Atypical Protein Kinase C in Neurodegenerative Disease II: 
PKC\(\gamma/\lambda\) in Tauopathies and \(\alpha\)-Synucleinopathies

Charles Y. Shao, MD, PhD, John F. Crary, PhD, Chandrakant Rao, MD, Todd C. Sacktor, MD, and Suzanne S. Mirra, MD

Abstract
To study the role of atypical protein kinase C (aPKC) in neurodegenerative disease, we investigated the distribution of PKCs/\(\gamma/\lambda\), an aPKC isoform, in a variety of tauopathies and \(\alpha\)-synucleinopathies. Immunohistochemical study revealed PKCs/\(\gamma/\lambda\) within tau-positive neurofibrillary inclusions in Alzheimer disease (AD), progressive supranuclear palsy, corticobasal degeneration (CBD), and Pick disease (PiD), within \(\alpha\)-synuclein-positive Lewy bodies in idiopathic Parkinson disease and dementia with Lewy bodies, as well as within glial inclusions in multisystem atrophy. We also observed PKCs/\(\gamma/\lambda\) label of actin-rich Hirano bodies in AD, PiD, and elderly individuals. Double immunolabeling and fluorescence resonance energy transfer demonstrated close physical association between PKCs/\(\gamma/\lambda\) and phospho-tau or \(\alpha\)-synuclein in some neurofibrillary tangles and Lewy bodies. Furthermore, PKCs/\(\gamma/\lambda\) colocalized with p62, a chaperone protein that binds to both aPKC and ubiquitin, in most of these inclusions. PKCs/\(\gamma/\lambda\) also closely associated with the inactivated form of glycogen synthase kinase-3\(\beta\) (GSK-3\(\beta\)[\(\text{ser}\ 9\)]). Together, these findings suggest that PKCs/\(\gamma/\lambda\) may play a role in common mechanisms involving the pathogenesis of neurodegenerative disease.

Key Words: Alzheimer disease, Atypical protein kinase C, \(\alpha\)-synucleinopathy, Tauopathy, Ubiquitin.

INTRODUCTION
Abnormal filamentous protein aggregates (intracellular inclusions) are hallmarks of many neurodegenerative diseases. Hyperphosphorylated tau comprises paired helical filaments (PHFs) in neurofibrillary tangles (NFTs) in Alzheimer disease (AD); globoe tangles and glial inclu-

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The second isoform, PKCγ/α, has been shown to play a critical role in growth factor-dependent survival and differentiation of cultured neuronal cells (26). Both isoforms may be important for establishing and maintaining cell polarity in a variety of cell types, including neurons (27). This article focuses on the distribution and potential role of PKCγ/α in neurodegenerative disease.

MATERIALS AND METHODS

Postmortem Human Brain Tissue

Autopsy brain tissue was derived from neuropathologically confirmed cases of neurodegenerative diseases and controls listed in Table 1. The 6 cases of “pure” AD as well as the 14 cases of AD and dementia with Lewy bodies all met CERAD neuropathologic criteria for definite AD (28, 29). The 3 PiD cases had classic neuropathologic features of this disorder, including frontotemporal atrophy, abundant tau-positive Pick bodies within neurons, including the granular cell and pyramidal cell layer of hippocampus, and Pick cells (30). The 5 cases of CBD, previously published (7), met current neuropathologic criteria for this disorder (1) with abundant tau-immunoreactive lesions, including astrocytic plaques, degeneration of cortex and nigra, and neurofilament protein-positive ballooned neurons. The 9 cases of PSP displayed the classic histopathologic features of PSP, including tau-positive globose NFTs (2); these changes occurred in a stereotypical distribution (31). The neuropathologic diagnoses of dementia with Lewy body cases conformed to consensus criteria of McKeith et al (32). The 2 cases of idiopathic PD presented clinically with classic L-dopa-responsive parkinsonism, and autopsy demonstrated nigral degeneration with α-synuclein-positive Lewy bodies in the substantia nigra and other sites. The 3 cases of MSA all exhibited striatonigral degeneration with 2 of the 3 also showing olivocerebellar pontine atrophy. The classic ubiquitin and α-synuclein-positive but tau-negative glial cytoplasmic inclusions within oligodendroglial cells (33) were found in all 3 cases. The 5 control cases were derived from patients aged 60 to 96 years with no clinical history of dementia as described in the accompanying paper (22). One case was Braak stage I, 2 cases were Braak stage II, and 2 were Braak stage III–IV. Sections of hippocampus, entorhinal cortex, amygdala, and temporal cortex from these control cases were used in this study.

Antibodies

Table 2 lists the primary antibodies used in this study. The mouse anti-PKCγ (human) and mouse anti-PKCα (rodent), widely used by others in rodent tissue (26, 34, 35), both recognize human PKC protein and showed identical results in this study. Thus, these antisera are referred to collectively as “anti-PKCγ/α” in this article. As we illustrate in the Western blot in the accompanying report (22), human brain homogenates from the superior temporal cortex, hippocampus, caudate nucleus, and cerebellum, the PKCγ/α antibody recognized a single 72-kD band compared with the

Table 2. Primary Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen</th>
<th>Species</th>
<th>Source</th>
<th>Dilution</th>
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<tbody>
<tr>
<td>PKCγ</td>
<td>PKCγ catalytic domain</td>
<td>Mouse IgG</td>
<td>Transduction Laboratories, San Jose, CA</td>
<td>1:200</td>
</tr>
<tr>
<td>PKCα</td>
<td>PKCα catalytic domain</td>
<td>Mouse IgG</td>
<td>Transduction Laboratories</td>
<td>1:200</td>
</tr>
<tr>
<td>pan-aPKC</td>
<td>aPKC C-terminal</td>
<td>Rabbit antiserum</td>
<td>T. C. Sacktor*</td>
<td>1:400</td>
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<tr>
<td>PHF1</td>
<td>Phospho-tau</td>
<td>Mouse IgG</td>
<td>P. Davies†</td>
<td>1:500</td>
</tr>
<tr>
<td>tau</td>
<td>Human tau</td>
<td>Rabbit antiserum</td>
<td>DAKO Corp., Carpinteria, CA</td>
<td>1:1000</td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>Ubiquitin</td>
<td>Rabbit antiserum</td>
<td>DAKO Corp.</td>
<td>1:500</td>
</tr>
<tr>
<td>p62</td>
<td>Zeta interacting protein (ZIP)</td>
<td>Goat antiserum</td>
<td>Santa Cruz Biotechnology, Inc., Santa Cruz, CA</td>
<td>1:400</td>
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<tr>
<td>GSK-3β[ser9]</td>
<td>Phospho-serine 9</td>
<td>Rabbit antiserum</td>
<td>Oncogene Research Products, San Diego, CA</td>
<td>1:1000</td>
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* Generated in Dr. Sacktor’s laboratory (23).
† Gift from Dr. Peter Davies, Department of Pathology and Neuroscience, Albert Einstein College of Medicine, Bronx, NY.
PKC, protein kinase C; GSK, glycogen synthase kinase.
rabbit antiserum to pan-aPKC C-terminal that recognized the 72-kD band, as well as a 55-kD band, representing PKMε in these regions.

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded sections (8 µm) were deparaffinized in xylene and rehydrated. To enhance antigen retrieval, sections were microwaved in a microwaveable pressure cooker (Black and Decker, Hampstead, MD) in 0.1 M citrate buffer at pH 6.0 until the pressure cooker reached full pressure. The sections were microwaved for an additional 5 minutes, kept standing until the solution reached room temperature, and then treated with 95% formic acid for 2 minutes. Endogenous peroxidase activity was blocked by incubating the sections in 3% H2O2 in phosphate-buffered saline for 20 minutes. The sections were incubated for 30 minutes in blocking solution comprised of 10% horse serum in a detergent of phosphate-buffered saline with surfactant (Optimax Wash Buffer; BioGenex, San Ramon, CA). Then, the sections were incubated overnight with the respective primary antibodies in buffer at dilutions listed in Table 2. After 3 5-minute washes in buffer, the sections were incubated with the secondary horse antirabbit/mouse IgG (Vector Laboratories, Burlingame, CA) in buffer for 30 minutes. The sections were washed as previously described and incubated in R.T.U. Vectastain Elite ABC reagent (Vector Laboratories) for 30 minutes, washed in buffer, and developed with 6.7% diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, MO) reaction in the presence of 0.03% H2O2. The sections were then counterstained with hematoxylin, cleared in xylene, mounted with Cytoseal 60 mounting medium (Richard-Allan Scientific, Kalamazoo, MI), and coverslipped.

For double-immunofluorescence, sections were incubated overnight at 4°C in a cocktail of the mouse anti-PKCζ/λ antibody and one of the following polyclonal antibodies: rabbit anti-phospho-tau, anti-α-synuclein, anti-ubiquitin, or anti-GSK-3β. After the incubation, a combination of secondary antibodies conjugated with Alexa Fluor 488 and Alexa Fluor 568 (Molecular Probe Inc., Eugene, OR) was applied in 1:200 dilutions for 30 minutes at room temperature. After staining, sections were incubated briefly in 1% Sudan Black B in 70% ethanol to eliminate lipofuscin-generated autofluorescence (36).

**Fluorescence Resonance Energy Transfer**

To ascertain the proximity between 2 molecules (e.g. PKCζ/λ and phosphotau), we took advantage of the fluorescence resonance energy transfer (FRET) technique as applied by Hyman and colleagues (37, 38). This method depends on the ability of 2 fluorochromes (labeling 2 different antigens) to act as fluorescent donor–acceptor pairs. For this

![FIGURE 1](http://jnen.oxfordjournals.org/)  
**FIGURE 1.** Association of PKCζ/λ with tauopathies and α-synucleinopathies. The PKCζ/λ antibody weakly labels the cytoplasm of hippocampal neurons and the neuropil in a control case (A), while strongly labeling neurofibrillary tangles (B) and Hirano bodies (C) in Alzheimer disease cases. The antibody also labeled Pick bodies in Pick disease as shown in dentate gyrus (D), globose tangles (E) and tufted glial cells (F) as shown in the subthalamic nucleus in progressive supranuclear palsy; and cortical astrocytes (G) in corticobasal degeneration. In α-synucleinopathies, the antibody strongly labels classic Lewy bodies in pigmented substantia nigra neurons (H) and Levy bodies in amygdala (I) and cortex in Parkinson disease and dementia with Levy bodies. In multisystem atrophy, glial inclusions are also immunoreactive as seen within the pons (J). Omission of the primary antibody yields no staining of any inclusions such as these nigral Levy bodies (K, arrow).
to work, the absorption spectra of the acceptor, in this study Alexa Fluor 568, must overlap the emission spectra of the donor, in this study, Alexa Fluor 488. If the 2 fluorochromes are in close proximity (less than 10 nm), photons from excited donor molecules will transfer to acceptor molecules, quenching the fluorescent emission of the donor. FRET is demonstrated, in principle, when an increase in donor intensity follows photobleaching of the acceptor.

Double-immunofluorescence-labeled sections were examined with a BioRad MRC-1024 confocal microscopic system equipped with a krypton/argon laser. After taking initial images at the 2 wavelengths, a portion of the Alexa Fluor 568 image was magnified and subjected to higher laser power bleaching. Then, comparable images were collected again at both wavelengths. FRET was demonstrated when photobleaching of the acceptor produced an increase in intensity of the donor. Control sections stained only with Alexa Fluor 488 failed to elicit FRET.

RESULTS

PKC\(\alpha\)/\(\lambda\) Immunoreactivity in Tauopathies and \(\alpha\)-Synucleinopathies

PKC\(\alpha\)/\(\lambda\) immunoreactivity, of mild intensity, in a finely granular pattern was noted within neuronal cell bodies and neuropil in a widespread distribution (Fig. 1A) in control tissues as well as in uninvolved areas of disease cases. Ependymal cells and choroid plexus epithelial cells were also immunoreactive; astrocytes and corpora amylacea failed to label (data not shown). This pattern is similar to that reported in rodents (35).

In AD, antibody to PKC\(\alpha\)/\(\lambda\) strongly labeled many NFTs in the hippocampus (Fig. 1B), neocortex, subcortical gray matter, and brainstem. The antibody also labeled most Hirano bodies in CA1 of the hippocampus (Fig. 1C). PKC\(\alpha\)/\(\lambda\)-positive NFTs exhibited classic flame-shaped appearances as well as “early” perinuclear configurations; “extracellular” or “ghost” tangles failed to label. Neurofil plaques, senile plaques, and granulovacuolar bodies in hippocampal neurons were generally immunonegative.

The PKC\(\alpha\)/\(\lambda\) antibody uniformly labeled Pick bodies in neurons of hippocampal dentate gyrus and neocortex of PiD (Fig. 1D). In cases of PSP, the antibody labeled globose tangles and tubbed astrocytes in the subthalamic, mesencephalic, inferior olivary, and cerebellar dentate nuclei (Fig. 1E, F). In CBD, tau-immunoreactive astrocytic inclusions in cerebral cortex, as well as neuronal and glial inclusions in the basal ganglia, were immunopositive (Fig. 1G). In the \(\alpha\)-synucleinopathies, the PKC\(\alpha\)/\(\lambda\) antibody strongly labeled all classic Lewy bodies found in the substantia nigra as well as the majority of Lewy bodies in the cerebral cortex and amygdala in PD and DLB (Fig. 1H, I). Lewy neurites in the amygdala, hippocampus CA2/3, and midbrain were not labeled. The PKC\(\alpha\)/\(\lambda\) antibody also labeled some glial \(\alpha\)-synuclein-positive inclusions in MSA (Fig. 1J). Omission of the primary antibody resulted in no immunolabeling of any of these structures (Fig. 1K).

The labeling of the PKC\(\alpha\)/\(\lambda\) antibody was similar to that of the pan-aPKC antibody, which further confirmed the presence of aPKC in tauopathies and \(\alpha\)-synucleinopathies (Table 3). The pan-aPKC antibody also labeled dystrophic neurites and Lewy neurites, likely reflecting the distribution of PKM\(\gamma\) present in a subset of NFTs and neurites as described in the accompanying report (22).

<table>
<thead>
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<th>TABLE 3. Immunohistochemical Findings</th>
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<td>Parkinson disease and Dementia with Lewy bodies</td>
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<td>Multisystem atrophy</td>
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Pan-aPKC, recognizing PKC\(\alpha\)/\(\lambda\) and PKM\(\gamma\); phospho-tau, labeled by PHF1 antibody; \(\alpha\)-syn, \(\alpha\)-synuclein; Ub, ubiquitin; +/-, inconsistent observations.
identical to that of phospho-tau; in such instances, FRET was demonstrated (Fig. 2E–H), suggesting a close proximity between the 2 molecules. In other inclusions in these tauopathies, however, the distribution and colocalization of PKCζ/λ and phospho-tau immunoreactivity were more variable.

PKCζ/λ immunoreactivity also was associated with α-synuclein and ubiquitin immunoreactivity in Lewy bodies (Fig. 3A–D). Interestingly, classic Lewy bodies in the substantia nigra exhibited PKCζ/λ immunoreactivity in their “cores” with α-synuclein and ubiquitin immunoreactivity at the periphery (Fig. 3A, B). “Pale” Lewy bodies, seen occasionally in the midbrain and thought to represent “early” Lewy bodies (39), were diffusely labeled by both PKCζ/λ and α-synuclein antibodies (Fig. 3C, E–H). Similarly, cortical Lewy bodies exhibited diffuse labeling by both antibodies (Fig. 3D). The close association between PKCζ/λ and α-synuclein in the pale Lewy bodies was further demonstrated by FRET (Fig. 3E–H) in a manner similar to that between PKCζ/λ and phospho-tau shown for NFTs (Fig. 2E–H).

Colocalization of PKCζ/λ With p62 and Ubiquitin in Inclusions
p62, a ubiquitin-binding protein, has been demonstrated within various inclusions in tauopathies and α-synucleinopathies (40, 41). Because p62 is also an atypical PKC-interacting
protein (42), we examined the relationship of PKC\(\text{I/L}\) to p62 and ubiquitin. In AD cases, we found that PKC\(\text{I/L}\) colocalized with p62 and ubiquitin in NFTs (Fig 4A–D). Note that the neuropil threads label only with antibody to ubiquitin (Fig. C, green) but are negative for both PKC\(\text{I/L}\) and p62. An astrocytic plaque filling the field in neocortex of corticobasal degeneration is immunonegative for PKC\(\text{I/L}\) (E). This plaque shows colocalization of p62 and ubiquitin as judged by the merged blue–green image (H). Classic Lewy bodies in the substantia nigra in Parkinson disease contain all 3 antigens (PKC\(\text{I/L}\), p62, and ubiquitin) as displayed by the merged image (L). Scale bar in (L) for all = 50 \(\mu\)m.

FIGURE 4. Colocalization of PKC\(\text{I/L}\) (red) with p62 (blue) and ubiquitin (green) in various diseases. (A–D) A cortical neurofibrillary tangle from a case of Alzheimer disease exhibits colocalization of PKC\(\text{I/L}\), p62, and ubiquitin as displayed by the merged image (D). Note that the neuropil threads label only with antibody to ubiquitin (C, green) but are negative for both PKC\(\text{I/L}\) and p62. (E–H) An astrocytic plaque filling the field in neocortex of corticobasal degeneration is immunonegative for PKC\(\text{I/L}\). This plaque shows colocalization of p62 and ubiquitin as judged by the merged blue–green image (H). (I–L) Classic Lewy bodies in the substantia nigra in Parkinson disease contain all 3 antigens (PKC\(\text{I/L}\), p62, and ubiquitin) as displayed by the merged image (L). Scale bar in (L) for all = 50 \(\mu\)m.

Close Association of PKC\(\text{I/L}\) With Inactivated GSK-3\(\text{B}[\text{ser9}]\) in Neurofibrillary Tangles in Alzheimer Disease

Atypical PKC has been shown to inactivate GSK-3\(\text{B}\) by phosphorylation at its serine 9 residue in adipocytes (43).

FIGURE 5. Association of PKC\(\text{I/L}\) with inactivated GSK-3\(\text{B}[\text{ser9}]\). PKC\(\text{I/L}\) (green) was colocalized with GSK-3\(\text{B}[\text{ser9}]\) (red) in many neurofibrillary tangles in hippocampus (A–C) and other brain regions. Scale bar in (A) for all = 50 \(\mu\)m.
Because GSK-3β is regarded as a major kinase responsible for tau phosphorylation and PHF formation (44), we examined the relationship between PKC/α and GSK-3β. We found that PKC/α colocalized with the inactivated form of GSK-3β[ser9] in most NFTs in AD in all areas examined (Fig. 5). A close association between the 2 molecules was demonstrated by FRET (data not shown).

**DISCUSSION**

We have found that PKC/α, an isoform of aPKC, is associated with a wide array of intracellular inclusions and structures in neurodegenerative diseases, including 1) tau-positive neuronal and glial inclusions in tauopathies (i.e. AD, PSP, PiD, and CBD); 2) Lewy bodies and glial inclusions in α-synucleinopathies (i.e. PD, DLB and MSA); and 3) actin-rich Hirano bodies (Table 3). PKC/α is present in all classic Lewy bodies found in the substantia nigra and in many cortical Lewy bodies, Hirano bodies, Pick bodies, and NFTs. Interestingly, the presence of aPKC, including PKC/α and PKMζ, in NFTs is unique among PKC subgroups, because a panel of isoform-specific antibodies to conventional and novel PKCs failed to label any NFTs in AD (22, 45, 46).

The association of PKC/α with neuropathologic features of neurodegenerative disease is more widespread than that of other protein kinases such as GSK-3β, Cdk5, MAPKs, PKA, and CaMKII; most of these kinases are associated either with tauopathies or α-synucleinopathies. The distribution of PKC/α in pathologic structures in these disorders largely parallels that of ubiquitin, although ubiquitin has not been identified in Hirano bodies (47). The ubiquitin-binding protein, p62, has been shown to occur in inclusions of both tauopathies and α-synucleinopathies as well as in Mallory bodies in the liver (40, 41). Our data show that PKC/α and p62, along with ubiquitin, are colocalized in many inclusions, suggesting that PKC/α may interact with ubiquitin and ubiquitin-binding proteins during abnormal protein aggregation. Furthermore, the association between PKC/α and p62 within neuropathologic structures described in this study is intriguing given recent data showing that p62 acts as a scaffolding protein, mediating interactions between neurotrophin receptors and nuclear factor kappa B (NF-κB), a transcription factor thought to play a role in anti-apoptosis and neuronal survival (26, 42, 48).

The variable pattern of colocalization of PKC/α with phospho-tau or α-synuclein as well as with p62 and ubiquitin may reflect dynamic interactions between these molecules (49) and PKC/α. For example, “pale” Lewy bodies, often regarded as precursors to classic Lewy bodies (39), exhibit diffuse labeling by both PKC/α and α-synuclein, whereas classic Lewy bodies exhibit disparate localization of the 2 molecules. It is conceivable that PKC/α first interacts with α-synuclein, playing a role in its phosphorylation (50), and then segregates as Lewy body formation progresses.

Impairment of PKC/α activity may contribute indirectly to the formation of NFTs. It is known that PKC, including aPKC, are capable of phosphorylating and inactivating GSK-3β during insulin-dependent glucose transport (43). Because GSK-3β plays a key role in tau phosphorylation in neurons, its inactivation by aPKC may reduce tau phosphorylation. In an in vivo study, injection of PKC inhibitors into the lateral ventricles of rat brains led to overactivation of GSK-3β, hyperphosphorylation of tau, and impaired spatial memory (51). Indeed, because aPKC are the only isoforms within the PKC family present in NFTs, aPKC is a likely candidate for mediating phosphorylation of GSK-3β on serine 9. Of the 2 aPKC isoforms, ζ and ι/ε, PKC/α is more likely to be the molecule regulating GSK-3β because PKCζ is undetectable in the forebrain region (35) and its truncated form, PKMζ, is present in smaller numbers of NFTs with a restricted distribution (22). The close association of PKC/α with the inactivated GSK-3β[ser9] in NFTs that we describe further supports this notion. Thus, PKC/α may belong to a group of kinases, including Akt/PKB and PKN, that occur in NFTs and are capable of phosphorylating GSK-3β (52, 53).

In addition to having an active role in inclusion formation, PKC/α also may be “entrapped” in various filamentous protein aggregates, thereby losing its ability to phosphorylate normal substrates. Whether sequestration of PKC/α by protein aggregates in neurodegenerative diseases impedes its response to growth factors and/or affects other critical functions in neurons remains to be explored.

In summary, we have demonstrated the association of PKC/α with a wide array of intracellular inclusions in tauopathies and α-synucleinopathies, suggesting that PKC/α plays a broad role in neurodegenerative disease. Further investigations are warranted to determine whether PKC/α contributes to phosphorylation during abnormal protein aggregation and whether interactions between PKC/α and various protein aggregates result in the disruption of the normal cellular functions of this important form of PKC.

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REFERENCES


