Munc13-1-mediated Vesicle Priming Contributes to Secretory Amyloid Precursor Protein Processing*

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The amyloid precursor protein (APP) gives rise to β-amyloid peptides, which are the main constituents of senile plaques in brains of Alzheimer’s disease patients. Non-amyloidogenic processing of the APP can be stimulated by phorbol esters (PEs) and by intracellular diacylglycerol (DAG) generation. This led to the hypothesis that classical and novel protein kinase Cs (PKCs), which are activated by DAG/PEs, regulate APP processing. However, in addition to PKCs, there are other DAG/PE receptors present in neurons that may participate in the modulation of APP processing. Munc13-1, a presynaptic protein with an essential role in synaptic vesicle priming, represents such an alternative target of the DAG second messenger pathway. Using Munc13-1 knockout mice and knock-in mice expressing a Munc13-1(H567K) variant deficient in DAG/PE binding, we determined the relative contributions of PKCs and Munc13-1 to PE-stimulated secretory APP processing. We establish that, in addition to PKC, Munc13-1 significantly contributes to the regulation of secretory APP metabolism.

The amyloid precursor protein (APP) is a transmembrane protein that can be processed by two independent proteolytic pathways. The generation of β-amyloid peptides, the main constituents of senile plaques in brain of Alzheimer’s disease patients, is initiated by β-secretase cleavage of APP (1, 2). The endoprotease catalyzing this APP cleavage at the N terminus of the β-amyloid peptide was recently identified (beta-site APP-cleaving enzyme 1; BACE1; Ref. 3). Proteolytic cleavage of APP within the β-amyloid sequence by α-secretase generates a secreted water-soluble 90–100-kDa protein and precludes the generation of β-amyloid peptides (4, 5). The α-secretase pathway of APP processing can be stimulated by phorbol esters (PE), which led to the hypothesis that PKC activation increases secretory APP processing (6–9). In the corresponding studies it was shown that treatment of neuronal cells with PEs (i) enhances the secretion of APP into the culture medium, (ii) increases the formation of non-amyloidogenic C-terminal APP-stubs (named F3-CT or C83), and (iii) reduces the generation of β-amyloid peptides. Competition between APP processing pathways occurs under conditions of robust activation of the α-secretase pathway, most likely because α- and β-secretase pathways utilize the same cellular pool of APP (10).

However, PKCs are not the only DAG/PE receptors present in neurons. Proteins of the Munc13 family (11), DAG/PE receptors with ligand affinities as high as those of PKCs (11). Therefore, DAG/PE binding site-specific drugs can not differentiate between effects mediated by PKCs or Munc13 proteins. In mammals, there are three homologous Munc13 genes, Munc13-1, Munc13-2, and Munc13-3, whose expression is largely brain-specific (12). Munc13-1 is expressed throughout the brain, whereas the expression of Munc13-2 and Munc13-3 is restricted to neocortical/hippocampal and cerebellar neurons, respectively (13, 14).

In the present study, we examined whether APP processing is regulated by Munc13-1, the dominant Munc13 isofrom in brain. For that purpose, we used brains from Munc13-1 knockout mice and from Munc13-1(H567K) knock-in mice that express a Munc13-1 variant deficient in DAG/PE binding instead of the wild-type protein. Because the homozygous knockout and the dysfunctional knock-in mice deteriorate within three to 5 h after birth, our analysis of APP metabolism relied on brains of newborn mice and on organotypic brain slice cultures established from brains of newborn mice. We also used human BE (2)-C neuroblastoma cells transfected with wild-type and mutant Munc13-1(H567K) constructs and monitored their effect on basal and PMA-stimulated secretory APP processing.

EXPERIMENTAL PROCEDURES

Mutant Mouse—Munc13-1 deletion mutant mice and Munc13-1(H567K) knock-in mutant mice were published previously (13, 15). Genotyping of mice was done according to published procedures (13, 15).

Western Blot Analysis—Neonatal mice were sacrificed by decapitation, the brains were removed rapidly from the skull and 10% homogenates (w/v) were prepared in Tris-buffered saline (0.1 M, pH 7.4). Aliquots of the homogenates were centrifuged at 4 °C and 100,000 g for 15 min, and the supernatants containing secretory DAG/PE-cleaved APP were stored at −80 °C. APP in tissue homogenates and supernatants as well as in conditioned medium of cultured cells/organotypic brain slice cultures was detected by Western blot analysis as described in ref. 16. Briefly, APP blotted onto nitrocellulose membranes was detected using a primary mouse antibody that recognizes an N-terminal APP epitope (22C11, Roche Applied Science, Mannheim, Germany, 1:500). In control experiments antibodies against the C-terminal fragment of APP (6E10 and 7H9, Chemicon, Deisenhofen, Germany; 1:1,000) were used to demonstrate that the supernatants did not contain full-length APP. Additionally, supernatants from tissue homogenates were run alongside with the membrane fraction and detected with the antibody 22C11 to show that full-length and secretory APP migrate at a different position in the gel system. To normalize uneven protein loading, actin detected with a polyclonal antisera (Sigma, A 2066; 1:1,000) served as internal control. Blots were digitized (ScanJet 6100C, Hewlett Packard) and images

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were evaluated by densitometric image analysis using the software package TINA 2.0 (Raytest, Straubenhardt, Germany). The sum of gray values over each individual band obtained by densitometry was normalized for actin immunoreactivity, and data were expressed as percentage change over corresponding control animal value and given as mean \( \pm \) S.E. Linearity between protein content and optical readings values was proved by running standard curves ranging from 10 to 50 \( \mu \)g of protein per lane.

**\( \alpha \)- and \( \beta \)-Secretase Activity Assays**—The \( \alpha \)- and \( \beta \)-secretase enzymatic activities in brains of mice with different genotype were measured using a commercial assay (R & D Systems, Wiesbaden-Nordenstadt, Germany) according to the manufacturer’s protocol. The assay is based on the cleavage of fluorochromated peptide substrates containing the \( \alpha \)- and \( \beta \)-secretase cleavage site, respectively. The enzymatic activity was monitored at 510 nm and expressed as percent of secretase activity in brains of wild-type mouse brain.

**BE (2)-C Cell Culture and Transfection**—The human neuroblastoma cell line BE (2)-C was cultured in Eagle’s minimum essential medium/Ham’s F-12 medium containing 15% fetal bovine serum and 2 mM glutamine in a humidified atmosphere of 5% CO\(_2\)/95% air at 37 °C. The growth medium was changed three times a week and the cells were split 1:3 weekly. For transfection, cells were seeded in 48-well plates at a density of 3 \( \times \) 10 \( ^4 \) (4) cells per well. The serum-containing medium was replaced by OptiMem, and cells were incubated with a mixture of 1 \( \mu \)g of the corresponding Munc13-1 or Munc13-1(H567K) plasmid DNA and 2 \( \mu \)l of the DMRIE-C transfection reagent (Invitrogen, Karlsruhe, Germany) per ml transfection medium for 3 h. Thereafter, the transfection medium was replaced by culture medium as above, cells were grown overnight, and then singularized and transfected cells were selected based on G418 resistance.

**Organotypic Brain Slice Cultures**—Organotypic brain slice cultures of newborn mouse brain were established as described recently (17). Briefly, brains were prepared and mounted in 1.5% agarose in distilled water, and 400-\( \mu \)m-thick sections were cut in the coronal plane using a vibratome. Brain sections were collected and maintained in culture plate inserts (Minicell CM, 30-mm diameter; Millipore, Bedford, MA) in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium containing 25% horse serum 0.2% D-glucose, 2 mM L-glutamine, and 0.3 mM HEPEs. The medium was changed every other day, and brain slices were cultured for 7 days before stimulation experiments with PMA were performed.

**PMA Stimulation**—BE (2)-C cells as well as organotypic brain slice cultures were stimulated with PMA (100 \( \mu \)M) or vehicle (Me\(_2\)SO) for 4 h. The PKC-specific inhibitor Go6983 was added 5 min before PMA treatment and was present in the incubation medium during the entire stimulation period. After stimulation, the medium was collected, concentrated 10-fold by centrifugation through amicon-30 filters (Millipore, Bedford, MA), and used for Western blot analysis of secretory APP molecules as described above.

**RESULTS AND DISCUSSION**

In the first set of experiments, total APP protein and secretory APP molecules from brains of newborn mice of all genotypes were quantified by Western blot analysis. There was no difference in total APP content between brains of wild-type mice, Munc13-1 knock-out mice, and Munc13-1(H567K) mice (Fig. 1). However, in brains of Munc13-1 knock-out mice there was a 20% reduction in secretory APP molecules measured in supernatants of tissue homogenates (Fig. 1). These APP molecules migrated at a lower molecular weight than full-length APP and did not display immunoreactivity for the intracellular C-terminal domain (not shown).
processing of APP in brains of Munc13-1 knock-out mice was not due to a reduced expression of the a-secretase ADAM-10 as judged from Western blot analysis (not shown).

However, the a-secretase enzymatic activity was reduced by 30% as compared with its activity in brains of wild-type mice, both in the absence and presence of 100 nM PMA (Fig. 2). Additionally, in brains from Munc13-1(H567K) mice, we observed a 30% reduction in PMA-stimulated, but not basal, a-secretase activity (Fig. 2). The enzymatic activity of β-secretase was similar in all genotypes, both in the presence and absence of PMA. This indicates a specific impairment of the a-secretase pathway of secretory APP processing.

We next examined the consequences of Munc13-1 knock-out and the Munc13-1(H567K) knock-in mutation on PMA-stimulated APP processing in organotypic brain slice cultures. Cultures were established from newborn mice of all genotypes and maintained for 7 days. Brain slices were then stimulated for 4 h with 100 nM PMA or vehicle, and the conditioned medium was collected and proteins larger than 30 kDa were enriched 10-fold by centrifugation through Amicon-30 filters. There was no cytotoxicity during the brief stimulation with 100 nM PMA as judged from lactate dehydrogenase release (not shown). The basal APP secretion from brain slices of all genotypes was very similar (Fig. 3a). However, PMA stimulation of brain slices from wild-type mice increased APP secretion by 160% as compared with basal APP secretion (Fig. 3a). This is a well known phenomenon described earlier and attributed to PKC activation (6, 8). The PMA-induced increase in APP secretion was much less pronounced in brain slices from Munc13-1 knock-out mice (increase of 70%), indicating that the effects of PMA rely to a considerable extent on the presence of Munc13-1 (Fig. 3a). A similar impairment in PMA-stimulated APP secretion was observed in brain slices from Munc13-1(H567K) knock-in mice (Fig. 3a). This observation demonstrates that not the presence of the Munc13-1 protein, but its DAG/PE receptor function is required for DAG/PE-dependent stimulation of secretory APP processing.

Our data on the reduced secretory APP processing under conditions of lacking or DAG/PE-insensitive Munc13-1 protein expression prompted us to perform another set of experiments where we transfected human BE (2)-C neuroblastoma cells with wild-type Munc13-1 or DAG/PE-insensitive Munc13-1(H567K) constructs. We followed secretory APP processing after stimulation with PMA for 4 h in the absence or presence of Go6983, a bisindolylmaleimide derivative that specifically interacts with the ATP binding site of PKCs but does not interfere with Munc13-1 function (15). In mock-transfected control BE (2)-C cells, PMA-stimulated APP secretion by 150% in the absence and by 70% in the presence of the PKC inhibitor Go6983 (3 μM; Fig. 3b). Overexpression of wild-type Munc13-1 in BE (2)-C cells robustly increased the PMA-stimulated APP secretion as compared with control BE (2)-C cells, providing additional evidence for a role of Munc13-1 in the PMA-stimulated secretory APP processing. This was supported by the observation that the counteracting effect of the PKC inhibitor Go6983 on PMA-induced APP secretion is much less pronounced in the Munc13-1 transfected BE (2)-C cells as compared with control BE (2)-C cells (Fig. 3b). Upon overexpression of DAG/PE-insensitive Munc13-1(H567K) protein, the PMA-stimulated APP secretion was significantly reduced as compared with control BE (2)-C cells, both in the absence and presence of Go6983 (Fig. 3b), most likely because overexpression of the DAG/PE-insensitive Munc13-1 protein compromises the function of the endogenous Munc13-1 protein.

The results presented here are in conflict with the view of an exclusive role for PKC in the PE-stimulated secretory APP processing. Published conclusions about the role of PKC in the modulation of APP processing are largely based on indirect pharmacological evidence using substances which are not PKC-specific but also target other proteins expressed by neurons and/or peripheral cells (18). Genetic approaches to characterize the role of PKC in cellular functions are hampered by the presence of multiple PKC isoforms with functional redundancy. Therefore, we used a genetic approach to interfere with the function of the Munc13-1 protein, a neuronal DAG/PE receptor, to reveal the contributions of PKC and Munc13-1 in PE-stimulated APP processing. This strategy revealed that Munc13-1 acts independently of and in parallel with PKC in the PE-stimulated APP processing pathway.

Our findings are consistent with a scenario according to which upon PE stimulation, APP is redistributed from the...
trans-Golgi network to secretory vesicles (19). If these vesicles lack functional Munc13-1 proteins, as is the case in knock-out cells, or if they express DAG/PE-insensitive Munc13-1, as in Munc13-1(H567K) knock-in cells, they will be less efficiently primed under basal and DAG/PE-stimulated conditions, respectively. As a consequence, neurotransmitter release as well as trafficking and insertion of APP into the plasma membrane after vesicular fusion are impaired. It is well established that APP present in the plasma membrane is the major source of the APP processed in the α-secretase pathway (5). Thus, diminished APP transport to the cellular membrane, e.g. due to interference with Munc13-1 function, reduces APP molecules available for α-secretase processing by extracellular matrix metalloproteinases such as ADAM-10 (20). As a result, secretory APP processing was found to be reduced in Munc13-1 mutants without reduction in the protein levels of its processing enzyme ADAM-10. However, α-secretase enzymatic activity in brain tissue homogenates, as measured by cleavage of a synthetic peptide, was reduced in Munc13-1 knock-out and Munc13-1(H567K) brains (see Fig. 2). This unexpected finding may be explained by the accumulation of full-length APP in secretory vesicles as described above and the subsequent disruption of the cellular membrane during the freeze/thaw cycle preceeding the α-secretase activity assay, which makes both the endogenous APP and the synthetic substrate available to competing for α-secretase. Alternatively, α-secretase activity harboring enzymes may be co-transported along with APP in secretory vesicles and, as in the case of APP, not released into the extracellular space by neurons lacking functional Munc13-1 protein. This novel regulatory mechanism of secretory APP processing appears to be specific for neuronal cells and does not occur in human embryonic kidney cells transfected with APP and Munc13-1 constructs.2

Our data demonstrate that Munc13-1-mediated vesicle priming is not only relevant for neurotransmitter release but is also functional in protein secretion/exocytosis as already shown recently for insulin exocytosis from pancreatic islet beta cells (21). Our observations also identify Munc13-1 as an alternative target to modulate APP processing pathways in Alzheimer’s disease. The development of activators of Munc13-1 function, such as PE derivatives specific for the Munc13-1 C1 domain or small molecules mimicking RIM1 (see Ref. 22) may prove useful as therapeutic agents to shift APP processing toward the non-amyloidogenic, secretory pathway. Independently of the clinical importance of our findings, this is the first and most likely not the last identification of a cellular function for Munc13-1 that was previously attributed exclusively to PKC.

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