Amyloid-\(\beta\) Decreases Cell-Surface AMPA Receptors by Increasing Intracellular Calcium and Phosphorylation of GluR2

Shi-Jie Liu, Robert Gasperini, Lisa Foa and David Henry Small*
Menzies Research Institute, University of Tasmania, Hobart, Tasmania, Australia

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Abstract. \(\alpha\)-Amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptors (AMPARs) are key regulators of synaptic function and cognition. In Alzheimer’s disease (AD), cell-surface AMPARs are downregulated, however the reason for this downregulation is not clear. In the present study, we found that \(\alpha\)\(\beta\) significantly decreased levels of the cell-surface AMPA-type glutamate receptor subunit 2 (GluR2), and increased the concentration of free cytosolic calcium ion ([Ca\(^{2+}\)]\(_i\)) in hippocampal neurons. Ion channel blockers (nifedipine, tetrodotoxin, SKF96365) decreased [Ca\(^{2+}\)]\(_i\) and increased the level of cell-surface GluR2, whereas Bay K 8644, an activator of L-type voltage-gated calcium channels increased [Ca\(^{2+}\)]\(_i\) and decreased cell-surface GluR2. \(\alpha\)\(\beta\) and Bay K 8644 increased phosphorylation of serine-880 (S880) on GluR2, whereas the nifedipine. tetrodotoxin and SKF96365 decreased S880 phosphorylation. Finally, we found that bisindolylmeimide I (GF 109203X, GFX), an inhibitor of protein kinase C (PKC) blocked both the decrease in cell-surface GluR2 and the increase in phospho-S880 induced by \(\alpha\)\(\beta\) and Bay K 8644. Taken together, these results demonstrate that \(\alpha\)\(\beta\) decreases cell-surface GluR2 by increasing PKC-mediated phosphorylation of S880. Our study supports the view that a rise in cytosolic [Ca\(^{2+}\)]\(_i\), induced by \(\alpha\)\(\beta\) could impair synaptic function by decreasing the availability of AMPARs at the synapse. This decrease in AMPARs may contribute to the decline in cognitive function seen in AD.

Keywords: Alzheimer’s disease, AMPA, amyloid-\(\beta\), calcium, GluR2, phospho-GluR2, PKC

INTRODUCTION

Alzheimer’s disease (AD) is the most common form of dementia in the elderly. It is estimated that 35 million people worldwide suffer from AD and that this number will increase four-fold by 2050. AD is characterized by two major pathological hallmarks: neurofibrillary tangles and neuritic plaques [1]. Neuritic plaques contain the amyloid-\(\beta\) protein (A\(\beta\)), which is thought to play a crucial role in the etiology of AD [1]. A\(\beta\) can induce a number of biochemical changes in cells including an increase in levels of cytosolic calcium [2] and the stimulation of kinases, such as glycogen synthase kinase-3\(\beta\) (GSK-3\(\beta\)) [3] and cyclin-dependent kinase-5 (CDK-5) [4], which contribute to the hyperphosphorylation of tau. Recently, it has been reported that A\(\beta\) deposits can impair default network function in older individuals even without clinical dementia symptoms [5]. Thus, A\(\beta\) may impair the function of neurons at an early stage in the development of AD.

Synaptic plasticity is a key component of memory and cognition, which regulates the strength of signaling between neurons [6]. Synaptic plasticity can be mediated by changes in the level of receptors on both pre- and post-synaptic membranes [6]. \(\alpha\)-Amino-
thereby downregulate AMP ARs on the cell-surface. Therefore, we hypothesized that phosphorylation leads to the removal of GluR2 from the cell surface [15,16]. However, the mechanism of this downregulation has not been elucidated. A recent study suggests that Aβ decreases cell-surface GluR1 by inhibiting Cu²⁺/calmodulin-dependent protein kinase II (CaMKII) [13]. However, AMPAR trafficking between the cell-surface and the cytoplasm is also known to be regulated by phosphorylation of GluR2 [7,14]. Serine-880 (S880) of GluR2 can be phosphorylated by protein kinase C (PKC) and this phosphorylation leads to the removal of GluR2 from the cell surface [15,16]. Therefore, we hypothesized that Aβ may stimulate PKC to phosphorylate GluR2 and thereby downregulate AMPARs on the cell-surface.

In the present study, we report that Aβ increases the level of cytosolic calcium in hippocampal neurons, and also increases phospho-S880 on GluR2. Our study suggests that Aβ-mediated effects on GluR2 phosphorylation could alter AMPAR activity and thereby attenuate synaptic activity.

**MATERIALS AND METHODS**

**Materials**

A rabbit polyclonal immunoglobulin G (IgG) (ab40878) which recognizes the N-terminus of GluR2 and a rabbit polyclonal antibody (ab52180) which recognizes GluR2 phosphorylated at S880 (anti-P-S880) were purchased from Abcam (Cambridge, UK) [13]. A mouse monoclonal IgG which recognizes the N-terminus of GluR2 (MAB397) was purchased from Millipore (Billerica, MA). Nifedipine, trypsin, Triton-X100, Nonidet P-40 (NP-40), sodium deoxycholate (DOC), sodium dodecyl sulfate (SDS) and poly-D-lysine-hydrobromide were purchased from Sigma-Aldrich Inc. (Saint Louis, MO). Cholera toxin B subunit conjugated to Alexa Fluor 594 was purchased from Molecular Probes (Eugene, OR). All Aβ peptides were purchased from rPeptide (Athens, GA). GF 109203X (GFX, bisindolylmaleimide I) was purchased from Merck KGaA (Darmstadt, Germany). Tetrodotoxin (TTX) and Bay K 8644 (Bay K) were purchased from Alomone Labs. (Jerusalem, Israel). SKF96365 (SKF) was purchased from Tocris Bioscience (Bristol, UK). Streptavidin-agarose beads and Fluo-4/AM were purchased from Invitrogen (Carlsbad, CA). Sulfosuccinimidyl-4(N,N-diethylamino)stilbene-2,4-disulfonate (Sulfo-NHS-LC-Biotin) and CL-X-films were from Thermo Scientific (Rockford, IL). Enhanced chemiluminescence detection kit was purchased from Millipore Corporation (Billerica, MA).

**Preparation of hippocampal neurons**

Dissociated hippocampal neurons were prepared from postnatal day 0 (P0) C57 mice using a modified protocol for rat tissue [17]. Hippocampi were dissected, digested by trypsin, the tissue was disrupted by trituration to produce isolated cells, and then the cells were seeded either on coverslips or directly on 6-well plates which had been previously coated with poly-D-lysine (10µg cells/well). The cultures prepared by this method contained at least 80% neurons and were used at 14 days in vitro (DIV) for all experiments.

**Preparation of Aβ peptides**

Aβ peptides were dissolved at a concentration of 1mM in dimethyl sulfoxide to make a stock solution that was stored in -80°C. After thawing stocks at 37°C, the Aβ solutions were diluted with culture medium to yield a final concentration of 1 µM and then used in immediately for all experiments. To analyze Aβ oligomers by western blotting, 24 µl of freshly prepared Aβ in culture medium was combined with 8 µl of 4 x dilution buffer (250 mM Tris-HCl, pH 6.8, 8% sodium dodecyl sulfate, 40%, v/v, glycerol, 0.02%, w/v, bromophenol blue and 10% (v/v) β-mercaptoethanol) and boiled for 5 min. Samples (20 µl) were loaded onto 16.5% Tris-tricine gels for electrophoresis. Proteins were then electrophysiologically transferred onto 0.22 µm nitrocellulose membranes, the membranes were air dried, boiled in phosphate-buffered saline (20 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl) for 5 min. The nitrocellulose membranes were incubated with an
anti-Aβ antibody (6E10, 1:1000 dilution) followed by a secondary peroxidase-conjugated anti-mouse IgG antibody (1:4000 dilution) and then bands were visualized by enhanced chemiluminescence on CL-X-ray film.

**Measurement of cell-surface GluR2**

Cell-surface proteins were labeled with biotin and cell-surface GluR2 was quantified as previously described [18]. Primary hippocampal neurons were washed three times with ice-cold PBS (pH 7.4) containing 1 mM CaCl₂, 1.3 mM MgCl₂ (PBS-Ca-Mg) on ice, and incubated with 1 mg/ml sulfo-NHS-LC-Biotin in PBS-Ca-Mg for 20 min at 4°C. Then, cells were rinsed in 100 mM glycine on ice to quench the biotin reaction and lysed in 500 µl of PBS containing 0.5% DOC (w/v), 0.5% NP-40 (v/v), 0.2% SDS (w/v), and a cocktail of protease inhibitors (Roche Applied Science, Mannheim, Germany) and phosphatase inhibitors (20 mM sodium β-glycerophosphate, 2 mM Na₃VO₄, 10 mM NaF, 5 mM Na₂P₂O₇). The lysates were centrifuged at 13,000 × g for 15 min at 4°C in a Heraeus Biofuge pico (DJB Labcare Ltd, Buckinghamshire, UK). An aliquot (100 µl) of the resulting supernatant fraction was removed to measure protein by DC protein assay kit (Bio-Rad Laboratories, Gladesville, NSW, Australia), and the remaining supernatant fraction was incubated with 30 µl streptavidin-agarose beads overnight at 4°C. The beads were centrifuged (500 × g, 5 min, 4°C) and then washed three times with lysis buffer, before being resuspended in 35 µl SDS sample buffer and boiled for 10 min. Western blotting was performed using anti-GluR2 (1:1000 dilution) or anti-P-S880 antibodies (1:1000 dilution). Bands were visualized by enhanced chemiluminescence and exposed on CL-x-ray film. Bands were scanned and the mean intensity of bands was quantified using NIH Image J software [19]. The ratio of immunoreactivity (I.R.) associated with cell-surface GluR2 to total GluR2 and P-S880 to total GluR2 was calculated and the results were expressed as a percentage of the average control value.

**Immunofluorescence staining**

Surface GluR2 was visualized in 14-DIV hippocampal cultures as previously described [20]. Cells were grown on poly-D-lysine-coated coverslips. After treatment with vehicle or drugs, the cells were chilled on ice, incubated with anti-GluR2 antibody (1:250 dilution) in culture medium for 20 min, washed three times with ice-cold PBS and fixed for 10 min with 4% paraformaldehyde. All staining was performed at the same time and with the same batch of chemicals. To visualize the cell surface, Alexa 594-labelled Cholera toxin B subunit (1:3000 dilution) was also added with the anti-GluR2 antibody into the medium. Cells were permeabilized with 0.1% (v/v) Triton-X100, blocked with 5% (v/v) goat serum in PBS, and then incubated with anti-P-S880 (1:300 dilution) in 5% goat serum for 2 h at room temperature. Primary antibody staining was visualized using an anti-mouse Alexa 488 antibody (for the cell-surface anti-GluR2 antibody) or an anti-rabbit Alexa 594 antibody (for anti-P-S880). Fluorescence was viewed with an Olympus BX-50 microscope equipped with an UplanSAPO 60× oil immersion objective, and images were acquired with a Magnafire CCD camera (Optronics, Goleta, CA). Mean pixel intensity of the region of interest (ROI) encompassing the cell body and neurites was quantitated with NIH Image J [19] and data were expressed as ratio of the fluorescence of surface GluR2 to cholera toxin B subunit or surface GluR2 to phospho-S880.

**Measurement of concentration of free cytosolic calcium ion ([Ca²⁺])**

Cells cultured in 96-well plates were incubated for 10 min at 37°C with 2 µM Fluo-4/AM in culture medium. The medium containing Fluo-4/AM was removed and cells were washed twice with Tyrode’s buffer (129 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 30 mM glucose, 25 mM HEPES-NaOH, pH 7.4) at 37°C. Fluorescence was measured on a Fluostar Optima plate reader (BMG Labtech GmbH, Offenburg, Germany). The excitation wavelength was 485 nm and the fluorescence emission was monitored at a wavelength of 520 nm. Changes in fluorescence were used as an index of changes in [Ca²⁺]. The ratio was calculated according to the formula \[ \frac{\Delta F}{F_0} = \frac{(F - F_0)}{F_0} \], where F is the fluorescence intensity of calcium and F₀ is average the fluorescence intensity before stimulation [21].

**Statistical analysis**

Statistical comparisons were made using one-way analysis of variance and a Least Significant Difference (LSD) post hoc test. A P value of 0.05 was used to determine statistical significance. Data were expressed at means ± S.E.M. Statistical analysis was performed with SPSS 10.00 software. All experiments were repeated 3 times with similar results in each experiment.
Fig. 1. Aβ reduces cell-surface GluR2 in primary cultured hippocampal neurons. A) Cell-surface proteins were labeled with biotin, affinity-purified using streptavidin-agarose, and then western blotting was performed with an anti-GluR2 antibody. After 24 h of incubation, Aβ40 and Aβ42 (both 1 µM) significantly reduced the cell-surface GluR2 immunoreactivity (I.R), but not total GluR2 I.R. \((n = 6 \text{ independent incubations, } ^* p < 0.05)\). B) Western blotting of freshly prepared Aβ40 and Aβ42 diluted into culture medium showed that both preparations of peptide contained oligomeric species. Arrows show expected positions of monomers (4 kDa), dimers (8 kDa) and tetramers (16 kDa). C) The effect of Aβ42 on cell-surface GluR2 was seen both at 4 h and 24 h after incubation. A scrambled sequence peptide (Scrambled Aβ42) did not influence the amount of cell-surface GluR2 I.R. \((n = 6 \text{ independent incubations, } ^* p < 0.05)\). D) Immunofluorescence microscopy of cell-surface GluR2 (green) also showed that there was a reduction in the level of cell-surface GluR2 by 1 µM Aβ40 and Aβ42 after 24 h incubation \((^* p < 0.05, n = 10 \text{ cells, compared with control})\). Cholera toxin B subunit was used as a cell-surface marker. Scale bar = 50 µm.

RESULTS

Aβ decreases cell-surface GluR2

We first investigated the effects of Aβ on cell-surface GluR2. Primary hippocampal neurons were prepared and cultured for 14 DIV, after which the cells were treated for 24 h with freshly prepared Aβ40 or Aβ42 (1 µM). Using a biotinylation approach to measure cell-surface GluR2, we found that Aβ40 and Aβ42 significantly decreased cell-surface GluR2 immunoreactivity to approximately 70% and 60% of control values, respectively. There was no significant change in the level of total GluR2 in the cells after the Aβ treatment (Fig. 1A). As oligomeric Aβ is generally considered to be the most toxic form of the peptide, we investigated whether oligomers were present in our Aβ preparation. The culture medium was analyzed after addition of Aβ by electrophoresis on a 16.5% SDS-Tris-tricine gel followed by western blotting for Aβ with a monoclonal an-
Cytosolic calcium regulates cell-surface GluR2

Cytosolic calcium is an important second messenger that plays a key role in regulating cell-surface AMPARs [20]. As Aβ is known to alter calcium levels in neurons [2], we investigated the role of calcium and Aβ on levels of cell-surface GluR2. In these experiments, we measured [Ca^{2+}]_{i} using the fluorescent calcium indicator Fluo-4 and we examined the effects of Aβ, channel blockers and agonists on [Ca^{2+}]_{i} (Fig. 2) and on the level of cell-surface GluR2 (Fig. 3).

Aβ_{40} and Aβ_{42} (both incubated at a concentration of 1 μM) significantly increased [Ca^{2+}]_{i} (Fig. 2A). Experiments with channel blockers supported the view that calcium is a key regulator of cell-surface GluR2. We found that drugs which lowered [Ca^{2+}]_{i} all increased the level of cell-surface GluR2. First, we investigated the effects of nifedipine, an L-type voltage-gated calcium channel blocker. Nifedipine (5 μM) significantly depressed calcium both in the presence and absence of Aβ (Fig. 2A). Nifedipine also significantly increased cell-surface GluR2 both in the absence and presence of Aβ (Fig. 3A). Like nifedipine, TTX (5 μM), a voltage-gated sodium channel blocker, de-
A/β decreases cell-surface GluR2

Fig. 3. A/β peptides decrease cell-surface GluR2, whereas nifedipine, SKF and TTX increase cell-surface GluR2. Cell-surface proteins were labeled with biotin, affinity-purified using streptavidin-agarose, and then western blotting was performed with an anti-GluR2 antibody. Figure shows a representative western blot for each experiment and quantitation of cell surface GluR2 and total GluR2 immunoreactivity (I.R.). A) Effect of nifedipine (5 µM) A/β40 and A/β42 (1 µM). B) Effect of TTX (5 µM), A/β40 (1 µM), and A/β42 (1 µM). C) Effect of SKF (5 µM), A/β40 (1 µM), and A/β42 (1 µM). D) Effect of Bay K (1 µM), A/β40 (1 µM), and A/β42 (1 µM). (n = 6 independent incubations, *p < 0.05).

pressed [Ca^{2+}]_i (Fig. 2B) and increased cell-surface GluR2 (Fig. 3B) both in the absence and presence of A/β. SKF (5 µM), a non-selective cation channel blocker, also significantly decreased [Ca^{2+}]_i (Fig. 2C) and increased cell-surface GluR2 (Fig. 3C) both in the absence and presence of A/β. Finally, to verify the central role of calcium on cell-surface GluR2, we examined the effect of Bay K, an activator of L-type voltage-gated calcium channels. We found that 1 µM Bay K significantly increased calcium (Fig. 2D), and decreased cell-surface GluR2 (Fig. 3D), both in the presence and absence of A/β.

Although our results suggested that A/β reduces cell-surface GluR2 by increasing [Ca^{2+}]_i, we found no evidence that the effect of A/β was mediated directly via L-type voltage-gated calcium channels, voltage-gated sodium channels or SKF-sensitive non-selective cation channels as the effect of A/β on calcium or cell-surface GluR2 was not blocked by any of the channel blockers.

A/β increases phosphorylation of S880 in GluR2

Phosphorylation of GluR2 on the C-terminus regulates trafficking of GluR2 between the cell surface and the cytoplasm [14–16]. Phosphorylation at S880 induces endocytosis of GluR2 and results in a decrease in cell-surface GluR2 [15,16]. To elucidate the mechanism by which A/β reduced cell-surface GluR2, we analyzed the phosphorylation of GluR2 using a phospho-S880-specific antibody. We found that 1 µM A/β40 or 1 µM A/β42 significantly increased phospho-S880 immunoreactivity to approximately 230% of control values, supporting the view that A/β increases phosphorylation of S880 on GluR2 (Fig. 4B). This effect on S880 phosphorylation was observed both at 4 hr and
Fig. 4. Aβ and Bay K increase while ion-channel blockers decrease phospho-S880 immunoreactivity. Figure shows a representative western blot and quantitation of phospho-S880 (P-S880) and total GluR2 immunoreactivity (I.R.) (n = 6 independent incubations, *p < 0.05). A) Aβ42 (1 μM) significantly increased the phospho-S880 I.R. both at 4 and 24 h after incubation. Scrambled Aβ42 peptide (1 μM) did not influence phospho-S880 I.R. (n = 6 independent incubations, *p < 0.05). In panels B-E, levels of phospho-S880 I.R. and total GluR2 I.R. were measured 24 h after addition of drugs and Aβ peptides. B) Effect of nifedipine (5 μM), Aβ40 (1 μM), Aβ42 (1 μM) on P-S880 I.R. C) Effect of TTX (5 μM), Aβ40 (1 μM), and Aβ42 (1 μM). D) Effect of SKF (5 μM), Aβ40 (1 μM), and Aβ42 (1 μM). E) Effect of Bay K (1 μM), Aβ40(1μM), and Aβ42 (1μM).

We next investigated the effect of channel blockers (nifedipine, TTX, and SKF) on phosphorylation of GluR2 at S880. We found that 5 μM nifedipine significantly decreased phospho-S880 immunoreactivity both in control incubations (lacking Aβ) and the Aβ-treated group (Fig. 4B). Both TTX (5 μM) and SKF (5 μM) also decreased the phospho-S880 immunoreactivity (Figs. 4C,D). In the same incubations, there was 24 hr after incubation (Fig. 4A). Scrambled Aβ had no significant effect on S880 phosphorylation.
no effect of nifedipine, TTX, or SKF on total GluR2 levels. These results demonstrated that calcium channel blockers decrease phosphorylation of S880 but do not block the Aβ-induced increase in phosphorylation of S880, providing further evidence that the effect of Aβ is not mediated by nifedipine-, TTX- or SKF-sensitive channels. Finally, to investigate the role of calcium in the phosphorylation of S880 further, we examined the effect of the L-type voltage-gated Ca^{2+} channel activator Bay K on the on the level of phospho-S880. We found that Bay K (1 µM) significantly increased the phospho-S880 immunoreactivity (Fig. 4E).

We also examined the effect of Aβ, Bay K, nifedipine, TTX and SKF on the localization of GluR2 by immunofluorescence microscopy (Fig. 5). We first stained cell-surface GluR2 on living cells using an ectodomain specific antibody. Then, the cells were fixed and permeabilized and incubated with the phospho-S880 antibody. Cell-surface GluR2 was detected using an Alexa-488 secondary anti-mouse IgG, whereas anti-phospho-S880 was detected using an Alexa-594 anti-rabbit IgG. Using this method, double labeling immunofluorescence showed that the ectodomain specific N-terminal GluR2 was stained on the surface of cells, whereas the anti-phospho-S880 antibody that recognizes C-terminus produced an intracellular labeling pattern (Fig. 5). Cell-surface total GluR2 did not colocalize with phospho-S880 immunoreactivity (Fig. 5). We found that 1 µM Aβ or 1 µM Bay K significantly decreased the ratio of cell-surface GluR2 immunofluorescence to phospho-S880 immunofluorescence, compared with controls (Fig. 5). On the other hand, 5 µM nifedipine, 5 µM TTX, and 5 µM SKF significantly increased this ratio. Thus, the immunofluorescence results confirmed the results of the biochemical studies and supported the view that the phosphorylation of GluR2 at S880 is regulated by the level of [Ca^{2+}].

**GFX decreases phosphorylation of GluR2 and blocks Aβ-induced decrease in cell-surface GluR2**

S880 in GluR2 is phosphorylated by protein kinase Cα (PKCα), which, in turn, can be activated by calcium [14,15]. To elucidate the role of PKC on Aβ-induced phosphorylation of GluR2 at S880 and on the level of cell-surface GluR2, we examined the effects of GFX, a well-characterized PKC inhibitor [22]. We found that 10 nM GFX significantly decreased phosphorylation of GluR2 at S880 and increased cell-surface GluR2, compared with controls (Fig. 6A). GFX also suppressed phosphorylation of S880 in the presence of Aβ. GFX increased the level of cell-surface GluR2, both in the presence and absence of Aβ (Fig. 6A). Furthermore, we found that 10 nM GFX completely suppressed an increase in phospho-S880 induced by 1 µM Bay K, and significantly increased the level of cell-surface GluR2, compared with Bay K treatment alone (Fig. 6B). These results suggest that GFX is able to block the effects of Aβ and Bay K on phospho-S880 and on the level of cell-surface GluR2.

To confirm the results of the western blot experiments, we measured cell-surface GluR2 and phospho-S880 by immunofluorescence microscopy. We found that 10 nM GFX significantly increased the ratio of surface GluR2 to phospho-S880 both in control and in Aβ and Bay K groups (Fig. 7). Therefore, the results from immunofluorescence experiments confirmed the results from the biotin-labeling experiments. Taken together, the results support the view that Aβ increases phospho-S880 by activating PKC.

**DISCUSSION**

AMPARs are involved in synaptic plasticity and memory formation [7,8]. Recent studies have shown that both the number of AMPARs and their activity are decreased in transgenic AD model mice [9–12]. Previously, studies have shown that Aβ can remove the AMPAR subunits GluR1 and GluR2 from the cell surface [12]. However, the mechanism by which this occurs is unclear. The present study confirms that Aβ downregulates cell-surface AMPARs and supports the view that this effect is due to phosphorylation of S880 on GluR2. Resende and colleagues [23] previously reported that an Aβ peptide (Aβ25–35) decreased the total level of GluR2. As Aβ25–35 perturbed calcium homeostasis, this study also supports the idea that calcium may be involved in the dysfunction of AMPA receptors induced by Aβ.

We hypothesized that calcium ion influx induced by Aβ and phosphorylation of S880 on GluR2 may be involved into the effects of Aβ on AMPAR levels and distribution. Gu and coworkers [13] found that Aβ impaired GluR1 trafficking and downregulated AMPARs currents by reducing CaMKII. Recently, inhibition of calcineurin, which is stimulated by calcium, was found to prevent endocytosis of AMPAR [24]. In the present study, we found that Aβ increased phosphorylation of S880 on GluR2 and also increased [Ca^{2+}]. Similar to the effects of Aβ, an activator of calcium channels (Bay K) decreased cell-surface GluR2, and increased phos-
Fig. 5. Aβ40 (1 µM) and Bay K (1 µM) decrease the ratio of cell-surface GluR2 to phospho-S880 in hippocampal neurons. Figure shows double immunofluorescence micrographs of cells in which cell-surface GluR2 (Surface GluR2) immunoreactivity was labeled with an anti-mouse Alexa 488 antibody and phospho-S880 immunoreactivity was labeled with an anti-rabbit Alexa 594 antibody. Figure also shows quantitation of fluorescence intensity (n = 10 neurons). Nifedipine (5 µM), TTX (5 µM), and SKF (5 µM) significantly increased the ratio of cell-surface GluR2 to phospho-S880 immunofluorescence (*p < 0.05, compared with control). Scale bar = 20 µm.

Fig. 6. The PKC inhibitor GFX blocks the decrease in cell-surface GluR2 and the increase in phospho-S880 induced by Aβ (A) and Bay K (B). Figure shows representative western blots of phospho-S880 (P-S880), cell-surface GluR2, and total GluR2 immunoreactivity (I.R.) as well as quantitation of immunoreactive bands (n = 6 independent incubations, *p < 0.05). A) GFX (10 nM) significantly increased cell-surface GluR2 and decreased phospho-S880. Furthermore, GFX blocked a decrease in surface GluR2 and an increase in the phospho-S880 induced by Aβ (n = 6). B) Bay K (1 µM) significantly increased phospho-S880 and decreased cell-surface GluR2, and 10 nM GFX blocked this effect (*p < 0.05, compared with control).
Fig. 7. The PKC inhibitor GFX blocks a decrease in the ratio of cell-surface GluR2 immunofluorescence to phospho-S880 immunofluorescence in hippocampal neurons induced by Aβ and Bay K. Aβ40 (1 µM) and Bay K (1 µM) significantly decreased the ratio of cell-surface GluR2 immunofluorescence to phospho-S880 immunofluorescence on hippocampal neurons, and 10 nM GFX significantly increased this ratio. Figure shows double immunofluorescence micrographs of cells in which cell-surface GluR2 (Surface GluR2) immunoreactivity was labeled with an anti-mouse Alexa 488 antibody and phospho-S880 immunoreactivity was labeled with an anti-rabbit Alexa 594 antibody. Figure also shows quantitation of fluorescence intensity (n = 10 neurons). GFX blocked the decrease in the ratio of cell-surface GluR2 immunofluorescence to phospho-S880 immunofluorescence induced by Aβ and Bay K. (*p < 0.05, compared with control). Scale bar = 20 µm.

 phosphorylation of S880 of GluR2 and [Ca^{2+}]. In contrast, blockers of Ca^{2+} channels increased cell-surface GluR2, decreased S880 phosphorylation and [Ca^{2+}]. Finally, we found that an inhibitor of PKC could block the effects of Aβ and Bay K on GluR2 and calcium. Taken together, these data demonstrate that Aβ decreases the amount of AMPARs on the cell surface by increasing phosphorylation of S880 on the GluR2 subunit.

The mechanisms of calcium influx induced by Aβ in hippocampal neurons are still unclear. In our studies, we used three different types of channel blockers in the present experiments: an L-type voltage-gated calcium channel blocker (nifedipine), a voltage-gated sodium channel blocker (TTX) and a non-selective cation channel blocker (SKF). All three calcium channel blockers decreased [Ca^{2+}], and S880 phosphorylation and increased cell-surface GluR2 when incubations were performed with Aβ. However, the blockers equally well decreased [Ca^{2+}], phosphorylation of S880 and increased cell-surface GluR2 irrespective of whether Aβ was present. Therefore, the results suggest that while calcium ion influx is important for the effect on cell-surface GluR2, the effect of Aβ is not directly mediated by L-type voltage-gated calcium channels, voltage-
gated sodium channels or SKF-sensitive non-selective cation channels.

It is generally believed that Aβ-induced calcium dysregulation is a critical step on impairing neuronal function in AD [2]. However, the mechanism by which calcium dysregulation may cause neuronal dysfunction is unclear. Our present study shows that calcium influx decreases the number of AMPARs on the cell surface. As AMPARs are key mediators of synaptic plasticity [7, 8], this suggests that the effects of Aβ on AMPARs are important for understanding Aβ-induced cognitive dysfunction.

AMPARs are assembled as heterotetramers. GluR2 is the most common subunit in heterotetrameric AMPARs [7]. The presence of GluR2 can prevent excitatory neurotoxicity because receptors containing GluR2 are impermeable to calcium [7]. Phosphorylation of S880 controls the balance of intracellular and cell-surface GluR2. Phosphorylation of S880 induces endocytosis of GluR2 and results in removal of GluR2 from the cell surface. It has been reported that PKCα, which belongs to conventional PKCs, is activated by calcium [15], and that the enzyme can phosphorylate S880 [16,17]. In the present study, we found that a PKC inhibitor blocked an Aβ-induced increase in S880 phosphorylation. The PKC inhibitor also blocked a decrease in cell-surface GluR2 induced by Aβ and the calcium channel activator Bay K. Therefore, our study strongly suggests that PKC mediates phosphorylation of S880 induced by Aβ.

As AMPARs are important for LTP [6]. Aβ-induced activation of PKC and phosphorylation of S880 might contribute to Aβ-induced inhibition of LTP [25]. However, the importance of this pathway for inhibiting LTP is not clear. For example, in addition to its action on AMPARs, PKC is known to stimulate LTP [26]. As PKC phosphorylates many different protein substrates, activation of PKC has many complex effects on signaling which are independent of AMPAR recycling. Furthermore, the effects of Aβ on LTP are likely to be mediated by mechanisms which are entirely independent of S880 phosphorylation.

In conclusion, our study suggests that Aβ-induced calcium entry leads to a decrease in cell-surface AMPARs by increasing phospho-S880 of GluR2. While this pathway will undoubtedly contribute to Aβ-mediated effects on synaptic signaling, it is likely that activation of other pathways will also be important. Identification of these other pathways will require further study.

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