDNA. The vector pcDNA3 (clone pc14(12)) was used for the study. FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Lys) was tagged at the C terminus of cDNA.

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Reverse Transcription-PCR—Rat 14-3-3-ζ has three protein variant forms (Met6Ala1106, Thr209Ala1106, and Thr209Arg1106) (27). To determine which 14-3-3-ζ isoform is expressed in PC12 cells, we performed reverse transcription-PCR. Total RNA from PC12 cells was isolated using RNA isolation kit (Ambion) and reverse-transcribed for cDNA synthesis by avian myeloblastosis virus reverse transcriptase using oligo-dT primer. Endogenous 14-3-3-ζ cDNA was amplified by PCR and sequenced.

Cell Culture and Differentiation by NGF—PC12 cells were cultured in collagen-coated dishes containing 15 ml of RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum in a humidified incubator (95% air, 5% CO2) at 37 °C (28).

RESULTS

Stable Expression of FLAG Epitope-tagged 14-3-3-ζ—Our previous study showed that there are several variants of 14-3-3-ζ cloned from mammalian brain. These variations are composed of the amino acids Metζ Thr at position 88, Alaζ Arg at 109, and Proζ Ala at 112. In the rat brain, Metζ Thrζ and Alaζ Argζ 14-3-3-ζ variants are present (27). Because the 14-3-3 protein binds to a variety of signaling molecules and acts as an adapter/scaffold protein, these variations may have effects on partner proteins for the interaction. To avoid the ambiguity due to the 14-3-3-ζ protein variants, we have identified the endogenous 14-3-3-ζ in PC12 cells by reverse transcription-PCR. This analysis showed that endogenous 14-3-3-ζ contains Metζ, Alaζ, and Proζ at the respective positions. We have added a FLAG epitope tag to this variant 14-3-3-ζ and expressed the variant in PC12 cells. To establish PC12 sub-cell lines that stably express the FLAG-epitope-tagged 14-3-3-ζ were selected based on the neomycin resistance. The transfected cells were grown in the presence of 500 μg/ml G418 for 6 days, and the surviving single cells were removed and expanded in a collagen-coated 12-well plate in serum-containing preconditioned RPMI 1640 media.

Immunoblot Analysis—Immunoblot analysis was carried out by a standard procedure (29). Anti-FLAG (Eastman Kodak Co, Sigma), anti-14-3-3-ζ (Santa Cruz), and isoform-specific anti PKC antibodies (Transduction Laboratories, Santa Cruz) were used for the study. Horseradish peroxidase-conjugated anti-mouse (Amresco, Santa Cruz) or anti-rabbit IgG (Amersham Biosciences) were employed as the secondary antibody, and the blot was visualized using the ECL method (Amersham Biosciences). In some experiments that required quantification, we directly measured chemiluminescence using FluorO Multi-imager (Bio-Rad) and analyzed by Quantity 1 program.

Immunoprecipitation—The following immunoprecipitation procedure was carried out at 4 °C. Cells grown on 100-mm collagen-coated dishes were washed with phosphate-buffered saline twice before lysis. RIPA buffer containing protease inhibitors (20 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml chymostatin, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride) were added for cell lysis and incubated for 30 min. The cell lysate was collected, triturated, and centrifuged at 1000 × g for 10 min. To precipitate the cell lysate, the supernatant was mixed with 20 μl of protein A beads (Invitrogen), incubated for 30 min while rocking, and centrifuged for 15 min at 10000 × g. Preclreated samples were incubated with a primary antibody for 2 h with rocking, and then protein A beads were added, incubated for 1 h, and centrifuged at 10000 × g. The immunoprecipitates were collected and washed three times with RIPA buffer.

PKC Assay for Immunoprecipitates—The activity of PKC of the immunoprecipitates with FLAG antibody from differentiated or undifferentiated cells was measured using two PKC assay systems that were optimized for Ca2+-dependent cPKC activity and for Ca2+-independent non-classical and atypical PKC activities (hereafter cPKC and nPKC assay, respectively). The FLAG immunoprecipitates for PKC assay were prepared from cell culture grown in 100-mm collagen-coated dish. The precipitated samples were washed 8 times with ice-cold dilution buffer (20 mM Tris-HCl, 1 mM EGTA, 1 mM diithiothreitol, pH 7.4), centrifuged at 12000 × g, and then diluted to 250 μl with ice-cold dilution buffer. Ten μl of the aliquot was assayed to measure PKC activity in the presence of 5 mM MgCl2, 1 mM EGTA, 20 mM Tris-HCl, pH 7.4, with the appropriate concentrations of Ca2+, PKC substrate, and PKC activators or inhibitors as specified below in the figure legends. For Ca2+-dependent cPKC assay, 400 μg/ml lysine-rich histone (Type III Sigma) was used for the assay at 500 μM free Ca2+. nPKC assay was performed in the absence of Ca2+ using 100 μg/ml peptide-e (Bacham) as a PKC substrate in the presence of 1 mM EGTA. The selection of PKC substrates was based on the difference in specificity between cPKCs and nPKCs. A lysine-rich histone is a good substrate of PKC-α, -β, and -γ and can be readily phosphorylated by these cPKCs, but it is a poor substrate for Ca2+-independent PKCs (30). In contrast, nPKC activity is poor toward histone, but it effectively phosphorylates peptide-e (30). For calcium dependence experiments, total Ca2+ concentrations to achieve appropriate free Ca2+ concentrations were controlled by taking account of multiple equilibria with EGTA, ATP, and Mg2+ (31). A phosphorylation reaction was initiated by adding [γ-32P]ATP (final concentration, 75 μCi; 150–200 cpm/pmol) to a reaction tube in a total volume of 50 μl. After a 20 min incubation at 30 °C, protein A-Sepharose beads in the reaction mixture were centrifuged by pulse spin, 30 μl of the supernatant was spotted onto phosphocellulose paper, and the radioactivity was counted after the wash.
ferentiated cells. In contrast, co-immunoprecipitation of PKC-ε and FLAG-14-3-3ζ cannot be detected from undifferentiated cells, even though both are present in these cells, and the levels of expression of 14-3-3ζ and PKC-ε remain constant during differentiation.

To examine whether PKC-ε associates not only with the recombinant 14-3-3ζ but also with the endogenous 14-3-3ζ in a manner dependent on neuronal differentiation, we prepared immunoprecipitation samples using anti-14-3-3ζ antibody from undifferentiated and NGF-differentiated PC12 cells. As shown in Fig. 2, endogenous 14-3-3ζ co-immunoprecipitates with PKC-ε only from NGF-differentiated cells, but the co-immunoprecipitation is not detected from undifferentiated PC12 cells. Thus, PKC-ε interacts with both the recombinant FLAG-14-3-3ζ as well as with the endogenous 14-3-3ζ in neurally differentiated PC12 cells but not in undifferentiated cells.

**Slow Onset of PKC-ε/14-3-3ζ Complex Formation**—One of the key questions regarding the association of PKC-ε with 14-3-3ζ is whether this complex formation is acutely induced by the receptor activation by NGF or if it is a reflection of the differentiated state of PC12 cells. To determine this, we measured the time course of the complex formation of PKC-ε and 14-3-3ζ by examining FLAG immunoprecipitates from PC12 B-3 cells treated with NGF with a time course of 5 min to 8 days. Co-immunoprecipitation of PKC-ε and FLAG 14-3-3ζ can be seen only after 5 days of NGF treatment, as shown in Fig. 3. This slow onset of the association indicates that the complex formation is not due to the acute effect of stimulation of NGF receptors, but it is associated at the final differentiation stage of PC12 cells.

**Differentiation-dependent and -independent Interaction between 14-3-3ζ and PKC**—We have tested whether the PKC-ε/14-3-3ζ interaction is specific to this PKC isoform. Among the immunoreactivity found in this sub-cell line, we have found that PKC-α, δ, ε, ζ, and μ isoforms also associate with FLAG-14-3-3ζ, as shown in Fig. 4. However, the interaction of these PKCζ is isoforms with 14-3-3ζ is different with respect to differentiation. PKCζ and μ isoforms were immunoprecipitated with FLAG antibody regardless of differentiation, suggesting that they interact with 14-3-3ζ constitutively. PKC-δ is also present in the FLAG immunoprecipitates from both undifferentiated and differentiated cells. However, the association of PKC-ε with FLAG-14-3-3ζ shows a consistent increase during differentiation (n = 3), suggesting that the interaction of this PKC isoform with 14-3-3ζ could also be sensitive to NGF-induced neuronal differentiation. Contrary to the PKCζ, μ, and δ isoforms, co-immunoprecipitation of PKC-α with 14-3-3ζ can be detected only when the cells were neurally differentiated. We could not detect PKC-α immunoreactivity, however, in the FLAG immunoprecipitate prepared from undifferentiated cells, indicating that either this isoform is not present in the immunoprecipitate or the level is too low for detection. Thus, the interaction between PKC-α and 14-3-3ζ in PC12-B3 cells depends on the state of differentiation. These experiments show that PKC isoforms bind to 14-3-3ζ in a differentiation-dependent as well as -independent manner. The results are summarized in Table I.

**Constitutive nPKC Activity in the FLAG Immunoprecipitate from Neuronally Differentiated Cells**—The FLAG immunoprecipitate contains several PKC isoforms, Ca²⁺-dependent PKC-α and Ca²⁺-independent PKC-δ, ε, ζ, and μ in NGF-differentiated PC12-B3 cells. To distinguish Ca²⁺-dependent and Ca²⁺-independent PKC activities, we developed assays that detect these PKC activities in the FLAG immunoprecipitates as described under “Experimental Procedures.” Using these assays, we measured the PKC activity in the FLAG immunoprecipitates from differentiated and undifferentiated PC12 B-3 cells. Parent PC12 cells that do not express FLAG epitope-tagged 14-3-3ζ was used as a negative control. As shown in Fig. 5, significant Ca²⁺-independent PKC activity was detected in the immunoprecipitate from NGF-differentiated cells, even in the absence of PKC activators. This activity can be further stimulated by TPA but inhibited to the basal level by a serine/threonine kinase inhibitor H7 or by GF109203X, a PKC-specific inhibitor (see Fig. 8). In contrast, the immunoprecipitate from undifferentiated cells has no detectable Ca²⁺-independent PKC activity. These results show that 14-3-3ζ-PKC complex in differentiated cells has constitutive and autonomous Ca²⁺-independent nPKC activity, whereas the complex in undifferentiated cells does not have detectable activity of nPKC.

**PKC-α Bound to 14-3-3ζ Is Inactive in Differentiated Cells**—PKC-α, a Ca²⁺-dependent cPKC, becomes associated with 14-3-3ζ during neuronal differentiation induced by NGF. However, there was no detectable Ca²⁺-dependent PKC activity in the immunoprecipitate regardless of differentiation as shown in Fig. 6. Furthermore, TPA has no effect on the PKC activity. The lack of activity in the tested samples is not due to a failure of the detection of cPKC activity, since purified PKC-α can be activated in this cPKC assay system as shown in the inset. Because PKC-β or -γ isoforms are not present in the FLAG immunoprecipitate, these results suggest that PKC-α associated with 14-3-3ζ in differentiated cells is inactive and insensitive to PKC activators. However, it is possible that the lack of cPKC activity could be simply due to an amount of PKC-α present in the immunoprecipitate that was too small for detection. To examine this, we have done parallel experiments in which we measure the amount of PKC-α in the immunoprecipitate by comparing with known quantities of purified PKC-α to determine the amount of the enzyme and cPKC kinase activity in the immunoprecipitate. As shown in Fig. 7, the immunoprecipitate used for cPKC assay (lanes 1 and 2 shown in duplicate) emitted chemiluminescence of 289 ± 142 (arbitrary unit), whereas there was no chemiluminescence observed for purified PKC-α up to 5 ng. The detection of purified PKC-α requires at least 5–10 ng of the enzyme (Fig. 7B). Based on these analyses, the amount of PKC-α in the immunoprecipitated sample used for cPKC assay was estimated to be ~6 ng/reaction tube. The activity of purified PKC-α could be clearly detected as low as 0.2 ng in the cPKC activity used for the study (data not shown). On the contrary, the 14-3-3ζ immunoprecipitate visualized in Fig. 7A is only at the background level (Table II). Based on these analyses, we conclude that the PKC-α is inactive in the immunoprecipitate from differentiated PC12 cells.

**nPKC Activity in the FLAG Immunoprecipitate Is Sensitive to Arachidonic Acid and Ca²⁺**—In addition to diacylglycerol, PKC can be activated by cis-unsaturated fatty acids such as arachi-
differentiated PC12 cells were used as a negative control. 

PKC-α is the likely candidate for the constitutive nPKC activity. Surprisingly, we found that the Ca^{2+}-independent PKC activity in the FLAG immunoprecipitate can be further enhanced by low (micromolar) concentrations of Ca^{2+}, but it is inhibited by higher (above 10 μM) Ca^{2+} concentrations. This raises an intriguing possibility that Ca^{2+} could regulate not only classical PKC but also non-classical novel or atypical PKC when it is associated with 14-3-3 ζ.

**DISCUSSION**

PKC has been indicated as one of the targets of 14-3-3, but the significance of the PKC association with 14-3-3 is still unclear. In this study, we have examined whether 14-3-3 ζ interacts with PKC in vivo in neuronal cells using PC12 and its derivative cell line B3 that stably expresses epitope-tagged 14-3-3 ζ. We have shown here that several PKC isoforms interact with 14-3-3 ζ in PC12-B3 cells. Association of PKC-α and -ε is differentiation-dependent; they do not bind with 14-3-3 ζ in undifferentiated cells but they become associated with 14-3-3 ζ when the cells are neuronally differentiated by NGF. In contrast to this differentiation-dependent association, PKC-δ, -ζ, and -μ isoforms were found to interact with 14-3-3 ζ regardless of the state of cell differentiation, although the association of PKC-δ with 14-3-3 ζ increases during differentiation to some extent. Significant Ca^{2+}-independent PKC activity is present in FLAG immunoprecipitate from differentiated cells, but it is not detectable in the immunoprecipitate from undifferentiated cells although PKC-δ, -ζ, and -μ isoforms are present.

These results indicate that the effect of 14-3-3 ζ on PKC in intact cells is different from those observed in vitro. The in vitro effect of 14-3-3 on PKC has been controversial as to whether it activates or inhibits the activity (18–21,39). Recently, in vitro activation of PKC by 14-3-3 was shown to be due to the acidic nature of 14-3-3, and it was suggested that in vitro activity does not correlate with in vivo regulation of PKC by 14-3-3 (40). Although in vivo demonstration of the functional association of 14-3-3 with PKC is rather limited, several 14-3-3 isoforms were shown to interact with PKC-ζ in COS cells and in fibroblasts,
resulting in the formation of a complex with Raf-1 (40). In T cells, 14-3-3 ζ (ζ) was shown to negatively regulate the activity of PKC-θ (θ) and PKC-µ (µ). Our study using neuronal cells shows that 14-3-3 ζ-bound form of PKC has different activities depending on the isoform. However, it should be noted that our data do not indicate whether the regulation of PKC, either activation or inhibition, is a result of the interaction between 14-3-3 ζ and PKC or precedes it.

The FLAG immunoprecipitate from differentiated PC12-B3 cells contains constitutive nPKC activity. This suggests that some of the non-classical PKC is active in cells when it is associated with 14-3-3. PKC-δ, -ζ, and -µ in addition to PKC-ε are the non-classical PKC isoforms found in the FLAG immunoprecipitate from differentiated PC12-B3 cells. Which PKC isoform(s) is then responsible for the constitutive nPKC activity detected in the FLAG immunoprecipitate? PKC-δ, -ζ, and -µ are also present in the FLAG immunoprecipitate from undifferentiated cells (Fig. 4). However, the PKC activity of the immunoprecipitate is not different from the background activity in the FLAG immunoprecipitate prepared from parent PC12 cells, which do not express FLAG epitope-tagged 14-3-3 (Fig. 5). This indicates that these PKC isoforms bound to 14-3-3 ζ are inactive or that their association with 14-3-3 ζ is too minute to detect the activity. It is possible that the activity of PKC-δ, -ζ, or -µ bound to 14-3-3 ζ become detectable in differentiated cells by an unknown mechanism. However, it is more likely that the activity found in the FLAG immunoprecipitate from differentiated cells is due to PKC-ε for the following reasons. PKC-ε and -δ are the isoforms that increase their association with 14-3-3 ζ during differentiation. Arachidonic acid potently activates and translocates PKC-ε, whereas PKC-δ is insensitive to arachidonic acid (36, 37). Our study shows that the constitutive nPKC activity is sensitive to arachidonic acid (Fig. 8), suggesting that PKC-ε is responsible for the constitutive nPKC activity in differentiated PC12 cells. Interestingly, purified PKC-ε has been shown to be selectively activated by 14-3-3 ζ over the other PKC isoforms in vitro (39), although it is not clear whether this in vitro effect is related to the in vivo activation of PKC-ε in the 14-3-3 ζ complex.

In addition to PKC-δ (24, 43, 44) and PKC-ζ (45), PKC-ε has been indicated to play a key role in neurite outgrowth in PC12 cells (22, 23, 26) as well as in neuroblastoma cells (46, 47). PKC activation by low concentrations (1–10 nM) of TPA has been shown to induce neuronal differentiation of human SH-SY5Y neuroblastoma cells (46). Parrow et al. (47) show that PKC-α...
and ε subtypes are the PKC isoforms highly enriched in the growth cones in differentiating SH-SY-5Y cells. They further showed that PKC-ε-expressing growth cones are fully capable of forming neurite outgrowth even when PKC-α was downregulated, indicating the involvement of PKC-ε for neurite outgrowth. In PC12 cells, chronic exposure of the cells with ethanol was shown to up-regulate PKC-ε as well as PKC-ζ and cPKC activity of immunoprecipitate sample from differentiated PC12 cells, immunoprecipitate samples and different amounts of purified PKC-ζ measured under the same conditions as in A. Ab, antibody.

Experimental Procedures.

Our study showed that nPKC activity in the 14-3-3 complex is constitutively active, but it is further stimulated by 10 μM arachidonic acid. Arachidonic acid at this concentration has been shown to stimulate neurite outgrowth and maximally increase the neurite length in PC12 and its sub-cell line (49–51). NGF has been shown to increase arachidonic acid release in PC12 cells (52, 53). Neural cell adhesion molecule-induced neurite outgrowth in PC12 cells was also shown to accompany arachidonic acid release (50). Because both NGF- and neural cell adhesion molecule-induced neurite outgrowth requires activation of PKC (22, 23, 45, 51), arachidonic acid stimulation of nPKC activity that is associated with 14-3-3 ζ shown in this study might have a significant role in neurite outgrowth.

Is the interaction of PKC with 14-3-3 ζ mediated by the well characterized 14-3-3 recognition motif containing phosphoserine? There are several consensus sequences identified for 14-3-3 binding; RXpXXpXXp(54), RXpXXpXXp(55), RXpXpXXp, (40), where pS is phosphoserine, and X is any amino acid. 14-3-3 can often recognize phosphothreonine in a place of phosphoserine within the motif (for example, Ref. 57). We searched for the 14-3-3 binding motif in PKC and found that it is present in PKC-γ, δ, ε, η, ζ, λ, and μ isoforms but is absent in PKC-α, β, β1, and θ. Our immunoprecipititation results show that among the PKC isoforms present in PC12-B3 cells, PKC-α, δ, ε, η, and μ are able to form a complex with 14-3-3 ζ. This does not correlate with the presence of these phosphorylation-dependent 14-3-3 binding motifs in PKC isoforms.

PKC-α does not have the motif and yet it associates with 14-3-3 ζ. PKC-θ, another isoform that does not have the 14-3-3 binding motif, was shown to bind to 14-3-3 θ(60) directly in Jurkat T cells, and it is inactivated (41). These observations suggest that 14-3-3 binding to certain PKC isoforms such as α and θ is mediated by a phosphorylation-independent mechanism, bridged by another partner protein or possibly the presence of an unknown motif. In addition to the well characterized phosphorylation-dependent 14-3-3 binding, 14-3-3 also binds to the partner proteins in a manner independent of phosphorylation. Phosphorylation-independent 14-3-3 binding has been found in proteins such as glycoprotein Ib (58) and Pseudomonas aeruginosa exoenzyme S (59). The binding sites within the 14-3-3 protein for phosphorylation-dependent and-independent interaction appear to be different. 14-3-3 binding and phosphorylation of PKC with 14-3-3 ζ in vivo is reported in the ameba Dictostelium (60). cAMP is a chemoattractant for starved Dictostelium, and this chemotaxis to cAMP requires a rapid cortical accumulation of myosin II and its phosphorylation (61). This phosphorylation was shown to be mediated by myosin II heavy chain-specific PKC, which is structurally homologous to classical PKC-α, β, and γ isoforms (62). Matto-Yelin et al. (60) show that the 14-3-3 binding to this Dictostelium PKC occurs through the C1 domain of the PKC and that this binding inhibits PKC activity. Interestingly, 14-3-3 binds to the C1 fragment expressed in the ameba in vivo even without the cAMP stimulation, and yet this cAMP does not bind to the wild type PKC-expressing cells to the release of PKC from 14-3-3 binding. This indicates that an additional mechanism may exist to regulate the 14-3-3 binding of PKC if the interaction is
mediated through the C1 domain of PKC. Because PKC-α, which does not have the consensus phosphorylation-dependent 14-3-3 binding motif, becomes associated with 14-3-3 ζ during differentiation of PC12-B3 cells, such a mechanism may regulate the association. Further studies are required to determine the mechanism of interaction between mammalian PKC and 14-3-3.

In summary, we have shown that several PKC isoforms interact with 14-3-3 ζ in PC12-B3 cells. Differentiation of PC12-B3 cells by NGF induces association of PKC with 14-3-3 ζ selective to α and ε PKC isoforms, and the association with 14-3-3 ζ has distinct effects on cPKC and nPKC activity.

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