Profilin II Regulates the Exocytosis of Kainate Glutamate Receptors*

Magali Mondin, Mario Carta, Elisabeth Normand, Christophe Mulle, and Françoise Coussen

From the Laboratoire Physiologie Cellulaire de la Synapse, CNRS UMR 5091, University of Bordeaux, 146 rue Léo Saignat, 33077 Bordeaux Cedex, France

The trafficking of ionotropic glutamate receptors to and from synaptic sites is regulated by proteins that interact with their cytoplasmic C-terminal domain. Profilin IIa (PfnIIa), an actin-binding protein expressed in the brain and recruited to synapses in an activity-dependent manner, was shown previously to interact with the C-terminal domain of the GluK2b subunit splice variant of kainate receptors (KARs). Here, we characterize this interaction and examine the role of PfnIIa in the regulation of KAR trafficking. PfnIIa directly and specifically binds to the C-terminal domain of GluK2b through a diproline motif. Expression of PfnIIa in transfected COS-7 cells and in cultured hippocampal neurons from PfnII-deficient mice decreases the level of extracellular of homomeric GluK2b as well as heteromeric GluK2a/GluK2b KARs. Our data suggest a novel mechanism by which PfnIIa exerts a dual role on the trafficking of KARs, by a generic inhibition of clathrin-mediated endocytosis through its interaction with dynamin-1, and by controlling KARs exocytosis through a direct and specific interaction with GluK2b.

Kainate receptors (KARs)² compose a family of ionotropic glutamate receptors, which play an important role in the regulation of synaptic transmission and neuronal excitability (1, 2). At variance with the closely related family of AMPA receptors, the main postsynaptic receptors involved in fast synaptic transmission, KARs exert their function by acting at either pre- or postsynaptic sites. Because their physiological functions critically depend on their specific localization as well as their density in these different neuronal compartments, it is important to better understand the mechanisms by which their trafficking is regulated in neurons. KARs are heterotetrameric receptor channels composed of various combinations of five subunits GluK1, GluK2, GluK3, GluK4, and GluK5 (formerly referred as GluR5, GluR6, GluR7, KA1, and KA2, respectively). The diversity of KARs is increased by the existence of splice variants for GluK1, GluK2, and GluK3 subunits. KAR subunits isoforms display different levels of expression at the plasma membrane depending on the alternative splicing of their C terminus (3–5). When expressed as homomers in heterologous cells or in cultured neurons, GluK2a is highly addressed to the plasma membrane, whereas GluK2b is present at low levels (3, 4, 6). The low level of expression of homomeric GluR6b can be explained by restricted export of the subunit from the endoplasmic reticulum (3). Native KARs likely exist as heteromers of different Glu subunits, as well as of different splice variants of the same GluK subunit, as for instance, in the case of GluK2a/GluK2b heteromers, which form native KARs (7). Oligomerization of KAR subunits plays a major role in their surface expression (4, 8). The regulation of ionotropic glutamate receptors trafficking to the cell surface is probably controlled by a combination of endoplasmic reticulum retention and export signals, as well as by domain-specific protein interactors. KAR subunits and splice variants diverge in their cytoplasmic C-terminal region, which opens the possibility that their trafficking and function are differentially regulated by proteins that interact specifically with subunit specific domains (7). GluK2a and GluK2b interact with two different sets of cytosolic proteins partners, some of which have been identified by proteomic analysis (7). Most reports have concentrated on the regulation of trafficking of homomeric GluK2a receptors by PDZ domain binding proteins (9), BTB–Kelch protein, and KRIp6 (10) and have not taken into consideration the fact that both GluK2a and GluK2b splice variants likely co-assemble in the brain.

Among proteins found to interact with GluK2b in a proteomic screen, profilin Ila (PfnIIa) appears as an interesting candidate for activity-dependent regulation of KARs (7). Mammalian profilins are actin-binding proteins, highly conserved with respect to their affinities for G-actin, poly-L-proline, and phosphoinositides. Profilins act as regulators of various cellular processes such as cytoskeletal dynamics and membrane transport (11). In mammals, several profilin isoforms have been identified. Whereas profilin I (PfnI) is ubiquitously expressed, PfnIIa is specifically expressed in the brain (12) and is a necessary element in key steps of neuronal differentiation and synaptic plasticity (13). PfnIIa interacts with proteins implicated in membrane trafficking, i.e., by interacting with and controlling dynamin-1 activity. Overexpression of PfnIIa inhibits endocytosis, whereas the lack of PfnIIa in neurons results in an increase in endocytosis and membrane recycling (14). Interestingly, profilins are targeted to dendritic...
Five hundred milligrams of brain or COS-7 cells were incubated with beads coupled to GST, GST-Cterm-GluK2a, GST-Cterm-GluK2b, or GST-Cterm-GluK2b/AA overnight at 4 °C. Beads were washed, and incubated overnight with the corresponding resins. For GST pulldown experiments, we performed using either the GluK2a C terminus domain (70 amino acids), the GluK2b C terminus domain (27 amino acids), or the GluK2b C terminus domain with mutations of two prolines into alanines. Cytosolic protein extracts from mouse brain or COS-7 cells were incubated with beads coupled to GST, GST-Cterm-GluK2a, GST-Cterm-GluK2b, or GST-Cterm-GluK2b/AA overnight at 4 °C. Beads were washed, suspended in loading buffer, run on SDS gels, and transferred onto membranes for immunoblotting.
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*Surface Biotinylation*—One day after transfection, COS-7 cells were washed with PBS (pH 8.0) and incubated with 0.5 mg/ml EZ-Link™ sulfo-NHS-S-LC-biotin (Pierce) in PBS for 30 min at 4 °C. After three washes, cells were scrapped in a lysis buffer containing 25 mM Hepes, 150 mM NaCl, 1% Triton X-100, and a mix of protease inhibitors. After centrifugation, the supernatant was incubated with immobilized streptavidin bead agarose overnight at 4 °C and washed extensively. Samples were analyzed by Western blot with antibodies corresponding to the proteins of interest.

*Antibodies*—The antibodies used in this experiment were as follows: anti-GluK2a/GluK3 (catalog no. 06-309, Upstate); polyclonal anti-myc (catalog no. 06-549, Upstate); monoclonal anti-myc (catalog no. 1-667-149, clone 9E10, Roche Applied Science); anti-dynamin-1 (catalog no. 05-319, Upstate); monoclonal anti-GFP (clone 7.1, Roche Applied Science); and polyclonal anti-GFP (catalog no. A-64-55, Molecular Probes). Anti-PfnI and anti-PfnIIa were kindly provided by Walter Witke (EMBL Monterotondo).

**RESULTS**

**Direct Interaction between PfnIIa and GluK2b**—We have previously shown that PfnIIa interacts with GluK2b in the brain by peptide pulldown and immunoprecipitation experiments (7). To further characterize the interaction between PfnIIa and GluK2b, we first determined whether this interaction was direct or whether it required an intermediate protein partner (Fig. 1). For this, we incubated either PfnI or PfnIIa proteins produced in *Escherichia coli* with GluK2a and GluK2b C-terminal peptides linked to Sepharose resins (Fig. 1A). This experiment indicated that the last 15 amino acids of GluK2b representing its C-terminal domain directly bind PfnIIa. This interaction was specific for PfnIIa because PfnI did not bind to GluK2b (as well as to GluK2a, data not shown) and was specific for the C-terminal domain of GluK2b as PfnIIa did not bind to the C-terminal domain of GluK2a. In control experiments, we found that GluK2a bound to the first two PDZ domains of PSD-95 (data not shown) (16).

Profilins interact with a wide variety of proteins via their poly-L-proline binding site. GluK2b C-terminal domain contains two consecutive prolines that could correspond to the consensus binding sequence to PfnIIa (Fig. 1B). To determine whether these amino acids are responsible for the binding of GluK2b to PfnIIa, we replaced the two prolines by alanines in GluK2b (GluK2b/AA). We expressed Myc-GluK2a, Myc-GluK2b, and Myc-GluK2b/AA with PfnIIa in COS-7 cells and tested interaction of PfnIIa by a co-immunoprecipitation assay (Fig. 1C). PfnIIa was co-immunoprecipitated with myc-GluK2b, but it was not detected in the Western blots after immunoprecipitation of Myc-GluK2a or of Myc-GluK2b/AA. These results confirm the specific interaction of PfnIIa with the C-terminal domain of GluK2b, but not of GluK2a, and show that the diproline motif in the C-terminal domain of GluK2b is essential for its direct interaction with PfnIIa.

*Plasma Membrane Localization of KARs Co-expressed with PfnIIa in COS-7 Cells and Neurons*—A number of profilin-interacting molecules identified in the mouse brain are part of the secretary machinery and could be involved in membrane trafficking (18). We thus examined whether the interaction between PfnIIa and GluK2b could affect the expression of GluK2b-containing receptors at the plasma membrane (Fig. 2). We performed biotinylation experiments in COS-7 cells expressing either Myc-GluK2b or Myc-GluK2a, with or without PfnIIa (Fig. 2, A–C, n = 5 for each condition). Only 4% (±1%) of Myc-GluK2b was present at the plasma membrane when expressed alone. Expression of PfnIIa further decreased membrane localization of Myc-GluK2b to 2% (±1%) (Fig. 2A), but did not affect plasma membrane expression of GluK2a alone (75 ± 4% without PfnIIa and 80 ± 6% with PfnIIa) (Fig. 2B). Heteromerization regulates KAR trafficking to the plasma membrane, and native GluK2-containing KARs are thought to comprise both GluK2a and GluK2b (7). We thus examined whether the interaction between PfnIIa and GluK2b affected the plasma membrane expression of heteromeric GluK2a/GluK2b receptors expressed in COS-7 cells by biotinylation experiments. Expression of GluK2a increased the amount of GluK2b at the plasma membrane (from 4% ± 1.2% to 32 ± 5%, n = 5) (Fig. 2A). Conversely, expression of GluK2b decreased the amount of GluK2a at the plasma membrane (from 75 ± 4% without GluK2b to 41 ± 10% with GluK2b, n = 5) (Fig. 2B). Expression of PfnIIa decreased the amount of heteromeric KARs at the plasma membrane by a
factor 2 (to 14 ± 3% when monitoring GluK2b and to 19 ± 10% when monitoring GluK2a, n = 5 for each condition) (Fig. 2, A and B). Importantly, PfnIIa did not affect expression of Myc-GluK2b/AA at the plasma membrane when transfected alone or with GluK2a (Myc-GluK2b/AA alone, 5 ± 1%; with PfnIIa, 5 ± 1%; myc-GluK2b/AA with GluK2a, 20 ± 1% without PfnIIa, 20 ± 3% with PfnIIa, n = 4). GluK2b/AA expressed with GluK2a is less exported than GluK2b (Myc-GluK2b/AA with GluK2a, 15 ± 1%; Myc-GluK2b with GluK2a, 34 ± 1%) (Fig. 2C).

We confirmed these results using immunocytochemistry experiments in COS-7 cells expressing the different combinations of subunits with or without PfnIIa (Fig. 2D, n = 4). Surface labeling of receptors was measured and normalized to the level of expression for each cell. This level was measured as the GFP signal corresponding either to pEGFP or to PfnIIa-GFP plasmids. As expected, the labeling corresponding to extracellular Myc-GluK2b expressed alone was very low (< 1%) and was undetectable in cells co-expressing PfnIIa. The relative amount of Myc-GluK2a at the surface was not

FIGURE 2. PfnIIa decreases surface localization of GluK2b-containing KARs. A–C, biotinylation experiments in COS-7 cells transfected with the different subunits with or without PfnIIa. Left panel, representative Western blots probed either with anti-Myc antibodies for the Myc-GluK2b (mK2b) subunit or with anti-GluK2a (K2a). Right panel, quantification of the Western blots corresponds to the ratio between EC and total (EC + IC) receptors (n = 5). Hatched bars represent expression of receptors with PfnIIa. Data represented as mean ± S.E. were compared using paired t test (*, p < 0.05; ***, p < 0.001). ns, not significant. D, extracellular labeling of receptors composed of different GluK2 subunits expressed in COS-7 cells. Extracellular KARs were detected with an anti-Myc antibody on live cells and an anti-rabbit Alexa Fluor 568 on COS-7 cells expressing the different receptor subunits as indicated. The right panel shows the quantification (n = 50–60 cells, four different experiments. Scale bar, 10 µm. Data represented as mean ± S.E. were compared using unpaired t test (***, p < 0.001). ns, not significant. Expression of PfnIIa decreases plasma membrane localization of GluK2b alone or in heteromeric GluK2a/GluK2b receptors and does not affect surface expression of receptors containing GluK2b/AA (K2b/AA).
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affected by co-expression of PfnIIa (32 ± 11% without PfnIIa, 25 ± 10% with PfnIIa, n = 40 cells). However, PfnIIa decreased the relative amount of Myc-GluK2a when expressed in combination with GluK2b (34 ± 4% without PfnIIa, 21 ± 2% with PfnIIa, n = 40 cells). The decrease of surface expressed KARs was even more pronounced when monitoring the Myc-GluK2b subunit in the heteromer (15 ± 2% without PfnIIa, 6 ± 1% with PfnIIa, n = 70 cells) (Fig. 2D, right panel). PfnIIa had no effect on the plasma membrane expression of receptors composed of GluK2a/Myc-GluK2b/AA subunits (15 ± 1% without PfnIIa, 18 ± 2% with PfnIIa, n = 50 cells). These results indicate that PfnIIa decreases the amount of homo- and heteromeric KARs containing GluK2b at the plasma membrane through a direct interaction between GluK2b and PfnIIa in COS-7 cells.

To study whether PfnIIa also affected surface trafficking of KARs in hippocampal neurons in culture, we took advantage of cultures derived from PfnIIa knock-out mice (13). As previously described in wild-type hippocampal neurons, endogenously expressed GluK2a was able to heteromerize with GluK2b and promoted forward trafficking of GluK2b to the neuronal plasma membrane (3). We thus expressed Myc-GluK2b (or Myc-GluK2b/AA) with or without PfnIIa in hippocampal neurons and performed live labeling of surface receptors composed of GluK2a/Myc-GluK2b/AA subunits in combination with GluK2b (or Myc-GluK2b/AA) with or without PfnIIa in hippocampal neurons and performed live labeling of surface receptors composed of GluK2a/Myc-GluK2b/AA subunits (15 ± 1% without PfnIIa, 18 ± 2% with PfnIIa, n = 50 cells).

We checked whether these mutations affected the binding of PfnIIa with GluK2b subunit (Fig. 4). We examined the binding of dynamin-1 and GluK2b to PfnIIa mutants, by GST pulldown experiments (Fig. 4A, n = 3). As expected, we did not observe any pulldown of dynamin-1 with the PfnIIa mutants, after incubation of the GST beads with a brain extract and quantification of anti-dynamin-1 labeling in Western blots. We next incubated extracts from COS-7 cells expressing the Myc-GluK2b subunit with GST beads. We found that PfnIIaY or PfnIIaD bind GluK2b, as well as wild-type PfnIIa (n = 5). Conversely, we performed a peptide pulldown assay with the C terminus peptide of GluK2b incubated with extracts of COS-7 cells expressing either PfnIIa, PfnIIaY, or PfnIIaD (Fig. 4B, n = 2). We observed that the same amount of PfnIIa and PfnIIa mutants were pulled down with the GluK2b C terminus peptide. These results were confirmed by immunoprecipitation experiments on extracts from COS-7 cells expressing Myc-GluK2b and PfnIIa (Fig. 4C, n = 3). Myc-GluK2b was immunoprecipitated with an anti-Myc antibody, and Western blots were probed with an antibody against PfnIIa. Similar amounts of PfnIIa, PfnIIaY, or PfnIIaD were co-immunoprecipitated with the Myc-GluK2b subunit. Thus, although PfnIIaY and PfnIIaD fail to interact with dynamin-1,

FIGURE 3. PfnIIa decreases surface expression of KARs composed of GluK2b in hippocampal neurons from PfnIIa knock-out mice. A, representative images of hippocampal neurons transfected with Myc-GluK2b (mK2b) or Myc-GluK2b/AA (mK2b/AA) and GFP or PfnIIa-GFP as indicated. Neurons were labeled with anti-Myc antibody and Alexa Fluor 568-conjugated secondary antibody (surface receptor). GFP images allowed us to define the total surface of the neurons. Scale bar, 10 μm. B, quantification of the EC receptors in arbitrary units. Data represented as mean ± S.E. were compared using unpaired t test (***, p < 0.001). Expression of PfnIIa decreases surface expression of GluK2b (K2b) but not of GluK2b/AA (K2b/AA).
these two mutations do not appear to affect the binding of PfnIIa to the GluK2b subunit. PfnIIa associates with dynamin-1 via its C-terminal proline-rich domain and thus probably associates with GluK2b via another domain.

Using these mutants, we could then differentiate between a specific function for the PfnIIa-GluK2b interaction and a more general effect of PfnIIa on endocytosis of membrane proteins. We first performed biotinylation experiments in COS-7 cells expressing heteromeric GluK2a/Myc-GluK2b receptors with or without PfnIIaY or PfnIIaD (Fig. 5A, n = 3). Extracellular and intracellular pools of receptors were purified on streptavidin beads, and samples were run on SDS gels, blotted with anti-Myc antibody, and quantified. Expression of PfnIIaY or PfnIIaD decreased the relative surface levels of GluK2b containing KARs to similar levels as with PfnIIa (Myc-GluK2b in GluK2a/GluK2b receptor expressed with PfnIIa, 18 ± 4%; with PfnIIaY, 18 ± 6%; with PfnIIaD, 16 ± 5%). Quantification of the relative amount of surface receptors by immunocytochemistry in COS-7 cells expressing PfnIIaY or PfnIIaD yielded similar results (Fig. 5B). By both biochemical and immunocytochemical methods, we found that mutated forms of PfnIIa did not affect membrane trafficking.
FIGURE 5. Mutated forms of PfnIIa decrease surface expression of KARs composed of GluK2b subunit as well as PfnIIa WT. A, biotinylation experiments on COS-7 cells transfected with GluK2 subunits (GluK2a with Myc-GluK2b (mK2b, left), or Myc-GluK2b/AA (mK2b/AA with GluK2a (right)) and GFP (control), PfnIIa, or the mutants as indicated. After biotinylation and purification of IC and EC pools of KARs on streptavidin-agarose, samples were run on SDS gels, and Western blots were probed with anti-Myc antibodies. EC localization of GluK2b subunits was quantified as the ratio of EC by total (EC/IC) receptors (n = three experiments). Data represented as mean ± S.E. were compared using unpaired t test (***, p < 0.001).

B, quantification of the extracellular labeling of KARs on COS-7 cells (30 cells, n = 2 experiments) expressing GluK2a/mGluK2b (left panel) or GluK2a/mGluK2b/AA (right panel) with PfnIIa WT, S134Y (Y), or S138D (D) mutants. Data represented as mean ± S.E. were compared by one-way ANOVA and Bonferroni’s post test (**, p < 0.01).

C, right, representative images of extracellular labeling of myc-GluK2b with the different mutants of PfnIIa expressed in PfnIIa knock-out hippocampal neurons. Left, quantification of EC labeling reported to the surface of the neurons (30 cells, n = 3), arbitrary units. Data represented as mean ± S.E. were compared by one-way ANOVA and Bonferroni’s post test (***, p < 0.001). Scale bar, 10 μm. Expression of the mutant forms of PfnIIa that still bind GluK2b subunit inhibits surface expression of KARs containing GluK2b at the same level as PfnIIa WT.
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Endocytosis Pathways of KARs Co-expressed with PfnIIa—We directly tested this hypothesis by examining the mechanisms and the rate of endocytosis of GluK2b-containing receptors expressed in COS-7 cells and the influence of PfnIIa (Fig. 6). Transferrin is internalized through the classical receptor-mediated endocytosis pathway involving clathrin-coated pits and dynamin (19). We analyzed the pathway for endocytosis of KARs by incubating the cells for 30 min at 37 °C with transferrin labeled with Alexa Fluor 488 (Fig. 6, A and B). When expressed alone, GluK2b was detected in intracellular compartments as big aggregates corresponding to endocytic events, and it was in great part colocalized with transferrin (76 ± 1%, n = 12 cells). Intracellular aggregates containing GluK2b also contain transferrin, indicating that this subunit is recycled via clathrin-coated pits. However, quantification of the rate of endocytosis was difficult because of the very low level of GluK2b expressed at the plasma membrane. We thus evaluated the colocalization of transferrin and GluK2b that compose heteromeric GluK2a/GluK2b receptors. When co-expressed with GluK2a in COS-7 cells, Myc-GluK2b was colocalized with transferrin in 50 ± 4% (n = 15) of intracellular aggregates. Co-expression of either PfnIIa or PfnIIaY did not change the level of colocalization (48 ± 4 and 49 ± 5%, respectively, n = 15 cells). When expressed with GluK2a, Myc-GluK2b/AA also colocalized to the same extent with transferrin (48 ± 2% without PfnIIa; 55 ± 3% with PfnIIa). Overall, these results suggest that GluK2b (both as a homomer or as part of a heteromeric receptor) is endocytosed through the same pathway as transferrin, hence clathrin-mediated endocytosis. Expression of PfnIIa does not qualitatively change the internalization pathway.

Because PfnIIa has been shown to be a negative regulator of clathrin-mediated endocytosis (14), we next measured the rate of endocytosis of receptors composed of GluK2a/Myc-GluK2b in COS-7 cells and its regulation by PfnIIa (Fig. 6C). To do this, we incubated COS-7 cells expressing Myc-tagged receptors (Myc-GluK2b in heteromeric GluK2a/Myc-GluK2b, Myc-GluK2b/AA in heteromeric GluK2a/Myc-GluK2b/AA, or Myc-GluK2a in Myc-GluK2a as a control) for 30 min at 37 °C with an anti-Myc antibody. Receptors remaining at the cell surface were then labeled with a secondary antibody conjugated with Alexa Fluor 488. Cells were then permeabilized, and a secondary antibody conjugated with Alexa Fluor 488. After permeabilization, cells were incubated with a secondary antibody conjugated with Alexa Fluor 488. Graphs represent the percentage of endocytosed receptors in function of the different cDNAs expressed as indicated. Data represented as mean ± S.E. were compared by one-way ANOVA and Bonferroni’s post test. *p < 0.05, **p < 0.01, ***p < 0.001. ns, not significant. mk2b/AA, Myc-GluK2b/AA; mk2b, Myc-GluK2b; mk2a, Myc-GluK2a; k2a, GluK2a; Y, S134Y.

The endocytosis pathway of GluK2b is not dependent on the expression of PfnIIa. Cells expressing the different cDNAs as indicated were incubated with an anti-Myc antibody and transferrin (Alexa Fluor 488, green) for 30 min at 37 °C. After endocytosis, cells returned at 4 °C, and extracellular KARs were labeled with a secondary antibody coupled to Alexa Fluor 647 (blue) at saturating concentration (1/300). Cells were then fixed and permeabilized, and endocytosed receptors were labeled with a secondary antibody coupled to Alexa Fluor 568 (red). Scale bar, 10 μm. A, graphs represent the percentage of internalized receptors colocalized with transferrin for each condition. Data represented as mean ± S.E. were compared by one-way ANOVA and Bonferroni’s post test. C, quantification of the level of endocytosis of KARs depending on the expression of PfnIIa or its mutants. Cells expressing the different cDNAs as labeled were incubated with an anti-Myc antibody for 30 min at 37 °C to allow endocytosis (endo.). Cells were then fixed and incubated with a secondary antibody conjugated with Alexa Fluor 488. After permeabilization, cells were incubated with a secondary antibody conjugated with Alexa Fluor 568. Graphs represent the percentage of endocytosed receptors in function of the different cDNAs expressed as indicated. Data represented as mean ± S.E. were compared by one-way ANOVA and Bonferroni’s post test (***, p < 0.001). ns, not significant. mk2b/AA, Myc-GluK2b/AA; mk2b, Myc-GluK2b; mk2a, Myc-GluK2a; k2a, GluK2a; Y, S134Y.

of receptors containing GluK2b/AA, which does not bind PfnIIa. We repeated these experiments in hippocampal neurons cultured from PfnIIa knock-out mice expressing the Myc-GluK2b or Myc-GluK2b/AA with or without the mutated forms of PfnIIa (Fig. 5C). Again, we found that expression of the mutated forms of PfnIIa, that still bind the GluK2b subunit but not dynamin-1, decreased the surface expression of KARs containing GluK2b. If inhibition of endocytosis by PfnIIa was involved in the regulated membrane expression of KARs, we may have expected a greater decrease of surface receptors in the presence of PfnIIaY or PfnIIaD. Our results suggest that PfnIIa does not inhibit clathrin-dependent endocytosis of GluK2b.

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is abrogated (43 ± 2%, n = 50 cells). Because this mutant binds GluK2b as efficiently as wild-type PfnIIa, the direct interaction between PfnIIa and GluK2b is not likely to be involved in decreased endocytosis of GluK2b. In addition, the internalization of GluK2b/AA, which does not bind PfnIIa, is similarly decreased in the presence of PfnIIa (without PfnIIa internalization of GluK2b, which does not bind PfnIIa, involved in decreased endocytosis of GluK2b. In addition, the binding of GluK2b as efficiently as wild-type PfnIIa, the direct interaction between GluK2b and PfnIIa, which lacks binding to PfnIIa, displays decreased endocytosis but not increased surface expression in presence of PfnIIa, as would be expected. This may be in some way related to the lower surface expression of GluK2b/AA as compared with GluK2b, for reasons that are not clear. Overall, these results strongly suggest that the decreased endocytosis of GluK2b-containing KARs by PfnIIa probably reflects a general inhibition of clathrin-mediated endocytosis by PfnII, which is not specific for GluK2b.

Impact of GluK2b-PfnII Interaction on Exocytosis of KARs—These previous results raise the question of how the interaction between GluK2b and PfnIIa affects KAR trafficking. The generic inhibitory effect of PfnIIa on clathrin-mediated endocytosis should lead to an increased amount of surface GluK2b. This contradicts our observation that PfnIIa decreases amount of surface-expressed GluK2b, when expressed either in a homomeric or heteromeric form. We tested the hypothesis that PfnIIa inhibited the exocytosis of GluK2b-containing receptors. To do this, we tagged GluK2b with SEP, and we inserted a tobacco etch virus (TEV) protease cleavage site after the signal sequence of either GluK2b SEP-TEV-GluK2b or GluK2b/AA (SEP-TEV-GluK2b/AA) proteins (Fig. 7). Supercleptic pFluorin is a pH-sensitive derivative of green fluorescent protein that does not emit any fluorescent signal under acidic conditions, preventing fluorescence emission from acidic intracellular compartments (20). The protease TEV cuts proteins exposed at the cell surface that display a specific proteolysis site composed of seven amino acids flanked by spacers as described (17) and does not cut endogenous extracellular proteins. After 24 h of expression of the different receptors and PfnIIa, the TEV protease was directly applied on live cells to remove the SEP from surface expressed GluK2b (time 0). Cells were then washed in culture medium, returned at 37 °C, and fixed after different times of recovery. Only proteins newly exported at the cell surface during these time periods were labeled with a monoclonal anti-GFP (and a secondary anti-mouse antibody coupled to Alexa Fluor 568) incubated before permeabilization. Intracellular SEP-TEV subunits are labeled with a polyclonal anti-GFP (secondary anti-rabbit coupled to Alexa Fluor 488). We then quantified the amount of extracellularly expressed receptors relative to the amount of intracellular receptors. Results are expressed as percentage of recovery from a value of 100% corresponding to uncut receptors. To evaluate the efficiency of the cutting by TEV protease, we labeled the cells before and after incubation by the enzyme.

In COS-7 cells, ~80–90% of SEP-TEV subunits was cut after incubation with the TEV protease. In the absence of PfnII, the recovery SEP-TEV-GluK2b/GluK2a KARs at the plasma membrane of transfected COS-7 cells was completed within 1 to 2 h (recovery at 1 h, 86 ± 7%, n = 45 cells; at 2.5 h, 120 ± 8%, n = 40 cells) (Fig. 7A). When PfnIIa was co-expressed with SEP-TEV-GluK2b/GluK2a KARs, only 30% of KARs was recovered at the plasma membrane after 2.5 h (recovery at 1 h, 31 ± 4%, n = 45 cells; at 2.5 h, 32 ± 5%, n = 40 cells). The level of recovery was 60% after 3.5 h (recovery at 3.5 h, 63 ± 4%, n = 20 cells). We next verified that the decreased rate of exocytosis was directly linked to the specific interaction between PfnIIa and GluK2b, and not to a more general effect of PfnIIa through unknown interactions with cytosolic proteins. We first observed that PfnIIa did not affect the rate of recovery of fluorescence SEP-TEV-GluK2b/AA, which does not bind PfnIIa (recovery at 1 h without PfnIIa, 74 ± 5%, n = 45 cells; with PfnIIa, 86 ± 5%, n = 40 cells) (Fig. 7B). Interaction of PfnIIa with the endocytic machinery did not influence the rate of recovery of fluorescence at the cell surface. Indeed, PfnIIaY in which dynamin-1 binding is abrogated but still interacts with GluK2b, affected exocytosis of SEP-TEV-GluK2b/GluK2a at the same rate as PfnIIa (recovery at 1 h, 23 ± 4%, n = 40 cells; at 2.5 h, 38 ± 5%, n = 40 cells) (Fig. 7A). The fact that the rate of recovery reaches similar values after 2.5 h with PfnIIa and PfnIIaY may reflect the fact that exocytosis of GluK2a/GluK2b receptors is fully blocked by PfnIIa.

To verify whether exocytosis, in general, is affected by the expression of PfnIIa, we expressed SEP-TEV-GluK2a alone (Fig. 7C). GluK2a is readily expressed at the surface due to efficient targeting of a forward trafficking motif. Without PfnIIa, the recovery of GluK2a at the plasma membrane was fast (recovery at 0.5 h, 94 ± 7%, n = 30 cells). Addition of PfnIIa did not change the recovery time of SEP-TEV-GluK2a (recovery at 0.5 h, 103 ± 10%, n = 30 cells).

We then repeated these experiments in cultured neurons derived from PfnII knock-out mice (Fig. 7, D and E). We co-transfected GluK2a with the different GluK2b constructs as indicated, and we quantified exocytosis of KARs by evaluating recovery from TEV cleavage after 0.5 to 2.5 h. The recovery of SEP-TEV-GluK2b/GluK2a KARs at the plasma membrane was not complete after 2.5 h (recovery at 1 h, 47 ± 10%, n = 14 cells; at 2.5 h, 69 ± 10%, n = 18 cells) (Fig. 7D). The extent of recovery was markedly reduced when PfnIIa was co-expressed with SEP-TEV-GluK2b/GluK2a (recovery at 1 h, 26 ± 8%, n = 13 cells; at 2.5 h, 20 ± 12%, n = 11 cells). Co-expression of PfnIIaY, which does not interact with the endocytic machinery, also affected recovery of SEP-TEV-GluK2b/GluK2a to the same extent as PfnIIa (recovery at 1 h, 23 ± 6%, n = 7 cells; at 2.5 h, 24 ± 5%, n = 7 cells) (Fig. 7D). The recovery rate of fluorescence of SEP-TEV-GluK2b/AA, which does not bind to PfnIIa, was not affected by co-expression with PfnIIa (recovery at 2.5 h without PfnIIa, 61 ± 7%, n = 12 cells; with PfnIIa, 53 ± 9%, n = 7 cells) (Fig. 7E), indicating
FIGURE 7. Exocytosis of KARs directly depends on the interaction between GluK2b and PfnIIa both in COS-7 cells and hippocampal neurons. Cells expressing the different cDNAs (SEP-TEV-GluK2b (K2b) with GluK2a, SEP-TEV-GluK2b/AA, with GluK2a (K2a) or SEP-TEV-GluK2a, without or with PfnIIa or the S134Y mutant), were incubated with the TEV enzyme (10 min at 37 °C followed by 10 min at 20 °C) and then returned at 37 °C for different recovery times as indicated. Cells were fixed and labeled for extracellular receptors with a monoclonal anti-GFP antibody at saturating concentration (1/300). After permeabilization, intracellular KARs were labeled with an anti-GFP polyclonal antibody. Secondary antibodies were anti-mouse Alexa Fluor 568 (red, EC) and anti-rabbit Alexa Fluor 488 (green, IC). The level of exocytosed KARs was performed as the ratio between exocytosed receptors on total intracellular receptors (n = 50 cells for each conditions, three experiments for COS-7 cells, n = 7 to 25 cells for hippocampal neurons, three experiments). Results are expressed as percentage of recovery; 100% corresponds to the ratio EC/IC before adding the TEV protease, representing the uncut KARs. A, exocytosis of SEP-TEV-GluK2b + GluK2a without PfnIIa (red squares), with PfnIIa (green squares) or with PfnIIaY (blue squares). Left panel, representative image (in red EC labeling of GluK2b, in green IC labeling of GluK2b), Right panel, quantification of the corresponding experiments. B, exocytosis of SEP-TEV-GluK2b/AA + GluK2a without PfnIIa (red triangles) or with PfnIIa (green triangles). Inhibition of exocytosis of GluK2b containing KARs is induced by a direct interaction of PfnIIa with GluK2b subunit. Data were compared by two-way ANOVA and Bonferroni’s post test (***, p < 0.001). Scale bar, 10 μm. C, exocytosis of SEP-TEV-GluK2a without PfnIIa (red circles) or with PfnIIa (green circles). PfnIIa does not change the rate of exocytosis of GluK2a. Data were compared by two-way ANOVA and Bonferroni’s post test (***, p < 0.001; **, p < 0.01). Scale bar, 10 μm. D and E, exocytosis of SEP-TEV-GluK2b + GluK2a (D) or SEP-TEV-GluK2b/AA + GluK2a (E) without PfnIIa (red squares or triangles), with PfnIIa (green squares or triangles) or with PfnIIaY (blue squares) on hippocampal neurons from Pfn−/− mice. Left panels, representative images (in red EC labeling of GluK2b, in green IC labeling of GluK2b). Right panels, quantification of the corresponding experiments. Inhibition of exocytosis of GluK2b-containing KARs is induced by a direct interaction of PfnIIa with the GluK2b subunit. Data were compared by two-way ANOVA and Bonferroni’s post test (***, p < 0.001; **, p < 0.01). Scale bar, 10 μm.
that the inhibition of exocytosis was due to the specific interaction between GluK2b and PfnIIa.

**DISCUSSION**

In the present study, we demonstrate that the KAR subunit GluK2b directly and specifically interacts with PfnIIa through a diproline motif localized at the C-terminal domain of the KAR subunit. This interaction leads to decreased surface expression of GluK2b, either expressed as a homomer or as part of heteromeric GluK2a/GluK2b KARs, both in transfected COS-7 cells and in cultured hippocampal neurons derived from PfnIIa knock-out mice. We further provide mechanistic explanations for the regulation of KAR trafficking by PfnIIa. The general effect of PfnIIa on endocytosis, which was described previously (14), is also found for KARs composed of the GluK2b subunit that are endocytosed by clathrin-coated pits. Direct interaction of GluK2b with PfnIIa leads to a KAR-specific inhibition of exocytosis. Taken together, our results provide evidence that PfnIIa, an actin-binding protein that is driven to dendritic spines in an activity-dependent manner, controls exocytosis of a major population of KARs composed of the GluK2b splice variant.

The interaction between PfnIIa and the GluK2b subunit was identified through a proteomic screen based on peptide and GST pulldown experiments and on indirect immunoprecipitation of KARs from brain of Myc-GluK2a transgenic mice (7). As for other KAR-interacting proteins identified in the same screen, it is important to ascertain whether there is a direct binding between the C-terminal domain of GluK2b (and GluK2a) or whether this is an indirect interaction. Here, we demonstrate with a peptide pulldown approach and purified PfnIIa produced in *E. coli* that this interaction is direct, and we identify a diproline motif in the C-terminal domain of GluK2b necessary for this interaction. This is the first direct interactor of the GluK2b C-terminal domain to be firmly identified. Conversely, Y2H screens have provided evidence for a direct interaction between GluK2a and PDZ domain-containing proteins (PSD-95, syntenin, GRIP, and PICK1) (21, 22), through the last four amino acids of GluK2a. In addition, two proteins of the BTB-Kelch family, actinfilin and KRRIP6, bind to the C-terminal domain of GluK2a (10, 23), although the exact sequence of interaction has not been identified. Finally, a recent study has indicated that Neto2, identified by a Y2H screen, acts as an auxiliary protein for KARs (24). Given the variety of proteins directly or indirectly interacting with KARs, a number of studies have attempted to understand how they control either the functional properties or trafficking of KARs (reviewed in Ref. 9).

Here, we show that PfnIIa has a marked influence on the amount of GluK2b-containing KARs at the cell surface, in heterologous systems and in cultured hippocampal neurons. Whereas GluK2a is readily expressed at the surface due to efficient targeting of a forward trafficking motif, GluK2b is only detected at very low levels at the membrane (3, 6). In the case of homomeric GluK2b, one may question the functional significance of further decreasing the level of surface receptors. However, an important point of the present study is that PfnIIa, through its binding to the C-terminal domain of GluK2b, markedly decreases surface expression of GluK2a when it is co-assembled with GluK2b. This finding provides additional support to the notion that the heteromerization of the two splice variants of GluK2 (7), which only differ in their cytoplasmic C-terminal domain, is highly significant for the regulation of trafficking of KARs to and from the plasma membrane. This heteromerization was also shown to be important for the physiological regulation of KARs (7). This process could certainly be extended to other members of the glutamate receptor family, as, for instance, the NR1 subunits, which exist in eight splice variants derived from a single gene (25). The possibility that two distinct splice variants of NR1 co-exist in an NMDA receptor complex and whether this has important impact on receptor trafficking has not yet been addressed.

Profilins were described originally as actin-binding proteins, but are now regarded as regulators of complex cellular processes, including clathrin-mediated endocytosis (11). This regulation depends on an interaction of PfnIIa with dynamin-1 and appears relevant for the control of membrane trafficking of receptors. However, PfnIIa was shown to inhibit endocytosis in neurons, which is in apparent contradiction with our finding that PfnIIa decreases the level of surface expressed KARs. To provide insight into the mechanisms of action of PfnIIa, we took advantage of mutant forms of PfnIIa, which do not interact with the dynamin-1 pathway but still interact with GluK2b, and we examined whether PfnIIa regulates the endocytosis of GluK2b receptors. Interestingly, GluK2b homomers and GluK2b/GluK2a heteromers, but not GluK2a co-localize with transferrin in endocytosis experiments, indicating that endocytosis of GluK2-containing receptors is mediated by a clathrin-dependent pathway. As in the case of transferrin, PfnIIa effectively decreases the rate of endocytosis of GluK2b-containing receptors, in contrast with mutants of PfnIIa that fail to interact with this endocytic machinery (14) but still bind to GluK2b. The inhibition of endocytosis of GluK2b by PfnIIa does not require direct and specific binding of PfnIIa to GluK2b but appears to rely on a generic regulation of clathrin-mediated endocytosis.

This down-regulation does not match with decreased levels of GluK2b-containing KARs at the cell surface. In fact, we show that the regulation of endocytosis appears to be masked by the prevailing reduction of exocytosis of GluK2b-containing receptors that relies on the specific interaction between GluK2b and PfnIIa. We have thus characterized PfnIIa as a double agent that first controls exocytosis in a protein-specific manner through a direct interaction with GluK2b and then acts as a more generic regulator of clathrin-mediated endocytosis. The mechanism by which PfnIIa inhibits exocytosis of GluK2b is unknown but may be linked to its role in the organization of the cytoskeleton and possibly to the actin-dependent trafficking of export vesicles to the plasma membrane. At inhibitory synapses, PfnIIa participates in a complex with gephyrin, an essential component of the protein network that participates in the dynamic assembly of inhibitory receptors (26). Although this has not yet been defined, PfnIIa could be involved in connecting gephyrin to two cytoskeletal systems, microtubules and microfilaments. In addition it is not
yet known how PfnIIa regulates the clusterization and stabilization of inhibitory receptors at synapses.

PfnIIa is redistributed to postsynaptic sites upon stimulation of postsynaptic NMDA receptors and influx of extracellular Ca\textsuperscript{2+} in spines, with both elements being involved in long term changes of synaptic function and architecture (15). Activity-induced profilin accumulation in synapses has been also demonstrated in conditions of a physiological learning process, namely fear conditioning in the amygdala (27), where it is accompanied with an enlargement of the postsynaptic density. We can postulate that massive redistribution of PfnIIa following activity-dependent plastic changes could play a role in the dynamic regulation of synaptic KARs. Interestingly, PfnIIa knock-out mice display a presynaptic phenotype with higher vesicle release probability, suggesting that PfnIIa may also be subject to redistribution to presynaptic compartments. Because GluK2-containing KARs are present at presynaptic sites where they regulate neurotransmitter release (28), it is tempting to speculate that the density of presynaptic KARs could be controlled by PfnIIa-mediated interactions. In conclusion, this study extends the regulatory functions of profilins within the context of synaptic molecules, playing a role both as a generic regulator of membrane trafficking and as a component of specific glutamate receptor complexes.

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