Change in Membrane Permeability Induced by Amyloid β-Protein Fragment 25–35 in Brain Neurons Dissociated from Rats

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Abstract—Effects of amyloid β-protein fragment 25–35, AβP(25–35), on the membrane permeability of organic molecules were examined in the brain neurons dissociated from rats by using an argon laser (equipped in flow cytometer and laser microscope) and a combination of two fluorescent dyes, fluo-3-AM and ethidium bromide. AβP(25–35) at concentrations of 1 μM or greater induced both leakage of fluo-3 from the neurons and permeation of ethidium across the membrane in a dose-dependent manner, although both dyes are highly impermeant to the intact plasma membrane. Thus, AβP(25–35) seems to increase not only membrane permeability of inorganic ions such as Ca²⁺, Na⁺ and K⁺, as previously suggested, but also that of organic molecules. Therefore, the brain neuron membrane is suggested to lose its integrity in the presence of AβP(25–35) that leads to neuronal death.

Keywords: Amyloid β-protein, Brain neuron, Intracellular Ca²⁺, Membrane permeability, Viability

Amyloid β-protein is a constituent of senile plaques observed in the brains of patients with Alzheimer’s disease (1, 2). Electrophysiological studies have shown that amyloid β-protein fragment 25–35, AβP(25–35), which is proposed to be responsible for the cytotoxic activity of amyloid β-protein (3–5), produces an irreversible increase in non-specific membrane conductance of neurons dissociated from amphibian ganglion (6) and mammalian brain (7). Therefore, results suggest a sustained increase in the intracellular Ca²⁺ concentration ([Ca²⁺]), of brain neurons that leads to neuronal death (4, 8). However, AβP(25–35)-induced increase in membrane conductance of neurons measured by the electrophysiological technique (a patch clamp technique) reflects only ionic flows across the membrane (6, 7). Thus, there is no information about the effect of AβP(25–35) on the membrane permeability of organic molecules in mammalian brain neurons. In this study, we have examined the effects of AβP(25–35) on the membrane permeability of organic molecules, simultaneously with the effects on [Ca²⁺], and cell viability, in dissociated mammalian brain neurons by using an argon laser (equipped in a flow cytometer and laser confocal microscope) and two fluorescent dyes, fluo-3-AM (9, 10) and ethidium bromide (11–14).

Materials and Methods

Preparation

Experiments were performed on cerebellar neurons enzymatically dissociated from 2-week-old Wistar rats (obtained from Nissin, Tokushima). The technique for enzymatic dissociation of rat cerebellar neurons was previously described (10, 15–17). In brief, the cerebellum was sliced at a thickness of 400 to 500 μm. Brain slices were treated with dispase (1000 protease units per ml; Godo Shusei, Tokyo) for 60 min in HEPES-buffered Tyrode’s solution which was oxygenated at a temperature of 36°C. After enzymatic treatment, the brain slices were triturated in Tyrode’s solution to dissociate single neurons. Tyrode’s solution containing dissociated neurons was passed through a mesh (diameter of 53 μm) to remove residues. Since the neurons (more than 90%) in the filtered cell suspension were characterized by their small size (diameter of about 10 μm or less) and spherical shape under microscopic observation, the cells were likely cerebellar granule neurons. Furthermore, neurons could be selected from the cytogram (forward-angle light scatter versus side light scatter, cell diameter versus cell density) for fluorescence measurement (13). Therefore, of all neu-
rons freshly dissociated from mammalian brain, cerebellar granule neurons are most suitable for flow-cytometric analysis at present.

**Fluorescence measurements and analysis**

To estimate the membrane permeability of organic molecules in the presence of AβP(25–35), two fluorescent probes, fluo-3-AM (Dojindo Laboratory, Kumamoto) (9, 12) and ethidium bromide (Molecular Probe Inc., Eugene, OR, USA) (12–14), were used. The respective dye was added into the cell suspension to achieve a final concentration of 300 nM for fluo-3-AM or 2.5 μM for ethidium bromide (10, 13, 15–17). Before any measurement of fluo-3 fluorescence, neurons were incubated with fluo-3-AM for 60 min. Fluorescence obtained from neurons incubated with ethidium bromide was measured at an appropriate time (2 min at least) after the dye application.

Simultaneous measurements for fluo-3 and ethidium fluorescences from brain neurons were made by a flow cytometer (Cyto ACE-150; Japan Spectroscopic Co., Tokyo) and confocal laser microscope (Meridian Instruments Far East, Inc., Tokyo). The excitation wavelength for fluo-3 and ethidium was 488 nm produced by an argon laser. In experiments using a flow cytometer, emissions were detected at a wavelength of 530±20 nm for fluo-3 and 600±20 nm for ethidium. Fluorescence cytograms (fluo-3 fluorescence intensity versus ethidium fluorescence intensity) obtained from a programmed number of neurons was analyzed by software (Jasco Ver. 3XX; Japan Spectroscopic Co., Tokyo) and a personal computer (PC-9801 RX; NEC, Tokyo). When a confocal laser microscope was used, the fluorescences were measured at a wavelength of 530±15 nm for fluo-3 and 600 nm or longer for ethidium. Fluorescence obtained from

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**Fig. 1.** Flow-cytometric measurements of fluo-3 and ethidium fluorescences from a programmed number of dissociated brain neurons (5000 neurons) in the cell suspension to which 300 nM fluo-3-AM and 5 μM ethidium bromide were added. A: schemes of permeation of fluo-3-AM, fluo-3 and ethidium across the membranes of dissociated brain neuron. The left scheme shows a neuron having an intact plasma membrane (live neuron). The middle one shows a neuron that is damaged or has a compromised membrane. The right one shows a dead neuron. B: the fluorescence cytograms, fluo-3 fluorescence (abscissa) versus ethidium fluorescence (ordinate), before (left panel, Control) and after application of 2.5 μM ionomycin (middle panel, Ionomycin) and 20 μM digitonin (right panel, Digitonin). Each cytogram was obtained from 5000 neurons. Part a indicates the neurons predominantly stained with fluo-3. Part b indicates those stained with both fluo-3 and ethidium. Part c shows those intensively stained with ethidium.
individual neurons were analyzed by software (InSIGHT-IQ, Meridian Instruments Far East, Inc.) and a computer (Evolution V-Q system; Advanced Logic Research, Inc., Irvine, CA, USA). Tentative calibration of fluo-3 fluorescence for the \([\text{Ca}^{2+}]_i\) was made by a method using ionomycin and Mn\(^{2+}\) (9). Statistical analyses were performed with the two-sample \(t\)-test with and without Welch’s correction. A \(P\) value of <0.05 was considered to be significant.

**Chemicals**

Amyloid \(\beta\)-protein fragment 25–35, A\(\beta\)P(25–35), was purchased from Sigma Chemical Co. (St. Louis, MO, USA). A\(\beta\)P(25–35) and ethidium bromide were initially dissolved in distilled water. Fluo-3-AM and ionomycin were dissolved in dimethyl sulfoxide and ethanol, respectively. Solvents at the final concentration of less than 0.3% did not affect the fluorescence measurement. All stock solutions were stored in the freezer and used within 1 month after preparation. Other chemical reagents except for fluorescent dyes were obtained from Katayama Chemical Industries (Osaka).

**RESULTS**

**Effect of ionomycin and digitonin on fluorescence cytograms of fluo-3 fluorescence versus ethidium fluorescences in brain neurons**

As shown in Fig. 1A, fluo-3-AM passively crosses neuronal membranes, and then is converted by intracellular esterase to fluo-3 which is retained by neurons with intact plasma membranes. Fluo-3 is predominantly used for monitoring \([\text{Ca}^{2+}]_i\), because this dye would be fluorescent if bound to intracellular \(\text{Ca}^{2+}\) (9, 12). However, neurons with a compromised membrane rapidly release fluo-3, even if neurons retain some residual esterase activity (12). Therefore, assays of leakage of fluo-3 from neurons may be used to determine the membrane permeability of organic molecules and/or the membrane integrity in neurons affected by cytotoxic substances. Furthermore, there is no fluo-3 fluorescence in dead neurons since dead neurons have no esterase activity. On the contrary, ethidium which is highly impermeant to intact plasma membranes stains only neurons that are dead or have compromised membranes. Therefore, simultaneous measurements of fluo-3 and ethidium fluorescences may provide useful information about the membrane permeability of organic molecules and/or the membrane integrity in brain neurons affected by cytotoxic substances.

Figure 1B shows the fluorescence cytograms (fluo-3 fluorescence versus ethidium fluorescence) obtained from the cell suspension to which fluo-3-AM and ethidium bromide were added. As shown in the control fluorescence cytogram (Fig. 1B), there were three cell populations: one was predominantly stained with fluo-3 (Part a of Fig. 1B), another was stained with both fluo-3 and ethidium (Part b of Fig. 1B), and the remaining one was intensively stained with ethidium (Part c of Fig. 1B). Ionomycin, a \(\text{Ca}^{2+}\) ionophore, at a concentration of 2.5 \(\mu\)M greatly augmented fluo-3 fluorescence of neurons only belonging to Part a, indicating an increased \([\text{Ca}^{2+}]_i\) (Figs. 1B and 3). The effect of ionomycin on the cell population in the cell suspension was estimated at 5 min after application. As shown in Figs. 1 and 2A, ionomycin did not significantly affect the respective cell population, although the \([\text{Ca}^{2+}]_i\) of neurons in Part a was greatly increased (Fig. 3). Effects of 20 \(\mu\)M digitonin on the fluorescence cytograms were examined since digitonin at 20 to 40 \(\mu\)M was reported to be sufficient to release essentially
all of the lactic dehydrogenase, a standard cytosolic marker (9). As shown in Fig. 2B, treatment of neurons with 20 μM digitonin decreased the number of neurons stained predominantly with fluo-3 (Parts a and b of Fig. 1B), but increased that with ethidium (Part c of Fig. 1B). Thus, lysis of the neurons with digitonin released fluo-3 and permitted ethidium to permeate the membranes. However, ethidium fluorescence from neurons gradually augmented after application of digitonin, although the fluo-3 fluorescence of neurons was transiently attenuated, indicating that it took some time to stain neurons with ethidium.

The result obtained from the fluorescence cytograms of fluo-3 fluorescence versus ethidium fluorescence seems to indicate that the cell suspension consists of neurons having intact membranes (Part a of Fig. 1B, live neurons), neurons having compromised membranes (Part b of Fig. 1B, presumably damaged neurons) and dead neurons (Part c of Fig. 1B). Therefore, it is likely that simultaneous measurements of fluo-3 and ethidium fluorochromes are useful for estimating the membrane permeability of organic molecules and/or the membrane integrity in brain neurons affected by cytotoxic substances, simultaneously with estimating cell viability and [Ca²⁺], of live neurons.

**Effect of AβP(25–35) on fluorescence cytograms of brain neurons**

Figure 4 shows the effect of 30 μM AβP(25–35) on the fluorescence cytogram obtained from 5000 neurons. AβP(25–35) greatly increased the number of neurons that were stained with ethidium in a time-dependent manner, indicating an increased number of damaged and dead neurons, although AβP(25–35) increased the intensity of fluo-3 fluorescence, indicating an increased [Ca²⁺], in some live neurons. Figures 5 and 6 show the time- and dose-dependent effects of AβP(25–35) on the cell population. AβP(25–35) time-dependently increased the numbers of dead and damaged neurons and decreased the number of live neurons (Fig. 5). The effect of AβP(25–35) reached a steady-state level within 30 min after application. Therefore, dose-dependent effects of AβP(25–35) on the fluorescence cytogram were examined at 30 min after application. AβP(25–35) at 0.3 μM did not exert any action on the fluorescence cytogram. Further increases in the concentration (up to 30 μM) of AβP(25–35) produced a dose-dependent decrease in the number of live neurons and an increase in the number of dead and damaged neurons. Dose-dependent changes in cell populations of dead, damaged and live neurons are

![Fig. 3. Effects of ionomycin on the mean intensity of fluo-3 fluorescence of the respective cell populations (parts a, b and c). Each column and bar indicate average of the mean intensity and S.D. of four experiments. Asterisk (*) shows a significant change (P<0.05) vs the control.](image)

![Fig. 4. Effects of 30 μM AβP(25–35) on the fluorescence cytogram, fluo-3 fluorescence (abscissa) versus ethidium fluorescence (ordinate), obtained from a programmed number of neurons (5000 cells). Cytograms were obtained before (left panel, Control) and 1 min, 3 min and 10 min after application of 30 μM AβP(25–35) (right three panels, AβP(25–35): 1 min, 3 min and 10 min, respectively).](image)
shown in Fig. 6. In the case of a continued application of 2.5 μM ionomycin, the number of live neurons also decreased from the control level (85.2±6.1%) to 68.8±6.7% (mean±S.D. of six experiments) during the 30-min period after application.

**Effect of AβP(25–35) on the [Ca^{2+}]_i of live neurons**

As shown in Fig. 4, the [Ca^{2+}]_i in some of the live neurons was increased by application of AβP(25–35) (11). Therefore, we further examined the effect of AβP(25–35) on the [Ca^{2+}]_i of live neurons. Fluo-3 fluorescence was obtained only from live neurons. Superimposed histograms were respectively obtained before (Control) and 1 min, 3 min and 5 min after application of 30 μM AβP(25–35). The bar under the fluo-3 fluorescence histogram indicates the region for high [Ca^{2+}]_i, described in the text and Fig. 8.

**Fig. 6.** Dose-dependent changes, induced by AβP(25–35), in the populations of live (open squares), damaged (open circles) and dead (filled circles) neurons. Dotted lines indicate the respective control level. Each symbol and bar show the mean percentage and S.D. of four experiments. Asterisks show significant changes (*P<0.05, **P<0.01 and ***P<0.005) vs respective control.

**Fig. 7.** Effects of 30 μM AβP(25–35) on the histogram of fluo-3 fluorescence obtained only from live neurons. Superimposed histograms were respectively obtained before (Control) and 1 min, 3 min and 5 min after application of 30 μM AβP(25–35). The bar under the fluo-3 fluorescence histogram indicates the region for high [Ca^{2+}]_i, described in the text and Fig. 8.

on the [Ca^{2+}]_i of live neurons. Fluo-3 fluorescence was obtained from only the neurons that were not stained with ethidium (live neurons). As shown in Fig. 7, when 30 μM AβP(25–35) was applied to live neurons, there were two peaks in the histogram of fluo-3 fluorescence, while there was a single peak in the control histogram. The calculated...
peak $[\text{Ca}^{2+}]_i$, in the control histogram was 169±48 nM (mean ± S.D. in four experiments). The peak $[\text{Ca}^{2+}]_i$ of neurons with higher intensity of fluo-3 fluorescence in the presence of 30 μM AzP(25–35) was not calculated because the intensity of fluo-3 fluorescence was similar to the maximum intensity (saturation). However, the peak $[\text{Ca}^{2+}]_i$ in all cases was greater than 10 μM at 1 min after application of 30 μM AzP(25–35). The number of neurons with higher $[\text{Ca}^{2+}]_i$ decreased time-dependently after application of AzP(25–35), although the number of neurons with normal $[\text{Ca}^{2+}]_i$ remained constant (Fig. 8). The result suggests that the decrease in the number of live neurons with higher $[\text{Ca}^{2+}]_i$ is due to a time-dependent increase in the number of damaged and dead neurons. In the case of 2.5 μM ionomycin, the agent increased the intensity of fluo-3 fluorescence in almost all live neurons (10, 11, also see Fig. 1B) and also produced saturation of fluo-3 fluorescence in the fluorescence histogram (11).

Effect of AzP(25–35) on fluo-3 and ethidium fluorescences of individual brain neurons

Since the results described above were obtained from a cell population consisting of 5000 neurons, AzP(25–35)-induced changes in membrane permeability of individual neurons were unknown. Therefore, we have examined the effect of 30 μM AzP(25–35) on individual neurons. The typical change in fluo-3 and ethidium fluorescences obtained from a single neuron is shown in Fig. 9. Although the intensity of fluo-3 fluorescence greatly increased immediately after the application of AzP(25–35), the fluo-3 fluorescence of the neurons rapidly attenuated. Furthermore, after the start of the attenuation of fluo-3 fluorescence, the neuron could be stained with ethidium. Therefore, the result obtained from the individual neuron confirms those obtained from a large cell population.

DISCUSSION

Dual measurement of fluo-3 and ethidium fluorescences as an useful tool for estimating cytotoxic action on brain neurons

There are many benefits from dual measurement of fluo-3 and ethidium fluorescences. Fluo-3-AM is a membrane-permeable esterase substrate that is converted by an intracellular esterase to fluo-3 that is retained by neurons with intact membranes. Fluo-3 is fluorescent when it binds to intracellular $\text{Ca}^{2+}$ (9). Therefore, the fluo-3 fluorescence can be also used to determine live neurons. In addition, the leakage of fluo-3 from neurons, resulting in an attenuation of fluo-3 fluorescence, is used to determine one aspect of cytotoxicities since neurons with compromised membranes cannot retain fluo-3. In this study, fluo-3 fluorescence was utilized in a combination with ethidium fluorescence because ethidium is highly impermeant to membranes of intact neurons (12). Thus, ethidium is permeant following membrane disruption, resulting in augmentation of ethidium fluorescence. Alternatively, when the membranes are compromised by cytotoxic substances fluo-3 passes cell membrane, going from the inside to the outside, while ethidium does so from the outside to the inside. Therefore, neurons with membranes compromised by cytotoxic substances are expected to exhibit simultaneously attenuation of fluo-3 fluorescence and augmentation of ethidium fluorescence. In fact, as shown in Figs. 1 and 2, the treatment of neurons with digitonin greatly increased the number of neurons that were predominantly stained with ethidium and decreased the number of those stained with fluo-3. Furthermore, dual measurement of fluo-3 and ethidium fluorescences is very suitable for monitoring $[\text{Ca}^{2+}]_i$ of neurons affected by cytotoxic substances because it enables us to determine $[\text{Ca}^{2+}]_i$ of neurons that retain sufficient fluo-3 by neglecting neurons stained with ethidium from the total cell populations examined (Fig. 7). Neurons stained with ethidium lose fluo-3, resulting in a decreased intensity of fluo-3 fluorescence that leads to underestimation of $[\text{Ca}^{2+}]_i$.

Actions of AzP(25–35) on brain neurons

Electrophysiological studies have shown that AzP(25–35) increases non-selectively membrane conductance in frog ganglion neurons (6) and rat brain neurons (7). If so, it would increase the $[\text{Ca}^{2+}]_i$ in brain neurons because $\text{Ca}^{2+}$ has the largest ionic gradient across the membrane. Also in this study, 30 μM AzP(25–35) greatly
increased the \([\text{Ca}^{2+}]\) in some, but not all, of the live neurons (Fig. 7). Therefore, \(\text{A}\beta\text{P}(25-35)\) is suggested to induce a destabilized intracellular \([\text{Ca}^{2+}]\) homeostasis that leads to neuronal death \((4, 8)\). However, it is likely that \(\text{A}\beta\text{P}(25-35)\) increases not only the membrane permeability of inorganic ions such as \([\text{Ca}^{2+}], \text{Na}^+\) and \([\text{K}^+]\) \((6, 7)\) but also that of organic molecules. As shown in Figs. 4 and 9, \(\text{A}\beta\text{P}(25-35)\) induced both the leakage of fluo-3 from neurons and permeation of ethidium across the membrane, although both dyes are highly impermeant to the intact plasma membrane. One may argue the possibility that an initial increase in \([\text{Ca}^{2+}]\), induced by \(\text{A}\beta\text{P}(25-35)\) triggers compromising of the membrane, resulting in an increase in membrane permeability of organic molecules. However, this may be unlikely because of the following observations: First, the increase in the number of neurons stained with ethidium after application of 2.5 \(\mu\text{M}\) ionomycin, a \(\text{Ca}^{2+}\) ionophore, was less than that after the application of 30 \(\mu\text{M}\) \(\text{A}\beta\text{P}(25-35)\), although ionomycin increased \([\text{Ca}^{2+}]\), to a greater degree than \(\text{A}\beta\text{P}(25-35)\). Secondly, \(\text{A}\beta\text{P}(25-35)\) also exerted a similar cytotoxic action on neurons that were treated with \(\text{BAPTA-AM}\), a chelator for intracellular \([\text{Ca}^{2+}]\) \((\text{Oyama et al.}, \text{ unpublished observation})\). Therefore, \(\text{A}\beta\text{P}(25-35)\) may also exert cytotoxic action through a mechanism unrelated to an increased \([\text{Ca}^{2+}]\). One may argue that the increase in \([\text{Ca}^{2+}]\), induced by \(\text{A}\beta\text{P}(25-35)\) is responsible for increasing membrane permeability because augmentation of fluo-3 fluorescence (increase in \([\text{Ca}^{2+}]\)) preceded the increase in permeation of ethidium across the membrane (Figs. 8 and 9). At present, we can not rule out this possibility. However, it is noted that it took some time to stain neurons with ethidium when the neurons were treated with 50 \(\mu\text{M}\) digitonin which produced a non-specific increase in membrane permeability immediately after application. Therefore, the time required for staining neurons with ethidium should be considered before reaching a final conclusion.

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