Nonspecific Interaction of Prefibrillar Amyloid Aggregates with Glutamatergic Receptors Results in Ca$^{2+}$ Increase in Primary Neuronal Cells*

Received for publication, May 26, 2008, and in revised form, July 31, 2008. Published, JBC Papers in Press, August 1, 2008, DOI 10.1074/jbc.M803992200

Francesca Pellistrì, Monica Bucciantini, Annalisa Relini, Daniele Nosi, Alessandra Gliozzi, Mauro Robello, and Massimo Stefani.

From the †Department of Physics, University of Genoa, Via Dodecaneso, 33, I-16146 Genoa, ‡Research Centre on the Molecular Basis of Neurodegeneration, §Department of Biochemical Sciences, University of Florence, Viale Morgagni, 50, 50134 Florence, and the †Department of Anatomy, Histology, and Forensic Medicine, University of Florence, Florence 50134, Italy

It is widely reported that the Ca$^{2+}$ increase following nonspecific cell membrane permeabilization is among the earliest biochemical modifications in cells exposed to toxic amyloid aggregates. However, more recently receptors with Ca$^{2+}$ channel activity such as α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), N-methyl D-aspartate (NMDA), ryanodine, and inositol 1,4,5-trisphosphate receptors have been proposed as mediators of the Ca$^{2+}$ increase in neuronal cells challenged by β-amyloid peptides. We previously showed that prefibrillar aggregates of proteins not associated with amyloid diseases are toxic to exposed cells similarly to comparable aggregates of disease-associated proteins. In particular, prefibrillar aggregates of the prokaryotic HypF-N were shown to be toxic to different cultured cell lines by eliciting Ca$^{2+}$ and reactive oxygen species increases. This study was aimed at assessing whether NMDA and AMPA receptor activations could be considered a generic feature of cell interaction with amyloid aggregates rather than a specific effect of some aggregated protein. Therefore, we investigated whether NMDA and AMPA receptors were involved in the Ca$^{2+}$ increase following exposure of rat cerebellar granule cells to HypF-N prefibrillar aggregates. We found that the intracellular Ca$^{2+}$ increase was associated with the early activation of NMDA and AMPA receptors, although some nonspecific membrane permeabilization was also observed at longer times of exposure. This result matched a significant co-localization of the aggregates with both receptors on the plasma membrane. Our data support the possibility that glutamatergic channels are generic sites of interaction with the cell membrane of prefibrillar aggregates of different peptides and proteins as well as the key structures responsible for the resulting early membrane permeabilization to Ca$^{2+}$.

Many human degenerative diseases are associated with the intracellular and/or extracellular deposition, in the affected tissues, of fibrillar aggregates arising from the ordered polymerization of specific proteins or peptides (1). Protein aggregation can occur either in the central nervous system, where it is associated with neurodegenerative disorders, including Alzheimer, Huntington, Parkinson, and prion diseases, or in peripheral tissues and organs, as in cardiac or systemic amyloidosis (2). Although in the peripheral amyloidoses the accumulation of huge amounts of amyloid deposits may be directly responsible for the clinical symptoms because of the physical disruption of tissue architecture, in neurodegenerative disorders the neurotoxic effects seem to arise directly from the presence of soluble protein oligomers produced early in the fibrillation path. Actually, early oligomers have been identified as the primary toxic species for a number of amyloid diseases (3) and shown to be cytotoxic to cells in culture (4) and in tissue (5, 6); on the contrary, mature fibrils are considered as stable and substantially harmless end products of the process (7), although in some cases they can be cytotoxic as well (8–10). Moreover, recent findings have shown that monomers can recycle within the fibril population (11), and amyloid fibrils may undergo fragmentation (12), increasing the concentration of free ends where further polymerization of misfolded monomers can occur. This view could support the widely accepted idea that, at least in most cases, amyloid fibrils are benign assemblies whose frequency of rupture could be related to their protective effect by clearing misfolded monomers, thus preventing their assembly into toxic oligomers.

Previous papers on amyloid cytotoxicity suggest that oligomers formed by proteins and peptides not associated with any amyloid disease display a toxic behavior indistinguishable from that of comparable oligomers of proteins and peptides associated with disease (7, 13). These findings suggest that, at least in most cases, cell damage by protein/peptide oligomers follows a general mechanism associated with early amyloid assemblies in the path of aggregation (14). Such a view supports the notion of amyloidoses as “conformational diseases,” whereby a common mechanism of pathogenesis involves shared conformational features of amyloid assemblies of structurally different peptides and proteins. Accordingly, disease-unrelated proteins can provide a useful experimental model to study the interaction between protein oligomers and cells to...
better understand the generic molecular mechanisms of their cytotoxicity.

It is commonly accepted that disruption of intracellular calcium homeostasis and oxidative stress are among the earliest biochemical modifications following the interaction of amyloid prefibrillar assemblies with the cell membranes (7, 15–19). We have previously shown that early aggregates of HypF-N, the N-terminal domain of the Escherichia coli hydrogenase maturation factor, a protein not associated with any amyloid disease that displays a fibrillogenic pathway (7, 20), are cytotoxic to a variety of cultured cells by increasing free Ca\(^{2+}\) and reactive oxygen species levels (13). We have also reported that the same aggregates can induce Ca\(^{2+}\) influx in liposomes and disrupt the ordered structure of supported lipid bilayers (20, 21). On the other hand, recent reports support the idea that the increase of the intracellular Ca\(^{2+}\) levels can result as a consequence of the interaction of the β-amyloid (Aβ)\(^2\) peptides with the glutamatergic NMDA receptors (NMDA-Rs) (22). Aβ can also drive the loss from the cell surface of both NMDA-Rs and AMPA receptors (AMPA-Rs) (23), whereas Ca\(^{2+}\)-permeable AMPA-Rs are involved in the cell death associated with neurodegenerative disorders such as amyotrophic lateral sclerosis and Alzheimer disease (24). The interaction of amyloidogenic peptides and, possibly, their early aggregates with cell surface receptors such as those involved in Ca\(^{2+}\) trafficking can account, at least in part, for the alteration of the ion distribution across the cell membrane, although not excluding some nonspecific membrane permeabilization.

In this paper we sought to elucidate some of the above issues. In particular, we investigated whether cultured rat cerebellar granule cells are damaged by HypF-N amyloid aggregates, whether cell damage is accomplished via a nonspecific pathway or through aggregate interaction with specific cell surface receptors, and whether NMDA-Rs and AMPA-Rs are involved in the biochemical modifications resulting from cell exposure to HypF-N or muscle acylphosphatase (AcP, a related protein) aggregates. To this purpose, we measured the Ca\(^{2+}\) influx following aggregate interaction with our experimental cell model by blocking selectively the possible permeation pathways. We also investigated whether the immunoreactivity of the AMPA-Rs and/or NMDA-Rs at the cell membrane was reduced in cells exposed to the aggregates for 1 h.

Our results indicate that an influx of the external Ca\(^{2+}\) in the exposed cells did occur and that such influx displayed two different kinetics. The co-localization experiments confirmed that the Ca\(^{2+}\) influx could substantially be attributed to the interaction of the early HypF-N aggregates with the glutamatergic NMDA-R and AMPA-R, similarly to previous data concerning Aβ peptides. Finally, we found a significant reduction of AMPA-R immunoreactivity in cells exposed to the aggregates for 1 h, suggesting receptor internalization following receptor-aggregate interaction.

**EXPERIMENTAL PROCEDURES**

**Cell Preparation**—Cerebellar granule neurons were prepared from 8-day-old Sprague-Dawley rats (Morini, Italia), as published previously (25). Cells were plated at a density of 1 × 10⁶ per dish on 20-mm poly-L-lysine-coated glass coverslips and maintained in Basal Eagle’s culture medium, containing 10% fetal calf serum, 100 μg/ml gentamicin, and 25 mM KCl at 37 °C in a humidified 95% air, 5% CO₂ atmosphere. Cultures were treated with 10 μM cytosine arabinoside from day 1 to minimize proliferation of non-neuronal cells. Experiments were performed in cultures between day 6 and 10 after plating.

**Preparation of Prefibrillar Aggregates**—HypF-N and AcP prefibrillar aggregates were obtained by incubating each protein at room temperature at a concentration of 29 and 27 μM, respectively, in 30% trifluoroethanol (TFE), 17 mM ammonium acetate, 2.0 mM dithiothreitol, pH 5.5. Aggregation times of 12, 24, and 48 h and 7 and 8 days were considered. Sample aliquots were withdrawn at different aggregation times and added to the cell culture media (0.2–2.0 μM final protein concentration range), after TFE removal. To remove TFE, which could give rise to artifacts, the volume of the protein aliquot was reduced to 70% of its initial value by evaporation under a stream of N₂ and then restored to 100% by adding the required amount of external solution. The amount of TFE still present in the sample after this treatment was about 1% of the initial value, as determined by mass spectrometry. The final TFE concentration in the cell culture media after aggregate addition was about 0.01%. Control experiments showed that such low TFE levels did not affect cell viability and did not influence the cell responses induced by protein aggregates.

**Fluorescence Measurements**—Neurons were incubated at 37 °C for 40–45 min in a 6.0 μM solution of the cell-permeant AM ester of Oregon Green (Molecular Probes, Eugene, OR) and then washed several times with 135 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, 5.0 mM Hepes, 10 mM glucose, pH 7.4, at room temperature. Then cultures were transferred to a recording chamber mounted onto a Nikon Eclipse TE300 inverted microscope. Cells were continuously perfused with the appropriate solution and were visualized using a ×100 objective in oil (N.A. 1.3).

The fluorescence signal, including real time changes of the internal calcium concentration after the addition of protein aggregates, was detected using a Hamamatsu digital CCD camera with a 450–490-nm excitation filter, a 505-nm dichroic mirror, and a 520-nm emission filter (Nikon Italia, Florence, Italy). Images were acquired with the Simple PCI software (Compix Imaging Systems, Hamamatsu Corp., Sewickley, PA). Fluorescence intensity was calculated in arbitrary units by evaporation under a stream of N₂ and then restored to 100% by adding the required amount of external solution. The amount of TFE still present in the sample after this treatment was about 1% of the initial value, as determined by mass spectrometry. The final TFE concentration in the cell culture media after aggregate addition was about 0.01%. Control experiments showed that such low TFE levels did not affect cell viability and did not influence the cell responses induced by protein aggregates.

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of antagonists of glutamatergic receptors were performed using memantine, CNQX, and APV purchased from Sigma.

Confocal Fluorescence Measurements—The co-localization of HypF-N prefibrillar aggregates with NMDA-Rs or AMPA-Rs was tested in cerebellar granule neurons treated for 10 min with 24-h aged protein aggregates (0.2 µM soluble protein concentration) at 37 °C. Cells were rinsed with phosphate-buffered saline, pH 7.4, and fixed with 2.0% paraformaldehyde for 10 min at 22 °C. After rinsing, cells were incubated with a blocking solution (0.2% gelatin, 0.5% bovine serum albumin in phosphate-buffered saline) for 20 min at 22 °C. Cells requiring membrane permeabilization were kept in ice-cold acetone for 1.0 min before the blocking step or incubated with the blocking solution supplemented with 0.2% saponin for 20 min. Neurons were then incubated with a monoclonal mouse anti-NMDA-R type 2B antibody (1:800) (MAI-2014, Affinity Bioreagents, Golden, CO), or a monoclonal mouse anti-GluR2 AMPA-R subunit antibody (1:800) (MAB397, Chemicon International Inc.), or a polyclonal goat anti-gp91 phox (sc-5827, Santa Cruz Biotechnology Inc.), together with a polyclonal rabbit anti-HypF-N antibody (1:100) (provided by Primm S.r.l. Milan, Italy) for 1.0 h at room temperature. After repeated washing, the coverslips were incubated with a secondary Alexa Fluor 488-conjugated anti-rabbit antibody (1:400) (Molecular Probes) (for HypF-N aggregates), or a secondary Alexa Fluor 546-conjugated anti-mouse antibody (1:400) (Molecular Probes) (for NMDA-R and AMPA-R), or a secondary Cy3-conjugated anti-goat antibody (1:400) (Molecular Probes) (for gp91 phox) for 1.0 h at room temperature. In control experiments, the primary antibodies were omitted from the labeling process. After repeated washing, the coverslips were mounted on the stage of a Leica TCS SP5 confocal scanning microscope (Leica, Mannheim, Germany) equipped with a HeNe/Ar laser source for fluorescence measurements. The observations were performed using a Leica Plan Apo 63× oil immersion objective. To minimize cross-talk between fluorochromes, sequential scanning was performed at excitation wavelengths of 488 and 543 nm. Series of optical sections (1024 × 1024 pixels, pixel size 204 nm) 0.5 µm in thickness were taken through the cells at 0.4-µm intervals. Confocal stacks were then resized to 4096 × 4096 pixel size (pixel size 51 nm) and then filtered with an FFT-based bandpass filter to cut background noise (structures down to 20 µm and up to 153 nm). For each sample, 20 optical sections were superimposed into a single composite image. For all channels a threshold value was used to improve the signal-to-noise ratio. The co-localization of NMDA-R, AMPA-R, and gp91 phox with HypF-N aggregates on the cell membrane was estimated on regions of interest (70-µm side, 12–13 cells per selected area) in three-dimensional confocal stacks, using a specially developed algorithm (28). In particular, we used ImageJ (NIH, Bethesda) and JACOP plugin (rsb.info.nih.gov) software.

To measure a possible HypF-N-induced reduction of cell surface receptors, cells were treated for 1 h with prefibrillar aggregates. Neurons were counterstained for the cell membrane with TRITC-conjugated wheat germ agglutinin for 20 min at room temperature, fixed, permeabilized, and immunostained for NMDA-Rs or AMPA-Rs as described above. The cells were then washed and incubated with a Cy2-conjugated anti-mouse secondary antibody (Molecular Probes) diluted 1:500. Finally, cells were processed for confocal microscopy as indicated above.

Cell surface-associated receptor fluorescence was quantified after cell borders were defined by using the contrast phase and the counterstaining images. Twenty or more cells from at least two independent preparations were analyzed for each experimental condition. The data expressed as arbitrary units of fluorescence intensity are reported as means ± S.E. For each set of experiments, the Student’s t test was used for statistical analysis. In all cases p values < 0.001 were considered to be significant.

Aggregate internalization was detected by incubating cultured cells for 24 h in the presence of protein aggregates labeled with Texas Red as reported previously (13). Briefly, 1.0 µl of a 10 mg/ml Texas Red solution in DMSO was added to 0.1 mg of protein aggregates dissolved in 10 µl of 0.1 M sodium bicarbonate buffer, pH 8.5. After 1 h of incubation at room temperature with continuous stirring, the reaction was stopped by adding 1.0 µl of 1.5 M hydroxylamine, pH 8.5. Series of optical sections were acquired at an excitation wavelength of 546 nm.

Patch Clamp Measurements—In the patch clamp experiments, the membrane potential was kept at −80 mV, and membrane currents were measured with the standard whole cell patch clamp technique as described previously (25). The internal solution contained 124 mM CsCl, 4.0 mM MgCl₂, 5.0 mM EGTA, 10 mM Hepes, and 3.0 mM ATP. The pH was adjusted to 7.4 with Trizma (Tris base). The external solution contained 120 mM tetraethylammonium chloride, 20 mM BaCl₂, 1.0 mM MgCl₂, 10 mM Hepes, 10 mM glucose, and 4.0 mM 4-aminopyridine, pH 7.4.

Patch electrodes were manufactured from borosilicate glass capillaries (Hilgenberg GmbH, Malsfeld, Germany) with a programmable Sachs and Flaming puller PC-84 (Sutter Instruments, Novato, CA). The final electrode resistance was 4.0–10 megohms when filled with the internal solution. Cell responses were filtered at 3.0 kHz and sampled at 100 Hz. Voltage stimulation and data acquisition were performed by a Labmaster D/A-A/D converter driven by pCLAMP software (Axon Instruments, Burlingame, CA). Capacitance transient neutralization and series resistance compensation were optimized. Data analysis was performed using the pCLAMP and SigmaPlot software (Jandel Scientific, Ekraft, Germany).

RESULTS

Prefibrillar Aggregates Induce a Very Fast Increase of Intracellular Ca²⁺ Concentration—Cerebellar granule cells loaded with Oregon Green were exposed to 0.2 µM HypF-N either in the monomeric native form or aggregated for different lengths of time. Early aggregates obtained after 24 or 48 h of incubation in the aggregation medium consisted of globular structures 2–3 nm in diameter, whereas aggregates aged for 8 days consisted mainly of mature amyloid fibrils, as reported previously (20). In all the cells analyzed, we observed a very fast increase of the fluorescence intensity following the addition of HypF-N early aggregates to the cell external medium. Fig. 1a (frames 1–5) shows a time sequence of fluorescence images of the same region of the sample before (frame 1) and after (frames 2–5) the addition of HypF-N aggregates aged for 24 h; the fluorescence...
increase is particularly evident in the marked cell. Fig. 1a, frame 6, shows the typical time course of the average fluorescence intensity, which reached a maximum in about 30 s and then decreased in 70 s, indicating membrane permeabilization. A statistical analysis of the time duration of the fluorescence responses elicited by aggregates aged 24 and 48 h is reported in Fig. 1b, showing that the distribution for the latter is shifted to shorter times. c and d, addition of native HypF-N (c) and mature HypF-N fibrils (8 days (8d) aged aggregates) (d) does not induce any fast response. In both cases the subsequent addition of early HypF-N aggregates (24 h) induces a fluorescence response, indicative of sample integrity. Arrows correspond to the addition of 0.2 μM HypF-N.

FIGURE 1. Fast response of granule cells to HypF-N in aggregated and monomeric form. a, fluorescence images of granules loaded with Oregon Green at different times before (frame 1) and after (frames 2–5) the addition of prefibrillar HypF-N aggregates aged for 24 h; frame 6, fluorescence intensity of the region of interest (highlighted in white in frames 1–5) as a function of time. Times corresponding to the capture of images 1–5 are indicated on the fluorescence curve. b, histograms of the duration of the fluorescence responses measured in the presence of 24-h (gray) and 48-h aged (dashed) Hyp-F-N aggregates, showing that the distribution for the latter is shifted to shorter times. c and d, addition of native HypF-N (c) and mature HypF-N fibrils (8 days (8d) aged aggregates) (d) does not induce any fast response. In both cases the subsequent addition of early HypF-N aggregates (24 h) induces a fluorescence response, indicative of sample integrity. Arrows correspond to the addition of 0.2 μM HypF-N.

Fluorescence Increase in Exposed Cells Results from an Influx of Extracellular Ca²⁺ Not Involving Voltage-dependent Calcium Channels—To assess whether the increase of intracellular Ca²⁺ resulted from the influx of extracellular Ca²⁺ or from the release of Ca²⁺ from the intracellular stores, we repeated the experiments in a Ca²⁺-free medium after having checked cell integrity by inducing a fluorescence response with early HypF-N aggregates (Fig. 2). In the absence of Ca²⁺, no fluorescence increase was recorded in cells exposed to HypF-N aggregates aged for 24 h (Fig. 2, middle) or 48 h (not shown). This suggests that the intracellular fluorescence increase did result from an influx of extracellular Ca²⁺.

Next, we checked whether the intracellular Ca²⁺ increase in the exposed cells resulted from the activation of voltage-dependent calcium channels. To this purpose, we added to the external solution 50 μM Cd²⁺, a blocker of voltage-dependent
Ca\textsuperscript{2+} channels. We found that the fluorescence response induced by 24-h aged aggregates was the same in the absence (Fig. 2a, left) and in the presence of Cd\textsuperscript{2+} (Fig. 2a, right, and Table 1). This result rules out the possibility that voltage-dependent calcium channels play a direct role in the observed Ca\textsuperscript{2+} influx in the exposed cells. The patch clamp technique provided further evidence that voltage-dependent Ca\textsuperscript{2+} channels are not involved in the Ca\textsuperscript{2+} influx. Fig. 2b shows the calcium current-voltage curves measured in control cells and in cells incubated in the presence of protein aggregates aged 48 h. The calcium current exhibits the typical behavior previously reported for granule cells (29) without significant differences between cells treated with protein aggregates and control cells. Overall, the experiments reported in Fig. 2 indicate that voltage-dependent calcium channels are not involved in the permeabilization of the cell membrane induced by HypF-N prefibrillar aggregates.

Fast Ca\textsuperscript{2+} Increase in Exposed Cells Depends on the Activation of Glutamatergic Receptors—Having excluded that the fast fluorescence increase induced by HypF-N oligomers in cerebellar granule cells involved voltage-dependent calcium channels, we wondered whether it could involve the activation of calcium channels such as the glutamatergic receptors. To test this possibility, we added to the external solution memantine a low affinity, noncompetitive NMDA-R antagonist, and we repeated the experiments exposing the cells to HypF-N aggregates aged 24 h. The results shown in Fig. 3a indicate that, in the presence of 10 \textmu M memantine, the initial rise in intracellular Ca\textsuperscript{2+} is significantly attenuated, and the time course of the increase of the Ca\textsuperscript{2+} concentration is different as compared with the experiments carried out in the absence of memantine (Fig. 3a). The analysis of the fluorescence intensity at the peak value indicates that granule cells treated with 24-h aged HypF-N aggregates displayed a significant fluorescence increase (25%). Conversely, the same treatment in the presence of memantine resulted in a reduced fluorescence change (14%). A similar behavior was observed in the presence of APV, an NMDA-R competitive antagonist; in this case the fluorescence change was even more reduced (9%) (Table 1). Overall, these results indicate that NMDA-Rs are involved in the Ca\textsuperscript{2+} influx following cell exposure to HypF-N aggregates. However, the time course of the increase of the cytosolic Ca\textsuperscript{2+} concentration resulting from the HypF-N aggregate-NMDA-R interaction is different from that recorded upon NMDA-R activation by 100 \textmu M NMDA in the absence of HypF-N aggregates (Fig. 3b). In the latter case the fluorescence signal reached a constant value and decayed only upon NMDA removal. The same experiment was performed after pretreatment of the cells (n = 23) with 24-h aged HypF-N aggregates; in this case the percentage of fluorescence increase (34 \pm 3) was about half that measured without pretreatment (60 \pm 4).

To further investigate the involvement of glutamatergic receptors in the Ca\textsuperscript{2+} influx triggered by HypF-N aggregates, we performed experiments in the presence of the AMPA-R antagonist CNQX. CNQX hinders membrane depolarization and hence Mg\textsuperscript{2+} removal from NMDA-Rs, which remain blocked. In the presence of CNQX, we did not observe the fast response in fluorescence associated with the increase of cytosolic Ca\textsuperscript{2+} in cells exposed to HypF-N aggregates (Table 1), suggesting that the latter affect the functionality of more than one type of glutamatergic receptors.

**Table 1**

| Protein aggregation time | % fluorescence increase | n  |
|--------------------------|------------------------|----|
| 0                        | 0                      | 10 |
| 24 h                     | 25 \pm 2               | 52 |
| 24 h + 10 \textmu M memantine | 14 \pm 2       | 30 |
| 24 h + 100 \textmu M APV  | 9 \pm 3                | 10 |
| 24 h + 10 \textmu M CNQX | 0                      | 18 |
| 24 h + 50 \textmu M Cd\textsuperscript{2+} | 23 \pm 2    | 20 |
| 48 h                     | 22 \pm 2               | 57 |
| 8 days                   | 0                      | 10 |

**FIGURE 2.** The fast response is because of a Ca\textsuperscript{2+} influx and does not involve voltage-dependent Ca\textsuperscript{2+} channels. a, effect of the external solution on the fluorescence response of a granule cell after the addition of 0.2 \textmu M HypF-N aggregates aged for 24 h. The response was measured in external standard solution (left), in the absence of calcium (middle), and in the presence of 50 \textmu M cadmium (right). b, representative calcium current-voltage relationships measured by the patch clamp technique in the absence of protein (straight line) and after incubation for 24 h with protein aggregates aged for 48 h (dashed line). The data are indicative of the behavior of several observed cells.
Amyloid Aggregate-induced Ca\(^{2+}\) Increase in Granule Cells

GluR2 AMPA-Rs by co-localization experiments carried out by double labeling confocal microscopy, as reported under “Experimental Procedures” (Fig. 4). As a control, we also performed the same co-localization experiment with the NADPH oxidase subunit gp91 phox, a plasma membrane protein found in many cell systems, including those in the central nervous system (30). In fact, there is no evidence that gp91 phox interacts directly with HypF-N or other amyloid proteins.

The red channel in Fig. 4 shows the distributions of AMPA-Rs (Fig. 4a), NMDA-Rs (b), and gp91 phox (c). The green channel in Fig. 4 shows the localization of the HypF-N aggregates in the three different experiments. When images were merged, a number of yellow areas were noted, representing the co-localization of the membrane-bound HypF-N aggregates with the glutamatergic receptors and the gp91 phox recognized by their specific antibodies. The scatter plots of fluorescence signals over the highlighted areas are shown on the right of Fig. 4. The Pearson’s correlation coefficient, the overlap coefficient according to Manders, and the co-localization rate were examined; these different algorithms gave similar results. In particular, the analysis over three different experiments yielded a significant degree of co-localization of AMPA-Rs and NMDA-Rs with membrane-bound HypF-N aggregates (78 and 50%, respectively). It is worth noting that a random distribution of red and green spots in the same conditions corresponds to a co-localization of 10%. In addition, we found a degree of co-localization of 25% of gp91 phox with HypF-N aggregates. Overall, these results indicate a significant difference between the co-localization patterns of HypF-N aggregates with glutamatergic receptors compared with gp91 phox. These data are consistent with the hypothesis of a privileged interaction of HypF-N aggregates with NMDA-Rs and AMPA-Rs located on the plasma membrane of the neuron.

To gain more insight into the putative mechanism of NMDA-R or AMPA-R endocytosis, we treated the neurons with HypF-N prefibrillar aggregates for a longer incubation time (1 h). The cells were fixed, counterstained, and immunostained for AMPA-Rs or NMDA-Rs, as described under “Experimental Procedures.” Compared with untreated cells, we did not find any significant fluorescence decrease in cell surface-associated NMDA-Rs (Fig. 5, a and b); in contrast, a significant removal of the surface AMPA-Rs was observed (Fig. 5, c and d). The quantitative analysis showed that HypF-N prefibrillar aggregates reduced AMPA-R staining by ~40%, suggesting receptor internalization (Fig. 5e). A similar result was obtained by Hsieh et al. (23) in the presence of A\(\beta\) and was attributed to endocytosis of synaptic receptors.

**Long Term Effect on Membrane Permeability**—To test the effects induced by protein aggregates on membrane permeability at longer times of exposure, granule cells were incubated for 24 h in the presence of either native HypF-N or pre-formed HypF-N aggregates obtained at different aggregation times; measurements in the presence of memantine and CNQX were also performed (Table 2). We found that at the concentration of 0.2 \(\mu M\), early protein aggregates aged for 24 or 48 h induced a fluorescence increase; the percentage of cells exhibiting such an effect was higher in the case of 24-h aged aggregates. Although memantine did not alter significantly the long term cell response, CNQX drastically reduced the fluorescence increase from 53 to 14%. Cell fluorescence was unchanged in the presence of both the monomeric native protein and mature fibrils. Typical fluorescence images obtained before and after cell exposure to the protein aggregates are shown in Fig. 6, a and b. Under the same conditions, a 2.0 \(\mu M\) concentration of 12, 24, or 48 h-aged aggregates caused cell death (Fig. 6c), whereas the protein in the native state or aggregated into mature fibrils had no effect at this concentration. Cell death was found even when the time of cell exposure to early protein aggregates was reduced to 12 h (data not shown).

It is interesting to compare HypF-N aggregate cytotoxicity with that of a less hydrophobic protein. To this purpose, a set of experiments was performed in the presence of AcP, a protein with significant sequence and structural homology with HypF-N (31). In the presence of TFE, AcP forms in a few hours mature amyloid fibrils after several days of incubation (32). At variance with the behavior observed in the presence of HypF-N,
granule cells exposed to 2.0 μM AcP aggregates obtained at different aggregation times were still alive after 24 h. However, a significant Ca\(^{2+}\) increase was induced by AcP aggregates aged for 12, 24, and 48 h; in particular, in the latter case, the fraction of cells displaying increased fluorescence was 85% (Table 3 and Fig. 6d), whereas it was reduced to nearly 60% in the presence of 12- and 24-h aged aggregates. The maximum percentage of fluorescence increase (121 ± 5) was found upon cell exposure to 12-h aged aggregates (Table 3). As observed for HypF-N, neither soluble monomeric AcP nor its fibrillar polymers obtained upon protein aging for 7 days in the presence of TFE caused any increase of Ca\(^{2+}\) concentration. The experiments in the presence of AcP and HypF-N gave similar results, although at different protein concentrations, possibly because of the lower hydrophobicity of AcP with respect to HypF-N.

To test whether aggregate internalization was somehow involved in the cytotoxic effects of HypF-N early aggregates, at least the long term ones, we imaged cells exposed to Texas Red-labeled aggregates using confocal fluorescence microscopy. Fig. 7 shows a sequence of optical sections through a cell at different depths. In the imaged area, the absence of fluorescence spots in the lower (Fig. 7, left) and upper (Fig. 7, right) sections and their presence only in the middle section indicates that aggregates are really located inside the cell body, confirming the ability of these assemblies to translocate across the cell membrane after their adsorption to the cell surface, as shown previously for HypF-N (13) and other protein (33, 34) aggregates. We have not investigated whether aggregate internalization involved the endocytic pathway or it resulted merely from passive translocation of the aggregates through the phospholipid bilayer. However, the finding of a reduced presence of AMPA-Rs in exposed cells compared with control cells (see above) and the data previously reported on AMPA-Rs receptor internalization by Aβ (23) support the idea that the aggregates can be internalized by endocytosis together with the glutamatergic receptors with which they interact.

**DISCUSSION**

Interaction with and disassembly of the cell membrane resulting in Ca\(^{2+}\) deregulation have been proposed as basic shared mechanisms of cytotoxicity of amyloid aggregates both in cell cultures (13, 35) and in tissue in a number of amyloid diseases (36). In this study we have investigated the interaction of rat cerebellar granule cells with HypF-N, a prokaryotic protein domain not associated with any amyloid disease, in its native form or at different stages of aggregation. Our aim was to provide insights into the specific pathways involved in the disruption of Ca\(^{2+}\) homeostasis, one of the biochemical modifications underlying amyloid cytotoxicity. We used a protein not associated with amyloid disease to further support the idea that, at least in most cases, amyloids share general mechanisms of cytotoxicity even for what the features of their interaction with cell membranes are concerned (4, 14, 36).

We found that early HypF-N aggregates cause an increase of the cytosolic Ca\(^{2+}\) in granule cells, whereas comparable amounts of their monomeric and fibrillar forms did not induce detectable changes; the same behavior was observed with AcP, a structurally related mammalian protein not associated with amyloid diseases. These results agree with previous finding showing that oligomeric aggregates, but not mature fibrils, of HypF-N cause membrane permeabilization in lipid bilayers and cytotoxicity in cultured NIH-3T3 and PC12 cells (7, 13, 20, 21). The increase of cytosolic Ca\(^{2+}\) concentration was the result of the addition to the cell external medium of prefibrillar HypF-N aggregates arising from protein incubation in 30% TFE for 24 or 48 h.

Fluorescence measurements indicated that the increase of intracellular Ca\(^{2+}\) in the exposed cells was associated with two
different phenomena as follows: a fast (in the time scale of a hundred of seconds) and transient increase, and a long term (24 h) sustained increase. In the fast response, the lack of fluorescence increase upon Ca\textsuperscript{2+} removal from the cell external medium indicates that the oligomer-induced Ca\textsuperscript{2+} rise results from an influx from the outside rather than from a release from the intracellular stores. The Ca\textsuperscript{2+} influx into neurons could occur either following nonspecific membrane permeabilization and/or through other specific pathways, such as those involving voltage-gated and/or glutamate-activated calcium channels. The substantial invariance of the Ca\textsuperscript{2+} signals in the presence of 50 \mu M cadmium, a specific blocker of voltage-gated calcium channels, allowed us to exclude that, in our model, these channels are involved in the Ca\textsuperscript{2+} influx. This result agrees with our finding that the current-voltage features of the voltage-gated Ca\textsuperscript{2+} channels are not affected by protein aggregates (Fig. 2b). It has previously been reported that voltage-gated Ca\textsuperscript{2+} channels are involved in the Ca\textsuperscript{2+} influx in human SH-SY5Y cells exposed to transthyrethin amyloid oligomers (35). However, that study was carried out on a different cell line; in addition, it did not show any direct association between transthyrethin oligomers and voltage-gated Ca\textsuperscript{2+} channels; hence, the effect described could be attributed to some molecular features of the cell model used and/or to indirect modifications of channel functionality following aggregate interaction with the cell membrane.

In our investigation, we found a considerable reduction (about 50% of its maximum value) of the fast fluorescence rise in granule cells exposed to 10 \mu M memantine (Fig. 3a). The latter is a low affinity uncompetitive antagonist of the glutamatergic NMDA-Rs currently prescribed as a memory-preserving drug for patients with Alzheimer disease. We thus propose that amyloid oligomers exert an immediate action on the cell membrane permeability by interacting with NMDA-Rs; the results of the co-localization experiments (Fig. 4) agree with such idea and with recently reported findings indicating a protective effect of memantine against Aβ oligomer toxicity to rat hippocampal neuronal cultures (37). The co-localization experiments (Fig. 4) showed that AMPA-Rs are also involved in the fast response. In fact, AMPA-Rs activation is required to remove the Mg\textsuperscript{2+} block of NMDA-Rs.

In the absence of HypF-N aggregates, memantine blocks completely the NMDA-activated Ca\textsuperscript{2+} currents in granule cells

| Protein aggregation time | % fluorescence increase | % of cells with fluorescence increase |
|--------------------------|-------------------------|--------------------------------------|
| 0                        | 0                       | 0                                    |
| 24 h                     | 53 ± 3                  | 69 ± 8                               |
| 24 h + 10 \mu M memantine| 51 ± 3                  | 71 ± 8                               |
| 24 h + 10 \mu M CNQX     | 14 ± 3                  | 69 ± 8                               |
| 48 h                     | 60 ± 6                  | 50 ± 7                               |
| 8 days                   | 0                       | 0                                    |

The Ca\textsuperscript{2+} influx into neurons could occur either following nonspecific membrane permeabilization and/or through other specific pathways, such as those involving voltage-gated and/or glutamate-activated calcium channels. The substantial invariance of the Ca\textsuperscript{2+} signals in the presence of 50 \mu M cadmium, a specific blocker of voltage-gated calcium channels, allowed us to exclude that, in our model, these channels are involved in the Ca\textsuperscript{2+} influx. This result agrees with our finding that the current-voltage features of the voltage-gated Ca\textsuperscript{2+} channels are not affected by protein aggregates (Fig. 2b). It has previously been reported that voltage-gated Ca\textsuperscript{2+} channels are involved in the Ca\textsuperscript{2+} influx in human SH-SY5Y cells exposed to transthyrethin amyloid oligomers (35). However, that study was carried out on a different cell line; in addition, it did not show any direct association between transthyrethin oligomers and voltage-gated Ca\textsuperscript{2+} channels; hence, the effect described could be attributed to some molecular features of the cell model used and/or to indirect modifications of channel functionality following aggregate interaction with the cell membrane.

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Amyloid Aggregate-induced Ca\(^{2+}\) Increase in Granule Cells

![Image](https://example.com/image)

**FIGURE 6. Long term effect of protein aggregates on granule cells.** Fluorescence images of granules loaded with Oregon Green are shown as follows. a, in control conditions; b, after incubation for 1 day with 0.2 \(\mu M\) HypF-N aged for 48 h; c, after incubation for 1 day with 2 \(\mu M\) HypF-N aged for 48 h; d, after incubation for 1 day with 2 \(\mu M\) AcP aged for 48 h. The scale bar is the same for all images.

### TABLE 3

**Long term response of granule cells to AcP aggregates**

Fluorescence increase (corresponding to Ca\(^{2+}\) influx) was recorded after 24 h of cell incubation in the presence of 2.0 \(\mu M\) AcP at different aggregation times. The values reported are the average over the number \(n\) of cells observed. The percentage of cells exhibiting the fluorescence increase is also reported.

| Protein aggregation time | % fluorescence increase | % of cells with fluorescence increase | \(n\) |
|--------------------------|------------------------|--------------------------------------|------|
| 0                        | 0                      | 0                                    | 50   |
| 12 h                     | 121 \(\pm\) 5           | 59 \(\pm\) 8                         | 170  |
| 24 h                     | 82 \(\pm\) 5            | 58 \(\pm\) 8                         | 120  |
| 48 h                     | 92 \(\pm\) 5            | 85 \(\pm\) 9                         | 130  |
| 7 days                   | 0                      | 0                                    | 40   |

at the resting potential,\(^3\) whereas a residual Ca\(^{2+}\) influx is observed, under these conditions, in cells exposed to HypF-N aggregates (Fig. 3a). The latter effect could be due to a modest membrane depolarization, known to be able to remove the memantine block (38). Such depolarization could result from the interaction of the aggregates with the AMPA-Rs, as supported by the co-localization experiments (Fig. 4). The complete suppression of the fast response by the AMPA-R antagonist CNQX supports this hypothesis.

It has recently been reported that, in cultured rat hippocampal neurons, oligomeric A\(\beta\) induces a significant decrease of dynamin 1, a protein that plays an important role in synaptic function (22). The first step of this process results from an NMDA-R-mediated Ca\(^{2+}\) influx resulting in A\(\beta\)-induced calpain activation and dynamin 1 degradation, excluding any depolarization mediated by Na\(^+\) channels. The A\(\beta\)-induced Ca\(^{2+}\) rise was blocked by the NMDA-R antagonist MK801. It has also been reported recently that A\(\beta\) oligomers are able to induce Ca\(^{2+}\) rise and oxidative stress in rat hippocampal neuronal cultures by NMDA-R activation and that such an effect is removed by the NMDA-R inhibitor memantine (37). Our data suggest that a comparable effect through a similar mechanism could be elicited by pre-fibrillar aggregates of the non-pathological protein HypF-N. This conclusion is supported by the inhibitory effect of memantine and CNQX on HypF-N aggregate-induced Ca\(^{2+}\) rise and by our co-localization experiments showing a significant correlation between the cell surface distribution of HypF-N aggregates and that of both glutamate receptors.

Overall, our data suggest that amyloid aggregates of proteins with different amino acid sequences and three-dimensional structures share a common mechanism of early Ca\(^{2+}\) homeostasis deregulation, including some physical and/or functional interference with glutamatergic ion channels.

For the long term responses, we found a sustained Ca\(^{2+}\) increase in granule cells exposed to HypF-N amyloid aggregates for 1 day. The long term increase of the fluorescence intensity in granule cells exposed to the aggregates was not affected by the presence of memantine; by contrast, the fluorescence increase was heavily reduced, but not suppressed, in the presence of CNQX (Table 2). Comparing the data reported in Table 2, about one-third of the fluorescence increase is not because of a sustained activation of the glutamatergic receptors. We have not assessed whether, under these conditions, the NMDA-R distribution is reduced, possibly following endocytosis, although the data relative to a 1-h exposure seem to rule out this possibility. Therefore, it can be proposed that the long term response results, at least in part, from a receptor-independent nonspecific increase of membrane permeability rather than from a sustained glutamatergic receptor activation. This view supports our recent data showing that early oligomers of HypF-N interact with lipid monolayers, destabilize supported lipid bilayers (21), and induce Ca\(^{2+}\) leakage from synthetic lipid vesicles (20).

Recent data show the following: (i) rat hippocampal neurons treated with natural A\(\beta\) oligomers after several days displayed significant dendritic spine loss mediated by activation of a signaling cascade involving NMDA-R blockade and reduction of Ca\(^{2+}\) influx (39); and (ii) 1 h of treatment with the same A\(\beta\) oligomers induced reduction of the surface expression of NMDA-Rs following enhanced receptor endocytosis (40). It

\(^3\)C. Marchetti, personal communication.
Amyloid Aggregate-induced Ca\(^{2+}\) Increase in Granule Cells

could well be that the very initial NMDA-R activation by A\(\beta\) oligomers is followed by receptor endocytosis; these effects could be followed, at later times, by more severe morphological and functional changes such as loss of dendritic spines and synaptic dysfunction with long term potentiation and long term depression alterations. Structural and synaptic abnormalities following AMPA-R inactivation and glutamatergic transmission reduction via receptor endocytosis have also been reported in amyloid precursor protein-overexpressing pyramidal neurons (23).

We found a significant reduction of AMPA-R, but not NMDA-R, immunoreactivity at the surface of rat cerebellar granule cells exposed for 1 h to the HypF-N prefibrillar aggregates. These data suggest AMPA-R internalization at this time of exposure. The apparent lack of NMDA-R reduction could result from a differing timing of NMDA-R internalization; alternatively, a different type of aggregate-receptor interaction could be proposed for AMPA-Rs and NMDA-Rs. Actually AMPA-Rs and NMDA-Rs are co-localized with HypF-N aggregates at a different extent (78 and 50%, respectively).

Recently published data indicate that cells expressing NMDA-type glutamate receptors with NR1/NR2A subunit composition are more vulnerable to A\(\beta\) aggregates (42), confirming the involvement of these receptors in amyloid aggregate cytotoxicity. Neuronal activity and neuronal insults have also been reported to dynamically remodel AMPA-Rs (24, 43). Moreover, in neurons expressing Ca\(^{2+}\)-permeable AMPA-Rs, A\(\beta\) causes dendritic dystrophy through perturbations of the Ca\(^{2+}\) homeostasis (44), whereas A\(\beta\) toxicity associated with increased intracellular Ca\(^{2+}\) exploits signaling pathways of long time depression to drive synaptic AMPA-R endocytosis (23). Our results support AMPA-R endocytosis even following exposure to HypF-N prefibrillar aggregates suggesting a direct interaction of the cross-\(\beta\) fold at least with such receptor. Finally, AMPA-Rs lacking the GluR2 subunit, and hence permeable to divalent cations, including Ca\(^{2+}\), have been found in neurodegenerative diseases, including Alzheimer disease (41). Although granule cells do not seem to possess AMPA-Rs lacking GluR2 subunits, it must be assessed whether their subunit composition is modified upon cell exposure to amyloid aggregates. The decrease of the Ca\(^{2+}\) fluorescence associated with glutamatergic receptors, observed in our cells exposed to HypF-N aggregates, supports the latter possibility.

Our data agree with the possibility that glutamatergic ligand-gated calcium channels are preferential, yet nonspecific, sites of the interaction of pre-fibrillar aggregates of different peptides and proteins with the cell membrane; they also support the idea that such receptors, when present, are involved in the early events following aggregate interaction with the cell membrane, leading to Ca\(^{2+}\) increase, endoplasmic reticulum stress, and mitochondria dysfunction eventually culminating with cell death. Finally, they provide support to the hypothesis that such biochemical modifications raised in the exposed cells by pre-fibrillar amyloid aggregates, as well as the ability of the latter to interact with cell membrane sites are generic, being apparently independent of the type of aggregated protein/peptide but, possibly, associated with a shared basic fold in the aggregates.

Further research is needed to better describe the molecular features of the receptor-aggregate interaction. In particular, a study is presently under way to assess the following: (i) whether amyloid aggregates bind directly to the receptors or to membrane subdomains in close proximity to the receptors themselves; (ii) whether such an interaction results in any modification of the receptor subunit composition and/or it favors receptor endocytosis and the timing of the latter; (iii) whether aggregate internalization following interaction with the cell membrane is passive or is mediated by the endocytotic pathway; and (iv) the dependence of such responses on membrane lipid composition mainly for what the cholesterol content is concerned.

Acknowledgments—We thank Andrea Gallo for help in part of the fluorescence experiments, Marco Raimondo for technical help, and Dr. Carla Marchetti (Institute of Biophysics, CNR, Genoa, Italy) for helpful discussions.

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Amyloid Aggregate-induced Ca\(^{2+}\) Increase in Granule Cells

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