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Identification of Microglial Signal Transduction Pathways Mediating a Neurotoxic Response to Amyloidogenic Fragments of β-Amyloid and Prion Proteins

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Microglial interaction with amyloid fibrils in the brains of Alzheimer's and prion disease patients results in the inflammatory activation of these cells. We observed that primary microglial cultures and the THP-1 monocytic cell line are stimulated by fibrillar β-amyloid and prion peptides to activate identical tyrosine kinase-dependent inflammatory signal transduction cascades. The tyrosine kinases Lyn and Syk are activated by the fibrillar peptides and initiate a signaling cascade resulting in a transient release of intracellular calcium that results in the activation of classical PKC and the recently described calcium-sensitive tyrosine kinase PYK2. Activation of the MAP kinases ERK1 and ERK2 follows as a subsequent downstream signaling event. We demonstrate that PYK2 is positioned downstream of Lyn, Syk, and PKC. PKC is a necessary intermediate required for ERK activation. Importantly, the signaling response elicited by β-amyloid and prion fibrils leads to the production of neurotoxic products. We have demonstrated in a tissue culture model that conditioned media from β-amyloid- and prion-stimulated microglia or from THP-1 monocytes are neurotoxic to mouse cortical neurons. This toxicity can be ameliorated by treating THP-1 cells with specific enzyme inhibitors that target various components of the signal transduction pathway linked to the inflammatory responses.

Key words: Alzheimer's disease; β-amyloid; prion; microglia; THP-1 monocytes; signal transduction; tyrosine kinase; inflammation; neurotoxicity

A subset of neurodegenerative diseases is linked to the aberrant, extracellular deposition of fibrillar proteins collectively termed "amyloids." Amyloids are generated in a disease-specific manner from structurally unrelated proteins (Kisilevsky, 1997).

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive deposition of β-amyloid fibrils in the brain to form senile plaques (Braak and Braak, 1997). The amyloidogenic material is composed of β-amyloid peptides (βA) that are proteolytically derived from the amyloid precursor protein (Kang et al., 1987; Haas et al., 1992; Koo and Squazzo, 1994; Turner et al., 1996). The plaques are associated with reactive microglia and astrocytes as well as dystrophic neurites (Itagaki et al., 1989; Miyazono et al., 1991; Cotman et al., 1996).

The prion disorders are neurodegenerative diseases characterized by the accumulation of a pathological form of the prion protein (PrP^{sc}) (Prusiner, 1982; Kretzschmar et al., 1986; Borchelt et al., 1992; Stahl et al., 1993). PrP^{sc} is characterized by its infectious nature, partial resistance to proteolysis, and the capacity to aggregate extracellularly in the brain and to deposit as amyloid plaques in a subset of prion disorders (Prusiner et al., 1984; Oesch et al., 1985). Plaque formation is correlated with the appearance of reactive astrocytes and microglia as well as vacuolar cell loss (Miyazono et al., 1991; Guiroy et al., 1994; Jeffrey et al., 1994; Williams et al., 1994, 1997; Muhleisen et al., 1995; Betmouni et al., 1996; Brown and Kretzschmar, 1997; Kretzschmar et al., 1997).

Both types of fibrillar amyloid deposits share an invariant association with reactive glial cells, particularly microglia. There is abundant evidence of a microglial-derived inflammatory component in either disease. Microglia associated with amyloid plaques exhibit elevated expression of several cell surface markers indicative of a reactive state (McGeer et al., 1993; McGeer and McGeer, 1995). A variety of acute-phase proinflammatory proteins are also associated with the amyloid plaques (McGeer and Rogers, 1992; McGeer and McGeer, 1995). Moreover, several *in vitro* studies have now documented the ability of fibrillar βA and PrP peptides to induce microglial secretion of cytokines and neurotoxic reactive oxygen species (Forloni et al., 1993; Brown et al., 1996; Ii et al., 1996; Klegeris and McGeer, 1997; Klegeris et al., 1997; Kretzschmar et al., 1997; Lorton, 1997; McDonald et al., 1997). Consequently, maintained microglial contact with amyloid plaques could serve to initiate localized inflammatory responses in diseased brains.

We have identified a tyrosine kinase-based signaling cascade in microglial lineage cells that is activated by exposure of the cells to both βA and PrP fibrils and is directly responsible for the production of neurotoxic factor(s) by the activated cells. These intracellular signaling pathways are common to those used by these cells in response to classical inflammatory stimuli (Ghazizadeh et al., 1994, 1995; Marcilla et al., 1995; Crowley et al., 1997; Vonakis et al., 1997). We demonstrate that specific inhibition of enzymes within the activation pathway prevents the acquisition of

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a reactive, neurotoxic phenotype and offers novel interventive strategies.

MATERIALS AND METHODS

Materials. The anti-phosphotyrosine antibody 4G10 was from Upstate Biotechnology (Lake Placid, NY). Anti-paxillin and anti-PYK2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-ERK and anti-ERK antibodies were purchased from Promega (Madison, WI) and Santa Cruz Biotechnology, respectively. The anti-MAP2 antibody was from Sigma (St. Louis, MO). The anti-$Fc_\gamma R_{II}$ antibody (monoclonal antibody IV.3) was obtained from Medarex (Annendale, NJ). Goat anti-mouse $F(ab)_2$ was obtained from Cappel (West Chester, PA). Affinity-purified horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibodies were purchased from Boehringer Mannheim (Indianapolis, IN). Peptides corresponding to amino acids 25–35 and 1–40 of human β-amyloid protein and amino acids 106–126 of human prion protein were purchased from Bachem (Philadelphia, PA). Scrambled β-amyloid 25–35 peptide was synthesized at Gliatech (Cleveland, OH). Scrambled prion peptide was a generous gift from Dr. Gianluigi Forloni (Milano, Italy). β-Amyloid peptides were resuspended in sterile dH_20, and prion peptides were dissolved in sterile 200 mM sodium phosphate buffer, pH 7.0. Fibrillar β-amyloid 1–40 was prepared by reconstitution of the lyophilized peptide in sterile distilled water, followed by incubation for 1 week at 37°C. Acetylated low-density lipoprotein (LDL) was a kind gift from Dr. Frederick DeBeer (University of Kentucky). Lipopolysaccharide (LPS), nitroblue tetrazolium, 12-*o*-tetradecanoylphorbol 13-acetate (TPA), glycated BSA, dantrolene, verapamil, nifedipine, and concanavalin A (Con A) were purchased from Sigma. Piceatannol was purchased from Boehringer Mannheim. Go6976 was purchased from LC Laboratories. 2,5-Di-*tert*-butyl hydroquinone (DTBHQ), thapsigargin, BAPTA, and PP1 were purchased from Calbiochem (La Jolla, CA).

Tissue culture. THP-1 cells were grown in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated fetal calf serum (FCS), 5×10^{-5} M 2-mercaptoethanol, 5 mM HEPES, and 2 μg/ml gentamycin in 5% CO_2. Microglial cultures were derived from postnatal day 1–2 mouse brain as described previously (McDonald et al., 1997). Neurons were cultured from cortices of embryonic day 17 (E17) mice (C57Bl/6J). Meninges-free cortices were isolated and digested in 0.25% trypsin and 1 mM EDTA for 15 min at 37°C. The trypsin was inactivated with DMEM containing 20% heat-inactivated FCS. Cortices were transferred to Neurobasal media with B27 supplements, triturated, and plated onto poly-L-lysine (0.05 mg/ml)-coated tissue culture wells. Neurons were grown in Neurobasal media (4.0×10^4 neurons/well) with B27 supplement for 5–7 d *in vitro* before use. The use of Neurobasal medium provided highly purified cultures of neurons to decrease the confound of contaminating glial cells in the cultures (Brewer et al., 1993).

Cell stimulation. THP-1 cells and microglia were stimulated by first removing their respective medium and replacing it with HBSS for 30 min at 37°C before stimulation. Cells were stimulated in suspension ($5–10 \times 10^6$ cells/200 μl of HBSS). To determine the effects of specific enzyme inhibition on βA and PrP stimulation of the THP-1 cells, we preincubated the cells for 45 min in the absence or presence of the drugs. To condition media, we plated THP-1 cells onto peptides bound (48 pmole/mm^2) to 48 well tissue culture dishes as described previously (Lagenaur and Lemmon, 1987; McDonald et al., 1997). Briefly, tissue culture wells were coated with nitrocellulose, and peptides were added to the coated wells and allowed to dry. The wells were then incubated with sterile 3% BSA in dH_2O for 1 hr to block cellular interactions with nitrocellulose. The BSA was removed, and THP-1 cells were added (1.8×10^4 cells) to wells containing the bound peptides in 0.25 ml of Neurobasal media for 48 hr in the presence or absence of drugs. The media were collected and then added to neuronal cultures for 72 hr. Microglial-mediated neuronal toxicity experiments involved the coculture of microglia (1.8×10^4 cells) added directly to neuronal cultures (4.0×10^4 cells) for 48 hr in the absence or presence of 1 μM βA 25–35. Neurons were fixed and stained with an anti-MAP2 (1:500) antibody. All conditions were performed in duplicate and repeated a total of four times. A counting grid was placed over the wells to count the number of neurons from eight identical fields for each condition. The average number of neurons per field was calculated for each condition to evaluate neuron survival.

Western blotting and immunoprecipitations. Cells were lysed in 200 μl of ice-cold radioimmunoprecipitation assay (RIPA) buffer (1% Triton, 0.1% SDS, 0.5% deoxycholate, 20 mM Tris, pH 7.4, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, 1 mM EDTA, 1 mM EGTA, and 0.2 M PMSF), and insoluble material was removed by centrifugation at 10,000 × *g* at 4°C for 10 min. Protein concentrations were quantitated by the method of Bradford (1976). Proteins were resolved by 7.5% SDS-PAGE and Western blotted with primary antibody [4G10 (1:2000); anti-phospho-ERK (1:20,000); anti-ERK (1:2000); anti-PYK2 (1:1000); anti-paxillin (1:5000)] overnight at 4°C. Antibody binding was detected via enhanced chemiluminescence (Pierce, Rockford, IL). To reprobe blots, we stripped them using 0.2N NaOH with vigorous shaking for 10 min at 25°C (Suck and Krupinska, 1996). Immunoprecipitations were performed by incubation of aliquots of the cellular lysates with the primary antibody (1 μg of antibody/mg of protein lysate) and Protein A-agarose for 2 hr at 4°C. The immunoprecipitates were washed three times in RIPA buffer, then resolved by 7.5% SDS-PAGE, and Western blotted as described.

Respiratory burst. Intracellular superoxide production was assayed as described by measuring the reduction of nitroblue tetrazolium (NBT) (Pick, 1986; McDonald et al., 1997). Briefly, THP-1 cells (2.0×10^6 cells per condition) were removed from media and allowed to incubate in HBSS for 30 min at 37°C with or without drugs (5 μM PP1, 50 nM thapsigargin, or 2 μM Go6976) or vehicle (DMSO). Cells were removed from HBSS, resuspended in HBSS containing 1 μg/ml NBT, and incubated for 30 min. Cells were then collected by centrifugation and sonicated in RIPA buffer to collect the NBT precipitates. Reduction of NBT was measured by the change in absorbance at 550 nm. The assays were performed in duplicate.

Cytosolic free-calcium measurement. $[Ca^{2+}]$ was measured in THP-1 cells using the fluorescent indicator fura-2 in a thermostatically controlled luminometer with magnetic stirring as described by El-Moatassim and Dubyak (1992). Calcium concentrations were calculated based on the method of Di Virgilio et al. (1988).

RESULTS

βA and PrP fibrils activate a similar tyrosine kinase-based signaling pathway in microglia and THP-1 monocytes

Exposure of both primary mouse microglia and THP-1 cells to fibrillar PrP 106–126 and βA 25–35 activated a tyrosine kinase-based intracellular signaling cascade in both THP-1 cells (Fig. 1*A*) and microglia (Fig. 1*B*). Full-length βA 1–40 and 1–42 peptides initiated intracellular signaling events, and the biologically active domain was mapped to residues 25–35 (Fig. 1*A*) (McDonald et al., 1997). Similarly, we used the biologically active domain of human prion protein comprising residues 106–126. This domain is essential for the conversion of PrP^c to PrP^{sc} and forms amyloid fibrils *in vitro* (Gasset et al., 1992; Selvaggini et al., 1993; Tagliavanni et al., 1993; Chen et al., 1995). The cellular stimulation is specific to the fibrillar conformation of the peptides because scrambled, nonfibrillar forms of PrP 106–126 and βA 25–35 did not elicit any increase in protein tyrosine phosphorylation levels (Fig. 1*A*). This response was not mediated by the receptor for advanced glycation end products (RAGE) or scavenger receptors because ligands for these receptors (glycated BSA and acetylated LDL, respectively) did not alter protein tyrosine phosphorylation levels (Fig. 1*A*). The pattern of protein tyrosine phosphorylation elicited by the βA and PrP peptides was qualitatively similar; however, the relative phosphotyrosine content of the individual proteins varied between experiments (Fig. 1*C*). The fibril-stimulated protein tyrosine phosphorylation results in the phosphorylation of a number of proteins that are also phosphorylated after activation of classical immune receptors such as $Fc_\gamma R_{II}$ (Fig. 1*C*). THP-1 cells were typically stimulated by adding fibrillar PrP 106–126 and βA 25–35 directly to the cells in solution. However, stimulation of protein tyrosine phosphorylation was also observed when the THP-1 cells were plated directly onto fibrillar peptides bound to tissue culture wells (data not shown) (McDonald et al., 1997, 1998).

The persistent exposure of THP-1 cells and primary mouse microglia to βA 25–35 (Fig. 2*B*,*E*, respectively) and PrP 106–126

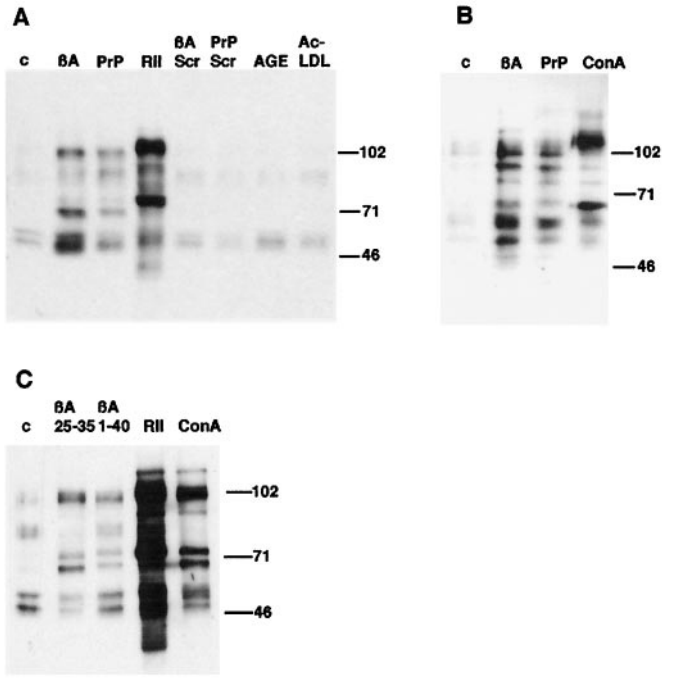

Figure 1. *βA* and *PrP* fibrils specifically activate tyrosine kinase signaling pathways in microglia and THP-1 monocytes. *A*, Protein phosphotyrosine levels were evaluated in THP-1 cells after stimulation with *PrP*, *βA*, scrambled *βA* (*Scr βA*) 25–35 (40 μM; 2 min), scrambled *PrP* (*Scr PrP*) 106–126 (80 μM; 5 min), 20 μg/ml glycated BSA + 20 μg/ml lactoferrin (*AGE*) (2 min; 37°C), or 20 μg/ml acetylated LDL (*Ac-LDL*) (2 min; 37°C). *B*, Protein phosphotyrosine levels were compared in primary mouse microglia stimulated in solution with the fibrillar peptides *PrP* 106–126 or *βA* 25–35 or with 60 μg/ml concanavalin A (*ConA*) to serve as a positive control. *C*, Protein tyrosine phosphorylation changes were compared when THP-1 cells were stimulated with full-length fibrillar *βA 1–40* peptide, *βA 25–35* peptide, or positive controls cross-linking of $Fc_\gamma R_{II}$ [25 μg/ml anti-$Fc_\gamma R_{II}$ antibody (Ab) + 100 μg/ml GαM Fab_2 (15 min on ice with Ab + 2 min at 37°C with Fab_2)] and Con A. Aliquots of the cell lysates were resolved by SDS-PAGE, Western blotted using the anti-phosphotyrosine antibody 4G10, and visualized by chemiluminescence.

(Fig. 2*C*,*F*, respectively) fibrils for 48 hr resulted in the elevation of phosphotyrosine immunoreactivity. These *in vitro* findings confirmed that sustained exposure of the cells to the fibrillar peptides evoked the prolonged activation of tyrosine kinase-based signaling events and functionally modeled the *in vivo* response of the plaque-associated microglia in the AD brain that exhibit high levels of phosphotyrosine (Wood and Zinsmeister, 1991).

The tyrosine kinases Lyn and Syk regulate MAP kinase activation

Lyn and Syk represent the principal catalytic components mediating the signaling response of cells of the monocytic lineage to immune stimuli (Ghazizadeh et al., 1994, 1995; Marcilla et al., 1995; Crowley et al., 1997; Vonakis et al., 1997). To test whether Lyn and Syk are involved as proximal elements in the signaling activation pathways stimulated by βA or PrP, we treated cells with a specific inhibitor of src family kinases, PP1, to block Lyn activity (Hanke et al., 1996) or with the Syk-selective inhibitor piceatannol (Oliver et al., 1994) and evaluated their effects on the activation of downstream signaling elements. Pretreatment of the THP-1 cells with PP1 and piceatannol inhibited ERK activation in response to fibrillar PrP 106–126 and βA 25–35 (Fig. 3*B–D*).

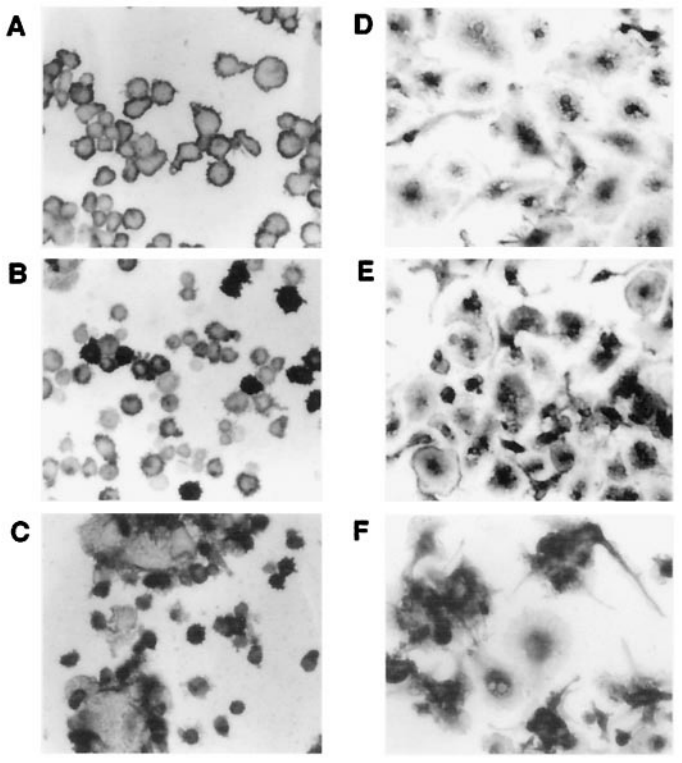

Figure 2. βA and PrP fibril stimulation of THP-1 cells and microglia induces increased phosphotyrosine staining. Primary mouse microglia (*D–F*) and THP-1 cells (*A–C*) were plated onto uncoated dishes (*A*, *D*) or dishes coated with βA 25–35 (*B*, *E*) or PrP 106–126 (*C*, *F*) and cultured for 48 hr. Cells were fixed in 4% paraformaldehyde and stained with the anti-phosphotyrosine antibody 4G10. Immunoreactivity was visualized using 3,3′-diaminobenzidine tetrahydrochloride as the chromogen.

As controls, the fibril-stimulated increase in protein tyrosine phosphorylation was also monitored after blocking Lyn and Syk activation. Inhibition of Lyn activity prevented the fibril-activated increase in protein tyrosine phosphorylation (Fig. 3*E*,*F*). Inhibition of Syk activation produced only a partial decrease in the changes in protein tyrosine phosphorylation (Fig. 3*G*), consistent with a scheme in which Syk is recruited to the receptor complex for activation downstream of Lyn.

βA and PrP fibril stimulation of THP-1 cells elicits a transient increase in intracellular calcium levels released from intracellular stores

Activation of monocyte immune receptor signaling pathways can involve an increase in intracellular calcium levels (Odin et al., 1991; Liao et al., 1992; Rankin et al., 1993; Shen et al., 1994). Moreover, it has been suggested that βA and PrP fibrils interact with cell membranes to alter specific calcium channel activities (Korotzer et al., 1995; Florio et al., 1996; Fraser et al., 1997; Herms et al., 1997; Lin et al., 1997).

We tested whether fibril stimulation of THP-1 cells altered levels of intracellular calcium. Fura-2–loaded THP-1 cells stimulated with either βA 25–35 or PrP 106–126 fibrils displayed a transient increase in intracellular calcium levels (Fig. 4*A*,*C*). The calcium was released from intracellular stores because elimination of extracellular calcium did not affect the fibril-stimulated increase in intracellular calcium levels (Fig. 4*B*,*D*). The depletion of extracellular calcium was confirmed by the inability of UTP to elicit an influx of calcium (data not shown).

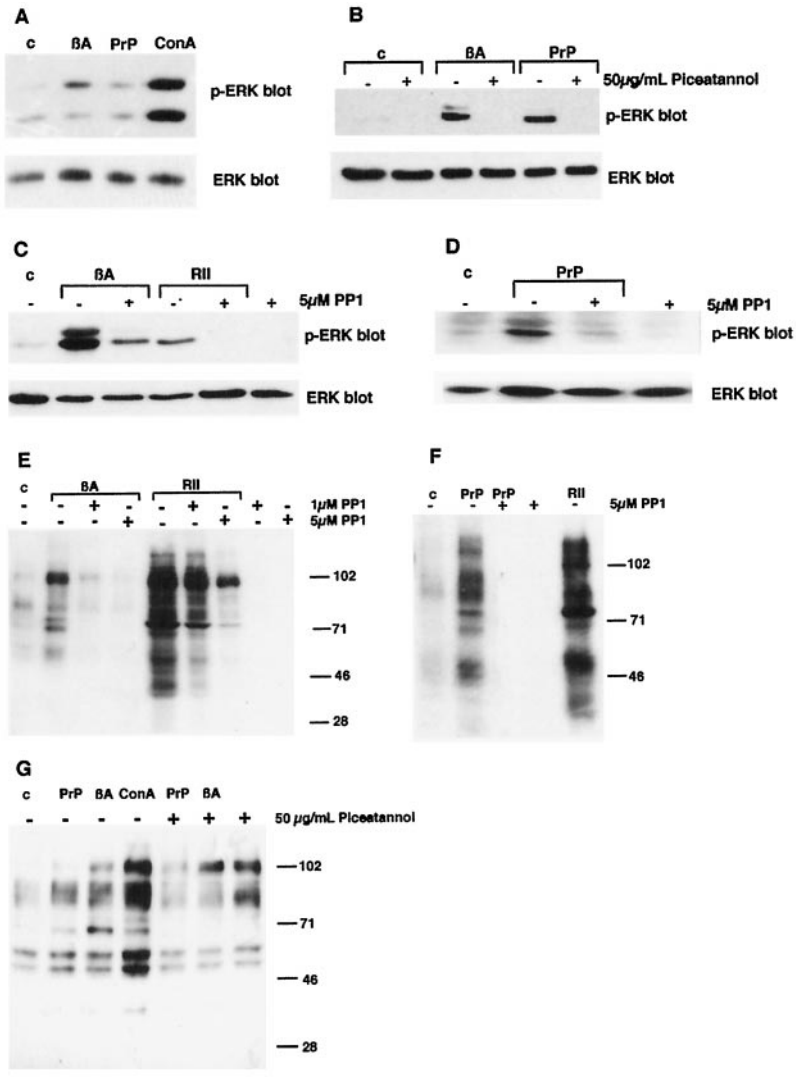

Figure 3. *βA* and *PrP* fibril stimulation of THP-1 cells requires activation of the tyrosine kinases Lyn and Syk to activate the MAP kinases. THP-1 cells were incubated in DMSO vehicle (*c*), 1 or 5 μM *PP1*, or 50 μg/ml piceatannol (45 min; 37°C for each) before incubation in *PrP* 106–126 (80 μM; 5 min) or *βA* 25–35 (40 μM; 2 min) or cross-linking of $Fc_\gamma R_{II}$ [25 μg/ml anti-$Fc_\gamma R_{II}$ Ab + 100 μg/ml GαM Fab_2 (15 min on ice with Ab + 2 min at 37°C with Fab_2)] or Con A (60 μg/ml; 5 min) as a positive control. Aliquots of the cell lysates were resolved by SDS-PAGE, Western blotted, and visualized by chemiluminescence. An antibody directed against the activated, phosphorylated forms of ERK1 and ERK2 (*p-ERK*) was used to monitor ERK activation. To normalize for protein load, we stripped the blots and reprobed them with anti-ERK antibody (*ERK*). Primary mouse microglia were stimulated with *PrP*, *βA*, or Con A, as described above. *A*, ERK phosphorylation was examined in primary mouse microglia stimulated with *PrP* 106–126 or *βA* 25–35. *B–D*, ERK phosphorylation and activation were also examined in THP-1 cells pretreated with 50 μg/ml piceatannol (*B*) or 5 μM *PP1* (*C*, *D*) before stimulation with *PrP* 106–126 and *βA* 25–35. *E*, *F*, Changes in protein tyrosine phosphorylation levels were examined using the anti-phosphotyrosine Ab 4G10, when THP-1 cells were pretreated with 1 or 5 μM *PP1* before stimulation with *βA* 25–35 (*E*) and *PrP* 106–126 (*F*). *G*, Changes in protein tyrosine phosphorylation levels were also monitored when THP-1 cells were pretreated with 50 μg/ml piceatannol before stimulation with *βA* 25–35 or *PrP* 106–126.

Monocyte immune receptor signaling pathways, such as those downstream of $Fc_\gamma R_{II}$, involve an increase in intracellular calcium levels via activation of phospholipase $C_{\gamma 1}$ ($PLC_{\gamma 1}$) (Odin et al., 1991; Liao et al., 1992; Rankin et al., 1993; Shen et al., 1994). Importantly, the βA and PrP fibril stimulation of THP-1 cells did not involve the activation of $PLC_{\gamma 1}$ and the subsequent IP_3-mediated intracellular calcium release. Neither βA nor PrP fibril stimulation led to the tyrosine phosphorylation and activation of $PLC_{\gamma 1}$, clearly demonstrating that these stimuli use pathways that are mechanistically distinct from those used by $Fc_\gamma R_{II}$ stimulation (Fig. 4*E*).

Intracellular calcium levels regulate the protein tyrosine phosphorylation of a subset of proteins

To establish the linkage between the increase in intracellular calcium levels and the activation of the tyrosine kinase signaling cascade, we evaluated levels of protein tyrosine phosphorylation in fibril-stimulated THP-1 cell lysates after manipulating the levels of both extra- and intracellular calcium. First, we confirmed that no influx of extracellular calcium was required for activation of the tyrosine kinase-dependent signaling pathway during stimulation with PrP 106–126 and βA 25–35 because stimulation of the THP-1 cells in calcium-free media had little affect on βA- and PrP-induced protein tyrosine phosphorylation changes (Fig. 5*A*).

Because there is some evidence that βA and PrP peptides interact with cell membranes and influence L-type calcium channel activity, we verified that calcium influx through L-type channels was not contributing to the changes in protein tyrosine phosphorylation levels (Korotzer et al., 1995; Florio et al., 1996; Fraser et al., 1997; Herms et al., 1997; Lin et al., 1997). Pretreatment of the cells with the specific L-type calcium channel antagonists verapamil and nifedipine had no affect on increased protein tyrosine phosphorylation after βA and PrP fibril stimulation (data not shown) (Carafoli, 1987; Palade et al., 1989). These data confirmed that extracellular calcium influx was not involved in tyrosine kinase activation by βA and PrP fibril stimulation.

We tested whether an increase in intracellular calcium levels was required for activation of a subset of the tyrosine kinase activities in response to the fibrillar peptides by depletion of the intracellular calcium stores and monitoring of the effect on fibril-induced protein tyrosine phosphorylation changes. Western blot analysis of THP-1 cell lysates stimulated with βA 25–35 or PrP 106–126 showed a decrease in tyrosine phosphorylation of several proteins after pretreatment with the calcium ATPase inhibitors DTBHQ and thapsigargin (Fig. 5*B*,*C*) (Charles et al., 1993; Khan et al., 1995). A similar decrease was observed when cells were pretreated with the ryanodine receptor inhibitor dantrolene as

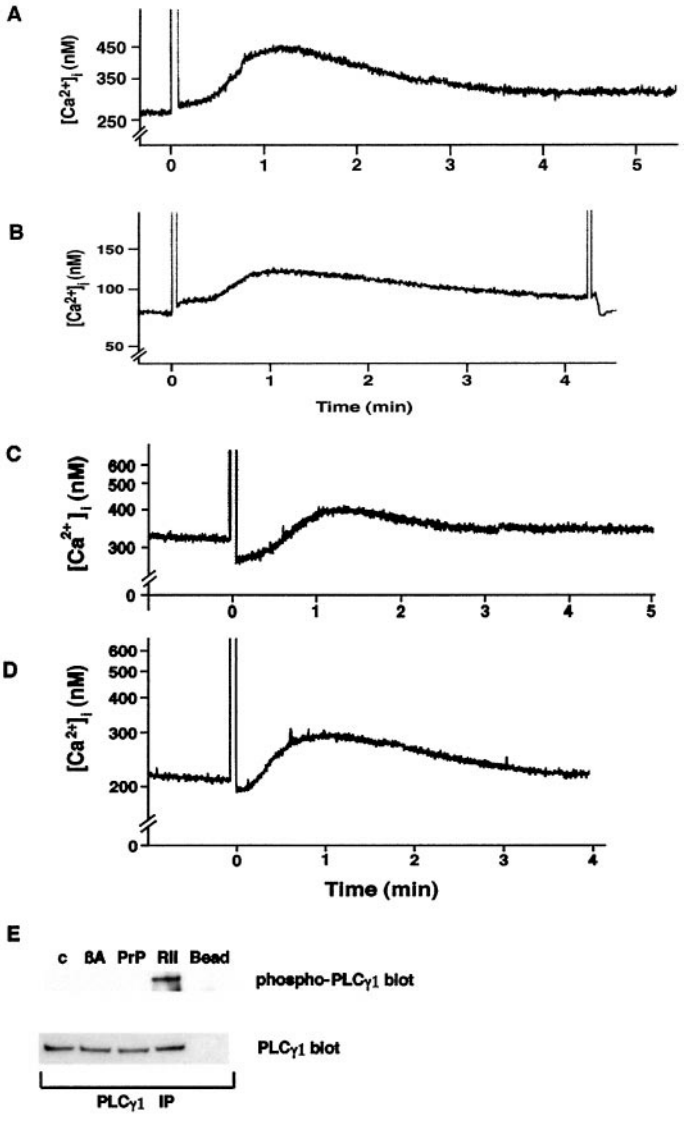

Figure 4. βA and *PrP* fibril stimulation of THP-1 cells leads to the release of intracellular calcium. THP-1 cells were preloaded for 45 min with 0.5 mM fura-2 AM, and basal levels of intracellular Ca were monitored for 3 min. *A–D*, At time 0, cells were stimulated with 40 μM β-amyloid (*A*) or 80 μM *PrP* 106–126 (*C*) (3.75×10^6 cells/condition) for ~5 min in a calcium-containing or calcium-free balanced salt solution. The effect of removing extracellular calcium on *βA* 25–35- (*B*) and *PrP* 106–126-induced (*D*) changes in intracellular calcium levels was compared. Data are representative of two independent experiments. *E*, $PLC_{\gamma 1}$ was immunoprecipitated from lysates of THP-1 cells stimulated with *PrP* 106–126 (80 μM; 5 min) or *βA* 25–35 (40 μM; 2 min) or with cross-linking of $Fc_\gamma R_{II}$ as a positive control [25 μg/ml anti-$Fc_\gamma R_{II}$ Ab + 100 μg/ml GαM Fab_2 (15 min on ice with Ab + 2 min at 37°C with Fab_2)]. Antibody specificities were verified by performing one immunoprecipitation (*IP*) in the absence of the immunoprecipitating antibody (*Bead*). The anti-phosphotyrosine antibody 4G10 was used to Western blot the immunoprecipitated protein. The blot was stripped and reprobed with the immunoprecipitating antibody to normalize for protein load.

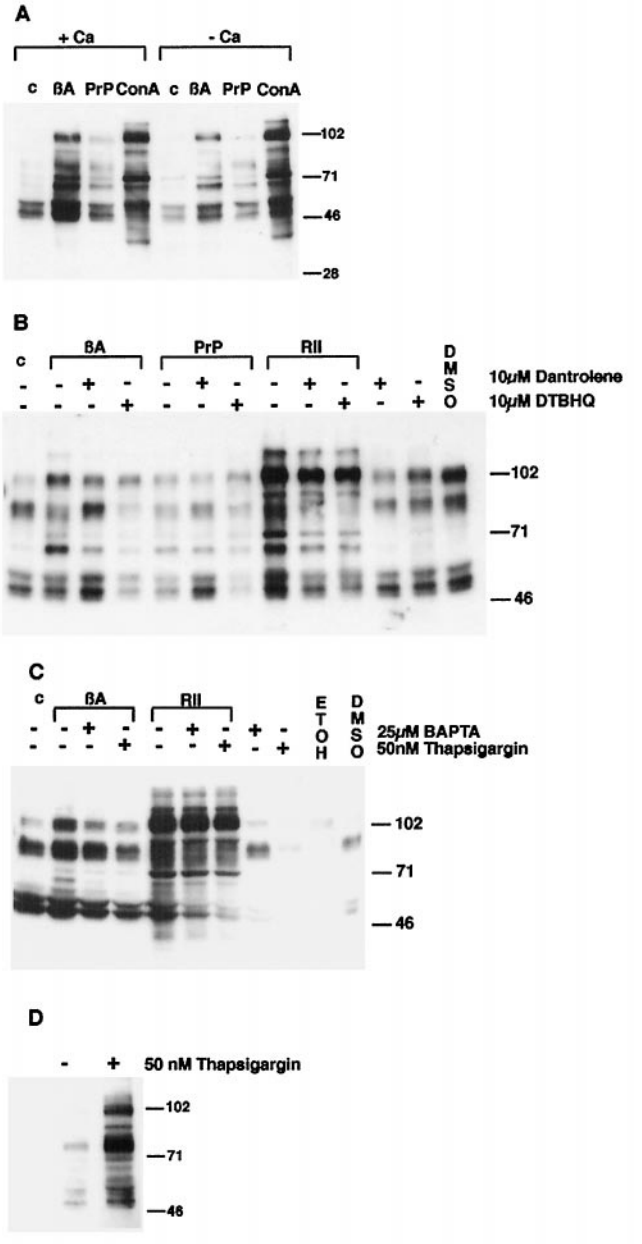

Figure 5. Release of intracellular calcium is required for specific tyrosine kinase activation during βA and PrP fibril stimulation of THP-1 cells. Aliquots of cell lysates were resolved by SDS-PAGE, Western blotted using the anti-phosphotyrosine antibody 4G10, and visualized by chemiluminescence. *A*, Protein phosphotyrosine levels were evaluated after THP-1 cells were stimulated with *PrP* 106–126 (80 μM; 5 min), with *βA* 25–35 (40 μM; 2 min), or with Con A (60 μg/ml; 5 min) as a positive control in either calcium-containing HBSS (+*Ca*) or calcium-free HBSS containing 1 mM EDTA and 1 mM EGTA (−*Ca*). *B*, *C*, Protein phosphotyrosine levels were then compared when THP-1 cells were incubated in *DMSO* vehicle (*c*), 10 μM *DTBHQ* (*B*), 10 μM dantrolene (*B*), 25 μM *BAPTA* (*C*), or 50 nM thapsigargin (*C*) (45 min; 37°C for each) before incubation in *PrP* 106–126 (80 μM; 5 min) or *βA* 25–35 (40 μM; 2 min) or cross-linking of $Fc_\gamma R_{II}$ as a positive control [25 μg/ml anti-$Fc_\gamma R_{II}$ Ab + 100 μg/ml GαM Fab_2 (15 min on ice with Ab + 2 min at 37°C with Fab_2)]. *D*, Protein phosphotyrosine changes were also examined when THP-1 cells were treated with *DMSO* vehicle or 50 nM thapsigargin for 10 min.

well as the intracellular calcium chelator BAPTA (Fig. 5*B*,*C*) (Charles et al., 1993; Bissonnette et al., 1994). These data confirmed that the increase in protein tyrosine kinase activity stimulated by βA and PrP fibril stimulation was mediated, in part, by changes in intracellular calcium. To address this further, we induced a transient release of calcium from intracellular stores by

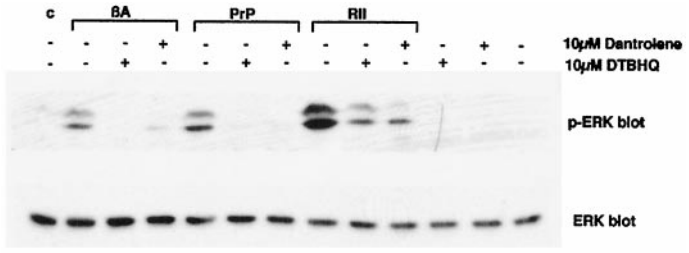

Figure 6. *βA* and *PrP* fibril stimulation of MAP kinase activity in THP-1 cells requires an intracellular calcium release. THP-1 cells were incubated in DMSO vehicle (*c*), 10 μM *DTBHQ*, or 10 μM dantrolene (45 min; 37°C for each) before incubation in *PrP* 106–126 (80 μM; 5 min) or *βA* 25–35 (40 μM; 2 min) or cross-linking of $Fc_\gamma R_{II}$ as a positive control [25 μg/ml anti-$Fc_\gamma R_{II}$ Ab + 100 μg/ml GαM Fab_2 (15 min on ice with Ab + 2 min at 37°C with Fab_2)]. Aliquots of the cell lysates were resolved by SDS-PAGE and Western blotted with an antibody directed against the activated, phosphorylated forms of ERK1 and ERK2 (*p-ERK*). Blots were stripped and reprobed with an anti-ERK antibody (*ERK*) to normalize for protein load.

treatment of THP-1 cells with thapsigargin. Short periods of thapsigargin treatment elicited an increase in protein tyrosine phosphorylation, demonstrating that intracellular calcium levels regulate a subset of tyrosine kinase activities in THP-1 cells (Fig. 5*D*)

MAP kinase activation in response to βA and PrP fibril stimulation requires intracellular calcium

The discovery of the fibril-stimulated elevation in intracellular calcium levels led us to investigate whether this was an obligatory intermediate step in signaling pathways leading to activation of the MAP kinases. Depletion of intracellular calcium stores with DTBHQ and dantrolene prevented the PrP 106–126- and βA 25–35-induced activation of ERKs (Fig. 6). These data establish that the elevation in the concentration of intracellular calcium ion is necessary for the downstream activation of the MAP kinases.

PKC activity is required for protein tyrosine phosphorylation and ERK activation in βA and PrP fibril-stimulated THP-1 cells

Classical PKC isoforms are well characterized effectors of calcium signals, and members of this family have been shown to be involved in immune receptor signaling (Shen et al., 1994; Karimi and Lennartz, 1995). We tested whether PKC was involved in fibril-stimulated signaling events by pretreatment of the THP-1 cells with a specific inhibitor to the calcium/phospholipid-dependent PKCs, Go6976 (Martiny-Baron et al., 1993). PKC inhibition resulted in a decrease in protein tyrosine phosphorylation induced by both βA 25–35 and PrP 106–126 treatment of THP-1 cells (Fig. 7*A*). Similar results were obtained with another PKC inhibitor, chelerythrine chloride (data not shown) (Herbert, 1990). These findings established that PKC activity was required for activation of a subset of tyrosine kinases. Control studies were performed to verify that PKC activity led to tyrosine kinase activation in these cells by treatment of the cells with a phorbol ester (TPA) that induced an increase in protein tyrosine phosphorylation levels (Fig. 7*B*).

Pretreatment of THP-1 cells with Go6976 prevented ERK activation by PrP 106–126 and βA 25–35 (Fig. 7*C*,*E*). Control experiments verified that Go6976 pretreatment also prevented a TPA-induced activation of the ERKs in THP-1 cells (Fig. 7*D*). These data demonstrate that PKC can drive a tyrosine kinase-dependent pathway required for ERK activation in response to fibril stimulation.

The calcium-sensitive tyrosine kinase PYK2 is activated by βA and PrP fibril stimulation of THP-1 cells

A recently described calcium-sensitive tyrosine kinase, PYK2, becomes activated after elevation of intracellular calcium levels (Lev et al., 1995; Li et al., 1998). The calcium-dependent regulation of PYK2 is indirect and is mediated via as yet unidentified elements. Treatment of THP-1 cells with βA or PrP fibrils resulted in the stimulation of PYK2 tyrosine phosphorylation (Fig. 8*A*), which is reflective of the enzymatic activation of this enzyme (Lev et al., 1995). PYK2 phosphorylation was blocked by pretreatment of the cells with inhibitors of Lyn and PKC (PP1 and Go6976, respectively) (Fig. 8*A*). Moreover, inhibition of intracellular calcium release by treatment of the cells with DTBHQ also blocked fibril-mediated PYK2 activation (Fig. 8*B*).

As an additional means of positioning PYK2 within this pathway, we used TPA to stimulate PKC activity directly to show that PYK2 was subsequently activated (Fig. 8*C*). The TPA-driven PYK2 phosphorylation was inhibited by the specific PKC inhibitor Go6976. These observations demonstrate that PYK2 phosphorylation is a consequence of calcium release and PKC activation in these cells.

We performed an additional study to verify that PYK2 was being activated by the βA and PrP fibril stimulation pathway. The cytoskeletal protein paxillin is a known substrate of PYK2 that is phosphorylated after PYK2 activation (Hiregowdara et al., 1997; Li and Earp, 1997; Ostergaard et al., 1998). Both PrP 106–126 and βA 25–35 stimulated an increase in paxillin phosphorylation that was inhibited by pretreating the cells with DTBHQ, PP1, and Go6976 (Fig. 8*D*,*E*). TPA stimulation of PKC activity and the subsequent PYK2 activation also led to tyrosine phosphorylation of paxillin (Fig. 8*F*).

These data support the conclusion that PYK2 activation in the βA and PrP pathway is a consequence of Lyn and Syk activation, intracellular calcium release, and PKC activation and is positioned downstream of these elements in the signal transduction pathway (see Fig. 11).

Distinct signaling pathways lead to βA and PrP fibril stimulation of respiratory burst and superoxide anion generation

Macrophages respond to immune stimuli by activation of a respiratory burst leading to the generation of intracellular superoxide anion (Chanock et al., 1994; Rosen et al., 1995). Extracellular diffusion of these reactive oxygen species acts as a toxin to surrounding cells. We observed that βA and PrP fibrils induced a respiratory burst in THP-1 cells measured as the reduction of NBT by intracellular superoxide anion (Fig. 9) (Pick, 1986). The fibril-stimulated respiratory burst was not inhibited by pretreating the cells with inhibitors of Lyn, PKC, or intracellular calcium release (PP1, Go6976, or thapsigargin, respectively). These data demonstrate that NADPH oxidase activation operates via signaling pathways that are mechanistically distinct from those required for ERK activation and stimulation of cytokine synthesis (Tannenbaum and Hamilton, 1989; Wood, 1994; Schmid-Alliana et al., 1998).

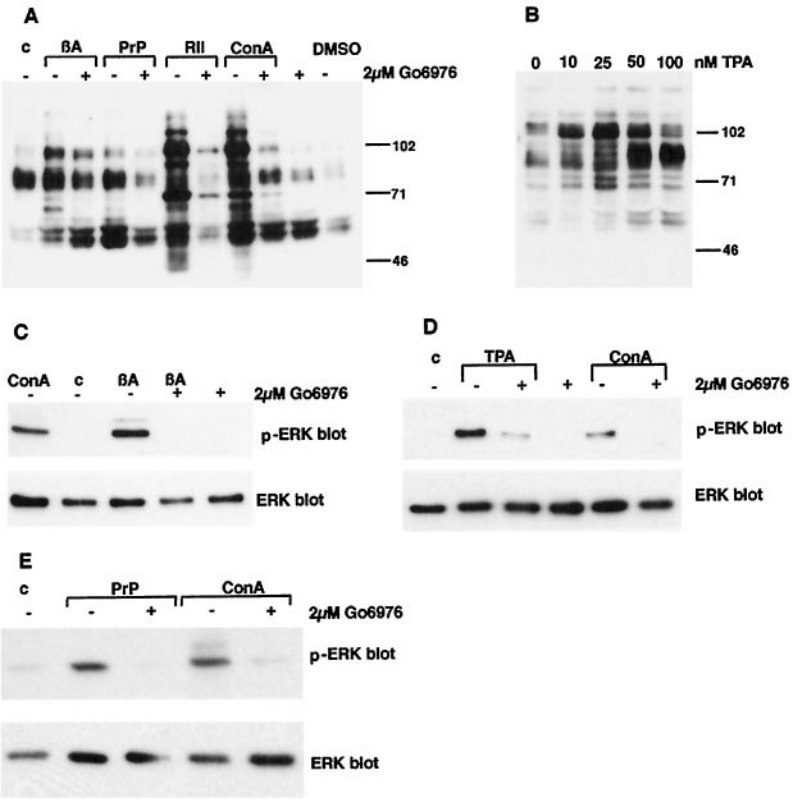

Figure 7. *βA* and *PrP* stimulation of THP-1 cells requires activation of PKC to activate downstream tyrosine kinases and the ERKs. THP-1 cells were incubated in *DMSO* or ethanol vehicle (*c*) or in 2 μM *Go6976* (45 min; 37°C) before incubation in *PrP* 106–126 (80 μM; 5 min) or *βA* 25–35 (40 μM; 2 min) or cross-linking of $Fc_\gamma R_{II}$ [25 μg/ml anti-$Fc_\gamma R_{II}$ Ab + 100 μg/ml GαM Fab_2 (15 min on ice with Ab + 2 min at 37°C with Fab_2)] or Con A (60 μg/ml; 5 min) as a positive control. Aliquots of cell lysate were resolved by SDS-PAGE, Western blotted, and visualized by chemiluminescence. *A*, Protein phosphotyrosine levels were compared, using the anti-phosphotyrosine antibody 4G10, when THP-1 cells were preincubated in 2 μM *Go6976* before stimulation with *PrP* 106–126 and *βA* 25–35. *B*, Changes in protein tyrosine phosphorylation were also monitored when THP-1 cells were incubated in ethanol vehicle (0 nM *TPA*) or in increasing concentrations of the phorbol ester *TPA*. Cell lysates were also Western blotted using an antibody directed against the activated, phosphorylated forms of ERK1 and ERK2. Blots were stripped and reprobed with an anti-ERK antibody to normalize for protein load. *C–E*, ERK phosphorylation and activation were examined in THP-1 cells pretreated with 2 μM *Go6976* before stimulation with *βA* 25–35 (*C*), *PrP* 106–126 (*E*), or 100 nM *TPA* (*D*).

The βA and PrP fibril-stimulated tyrosine kinase signaling pathway stimulates THP-1 monocytes to produce neurotoxic factor(s)

Numerous reports have described the ability of microglial lineage cells to generate neurotoxic products in response to treatments with βA or PrP peptides (Forloni et al., 1993; Giulian et al., 1995; Brown et al., 1996; Ii et al., 1996; Klegeris and McGeer, 1997; Klegeris et al., 1997; Kretzschmar et al., 1997; Lorton, 1997; McDonald et al., 1997; C. K. Combs, D. R. McDonald, and G. E. Landreth, unpublished observations). We used a tissue culture system that uses highly purified populations of primary mouse neurons either cocultured with purified mouse microglia or cultured in conditioned media from THP-1 cells to establish whether the elaboration of neurotoxic and proinflammatory products was dependent on the tyrosine kinase-based signaling pathways identified here. Purified mouse microglia and THP-1 cells were stimulated with PrP 106–126, βA 25–35, or βA 1–40 for 48 hr with or without the presence of selected, specific enzyme inhibitors. We observed that conditioned media from untreated THP-1 cells or microglia provoked only a low level of neuronal death (Fig. 10). However, incubation of neurons with conditioned medium from βA- and PrP-stimulated THP-1 cells or microglia resulted in a dramatically greater degree of neuronal death.

To evaluate whether the tyrosine kinase-based signaling pathways were responsible for generating the neurotoxic products, inhibitors of specific enzymes in the pathway were used to treat the THP-1 cells during the 48 hr period of βA and PrP fibril stimulation. Treatment of THP-1 cells with PP1, Go6976, and piceatannol (inhibiting Lyn, PKC, and Syk, respectively) inhibited the production of neurotoxic products (Fig. 10*C*,*D*). We also pretreated THP-1 cells with the MEK inhibitor PD98095 to inhibit ERK activation downstream of βA and PrP fibril stimulation (Alessi et al., 1995). A similar neuroprotective effect was observed. These data clearly demonstrate that the fibril-activated signaling pathways we have defined are directly responsible for the generation of neurotoxic products (Fig. 11).

DISCUSSION

There is compelling evidence that inflammatory processes play a significant role in the pathophysiology of AD (McGeer et al., 1993; McGeer and McGeer, 1995). Indeed, in a number of studies, treatment with nonsteroidal anti-inflammatory drugs (NSAIDS) has been shown to reduce dramatically the incidence of AD-related dementia (Rich et al., 1995; McGeer and McGeer, 1996; McGeer et al., 1996). Recently, Stewart et al. (1997) have documented in a longitudinal study that patient populations treated over extended intervals with these drugs were found to be at a substantially reduced risk of acquiring AD. The principal action of these drugs is to suppress the production of proinflammatory products that are primarily secretory products of microglia. NSAID use was correlated with a significant reduction in the number of activated microglia present in the AD brain (Mackenzie and Munoz, 1998). The association of reactive microglia with amyloid deposits in the brain is an invariant feature of AD, its animal models, and a subset of the prion diseases. Several studies have demonstrated an ability of both βA and PrP peptide fibrils to activate microglia to secrete cytokines, reactive oxygen species, and other neurotoxins (Forloni et al., 1993; Giulian et al., 1995; Brown et al., 1996; Ii et al., 1996; Klegeris and McGeer, 1997; Klegeris et al., 1997; Kretzschmar et al., 1997; Lorton, 1997; McDonald et al., 1997; Combs, McDonald, and Landreth, unpublished observations).

We have demonstrated that microglia can respond to fibrillar amyloid peptides by initiation of a complex signal transduction

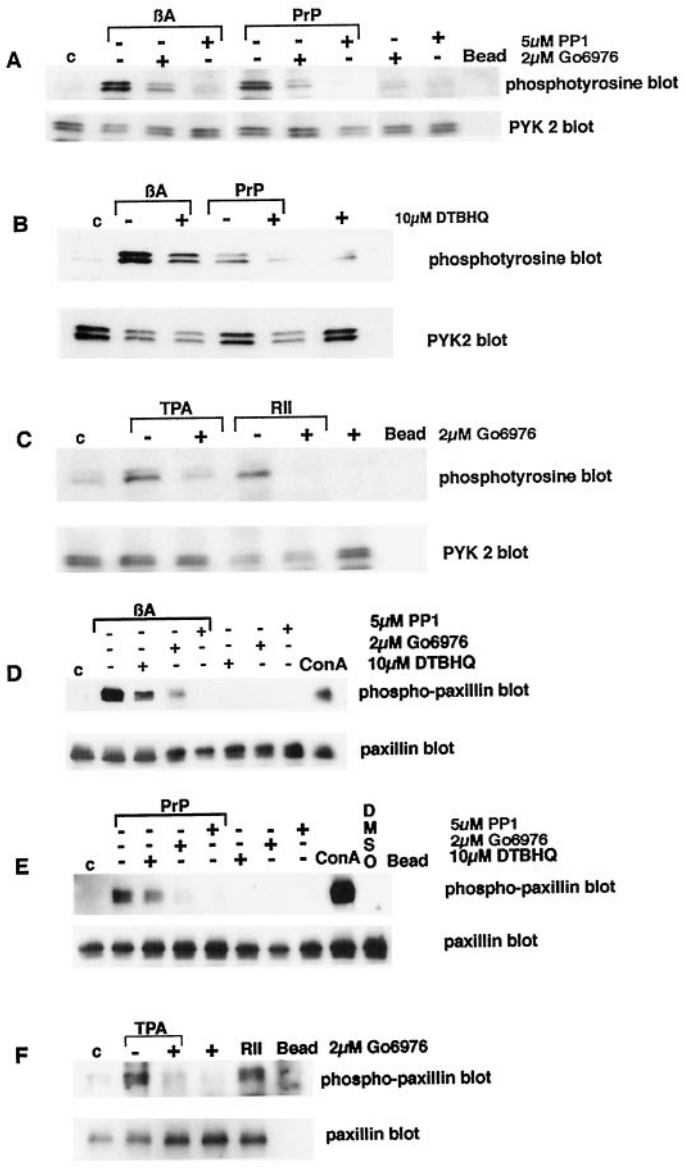

Figure 8. The calcium-sensitive tyrosine kinase *PYK2* is activated after *βA* and *PrP* fibril stimulation of THP-1 cells. THP-1 cells were incubated in *DMSO* vehicle (*c*), 50 nM thapsigargin, 10 μM *DTBHQ*, 5 μM *PP1*, or 2 μM *Go6976* (45 min; 37°C for each) before incubation in *PrP* 106–126 (80 μM; 5 min) or *βA* 25–35 (40 μM; 2 min) or cross-linking of $Fc_\gamma R_{II}$ [25 μg/ml anti-$Fc_\gamma R_{II}$ Ab + 100 μg/ml GαM Fab_2 (15 min on ice with Ab + 2 min at 37°C with Fab_2)] or Con A (60 μg/ml; 5 min) as a positive control. The tyrosine kinase *PYK2* and the cytoskeletal protein paxillin were immunoprecipitated from lysates of THP-1 cells and resolved by SDS-PAGE. The anti-phosphotyrosine antibody 4G10 was used to Western blot the immunoprecipitated proteins. The blots were stripped and reprobed with the immunoprecipitating antibodies to normalize for protein load. *A*, *B*, *D*, *E*, Changes in *PYK2* (*A*, *B*) and paxillin (*D*, *E*) tyrosine phosphorylation were observed after pretreatment of THP-1 cells with 10 μM *DTBHQ*, 5 μM *PP1*, or 2 μM *Go6976* before stimulation with *PrP* 106–126 and *βA* 25–35. *C*, *F*, Changes in *PYK2* (*C*) and paxillin (*F*) tyrosine phosphorylation were also observed after pretreatment of THP-1 cells with 2 μM *Go6976* before stimulation with 100 nM *TPA*.

cascade leading to the acquisition of a reactive phenotype and the synthesis of acute phase and proinflammatory products (McDonald et al., 1997). The microglia respond to either βA or PrP fibrils by activating a common tyrosine kinase-dependent signaling response (McDonald et al., 1997, 1998; Combs, McDonald,

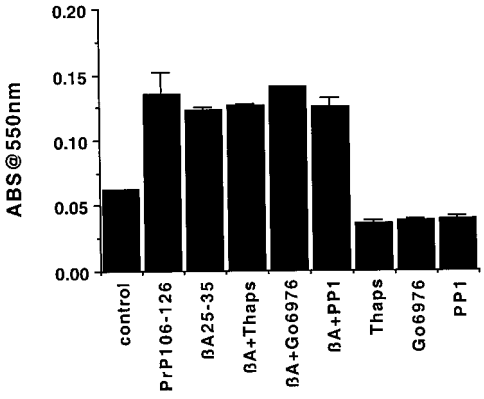

Figure 9. *βA* and *PrP* fibrils stimulate a respiratory burst in THP-1 cells. THP-1 cells were incubated in DMSO vehicle (*control*), 50 nM thapsigargin (*Thaps*), 5 μM *PP1*, or 2 μM *Go6976* (30 min; 37°C for each) before incubation in *PrP* 106–126 (40 μM) or *βA* 25–35 (40 μM). Cells were stimulated for 30 min in HBSS containing 1 μg/ml NBT. Cells were collected and sonicated in RIPA buffer. Generation of superoxide anion was measured by the change in absorbance at 550 nm. The mean absorbance values (± SEM) representative of three independent experiments are shown.

and Landreth, unpublished observations). The signaling pathways activated by amyloid fibrils are also used by classical immune receptors in cells of this lineage to elicit a proinflammatory response and acquisition of a reactive phenotype. This provides a mechanistic explanation for the functionally similar response to the two classes of stimuli.

The present study extends our previous work by identifying new elements that participate in the amyloid-induced signaling cascades and by establishing the position of the various signaling elements within the pathways. We have demonstrated previously that the tyrosine kinases Lyn and Syk are both activated as proximal components in this fibril-stimulated signaling cascade (McDonald et al., 1997, 1998; Combs, McDonald, and Landreth, unpublished observations). This is analogous to typical inflammatory signaling pathways such as those mediated via immune receptors like $Fc_\gamma R_{II}$. The present study has demonstrated that amyloid stimulation leads to the elevation of intracellular calcium levels as a consequence of its release from intracellular stores. Significantly, the fibril-induced calcium mobilization does not involve the action of $PLC_{\gamma 1}$ that mechanistically distinguishes this signaling event from that used by $Fc_\gamma R_{II}$. $Fc_\gamma R_{II}$ uses Syk to phosphorylate and activate $PLC_{\gamma 1}$ for phosphotidylinositol 4,5-bisphosphate cleavage, generation of IP_3, intracellular calcium release, and subsequent PKC activation (Liao et al., 1992; Rankin et al., 1993; Shen et al., 1994). Amyloid fibril exposure leads to the activation of calcium/phospholipid-dependent PKC isoforms. These events are necessary not only for the subsequent activation of the calcium-sensitive tyrosine kinase PYK2 but also for the downstream activation of the ERKs. It is unclear whether PYK2 activation is required for ERK activation in the βA and PrP fibril signaling pathway. In other cell types, however, PYK2 activation is clearly linked to ras/raf-dependent ERK activation via binding to the small adapter protein shc (Lev et al., 1995; Della Rocca et al., 1997).

In the AD brain, plaque-associated microglia exhibit elevated levels of tyrosine phosphoproteins, indicative of sustained activation of intracellular signaling pathways (Wood and Zinsmeister, 1991). We have now established, using an *in vitro* model, that the sustained contact of monocytes and microglia with fibrillar pep-

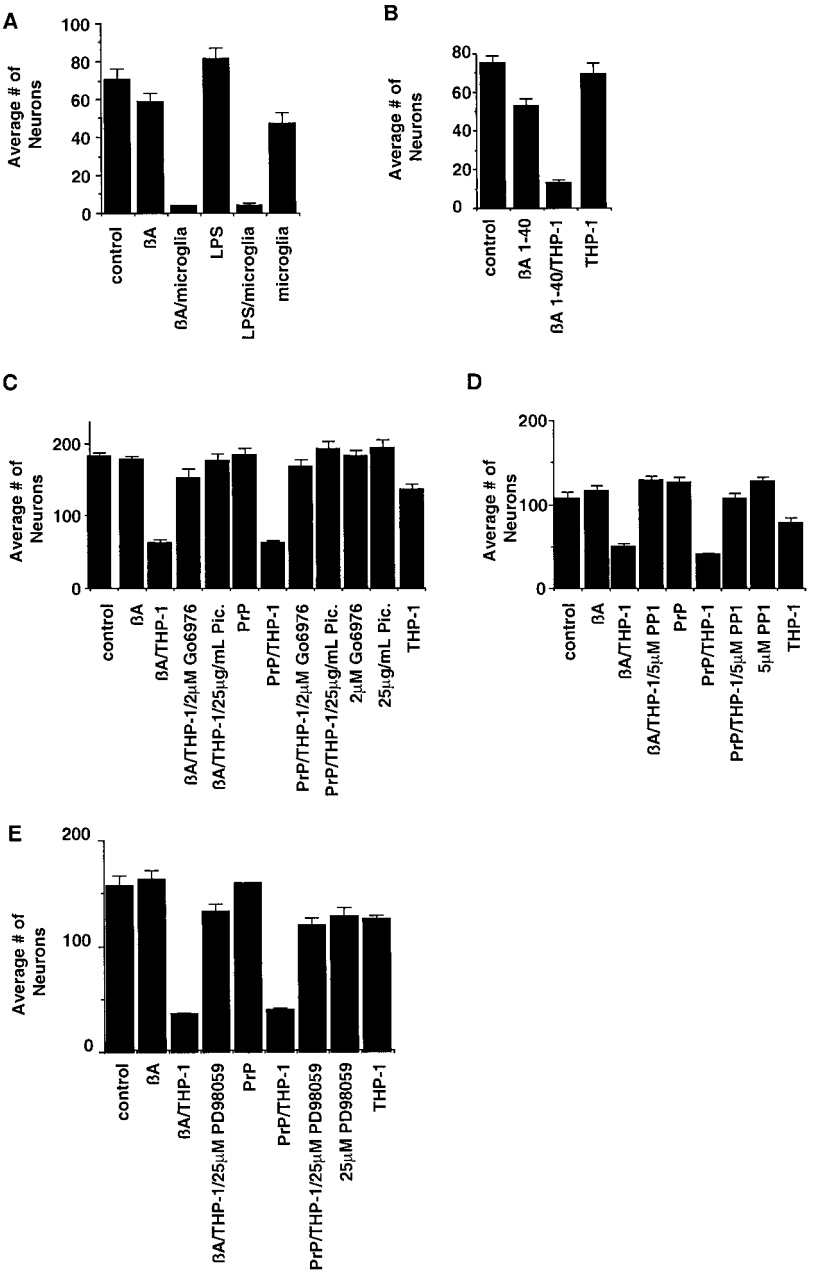

Figure 10. *βA* and *PrP* fibrils stimulate microglia and *THP*-1 monocytes to secrete neurotoxic products. Purified cultures of mouse cortical neurons (E17; 5–7 d *in vitro*) were cultured alone or in the presence of microglia or conditioned media from *THP*-1 cells (4.0×10^4 neurons/1.8×10^4 microglia or *THP*-1 cells). *A,* Microglia were added to neuronal cultures, along with the peptide fibrils *βA*25–35 (1 μM) or 1 μg/ml LPS as a positive control for 48 hr. *B–E*, *THP*-1 cells were also stimulated for 48 hr by plating into tissue culture wells coated with the peptides *PrP* 106–126, *βA* 1–40, and *βA* 25–35 (48 pmole/mm^2) in the presence of DMSO vehicle (*control*) or 25 μg/ml piceatannol (*Pic*.; *C*), 2 μM *Go6976* (*C*), 5 μM *PP1* (*D*), and 25 μM *PD98095* (*E*). Conditioned medium was obtained from wells in which *THP*-1 cells were incubated in the absence or presence of *βA* or *PrP* as well as control incubations of the medium alone or medium from wells containing only surface-bound *βA* or *PrP*. Evaluation of the effects of the various drugs included parallel incubations in the absence or presence of *THP*-1 cells. Conditioned medium was added to mouse cortical neuron cultures for 72 hr. Microglia–neuron cocultures were maintained for 48 hr. Neurons were then fixed, stained for neuron-specific MAP2 protein, and counted. Neurons from four fields per condition were counted in duplicate wells and averaged (± SEM). Graphs are a representative of four independent experiments.

tides results in constitutively elevated phosphotyrosine levels. This closely resembles the *in vivo* condition of plaque-associated microglia in AD brains. This cascade is specific for the fibrillar forms of the peptides and does not seem to involve peptide binding to scavenger or RAGE receptors (McDonald et al., 1997; Combs, McDonald, and Landreth, unpublished observations).

Numerous *in vitro* studies have demonstrated that βA and PrP fibril stimulation of microglia leads to the acquisition of a neurotoxic phenotype (Forloni et al., 1993; Giulian et al., 1995; Brown et al., 1996; Ii et al., 1996; Klegeris and McGeer, 1997; Klegeris et al., 1997; Kretzschmar et al., 1997; Lorton, 1997; McDonald et al., 1997; Combs, McDonald, and Landreth, unpublished observations). Although the amyloid-induced production of neurotoxins is well documented, the identity of the species responsible for neuronal death remains controversial (Forloni et al., 1993; Giulian et al., 1995; Brown et al., 1996; Ii et al., 1996; Klegeris and McGeer, 1997; Klegeris et al., 1997; Kretzschmar et al., 1997; Lorton, 1997; McDonald et al., 1997; Combs, McDonald, and Landreth, unpublished observations) but reflects the activation of a coordinated response pathway and the synthesis of numerous proinflammatory species. We have used a well established *in vitro* model to investigate whether the amyloid-stimulated signaling pathways we have characterized are linked to the production of neurotoxic products (Giulian et al., 1995). We report that treatment of microglia or THP-1 cells with inhibitors that target specific protein kinases that comprise the βA- and PrP-activated signaling pathway (e.g., Lyn, Syk, PKC, and ERK) effectively blocked the amyloid-stimulated production of neurotoxins and promoted neuron survival. These experiments verified that the tyrosine kinase-based inflammatory pathways were directly re-

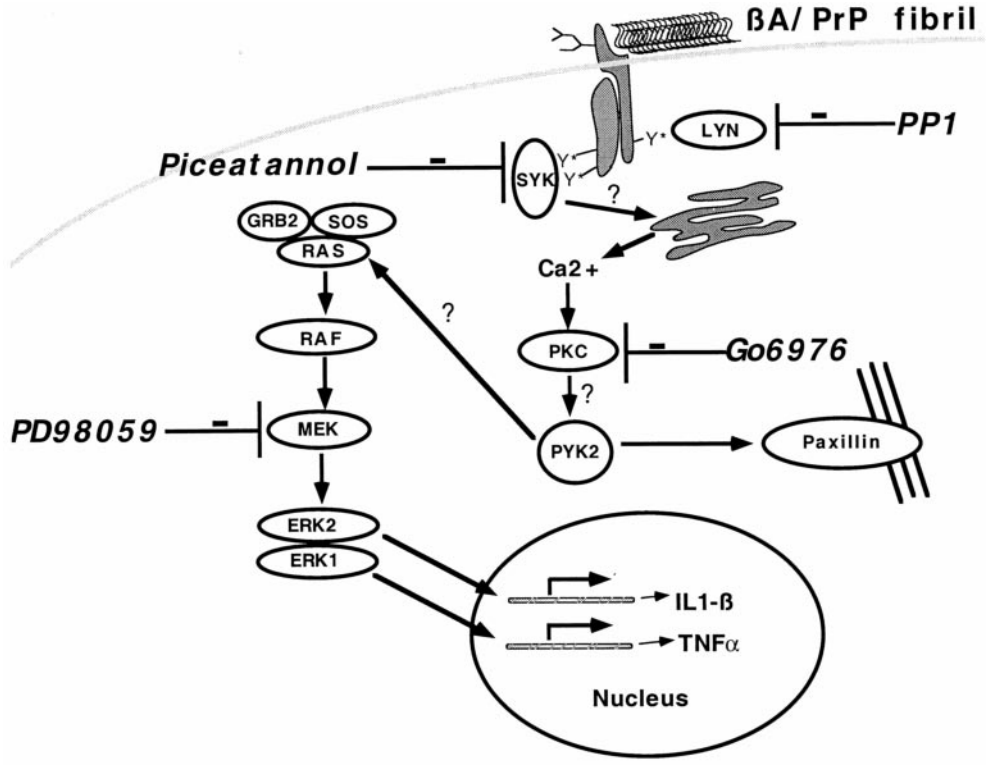

Figure 11. Mechanism of microglial activation by fibrillar *βA* and *PrP* peptides responsible for neurotoxicity. A schematic is shown detailing the defined tyrosine kinase-based signaling pathway by which *βA* and *PrP* fibrils activate microglia and monocytes to elicit production of neurotoxic factors. Indicated are points in the signaling pathway at which specific enzyme inhibition can alleviate production of the neurotoxic products. *IL1-β*, Interleukin-1-*β*; *TNFα*, tumor necrosis factor *α*; *Y*, tyrosine phosphorylation.

sponsible for production of neurotoxic factors and have validated the approach of using agents that specifically target elements of the signal transduction apparatus.

We have shown that microglia and other cells of this lineage respond to exposure to amyloid fibrils by initiation of complex signal transduction cascades. These signaling pathways are also activated in response to immune stimuli and effect a sophisticated and coordinated cellular response leading to the production of a diverse range of bioactive molecules and cellular behaviors. The efficacy of nonsteroidal anti-inflammatory drugs in reducing the incidence and progression of AD provides strong support for the critical involvement of inflammatory processes in the etiology of AD and related diseases. The molecular dissection of these pathways has allowed us to identify constituents of these cascades and to test directly whether the selective inhibition of these enzymes inhibits the production of proinflammatory products and ameliorates the neurotoxicity associated with amyloid exposure. The data have shown clearly that this strategy is effective in our *in vitro* model system. The detailed knowledge of the microglial intracellular signaling pathways may allow novel therapeutic approaches to AD and related diseases.

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