Alzheimer's Disease Amyloid β-Protein Forms Zn^{2+}-Sensitive, Cation-Selective Channels Across Excised Membrane Patches from Hypothalamic Neurons

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ABSTRACT We have previously shown that the 40-residue peptide termed amyloid β-protein (AβP[1–40]) in solution forms cation-selective channels across artificial phospholipid bilayer membranes. To determine whether AβP[1–40] also forms channels across natural membranes, we used electrically silent excised membrane patches from a cell line derived from hypothalamic gonadotrophin-releasing hormone GnRH neurons. We found that exposing either the internal or the external side of excised membrane patches to AβP[1–40] leads to the spontaneous formation of cation-selective channels. With Cs^+ as the main cation in both the external as well as the internal saline, the amplitude of the AβP[1–40] channel currents was found to follow the Cs^+ gradient and to exhibit spontaneous conductance changes over a wide range (50–500 pS). We also found that free zinc (Zn^{2+}), reported to bind to amyloid β-protein in solution, can block the flow of Cs^+ through the AβP[1–40] channel. Because the Zn^{2+} chelator *o*-phenanthroline can reverse this blockade, we conclude that the underlying mechanism involves a direct interaction between the transition element Zn^{2+} and sites in the AβP[1–40] channel pore. These properties of the AβP[1–40] channel are rather similar to those observed in the artificial bilayer system. We also show here, by immunocytochemical confocal microscopy, that amyloid β-protein molecules form deposits closely associated with the plasma membrane of a substantial fraction of the GnRH neurons. Taken together, these results suggest that the interactions between amyloid β-protein and neuronal membranes also occur in vivo, lending further support to the idea that AβP[1–40] channel formation might be a mechanism of amyloid β-protein neurotoxicity.

INTRODUCTION

Alzheimer's disease (AD) is a chronic dementia affecting the aging population and is characterized by the presence of amyloid plaques in the brain and intraneuronal neurofibrillary tangles (Neve et al., 1990; Hardy and Higgins, 1992; Selkoe, 1991). The major components of brain amyloid plaques are peptides, 39 to 42 residues long, termed amyloid β-proteins (Masters et al., 1985; Hass and Selkoe, 1993). These peptides are proteolytic products of the widely distributed amyloid precursor glycoprotein (APP_{751}), defined by a locus on chromosome 21 (Goldgaber et al., 1987; Tanzi et al., 1987). In particular, amyloid β-protein (AβP[1–40]) has been linked to the neurotoxic principle causing neuronal death in the disease, although the mechanism has remained elusive (Yankner et al., 1990; Malouf, 1992; Yankner, 1992; Frazer et al., 1997).

Cortical neurons exposed to AβP[1–40] exhibit elevated levels of intracellular free calcium ($[Ca^{2+}]_i$), suggesting that toxicity may result from amyloid β-protein-evoked Ca^{2+} entry (Matson et al., 1992). We have previously shown that when AβP[1–40] was incorporated into lipid bilayer membranes, the amyloid formed cation-selective (including Ca^{2+}) channels (Arispe et al., 1993a,b; Durell et al., 1994), and we suggested that the ability of AβP[1–40] to form these cation channels could be the molecular basis of amyloid neurotoxicity (Arispe et al., 1994). To determine whether direct incorporation of AβP[1–40] molecules leading to channel formation might also occur in neuronal membranes, we studied the deposit of AβP[1–40] molecules on the cell surface of cultured hypothalamic GnRH, GT1–7, neurons by immunocytochemical techniques and the AβP[1–40]-channel formation in excised membrane patches from these cells after exposure to amyloid β-protein. We found and report here that although AβP[1–40] molecules can interact with the excised membrane patch from either side, peptide incorporation leading to channel formation was faster from the inner aspect of the excised patch. As expected, we found that the reversal potential of the AβP[1–40]-specific channel currents followed the Cs^+ gradient, indicating that the channel was permeable to Cs^+ over Cl^-. We also noted that the AβP[1–40] channel incorporated into excised patches of GnRH neuronal membrane exhibits spontaneous conductance changes over a wide range (50–500 pS; Arispe et al., 1993a). To understand the mechanism of amyloid β-protein actions, we examined the ability of Zn^{2+}, known to induce amyloid β-protein aggregation (Bush et al., 1994), to bind to specific AβP[1–40] sites (Bush et al., 1993), and to modulate AβP[1–40] channel activity in planar bilayers (Arispe et al., 1996). We found that Zn^{2+} (50–500 μM) can block the

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AβP[1–40] channel incorporated into the neuronal membrane from either the external or the internal side, and the blockade can be reversed by the addition of *o*-phenanthroline, a specific Zn^{2+} chelator, to the side containing Zn^{2+}. These properties of the AβP[1–40] channel formed across natural membranes are similar to those observed in artificial bilayer membranes (Arispe et al., 1993a,b, 1996), supporting the idea that AβP[1–40] channel formation might be the mechanism of amyloid β-protein neurotoxicity (Arispe, 1994; Frautschy et al., 1991; Kowall et al., 1992; Fuson et al., 1996). Finally, to determine whether AβP[1–40] can also interact with the plasma membrane of viable GT1–7 neurons, and to gather information on the exact localization of AβP[1–40] molecules, we used confocal microscopy to localize two specific antibodies, one raised against a neuronal protein marker (MAP2), and the other against AβP[1–40]. Both antibodies reacted with the target proteins demonstrating the deposition of aggregates of AβP[1–40] on the surface of GT1–7 cells. From these data we conclude that free AβP[1–40] molecules in solution can spontaneously interact with both intracellular and extracellular aspects of the plasma membrane of GnRH neurons to form cation-selective channels. In addition, our studies of Zn^{2+}/AβP[1–40] channel interactions reveal that the transition element binds to specific domains of the AβP[1–40] channel molecules, leading to a blockade of the channel. Because the blockade occurs when the amyloid β-protein and Zn^{2+} are added either to the same side or to opposite sides of the membrane, these interactions could explain recent data showing that peptide deposition and AβP[1–40]-associated neurotoxicity are attenuated by Zn^{2+} (Fuson et al., 1996; May et al., 1996). A preliminary presentation of the data in an abstract form has been made to the Biophysical Society (Kawahara et al., 1996).

MATERIALS AND METHODS

Cell culture

Immortalized hypothalamic neurons (GT1–7; Mellon et al., 1990) were cultured under conditions described previously (Mellon et al., 1990; Spergel et al., 1995). Briefly, GT1–7 cells (provided by Dr. R. Weiner, University of California at San Francisco) were grown in a mixture of 50% Dulbecco's modified Eagle's medium and Ham's F-12 medium supplemented with 2.4 g/liter $NaHCO_3$, 0.1 g/liter gentamicin, and 100 ml/liter heat-inactivated fetal calf serum. The culture medium was changed every 2–3 days. Upon reaching confluence, the GT1–7 cells were dissociated by incubation for 5 min at 37°C in phosphate-buffered saline containing 0.5 g/liter trypsin, 0.15 g/liter deoxyribonuclease I, 5.4 μM EDTA, 5.95 mM $NaHCO_3$, 5–6 mM glucose, and 0.07 gentamicin. The cells were then centrifuged again, resuspended, counted, and plated in 35-mm Petri dishes at a density of 5×10^4 cells/dish. After plating, the cells were cultured for 2–3 days before use in patch-clamp experiments.

AβP channel current recordings

Patch-clamp experiments were performed as previously described (Rojas et al., 1990; Spergel et al., 1996), with minor modifications. Patch pipettes were made from microhematocrit capillary glass by using a microcomputer-controlled multistep puller (BB-CH; Mecanex, Geneva, Switzerland). Pipettes were coated to the tip with Sylgard (Dow Corning). When filled with a Cs^+ solution (in mM: 140 CsCl, 0.5 $CaCl_2$, 0.5 $MgCl_2$, 1 EGTA, 5 NaHEPES, pH 7.4), pipette tip resistance ranged from 8 to 12 MΩ. Channel current recordings were made with the same Cs^+ solution, both inside the pipette and in the bath, by means of an EPC-7 amplifier (List Electronics, Darmstadt, Germany). AβP[1–40] solutions were always prepared fresh in distilled water. The amyloid β-protein was added to the bath at a concentration of ~4.7 μM.

Membrane currents under voltage clamp conditions were recorded through a low-pass filter set at 10 kHz (eight-pole Bessel, model 902 LPF; Frequency Devices, Haverhill, MA) to the input amplifier of a 16-bit resolution digital magnetic tape recorder (PCM-VCR; Unitrade, Data Acquisition and Storage Systems, Philadelphia, PA).

Immunohistochemistry

GT1–7 cells were incubated for 24 h in the presence of AβP[1–40] (~4 μM) added to the culture medium. Next the GT1–7 cells were fixed by the addition of paraformaldehyde (4%). After fixation cells were thoroughly washed with medium. After this step specific antibodies against AβP[1–40] (αβ42; kindly donated by Dr. Mori, Tokyo Institute of Psychiatry) and against microtubule associated protein of dividing cells (MAP2) were added. After a prolonged exposure to the protein-specific antibodies, secondary antibodies, conjugated to the fluorescent probes fluorescein-5-isothiocyanate (FITC) and Texas Red, respectively, were used to identify the presence of the proteins in the cells using a confocal microscope.

Statistical evaluation and channel current analysis

Off-line analysis of the recorded AβP[1–40] channel activity was carried out with the software package pClamp 5.51 (Axon Instruments, Burlingame, CA). Data-base files were obtained from playbacks of the experimental records digitized with a 12-bit analog-to-digital converter (TL-1 DMA interface; Axon Instruments), using the Fetchex subroutine. At least 10 2048-point episodes were needed to achieve the minimum 2500 events required to construct histograms. These data files were analyzed using the subroutine TIP (Transit Interface Program; Baylor College of Medicine, Houston, TX). As a rule, the data base used for the open-time and close-time distributions corresponded to recordings with a signal-to-noise ratio of ≥5:1.

Four experimental paradigms were carried out on excised inside-out membrane patches:

AβP[1–40] added to the bath solution

For these experiments the excised membrane patch was exposed to symmetrical Cs^+ solution for a 10–30-min period before the addition of amyloid β-protein. 1) After the incorporation of AβP[1–40] channel activity, Zn^{2+} was added to the bath solution. 2) After the blockade by Zn^{2+} was apparent, the Zn^{2+} chelator, *o*-phenanthroline, was added to the bath solution, which contained AβP[1–40] and Zn^{2+}.

AβP[1–40] added to the pipette solution

To allow for a control period in the absence of amyloid β-protein, the pipette was first back-filled with a CsCl solution. Next, a small volume (~5 μl) of AβP[1–40] water solution was added to the distal end of the Cs^+ solution in the pipette. The time taken for the amyloid molecules to reach the outer aspect of the patch allowed us to verify that the membrane patch was electrically silent. 1) After the incorporation AβP[1–40] channel activity, Zn^{2+} was added to the solution in the dish. 2) The Zn^{2+} chelator, *o*-phenanthroline, was added to the solution in the dish after the blockade by Zn^{2+} was apparent.

Each paradigm was repeated at least three times with different cell preparations. The figures shown are representative of the records obtained.

Chemicals and reagents

Amyloid β-protein was obtained from Bachem California (Torrance, CA), *o*-phenanthroline was from Sigma (St. Louis, MO), and $ZnCl_2$ was from Fluka (Ronkonkoma, NY).

RESULTS

To detect endogenous channels that might be present in the patch, our standard protocol included an initial control period of recording at high gain (200 mV/pA). During this control period the membrane potential across the patch was held at different levels (from −40 to 40 mV) while the current was recorded. For pipette applications of AβP[1–40] (extracellular side of the cell membrane), the minimum time allowed for the control period was 5 min (see methods). For bath applications of AβP[1–40] (intracellular side of the cell membrane), the minimum control time was 15 min. Provided the membrane patch was electrically silent (i.e., endogenous channel current of amplitude smaller than 0.1 pA) during this time, the amyloid β-protein was added to the solution in the dish to a final concentration of 4.7 μM.

AβP[1–40] but not AβP[40–1] forms cation channels across neuronal membranes

Channel activity always appeared minutes after the exposure of the inner aspect of the membrane patch to AβP[1–40]. Records of such AβP[1–40] channel activity incorporated from the solution into an inside-out excised patch are

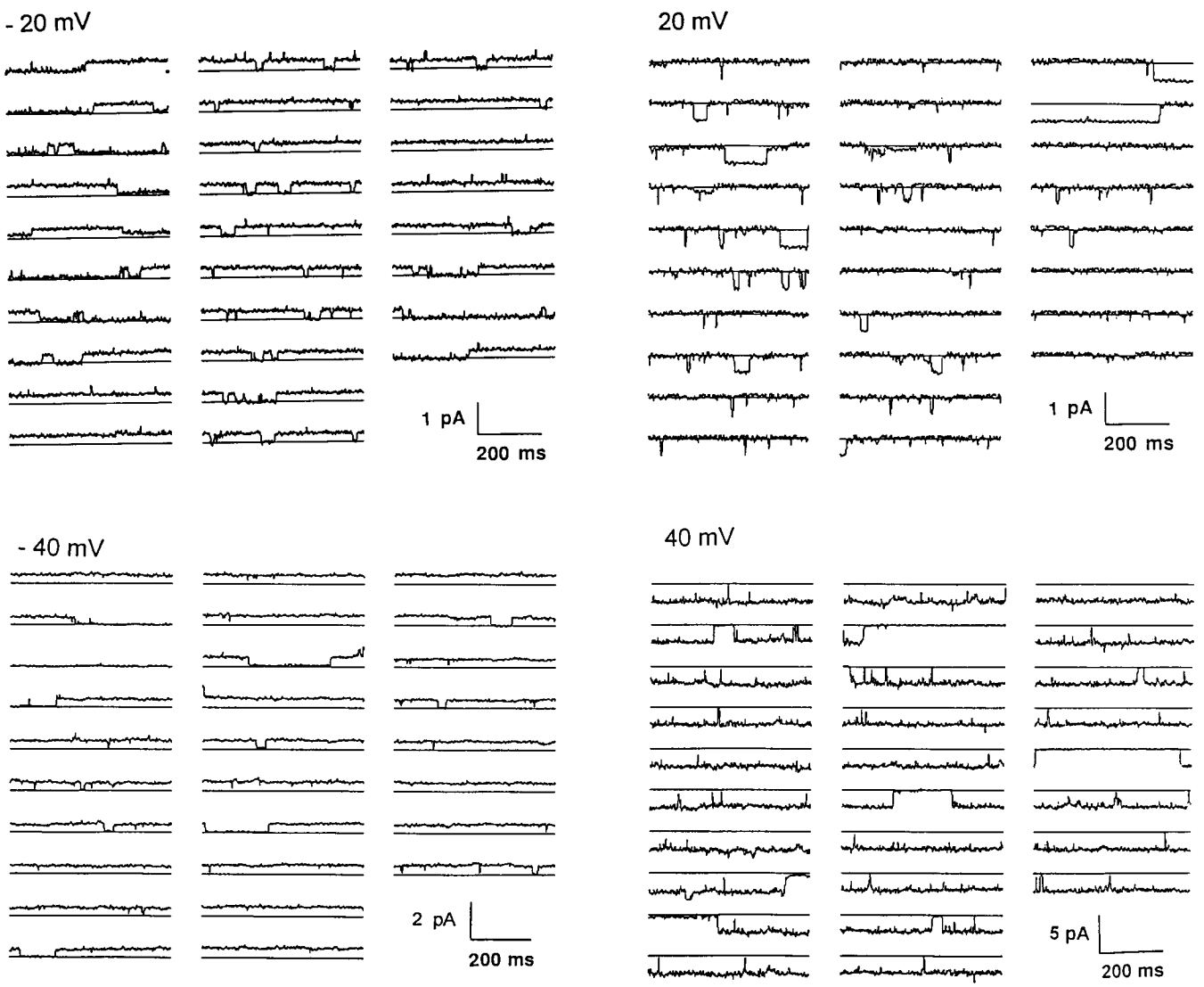

FIGURE 1 Direct incorporation of AβP[1–40] into membrane patches from the intracellular side (bath). All records shown here were made 14–25 min after the application of AβP[1–40] to the bath (~4.5 μM). (*Upper left*) Three sets of 10 segments of a continuous current record made at −20 mV. (*Upper right*) Records made at 20 mV. Current records acquired at −40 and 40 mV are depicted at the lower left and lower right, respectively.

shown in Fig. 1. The presence of spontaneous transitions between different levels of the current, together with the appearance of complete channel closures from different levels, suggests the presence of a single channel with several levels of conductance. Open-channel current levels as well as open- and closed-channel kinetics were analyzed as described previously (Arispe et al., 1996). To calculate the fraction of time at a given conductance level from records like those shown in Fig. 1, we obtained first open- and closed-time histograms (not shown). The fractional open time (estimated from records like those depicted in Fig. 1, *bottom*) remained unchanged at ~0.8, even though the transmembrane potential was changed from −40 mV (*left*) to 40 mV (*right*). By contrast, changing the potential from −20 mV (*top left*) to 20 mV (*top right*) reduced the open time of the channel from 0.75 to 0.25. Under the conditions of symmetrical solutions used here (140 mM CsCl on both sides of the membrane patch), the open-channel current was found to be close to zero, as expected, at a transmembrane potential of 0 mV. By contrast, in an asymmetrical CsCl solution system (pipette: 140 mM; bath: 70 mM), the reversal potential was found to be about −16 mV. This is close to the Nernst potential of −17.4 mV, assuming that the charge carrier is Cs^+. As illustrated in Fig. 1, the current changed from positive (*left*) to negative (*right*). By contrast, exposure of either the intracellular or the extracellular side of the plasma membrane to the peptide AβP[40–1] with the reverse amino acid sequence had no effect. Indeed, membrane patches ($n = 6$) remained electrically silent after prolonged (30–60 min) exposures to the peptide AβP[40–1] (not shown).

Several traits of the AβP[1–40] channel activity incorporated into artificial bilayers are also expressed in natural membranes. These include the presence of different levels of channel conductance, from ~5 pS to 3 nS (Arispe et al., 1993a), and the sensitivity to the transition element Zn^{2+} (Arispe et al., 1996), illustrated in the following section.

Interactions between Zn^{2+} and AβP[1–40] channels

We have previously shown that AβP[1–40] channels incorporated into artificial lipid bilayer membranes interact with Zn^{2+} in a dose-dependent manner (Arispe et al., 1996). At concentrations less than 250 μM, Zn^{2+} may induce profound changes in channel kinetics, and at higher doses, Zn^{2+} may even block the AβP[1–40] channel. To determine whether the AβP[1–40] channel activity in natural membranes can be modulated by Zn^{2+}, amyloid β-protein was added to the solution bathing either the extracellular side (Fig. 2) or the intracellular side (Fig. 3) of inside-out excised membrane patches. After the direct incorporation of AβP[1–40] molecules from the solution, which was revealed by the appearance of channel activity, Zn^{2+} was added to the chamber facing the inner aspect of the patch.

Fig. 2 shows the activity of the AβP[1–40] channel incorporated from the extracellular side of the patch (AβP[1–40] in the pipette). Traces represent segments of a continuous record of AβP channel activity recorded at different transmembrane potentials in the absence (*top*) and in the presence (*bottom*) of Zn^{2+}. In the absence of Zn^{2+}, regardless of the direction of the Cs^+ current, channel activity is rather irregular. At −40 mV, Cs^+ is expected to flow through the open channel from the bath to the pipette (*upper left*), and in the opposite direction at 40 mV (*upper right*). A few minutes after the addition of Zn^+ (250 μM) to the solution facing the cytoplasmic aspect of the membrane patch, channel activity was profoundly inhibited (*bottom*). It should be noted that the extent of the blockade induced by Zn^{2+} depends on the direction of the Cs^+ flow. Indeed, while at a pipette potential of −40 mV, there is an almost complete inhibition in the AβP[1–40] channel activity (Fig. 2, *lower left*). In contrast, at 40 mV, for a substantial fraction of time, there is channel activity expressed as well-defined events (Fig. 2, *lower right*). Because these records were made at a positive potential inside of the pipette, the electrical potential gradient should drive Cs^+ ions through the open channel. It is then possible that Cs^+ flowing along the pore, from the external side of the membrane, might displace Zn^{2+} from binding sites at the cytoplasmic end of the pore. In addition, before the application of Zn^{2+}, the fractional open time of the AβP[1–40] channel remained at ~0.9, even though the transmembrane potential was changed from −40 mV to 40 mV (Fig. 2, *top*). By contrast, in the presence of Zn^{2+} (≤250 μM), the fractional open time changed from ~0.05 at −40 mV (Fig. 2, *lower left*) to ~0.17 at 40 mV (Fig. 2, *lower right*).

From these data we conclude that the AβP[1–40] molecule can insert itself into the plasma membrane from the solution on the extracellular side. Because Zn^{2+} applied from the intracellular side can modulate the channel, the unavoidable conclusion is that AβP[1–40] molecules forming the channel (Durell et al., 1994) can span the hydrophobic region of natural membranes, making it possible for the transition element to interact with the channel from the intracellular side.

Zn^{2+} blockade is reversed by *o*-phenanthroline

We also studied the specificity of the interactions between Zn^{2+} and amyloid β-protein channels incorporated directly from the solution into the cytoplasmic side of the patch. In the symmetrical CsCl solution system, the potential across the patch was set at −20 mV, and a few minutes after the addition of the protein AβP[1–40] to the bath (4.7 μM), channel activity began (Fig. 3, *left*). Because the characteristic multilevel channel activity was observed, we concluded that incorporation of the amyloid β-protein had occurred (Fig. 3, *left*). Amplitude histograms of the activity revealed the multilevel character of the channel activity with the following levels: 1.0, 1.7, 2.6, 4.1, 5.9, 9.3, and 12.8 pA (not shown). Furthermore, exposure of the cytoplasmic side of the patch to Zn^{2+} (250 μM) induced a

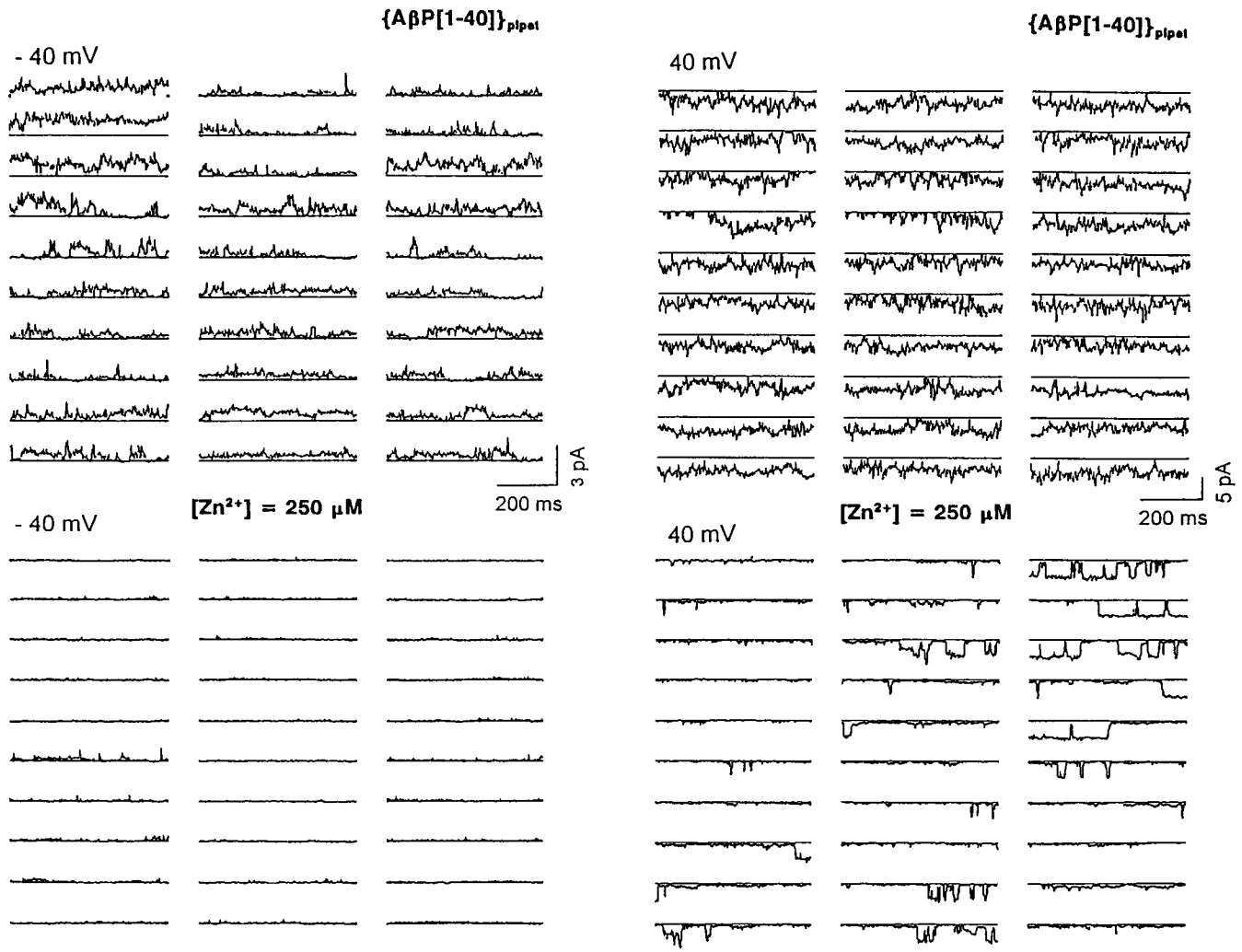

FIGURE 2 Addition of Zn^{2+} to the bath blocks the AβP[1–40]-channel incorporated from the solution in the pipette. (*Top*) Two sets of ten segments of a continuous current record made in the absence of Zn^{2+}, at −40 mV (*left*) and 40 mV (*right*), respectively. (*Bottom*) Two sets of 10 segments of a continuous current record made after the addition of Zn^{2+} (250 μM) to the bath at −40 mV (*left*) and 40 mV (*right*), respectively.

marked reduction of the amplitude of the channel current (Fig. 3, *center*). In the presence of Zn^{2+}, transitions between three tiny levels of channel current (0.69, 1.0, and 1.85 pA) occurred at a high frequency (Fig. 3, *center*), suggesting brief dissociations between the Zn^{2+} and the AβP[1–40] channel. These profound changes in the AβP[1–40] channel gating induced by Zn^{2+} led us to consider the possibility that Zn^{2+} might stabilize a few conformations of the AβP[1–40] aggregate forming the hypothetical pore (Durell et al., 1994). It should be mentioned here that these effects of Zn^{2+} on AβP[1–40] channel activity incorporated into neuronal membranes are similar to those observed in the artificial bilayer (Arispe et al., 1996). Finally, the addition of *o*-phenanthroline (2 mM), a specific Zn^{2+} chelator, effectively removed the free Zn^{2+}, leading to the recovery of the AβP[1–40] channel currents (Fig. 3, *right*). Amplitude histograms showed the following levels: 1.1, 1.7, 2.5, 3.9, 6.0, 8.8, and 12.8 pA. It should be noted that these levels follow the "3/2 rule" for channels with multilevels of conductance (Pollard et al., 1994).

Experiments in which AβP[1–40] was incorporated from the extracellular side of the patch, and Zn^{2+} was added to the bath facing the intracellular side of the patch, also showed the interactions between the channel and the transition element. The analysis of the AβP[1–40] channel activity recorded in the presence of Zn^{2+} revealed the occurrence of brief transitions between different single-channel current levels. Indeed, the four segments of a continuous record acquired at 40 mV shown in Fig. 4 on an expanded time base show brief closures of the AβP[1–40] induced by Zn^{2+}. It is apparent that the transitions also occurred between consecutive levels. It should not be forgotten that in the absence of divalent cations, AβP[1–40] channel gating is rather insensitive to the transmembrane potential (Fig. 1). By contrast, the frequency of the transitions between different levels (Fig. 4, *top*) changed at dif-

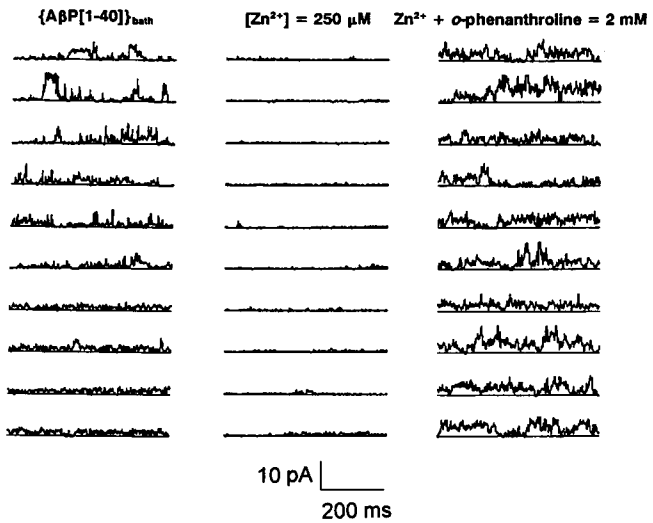

FIGURE 3 Direct incorporation of AβP[1–40] from the intracellular side of the membrane patch. (*Left*) Ten consecutive records of the AβP[1–40] channel activity at −20 mV. (*Center*) Ten consecutive records of the AβP[1–40] channel activity at −20 mV after the exposure of the intracellular aspect of the membrane patch to Zn^{2+} (250 μM) added to the bath solution. (*Right*) Records were made at −20 mV a few minutes after the addition of *o*-phenanthroline (2 mM) to the bath.

ferent transmembrane potentials. For example, the fractional open time at the smallest level (i.e., −0.69 pA; Fig. 4, *top*) decreased from 0.7 (at 40 mV) to 0.4 at 10 mV.

Identification of AβP[1–40] deposition by confocal immunocytochemical microscopy

To determine whether AβP[1–40] can also interact with the membrane of viable GT1–7 neurons and to gather information on the localization of the AβP[1–40] molecules, we used two specific antibodies, one raised against a neuronal marker (MAP2) and the other against AβP[1–40]. As shown in the confocal micrographs depicted in Fig. 5, both antibodies reacted with the target proteins, demonstrating the deposition of aggregates of AβP[1–40] on the surface of a substantial fraction of the cultured GT1–7 cells. It is also apparent that whereas MAP2 (pseudocolor green) is homogeneously distributed on the cell bodies and along the cell branches and processes, the amyloid β-protein marker covers only some areas on the surface of the neurons. Although it is impossible to determine the exact neuronal target structure with the resolution at hand (pseudocolors red-yellow), in enlarged images of selected zones of the plated GT1–7 cells, the density of the AβP[1–40] mark is higher than that of MAP (Fig. 5 *B*) and is by far less homogeneous (Fig. 5 *A*). It is possible that discrete areas of the GT1–7 cells are more susceptible to the deposition of amyloid β-protein.

DISCUSSION

The data presented here provide compelling evidence supporting the idea that free AβP[1–40] molecules in solution

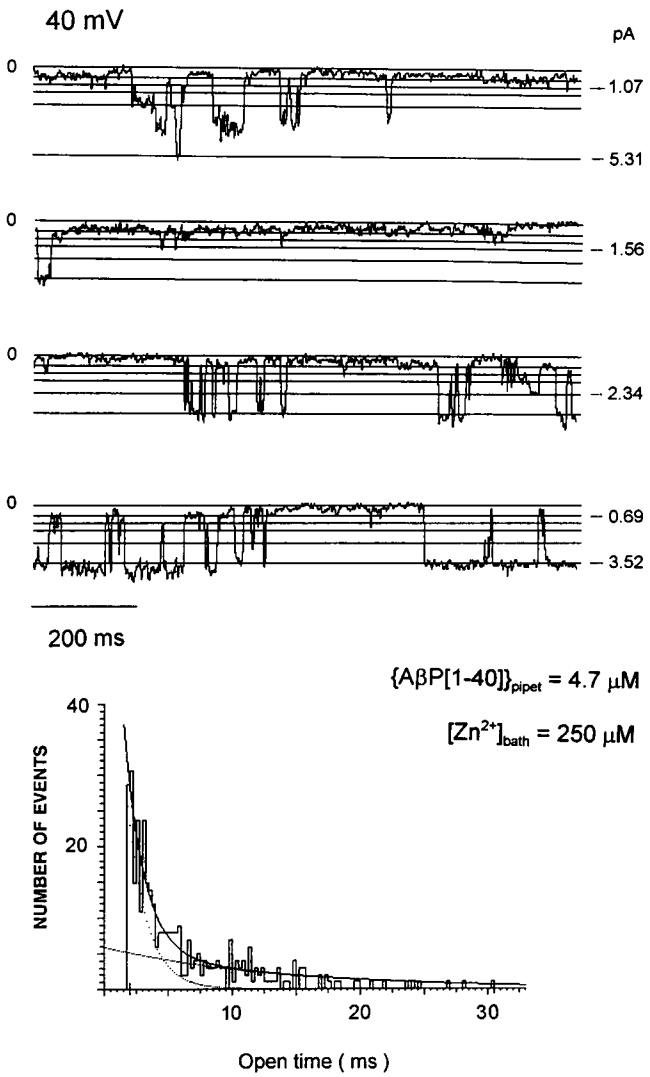

FIGURE 4 Multilevel AβP[1–40]-channel activity during Zn^{2+} evoked blockade. AβP[1–40] was added to the solution in the pipette (~4.7 μM). Zn^{2+} was added to the solution in the bath (250 βM). (*Top*) Records were made at 40 mV. Horizontal lines indicate the current level (*numbers on the right side in pA*) calculated using the "3/2 rule" (Pollard et al., 1994). (*Bottom*) Open-time distribution. The fitted curve was calculated with two time constants, 2.5 and 11.6 ms.

can spontaneously interact with the plasma membrane of GnRH neurons to form cation-selective channels. Furthermore, channel formation occurs when AβP[1–40] is present in the saline bathing either the intracellular or the extracellular aspect of the cell membrane. The characteristics of the AβP[1–40] channel activity in the natural membrane are rather similar to those described before in artificial lipid bilayers (Arispe et al., 1993a,b, 1996). By contrast, membrane patches remained electrically silent after exposure to the peptide AβP[40–1] with the reverse amino acid sequence for a period of 60 min. Thus it is possible that the peptide AβP[1–40] in solution can acquire a specific conformation that allows the incorporation of the protein to form the channel.

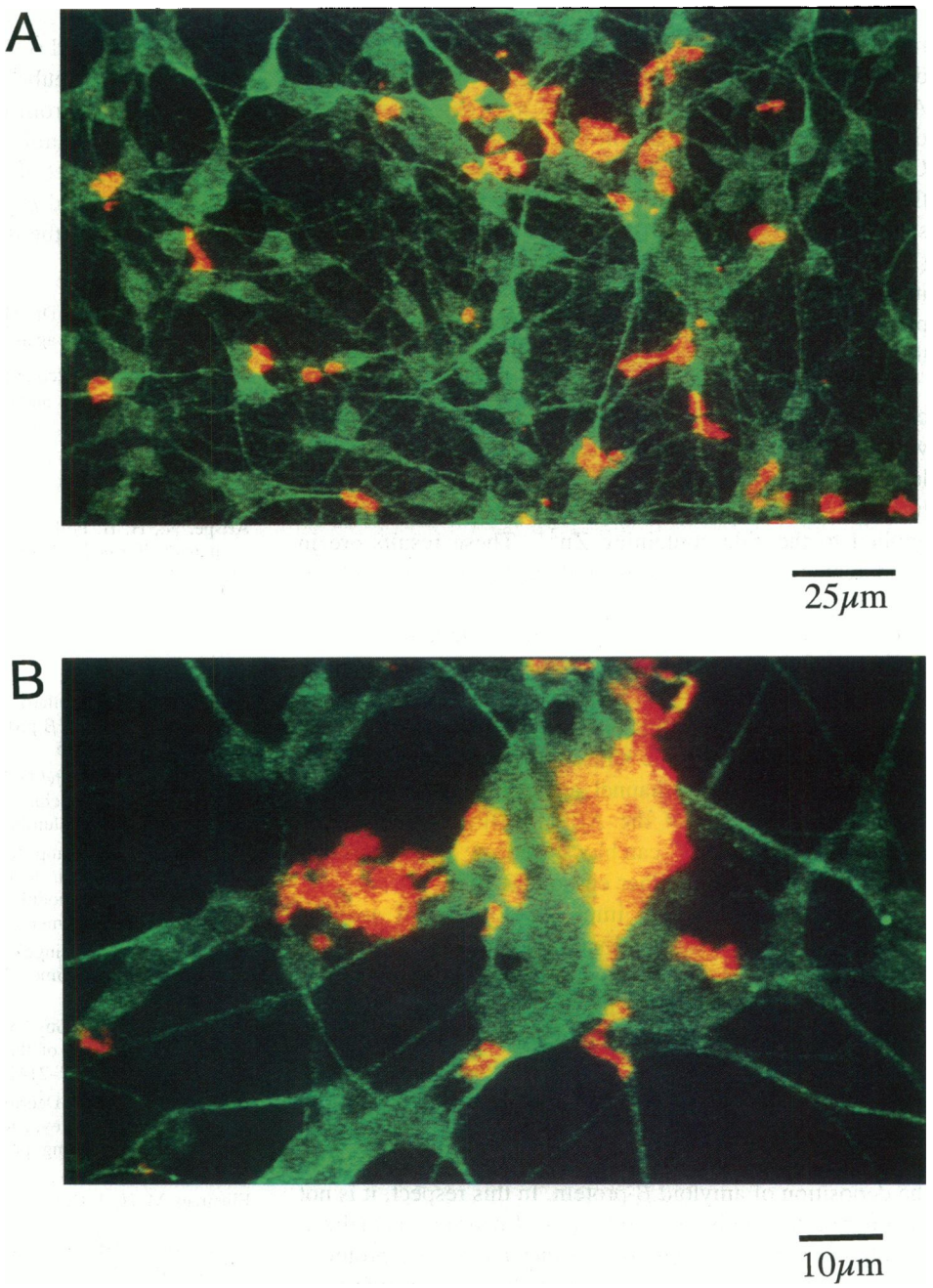

FIGURE 5 Immunocytochemical identification and localization of the AβP[1–40] in GnRH neurons. Confocal fluorescence microscopy of cultured GT1–7 after αβ42 binding (FITC fluorescence) followed by MAP2 binding (Texas red fluorescence). For contrast, red fluorescence from MAP2 was changed to green by using pseudocolor software.

We have also shown here that Zn^{2+} interacts with specific domains of the AβP[1–40] molecules incorporated into the excised membrane patch, leading to blockade of the channel. This blockade of the Aβ[1–40] channel occurs even when the transition element is added to the intracellular side (bath solution) and the channel protein is added to the extracellular side of the membrane patch. This result strongly suggests that the AβP[1–40] molecules incorporated into the membrane can span the entire membrane.

We have previously proposed that channel formation requires the aggregation of several AβP[1–40] molecules (Durell et al., 1994). Thus the marked decrease in AβP[1–40] channel current induced by the addition of Zn^{2+} on the cytoplasmic side of the excised membrane patch (Figs. 2 and 3) suggests that Zn^{2+} ions present in the solution can interact with specific domains of the AβP[1–40] molecules forming the pore. Thus AβP[1–40]/Zn^{2+} interactions can occur, despite the fact that the amyloid β-protein has been incorporated from the extracellular side (Fig. 2). Low-affinity association between Zn^{2+} and the AβP[1–40] channel could explain the fast blocking events (as depicted in Fig. 4). It is also possible that the low-affinity binding of Zn^{2+} to AβP[1–40] sites may induce rapid rearrangements of the molecules forming part of the channel aggregate (Durell et al., 1994). These conformational changes might in turn cause the observed flickering of the current. However, the

open time of the AβP[1–40] channel was found to be rather insensitive to the transmembrane potential under calcium-deficient conditions (Fig. 1). Thus voltage-dependent AβP[1–40] channel gating (Fig. 4, *top*) can be observed only under conditions that allow divalent cations, such as Ca^{2+} (Arispe et al., 1993b) and Zn^{2+} (this work), to enter the channel pore (Durell et al., 1994) and interact with specific binding sites. On the basis of our molecular model (Durell et al., 1994), we conclude that Zn^{2+}/AβP[1–40] interactions must occur in a high dielectric constant region, probably at the entrance of the AβP[1–40] channel, where the electric field can be assumed to be relatively constant.

Application of *o*-phenanthroline induced a complete recovery of the control AβP[1–40] channel activity, which was characterized by infrequent transitions among different levels of conductance (not shown). It should be mentioned here that *o*-phenanthroline can remove the blockade only if applied to the side containing Zn^{2+}. These results are in good agreement with recently published planar lipid bilayer data (Arispe et al., 1996).

In conclusion, our channel data indicate that AβP[1–40] molecules in solution can interact with both the external as well as the internal aspect of the plasma membrane and form cation-selective channels. The addition of Zn^{2+} to the bath solution (cytoplasmic side of the patch) blocked the multilevel AβP[1–40] channel activity, regardless of the side of incorporation of the AβP[1–40] molecules. This result suggests that Zn^{2+} can block the flow of Cs^{+} through the open channel from either end of the pore.

Finally, our preliminary immunocytochemical analysis showed that whereas the neuronal membrane marker MAP2 was homogeneously localized throughout the cell bodies and dendritic tree, the antibody αβ42 reacted with amyloid β-protein molecules deposited onto restricted regions of the cell bodies and processes (Fig. 5 *B*). Although we do not have an explanation for this remarkable difference in the localization of the two antibodies, it is possible that discrete membrane areas of the GT1–7 cells are more susceptible to the deposition of amyloid β-protein. In this respect, it is not uncommon to find in different types of neurons specialized membrane regions containing distinct membrane proteins. For example, immunolocalization studies in Purkinje neurons revealed a rather homogeneous localization of ryanodine and inositol 1,4,5-triphosphate (IP_3) receptor proteins throughout the cell body and dendritic tree. By contrast, IP_3 receptors were detected only in the dendritic spines (Ellisman et al., 1990, 1991; Martone et al., 1993; Sharp et al., 1993).

Taken together, the results presented here suggest that the interactions between amyloid β-protein and neuronal membranes also occur in vivo, lending further support to the idea that AβP[1–40] channel formation might be a mechanism of amyloid β-protein neurotoxicity (Arispe et al., 1994). The interactions of Zn^{2+} with amyloid β-protein molecules incorporated into neuronal membranes could even lead to attenuation of amyloid channel neurotoxicity. It should also be mentioned here that the concentration of amyloid β-protein used in this work was higher than the levels detected in cerebrospinal fluid (CSF) samples from normal and Alzheimer's patients (Seubert et al., 1992). However, analysis of CSF in samples from these subjects showed identical concentrations of soluble AβP[1–40] (i.e., <1 μM) in both groups (Tabaton et al., 1994), suggesting that the presence of soluble amyloid β-protein in the CSF cannot be considered a marker of the disease.

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