Early and Late Cytotoxic Effects of External Application of the Alzheimer's Aβ Result from the Initial Formation and Function of Aβ Ion Channels†

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ABSTRACT: Extracellular application of the Alzheimer's beta-amyloid (Aβ) peptide evokes a series of cellular responses that leads to the death of cells by apoptosis. Some responses to freshly prepared Aβ occur immediately, including changes in intracellular calcium concentration and changes in membrane permeability and phosphatidylserine asymmetry. We show here that the cytotoxic action of externally applied Aβ, such as caspase activation and apoptotic loss of cell viability, occurs and persists even several days after Aβ is removed from the medium. We find that the mechanism for this persistent cytotoxic action of extracellular Aβ is based on the sustained activity of active Aβ ion channels that remain incorporated in the cell membrane. To confirm this assessment, we blocked the late cytotoxic action of Aβ with the classically known Aβ channel blockers zinc and tromethamine. To further validate this conclusion, we developed a specific peptide segment from the sequence forming the mouth of the Aβ channel to block Aβ Ca^{2+} channels acutely and to block late Aβ effects on caspase activation and apoptosis. This is the first report of a specific Aβ channel blocker compound, NA4, which efficaciously and potently blocks the most known cellular responses to Aβ.

Beta-amyloid peptides (Aβs) have been implicated as the primary cause of cellular damage and degeneration. However, a consensus on the primary mechanisms that cause neuronal degeneration and death in Alzheimer's disease brain remains elusive. Numerous reports have provided evidence that cellular damage and degeneration may be caused by a toxic interaction between Aβs and nerve cells. The interaction between Aβs and the cell surface membrane is followed by activation of an intracellular signaling cascade that leads to the death of cells by apoptosis (*1*). Direct measurements of intracellular calcium show that cell exposure to Aβ disrupts calcium homeostasis (*2*) by generating an acute $[Ca^{2+}]_i$ increase (*3*, *4*). This acute calcium increase may be responsible for the initiation of pre-apoptotic events leading to cell degeneration (*5*). We have shown that the Alzheimer's disease peptide Aβ40 displays affinity for acidic phospholipid membranes (*6*, *7*) and spontaneously inserts into lipid bilayers and neuronal membranes to form high conductance zinc/tromethamine-sensitive calcium channels (*8*−*10*). This finding led to the Aβ ion channel hypothesis, which proposes that the intracellular calcium increased observed after cell exposure to Aβ is a consequence of the formation Aβ calcium channels (*9*−*11*).

In neuronal cells, it has been shown that Aβ evokes a variety of acute elevations in intracellular calcium that occur with time courses that are Aβ-concentration dependent (*3*). Short-term effects of Aβ40 and Aβ42 have also been shown in fibroblasts and in endothelial cells (*4*, *15*). Morphological and intracellular calcium changes in these cells are apparent within 10−15 min after treatment with freshly prepared Aβ solutions. These short-term effects of Aβ had been linked to the formation of nascent Aβ calcium channels (*3*, *4*, *15*) using Aβ ion channels blockers such as zinc and tromethamine (*8*, *9*).

We have noticed in preliminary experiments that the activation of apoptotic mechanisms produced by Aβ exposure continues up to the death of the cells despite early removal of Aβ from the culture media. To test for the involvement of initial Aβ channel function in the generation of this long-term Aβ channel effects, we have studied the effect of short time pulses of Aβ exposure for the generation of late pro-apoptotic processes and cell death. To test whether Aβ channels are responsible for these late cytotoxic effects in this continued action, we used Aβ channels blockers such as zinc, tromethamine, and a specific heptameric Aβ channel blocker peptide. We found that the presence of Aβ ion channel blockers in the extracellular medium after the Aβ pulse effectively prevents late pro-apoptotic processes from developing after Aβ has been removed from the medium. We interpret these data to suggest that both the early and the late cytotoxic effects of Aβ are due to the initial formation of Aβ channels.

METHODS

Cell Cultures. Early Effects of Aβ. To study the short-term effects of Aβ on cells, we used the immortalized cell line PC12, derived from a transplantable rat pheochromocy-

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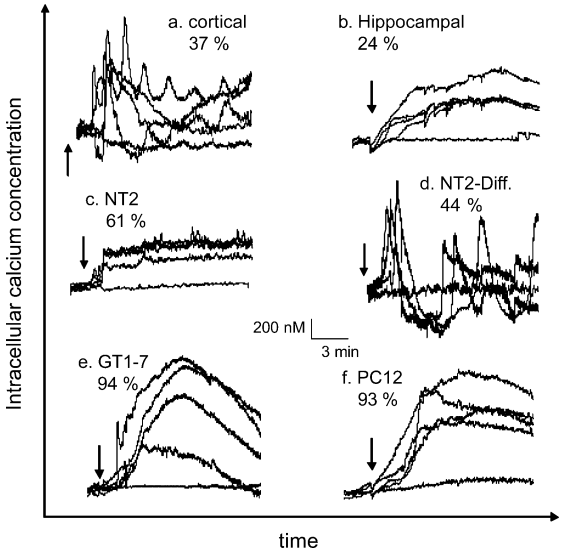

FIGURE 1: (a−f) External addition of Aβ induces acute changes in intracellular calcium concentration. Time course of $[Ca^{2+}]_i$ changes are observed after the addition of 20 μM Aβ42 to a variety of primary and permanent neuronal cells lines. The type of cell is indicated, and the arrows signal the moment of Aβ addition. The panels display only selected simultaneous records from five responding cells located at different positions of the optical field, and the numbers correspond to the percentage of cells that responded (n = 60−80 cells). As it is observed in the figure, the percentage of cells responding to Aβ, the amplitude and the time course of the $[Ca^{2+}]_i$ changes in each cell are highly diverse. However, for all types of cells responding, the calcium change occurs promptly within seconds after the addition of Aβ42.

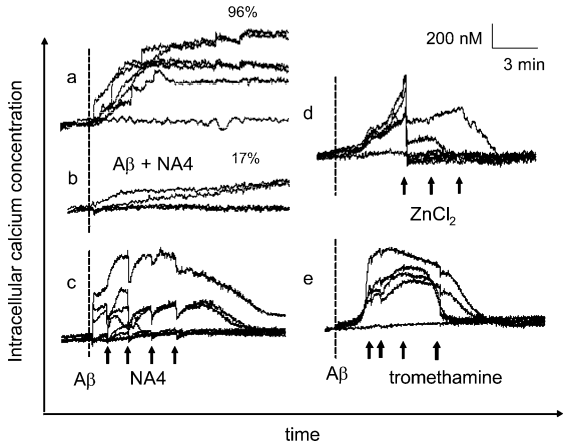

FIGURE 2: The acute calcium response to Aβ can be attenuated by Aβ ion channel blockers. (a) Freshly prepared Aβ was added to the growing chamber containing PC12 cells at the time indicated by the vertical dotted line. (b) The acute calcium response was recorded when 60 μg/mL of the specific Aβ channel blocker peptide NA4 was in the culture medium before the addition of 20 μM Aβ42. The numbers in panels a and b indicate the percentage of cells responding to the addition of 20 μM Aβ (n = 80 cells). (c, d, e) After the calcium response to Aβ was initiated small doses of the Aβ channel blockers NA4 (10 μg/mL), $ZnCl_2$ (5 μM), and tromethamine (10 mM) were consecutively added at the time indicated by the arrows. In all cases, the calcium responses were attenuated to control levels. Ca^{2+} calibrations were done with solutions containing known concentrations of free Ca^{2+}, 1 mM free Mg^{2+} (Calcium Calibration Buffer kit with Magnesium #2, Molecular Probes) and 5 μM FURA-2 penta-potassium salt (Molecular Probes) using InCyt IM2 software.

toma (ATCC), GT1−7, immortalized hypothalamic neurons (provided by Dr. M. Kawahara, NIH), differentiated and undifferentiated embryonal carcinoma NT2 cells (ATTC), and primary cultures of hyppocampal and cortical neurons from rat brains. Inmortalized cells were grown in the recommended ATCC media. Primary cultured cells were grown in neurobasal medium/B27 (GIFCO).

Protocol for Short-Time Pulses of Aβ. To study the late effect on cells observed after removal of Aβ, PC12 cells were cultured in D-MEM medium with Aβ42 (AnaSpec) for brief periods of up to 4 h. During these brief periods of time, we anticipated that Aβ might interact with the cell membrane and to form stable ion channels. Aβ was then removed from the culture medium, and cells were analyzed 24−72 h later for pro-apoptotic processes and survival.

Intracellular Free Calcium Measurements. Cells were plated on glass coverslips coated with collagen or poly-D-lysine/laminin and loaded with calcium-sensitive probe using media (BSS; in mM: 135 NaCl, 10 glucose, 5 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 10 NaHepes, pH 7.4) containing 2 μM Fura-2AM (Molecular Probes). After a loading period (approximately 30 min), cells were treated with Aβ42. The time course of changes in the emission from Fura-2AM was observed using an inverted epi-fluorescence/phase contrast microscope equipped with a low-light level integrating CCD camera + microphotometer assembly (InCyt I/P-2 TM Imaging & Photometry System, Intracellular Imaging INC).

Cell Viability Assays. Cell viability was measured using a colorimetric XTT assay (Cell Proliferation kit II from Roche Molecular Biochemicals). The release of lactate dehydrogenase (LDH) from the cytosol into the media was used to report on the integrity of the cell membrane. The Cytotoxicity Detection kit (LDH, Roche) was used to measure the lactate dehydrogenase (LDH) activity. Results were analyzed by analysis of variance (Anova; Origin). $p < 0.05$ was regarded as significant.

Apoptosis Assays. The progression of apoptosis by initiation of the caspase activation cascade was quantified by the CellProbe HT caspase 3/7 whole cell assay kit (Beckman Coulter). To determine surface membrane phosphatidylserine (PS) and membrane permeability, we used the Vybrant Apoptosis assay (Molecular Probe) and an EPICs XL-MCL cell analyzer. Suspensions of cells were stained with alexa Fluor 488-annexin 5 and propidium iodide (PI), followed by flow cytometric analysis. Results were analyzed by analysis of variance (Anova; Origin). $p < 0.05$ was regarded as significant.

AO Ion Channel Blockers. To block Aβ ion channels we used $ZnCl_2$ (Sigma), tromethamine (Sigma-tris) and the heptamer peptide NA4 (native sequence of $A\beta_{8-14}$ (SGYEVHH), (*13*). The peptide NA4 was synthesized using HBTU/DIEA activation of Fmoc amino acids. The purity of the peptide was confirmed by HPLC, and the sequence was confirmed by an automatic protein sequencer, Applied Biosystems model 476.

Flowcytometry. For flow cytometric analysis, cells were incubated in PBS with 0.3% BSA containing 1 and 10 μM Aβ42-FITC (AnaSpec) for 30 min and analyzed by an EPICs XL-MCL cell analyzer.

RESULTS

Short-Term Effects of Aβ on Cells. Intracellular Calcium Increase. Cells plated on glass coverslips were analyzed with a calcium imaging system, which enabled us to accurately

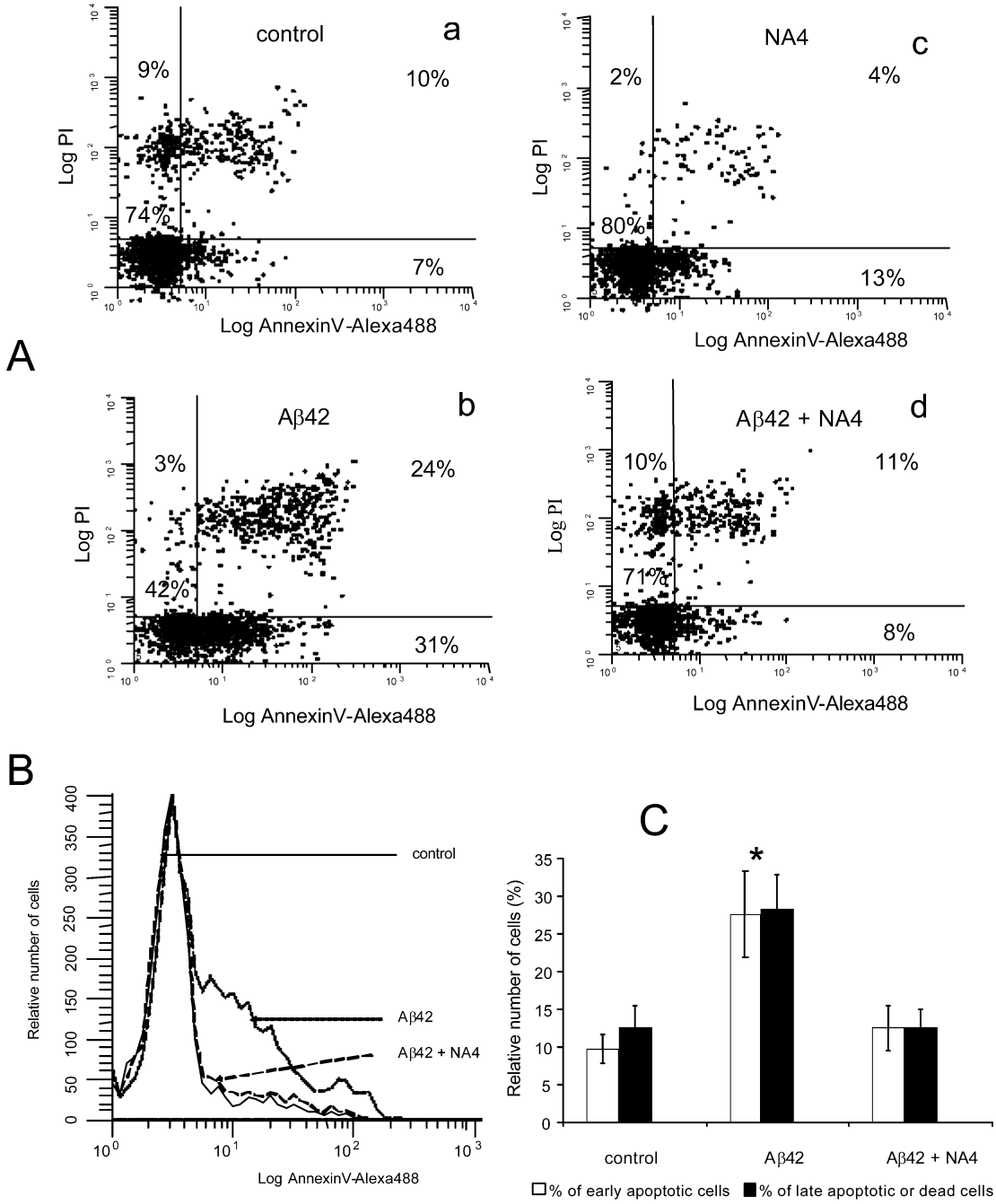

FIGURE 3: External addition of Aβ42 induces acute changes in membrane permeability and phosphatidylserine asymmetry. (A) Flow cytometric analysis of PC12 cells stained with alexa Fluor 488-annexin 5 and PI performed 2 h after cells were exposed to 20 μM freshly prepared Aβ42. The fluorescence signals from control conditions (a) redistribute toward the upper and right quadrants (b) indicating a significant increase in phosphatidylserine and propidium iodide positive cells. The numbers indicate the percentage of cells distribution per quadrant. The Aβ channel blocker peptide NA4 (60 μg/mL) is totally innocuous to PC12 cells (c) and totally prevented the redistribution of the fluorescence signal caused by Aβ42 (d). (B) Histograms of the alexa Fluor 488-annexin 5 signals distribution before (control) and 2 h after the addition of either 20 μM freshly prepared Aβ42, or 20 μM freshly prepared Aβ42 plus 60 μg/mL of the Aβ channel blocker peptide NA4. (C) Plots of the average distribution of intact cells, from four flow cytometric experiments, sorted in terms of the percentage of early (white bar), late apoptotic and dead cells (black bars) before (control) and 2 h after the addition of either 20 μM freshly prepared Aβ42, or 20 μM freshly prepared Aβ42 plus 60 μg/mL of the Aβ channel blocker peptide NA4. Significant blockage of induction of apoptosis was observed when the Aβ channel blocker peptide NA4 was in the medium (*$p < 0.00019$, for Aβ42 + NA4 compared to Aβ42, and $p = 1.0$ for Aβ42 + NA4 compared to control).

measure calcium changes from as many as 100 cells simultaneously. Figure 1 shows a gallery of typical records of the time courses of $[Ca^{2+}]_i$ changes observed after the addition of freshly prepared Aβ. The panels display only selected simultaneous records from five responding cells located at different positions of the optical field. Cells were considered as "responding" when the increase in the intracellular calcium induced by Aβ was higher than 25 nM. As indicated in the figure, the percentage of cells responding to the addition of an equal amount of Aβ varies depending on the type of cell. The amplitude and the time course of the $[Ca^{2+}]_i$ changes in each cell are highly diverse. However, for all types of cells responding, the calcium change occurs promptly within seconds after the addition of Aβ. The

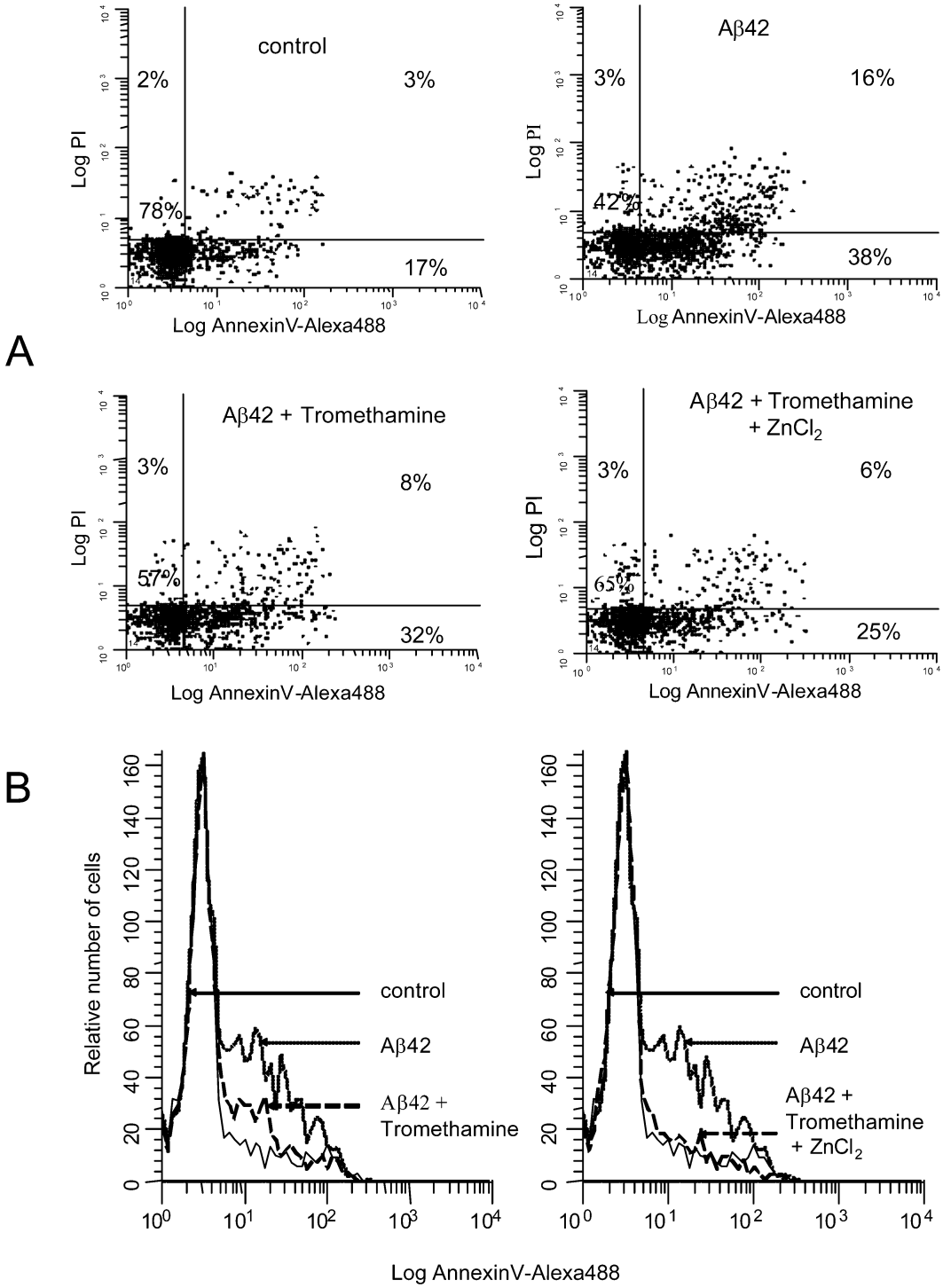

FIGURE 4: The acute changes in membrane permeability and phosphatidylserine asymmetry induced by Aβ can be prevented by Aβ ion channel blockers. (A) Flow cytometric analysis of PC12 cells stained with alexa Fluor 488-annexin 5 and PI performed 2 h after cells were exposed to 20 μM freshly prepared Aβ42. The redistribution of the fluorescence signals from control (a) induced by Aβ42 (b), is prevented by 40 mM tromethamine (c) and most effectively by the combination of 40 mM tromethamine and 5 μM $ZnCl_2$ (panel d). (B) Histograms of the alexa Fluor 488-annexin 5 signals distribution before (control) and 2 h after the addition of either 20 μM freshly prepared Aβ42 alone, or Aβ42 in combination with the Aβ channel blockers zinc (5 mM) and tromethamnine (40 mM). The additional peak in the fluorescence signals induced by Aβ42 is absent when the experiment is performed in the presence of the Aβ channel blockers zinc and tromethamine.

variation in the calcium responses in individual cells, from 50 to 700 nM, corresponds to what one should expect to be the response to the formation of a random number of multiconductance Aβ calcium channels. As shown in Figure 2 (panels a and b), the number of responding cells, and the magnitude of the $[Ca^{2+}]_i$ changes observed after the addition of freshly prepared Aβ to PC12 cells, were considerably reduced when the culture medium contained the specific Aβ channel blocker peptide NA4. The percentage of cells responding to the addition of 20 μM Aβ, was reduced from 96 to 17% when the medium contained 20 μg/mL of the NA4 peptide. Similarly, Figure 2 (panels c, d, and e) shows that addition of NA4 (10 μg/mL), zinc ions (5 μM) and tromethamine (10 mM), while the $[Ca^{2+}]_i$ was changing, detained $[Ca^{2+}]_i$ increase and produced a noticeably reduction of the $[Ca^{2+}]_i$ to normal concentration levels.

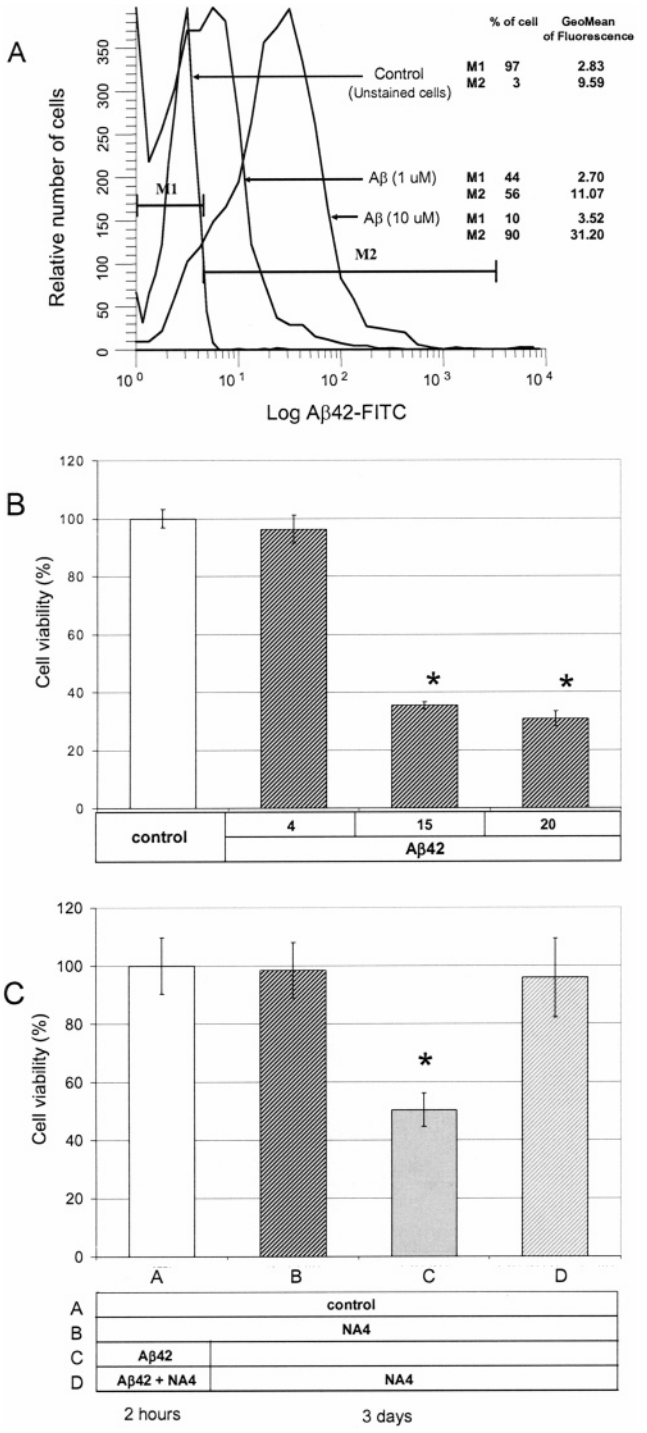

FIGURE 5: Late cytotoxicity after early removal of Aβ. (A) Flow cytometric analysis of PC12 cells after a short exposure to Aβ. Cells were exposed to Aβ42-FITC for 30 min, washed three times, and analyzed 1 h later. The histograms show a 19× and a 30× increase in the % of cells exposed to 1 and 10 μM Aβ42, respectively, indicating that after the washing process Aβ remains bound to the cells. (B) PC12 cells were exposed to 4, 15, and 20 μM of Aβ42 for 3 h. The media was then replaced with an Aβ42-free media and cell viability was evaluated 48 h later ($*p < 1.93 \times 10^{-8}$ for Aβ42 15 μM compared to control, and $*p < 0.017$ for Aβ42 15 μM compared to 20 μM). (C) PC12 cells were pretreated for 2 h with a medium containing 15 μM Aβ42. The medium was then replaced with an Aβ42-free media, with and without 16 μg/mL of the Aβ channel blocker peptide NA4. The Aβ42 pretreatment caused a considerable reduction in the percentage of viable cells ($*p < 7 \times 10^{-6}$, for A compared to C and for C compared to D). The presence of NA4 in the medium had no apparent effect on cell survival.

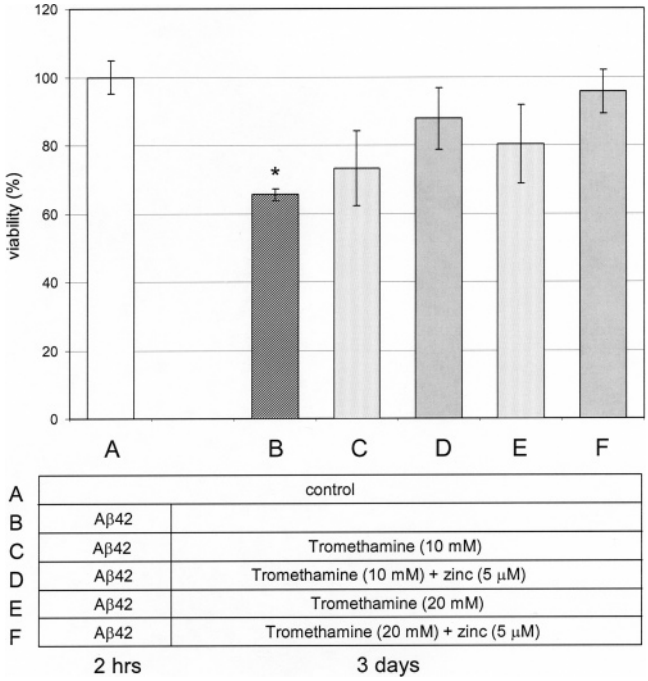

FIGURE 6: Lingering cytotoxicity after removal of Aβ is prevented with Aβ channel blockers. PC12 cells were pretreated for 2 h with a media containing 15 μM Aβ42. The media was then replaced with an Aβ42-free media, and cell viability was evaluated 72 h later. After this period of time, the percentage of viable cells was reduced to 62% of the control value ($*p < 3.93 \times 10^{-7}$ for A compared to B). However, this reduction was increasingly attenuated by complementing the Aβ42-free medium with the Aβ channel blockers tromethamine and zinc, at the concentrations indicated in the figure ($p = 0.26$ for F compared to A).

Membrane Permeability and Phosphatidylserine Asymmetry. PC12 cells were exposed to Aβ42 for a period of 2 h, washed twice in annexin 5 buffer, and then analyzed for changes in membrane permeability and asymmetry by flow cytometry. Propidium iodide (PI) permeability and increased extracellular phosphatidylserine (PS) are parameters that can be used to measure membrane permeability and membrane asymmetry, respectively. Damage of cells during mechanical harvesting for flow cytometry studies occurs and may cause moderate variation in the PI staining observed in the different conditions. However, the magnitude of this effect is low compared to the robust response of the cells to Aβ and the attenuation observed by the Aβ blockers. As shown in Figure 3A, flow cytometric analysis proves that Aβ causes PC12 cells to exhibit early proapoptotic processes. Two hours after exposure to Aβ42, the fluorescence signals from control conditions (panel a) redistribute toward the upper and right quadrants (panel b). Aβ42 significantly increased the percentage of PS positive cells, from 17 to 55%, as well as the percentage of PI positive cells, from 19 to 27%. When the cells are exposed to Aβ42 simultaneously with the Aβ channel blocker peptide NA4, the redistribution of the fluorescence signal caused by Aβ42 is totally blocked (panel d). As it is shown in panel c, 60 μg/mL of the Aβ channel blocker peptide NA4 is totally innocuous to PC12 cells. The histograms of the Alexa Fluor 488-annexin 5 signal distribution in Figure 3B show that, by comparison to the control, the Aβ-treated cells population shows an additional peak at higher intensity levels. This indicates that annexin 5 has detected higher amount of PS on the outer surface membrane

of these cells. The detection of this extra peak is suppressed when the experiment is performed in the presence of the Aβ channel blocker peptide NA4. The plots in Figure 3C are the average distribution of intact cells of four flow cytometric experiments. The plots show that by 2 h of exposure to Aβ42 the percentages of early (white bar) and late apoptotic plus dead cells (black bars) are already significantly increased ($*p < 0.00019$, for Aβ42 compared to control). In the presence of the Aβ channel blocker peptide NA4 the distribution of cells remains as in the control ($p = 1.0$ for Aβ42 + NA4 compared to control). Similar prevention of the redistribution of the fluorescence signals induced by Aβ42 is observed when the cells are exposed to Aβ42 simultaneously with zinc ions or tromethamine. The flow cytometric analysis in Figure 4A shows that the presence of these Aβ channel blockers maintained the distribution of the fluorescence signals at the same levels as that of the control. Figure 4B shows that the additional peak in the fluorescence signals observed at higher intensity levels from cells treated with Aβ42 is not observed when cells are treated with Aβ42 simultaneously with Aβ channel blockers. The combination of zinc ions and tromethamine is the most efficient in maintaining the distribution as in the control.

Long-Term Effects of Short-Term Exposure of Aβ on Cells. The results from the experiments described above suggest that cells need only a short exposure to Aβ to manifest responding signals. As shown in Figure 5A, a flow cytometric analysis performed 1 h after cells were exposed for one-half hour to Aβ-FITC confirms that Aβ remains on the surface of the cell membrane even after the cell culture medium is replaced by an Aβ-free medium. It is possible that the interaction of Aβ with the surface of the cell membrane can result in stable Aβ insertion in the membrane and the formation ion channels. Therefore, we anticipated that the damage to the cell induced by the action of the Aβ channels might continue despite the absence of Aβ in the media.

Cell Viability. PC12 cells were exposed to several concentrations of Aβ42 for 3 h. The media was then replaced with an Aβ42-free media, and cell viability was evaluated 48 h later. As shown in Figure 5B, 48 h after the removal of Aβ from the media, the percentage of viable cells showed a significant decrease ($p < 1.93 \times 10^{-8}$). Despite the absence of Aβ42 in the media, the magnitude of the reduction in the cell viability correlates with concentration of Aβ42 during the 3 h pretreatment ($p < 0.017$, 15 μM compared to 20 μM). The results shown in Figure 5C demonstrate that after the removal of Aβ, the addition of the specific channel blocker NA4 peptide significantly blocks the Aβ cytotoxicity ($p < 7 \times 10^{-6}$, for Aβ42 + NA4 compared to Aβ42). This result suggests that the late effect of early removal of Aβ42 is the result of the formation of NA4-sensitive channels.

To confirm this finding, similar experiments were performed using other long-known Aβ ion channel blockers such as zinc and tromethamine. PC12 cells were preincubated for 2 h in a 15 μM Aβ42-containing medium, followed by incubation for 72 h in an Aβ42-free medium containing various combinations of zinc and tromethamine. After that period of time, the percentage of viable cells was evaluated. The results displayed in Figure 6 shows that when an appropriate combination of the Aβ ion channel blockers was used (5 μM $ZnCl_2$ and 20 mM tromethamine) the cells were totally protected from the killing effect of 15 μM Aβ42 ($p = 0.26$ for A compared to F).

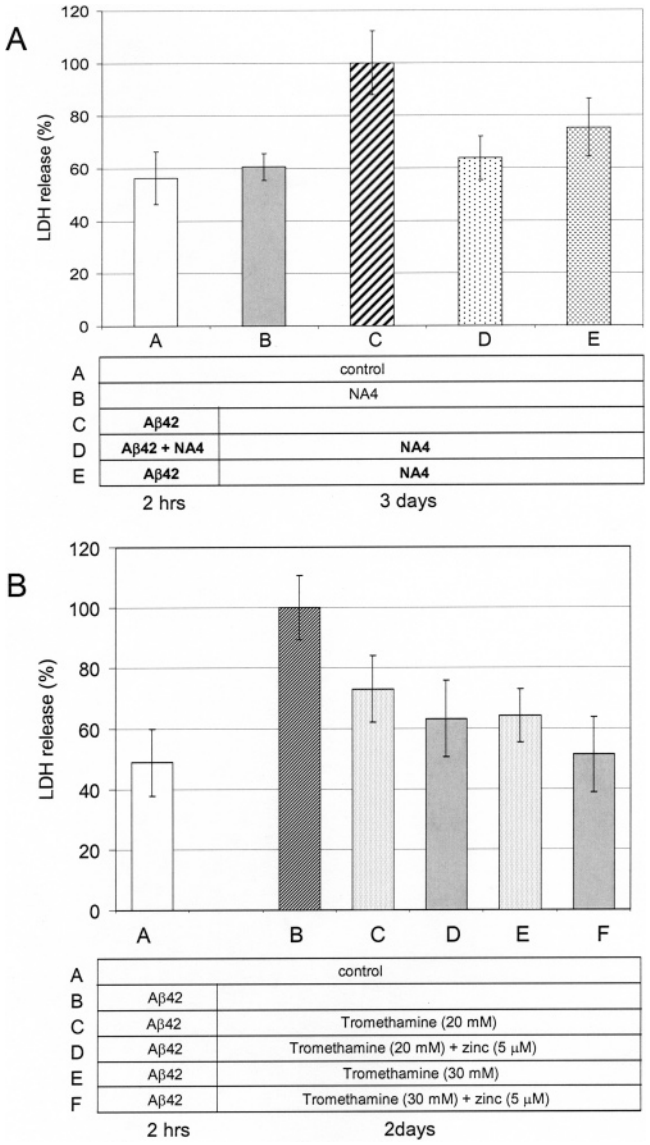

FIGURE 7: Lingering disruption of the cell membrane permeability after removal of Aβ is prevented by blocking the Aβ channels. (A) PC12 cells were pretreated for 2 h with a media containing 15 μM Aβ42. The media was then replaced with an Aβ42-free media and the amount of LDH release into the media was evaluated 3 days later. Significant blockage of the additional LDH release from the treated cells was observed when 16 μg/mL of the Aβ channel blocker peptide NA4 was in the medium ($*p < 0.03$ for D compared to C). The NA4 peptide is somewhat more efficient, although not significantly different ($p = 0.15$ for E compared to D), when it was present in the medium during the pretreatment with Aβ. (B) PC12 cells were pretreated for 2 h with a medium containing 15 μM Aβ42. The medium was then replaced with an Aβ42-free medium, and the amount of LDH released into the medium was evaluated 2 days later. The amount of LDH was plotted as percentage of the amount released by Aβ-treated cells. After this period of time, Aβ-treated cells had released twice LDH than untreated cells (control) ($*p < 3.4 \times 10^{-4}$ for A compared to B). However, this additional LDH release was attenuated by complementing the Aβ42-free medium with the Aβ channel blockers tromethamine and zinc, at the concentrations indicated in the figure ($p = 0.65$ for A compared to F).

Membrane Permeability. To test for the late effects on membrane permeability after short exposure to Aβ, we

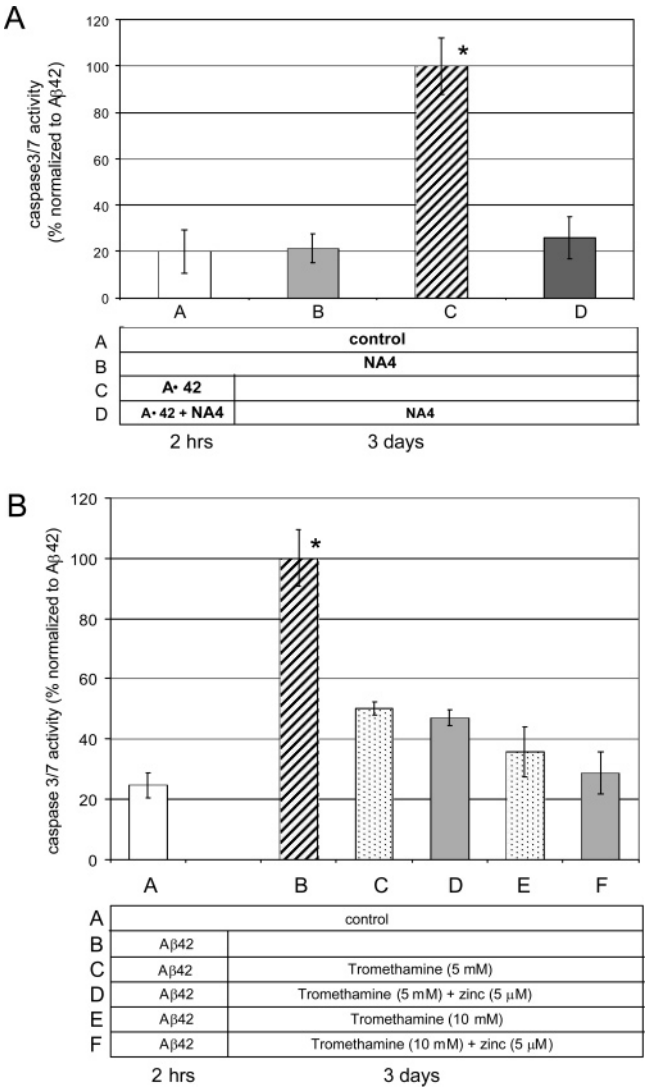

FIGURE 8: Late caspase activation after removal of Aβ is prevented by blocking the Aβ channels. (A) PC12 cells were pretreated for 2 h with a medium containing 9 μM Aβ42. The medium was then replaced with an Aβ42-free media and the level of caspase activation was evaluated 3 days after the pretreatment with Aβ. The caspase activity in the cells pretreated with Aβ was five times higher than the activity in the untreated control cells (*$p < 4.74 \times 10^{-5}$ for A compared to C). In the presence of the Aβ channel blocker NA4 in the medium, despite the pretreatment with Aβ, the activity of the caspases remained at the same level of the control cells ($p = 0.38$ for A compared to D). (B) PC12 cells were pretreated for 2 h with a media containing 20 μM Aβ42. The media was then replaced with an Aβ42-free media and the level of caspase activation was evaluated 3 days later. The Aβ42-free media was also complemented with combinations of the Aβ channel blockers tromethamine and zinc, at the concentrations indicated at the bottom of the plots. The caspase activity in Aβ-treated cells was almost five times higher than in untreated control cells (*$p < 6.48 \times 10^{-6}$ for A compared to B). Despite the pretreatment with Aβ, the complement of the media with the Aβ channel blockers tromethamine and zinc effectively maintained the caspase activity to the level of the untreated control cells ($p = 0.36$ for A compared to F).

measured lactate dehydrogenase (LDH) released from cells into the culture medium. In this experiment, we incubated PC12 cells in a medium containing Aβ42. After 2 h, the medium was replaced for an Aβ-free medium, and the LDH released from cells was measured 3 days later. As shown in Figure 7A, despite the absence of Aβ in the medium the cells pretreated with Aβ42 (15 μM) released almost double the amount of LDH into the medium than the untreated cells ($p < 0.01$ for A compared to C). However, this increased LDH release from the Aβ-treated cells was significantly blocked when the Aβ-free medium contained the specific Aβ channel blocker NA4 peptide (16 μg/mL) ($p < 0.003$ for C compared to D). The example illustrated in this figure shows that the blocking action of the NA4 peptide is somewhat more efficient, although not significantly different ($p = 0.15$ for E compared to D), when it was present in the medium during the pretreatment with Aβ. Experiments following a similar protocol were preformed this time adding various combinations of the Aβ ion channel blockers zinc and tromethamine to the Aβ-free incubation medium. Figure 7B shows that when an appropriate combination of the Aβ ion channel blockers is used (5 μM $ZnCl_2$ and 10 mM tromethamine) the cellular membrane integrity was totally protected from the effect of the pretreatment with 15 μM Aβ40 ($p < 3.4 \times 10^{-4}$ for A compared to B; $p = 0.65$ for A compared to F).

Caspase Activity. One of the intermediate processes that characterize the cell degeneration by apoptosis is a cascade of activation of downstream caspases. The caspases convey the apoptotic signal in a proteolytic cascade that leads to cell death. Aβ is known to induce cell death through a caspase-dependent apoptosis (*14*). We therefore tried to assess the lingering effects by measuring the activation of caspase 3/7 days after removal of Aβ. PC12 cells were incubated in a medium containing Aβ42. After 2 h, the medium was changed for an Aβ-free medium, and the activity of caspase 3/7 was measured 3 days later. As shown in Figure 8A, despite the absence of Aβ in the medium, the caspase activity in the cells pretreated with Aβ was five times higher than the activity in the untreated control cells ($p < 4.74 \times 10^{-5}$ for A compared to C). The figure also shows that the activity of the caspases remained at the same level of the control cells when the Aβ-free medium contained the specific Aβ channel blocker NA4 peptide ($p = 0.38$ for A compared to D). Similar experiments were performed using the Aβ ion channel blockers zinc and tromethamine. The results displayed in Figure 8B show that when the combination of 5 μM $ZnCl_2$ and 10 μM tromethamine was used, the caspase 3/7 activity was maintained at the same level as the control ($p = 0.36$ for A compared to F).

DISCUSSION

These data show that Aβ initiates both short-term and long-term effects on cytotoxicity. The long-term effects are established as soon as Aβ ion channels are formed on the cell membrane. These results are in agreement with the concept that interaction of Aβ with the surface membrane of the cell leads to the formation of permanent Aβ ion channels.

Short-Term Effects of Aβ Result from the Formation of Aβ Ion Channels. Freshly prepared solutions of Aβ have long been known to induce immediate intracellular calcium changes and morphological alterations in cultured endothelial cells (*4*), human fibroblasts (*15*), neuroblastoma cells (*18*), and hypothalamic neurons (*19*). These effects of fresh Aβ solutions have been linked to the rapid formation of soluble oligomeric Aβ aggregates, capable of forming ion channels. The disruption of membrane asymmetry and integrity, and

the changes in intracellular calcium that we observed in a variety of cells right after the application of fresh Aβ are analogous to the effects of fresh Aβ observed by these previous investigations. It has also been observed that Aβ soluble oligomers, formed after days of incubation of Aβ monomer solutions, produce calcium disregulation and membrane disruption (*20*). We propose that the toxic species of Aβ responsible for the short-term effects observed from freshly prepared Aβ solutions are probably ion channel-forming aggregates that form earlier than aggregates formed in aged Aβ solutions. These later aggregates produce a nonspecific increase in membrane permeability, which permits the permeation of molecules greater than the size cutoff for most ion channels (*20*).

The prevention of the morphological alterations and the changes in intracellular calcium utilizing zinc ions to block the Aβ channels (*4*, *15*, *19*) support the idea of the participation of Aβ ion channel-forming aggregates. The prevention of intracellular calcium changes in a variety of cells and the prevention of disruption of membrane asymmetry and integrity utilizing NA4, which is selective and very effective to block Aβ channels, add further corroboration to the participation of Aβ ion channels in the generation of these changes. The description of the architecture of the Aβ ion channel (*16*) provided a way for the designing of specific Aβ ion channel blockers. The peptide NA4, with a residue sequence similar to Aβ_{8-14}, was found to be very effective and selective to block Aβ ion channels in planar lipid bilayers (*16*). We found here that this peptide, totally innocuous to the studied cellular mechanisms, was equally effective to prevent the short-term effects produced by the interaction with membranes of freshly prepared Aβ. Thus it verifies that the acute effects of Aβ are produced as a consequence of the formation of ion channel in the surface of the cellular membrane. This assertion was further confirmed by the results from experiments using two other known Aβ channel blockers, zinc ions and tromethamine. These compounds, although not considered to be as specific to block Aβ channels, have been found to be very effective to stop ion currents through Aβ channels in artificial and natural membranes patches (*19*, *21*, *22*) and to prevent cell death induced by Aβ (*23*).

Long-Term Effects after Removal of Aβ Result from the Formation of Aβ Ion Channels. Cells incubated continuously in Aβ-containing media develop evidence of intracellular activation of apoptosis (*1*, *17*, *24*). Our study shows that even in the absence of Aβ in the culture medium cells that have previously been exposed for a short period of time to Aβ-FITC continue to develop evidence of intracellular activation of apoptosis. This late effect of Aβ on cells can only be explained if, after changing the incubation medium, Aβ remained in the cell, either forming part of the surface membrane or in the interior of the cell. In fact, our results from the flow cytometric analysis, and fluorescence microscopy (not shown), of cells exposed for a short period of time to Aβ-FITC show that effectively Aβ remains bound to the cells even after profound washing. The magnitude of the long-term effect should correspond to the amount of Aβ remaining in the cells. Our experiments show that as the concentration of Aβ during the pretreatment period is made higher, the percentage of viable cells measured after a determined period of time is smaller. The prevention of the long-term effect of Aβ by the external application of zinc, tromethamine and NA4 demonstrate that Aβ can be reached from the outside of the cell, and that the Aβ that remains in the cell after changing the bathing medium forms part of the surface membrane.

To investigate the molecular basis of the long-term effect of removal of Aβ, we chose three types of cellular responses that occur at different cellular locations and phases of the processes leading to cell death and have been proven to be related to Aβ toxicity (*1*, *17*, *25*). These are the perturbations of the membrane permeability that permit leakage of the cytosolic LDH representing one of the first cellular surface membrane responses, the downstream caspase 3 activation, which is an intermediate intracellular response, and XTT enzymatic activity as a measurement for cell viability. These three different cellular responses to Aβ were efficiently prevented by external addition of Aβ ion channel blockers, in particular, NA4, which is a very specific Aβ channel blocker (*16*). This confirms that Aβ remained in the surface membrane after being removed from the medium and that it is lodged in the membrane in the configuration of an ion channel.

In summary, the results from the experiments described in this work support the hypothesis that confirm that exposure of cells to Aβ results in the initial formation of oligomeric Aβ aggregates which assemble calcium channels as they interact with the cell surface membrane.

The results also show that to prevent the toxic effect of Aβ on cells after the cells have been exposed to Aβ it is not sufficient to remove Aβ from the solution bathing the cells. Rather, it is necessary to selectively prevent the ionic fluxes through newly formed Aβ channels. Relevantly, if the Aβ channel blockers have been present in the cell culture media prior the formation of Aβ channels, long-term cytotoxic effects fail to occur.

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