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Amyloid β-Peptide Impairs Ion-Motive ATPase Activities: Evidence for a Role in Loss of Neuronal Ca^{2+} Homeostasis and Cell Death

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The amyloid β-peptide (Aβ) that accumulates as insoluble plaques in the brain in Alzheimer's disease can be directly neurotoxic and can increase neuronal vulnerability to excitotoxic insults. The mechanism of Aβ toxicity is unclear but is believed to involve generation of reactive oxygen species (ROS) and loss of calcium homeostasis. We now report that exposure of cultured rat hippocampal neurons to Aβ1-40 or Aβ25-35 causes a selective reduction in Na^+/K^+-ATPase activity which precedes loss of calcium homeostasis and cell degeneration. Na^+/K^+-ATPase activity was reduced within 30 min of exposure to Aβ25-35 and declined to less than 40% of basal level by 3 hr. Aβ did not impair other Mg^{2+}-dependent ATPase activities or Na^+/Ca^{2+} exchange. Experiments with ouabain, a specific inhibitor of the Na^+/K^+-ATPase, demonstrated that impairment of this enzyme was sufficient to induce an elevation of $[Ca^{2+}]_i$ and neuronal injury. Impairment of Na^+/K^+-ATPase activity appeared to be causally involved in the elevation of $[Ca^{2+}]_i$ and neurotoxicity since suppression of Na^+ influx significantly reduced Aβ- and ouabain-induced $[Ca^{2+}]_i$ elevation and neuronal death. Neuronal degeneration induced by ouabain appeared to be of an apoptotic form as indicated by nuclear condensation and DNA fragmentation. The antioxidant free radical scavengers vitamin E and propylgallate significantly attenuated Aβ-induced impairment of Na^+/K^+-ATPase activity, elevation of $[Ca^{2+}]_i$ and neurotoxicity, suggesting a role for ROS. Finally, exposure of synaptosomes from postmortem human hippocampus to Aβ resulted in a significant and specific reduction in Na^+/K^+-ATPase and Ca^{2+}-ATPase activities, without affecting other Mg^{2+}-dependent ATPase activities or Na^+/Ca^{2+} exchange. These data suggest that impairment of ion-motive ATPases may play a role in the pathogenesis of neuronal injury in Alzheimer's disease.

[Key words: Alzheimer's disease, antioxidants, calcium-ATPase, free radicals, hippocampus, Na^+/K^+-ATPase, ouabain, reactive oxygen species, sodium–calcium exchange, synaptic membrane]

Although the cause of neuronal degeneration in Alzheimer's disease (AD) and other age-associated neurodegenerative disorders is not clear, increasing data implicate metabolic impairment, accumulation of ROS, and excitotoxicity (see Beal, 1992; Ames et al., 1993; Mattson et al., 1993a, for review). The major component of plaques in AD brain is the 40–42 amino acid amyloid β-protein (Aβ) which arises from the larger β-amyloid precursor protein (βAPP; see Selkoe, 1993, for review). In addition to the presence of Aβ in AD brain, its association with neurofibrillary pathology, and linkage of βAPP mutations to the disease, experimental data suggest that Aβ may contribute to the neurodegenerative process. Aβ can be toxic to cultured neurons and increases their vulnerability to excitotoxic and metabolic insults (Koh et al., 1990; Yankner et al., 1990; Mattson et al., 1992, 1993b). The neurotoxic activity of Aβ is known to be related to its ability to form insoluble aggregates (Pike et al., 1991; Busciglio et al., 1992; Mattson et al., 1993b; Pike et al., 1993) which accumulate on and/or in the plasma membrane (Mattson et al., 1993b). The mechanism of Aβ toxicity apparently involves dysregulation of cellular calcium homeostasis resulting in increased $[Ca^{2+}]_i$ and markedly increased $[Ca^{2+}]_i$ responses to excitatory amino acids and membrane depolarization (Mattson et al., 1992, 1993b; Hartmann et al., 1993). Neurons dying as the result of exposure to Aβ manifest key features of apoptosis including cell shrinkage, nuclear condensation, DNA fragmentation, and cell surface blebbing (Forloni et al., 1993; Loo et al., 1993; Cotman et al., 1994; Thompson, 1995).

Accumulation of ROS is implicated in the mechanism of Aβ neurotoxicity. Aβ induced peroxide accumulation in cultured neuroblastoma cells and primary hippocampal neurons and antioxidants protected against Aβ toxicity (Behl et al., 1994; Goodman and Mattson, 1994a; Goodman et al., 1994). In addition, Aβ induced lipid peroxidation in synaptosomes (Butterfield et al., 1994) and cultured cortical cells (Behl et al., 1994). Induction of ROS in neurons by Aβ may result from the free radical-generating capacity of the peptide itself (Hensley et al., 1994) or may be secondary to calcium influx (Mattson et al., 1995a). In light of these data and because antioxidants attenuated Aβ-induced elevation of $[Ca^{2+}]_i$ in cultured hippocampal neurons (Goodman and Mattson, 1994a; Goodman et al., 1994) we tested the hypothesis that Aβ impairs, by an oxidative mechanism, the function of plasma membrane proteins involved in regulation of ion homeostasis.

Three different protein complexes in the plasma membrane that play key roles in regulation of $[Ca^{2+}]_i$ are the Na^+/K^+-ATPase, the Ca^{2+}-ATPase and the Na^+/Ca^{2+} exchanger. The Na^+/K^+-ATPase is critically important in osmotic balance and cell vol-

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ume maintenance as well as in the maintenance of rest membrane potential and restoration of membrane potential following depolarization (see Stahl, 1986; Sweadner, 1989, 1991, for review). Ouabain, a selective inhibitor of Na^+/K^+-ATPase, causes alterations in neuronal ion homeostasis similar to those observed in excitotoxic paradigms (Smith et al., 1984; Mayer and Westbrook, 1987; Brines et al., 1993). The plasma membrane Ca^{2+}-ATPase is primarily responsible for maintaining rest $[Ca^{2+}]_i$, while the Na^+/Ca^{2+} exchanger provides a mechanism for rapidly removing Ca^{2+} following stimulation but can reverse direction with membrane depolarization. We report herein that Aβ markedly impairs Na^+/K^+-ATPase activity in cultured rat hippocampal neurons and compromises both the Na^+/K^+-ATPase and Ca^{2+}-ATPase in synaptosomes from postmortem human hippocampus. Impairment of these ion-motive ATPases appears to be a key step in the cell death process.

Materials and Methods

Cell culture. Primary hippocampal cell cultures were established from embryonic rats (day 18 of gestation) as detailed elsewhere (Mattson et al., 1995b). Cells were plated into polyethyleneimine-coated plastic or glass bottomed culture dishes at a density of 70–120/mm². The cultures were maintained in Eagle's Minimum Essential Medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (GIBCO), 20 mM KCl and 1 mM pyruvate. The atmosphere consisted of 6% CO_2, 94% room air and was maintained near saturation with water. Experiments were performed in cultures that had been maintained for 6–10 d. Using these culture conditions, approximately 80–90% of the cells are neurons and the remaining cells are astrocytes as judged by characteristic morphology and differential immunoreactivity with antibodies to neuron-specific (neurofilament, MAP2 and tau) and astrocyte-specific (glial fibrillary acidic protein and S-100β) proteins (Mattson et al., 1993b, 1995b).

Experimental treatments and quantification of neuronal survival. Synthetic Aβ25-35 was purchased from Bachem (lot #ZJ744), and Aβ1-40 (lot #ZK600) was a generous gift from Athena Neurosciences Inc. In preliminary studies we determined that both of these peptides exhibited rapid aggregation kinetics and therefore did not require "aging" prior to addition to cell cultures (cf., Mattson et al., 1993b; Pike et al., 1993). Peptides were stored lyophilized, and 1 mM stock solutions were prepared in sterile deionized water immediately prior to use. Ouabain and tetrodotoxin (Sigma) were prepared as 500–1000× stocks in saline. In some experiments, cells were incubated in Locke's solution which contained (in mM): NaCl 154; KCl, 5.6; $CaCl_2$, 2.3; $MgCl_2$, 1.0; $NaHCO_3$, 3.6; glucose, 10; Hepes buffer, 5 (pH 7.2). Na^+-deficient medium was prepared by complete replacement of NaCl with LiCl (pH 7.2). Ca^{2+}-deficient medium was prepared without added calcium. Cell survival was quantified by counting the number of viable neurons in premarked microscope fields prior to, and at indicated time points following exposure to experimental treatments as described previously (Mattson et al., 1992; 1995). Many neurons that died during the experimental period were not present at the end point. Remaining neurons were considered nonviable if their neurites were beaded and/or fragmented and if the soma was rough, swollen, vacuolated, and irregular in shape. Viable neurons had neurites that were smooth in appearance and cell bodies that were smooth and round or oval in shape.

Membrane preparation. Following experimental treatment cells were washed twice with PBS, and 300 μl of cell lysis solution (20 mM imidazole, 0.6 mM EGTA, 0.1 mM PMSF) was added per 60 mm dish. Cells were scraped, and the lysate was homogenized using a dounce homogenizer (8–10 strokes). Homogenate was centrifuged for 5 min at 1500 × *g*, and the supernate was was then centrifuged for 45 min at 120,000 × *g* (4°C). The pellet was suspended in a buffer consisting of 40 mM histidine and 40 mM imidazole (pH 7.1). Protein concentration was determined using a Pierce BCA kit.

ATPase activity assay. Membrane ATPase activities were assayed by a method adapted from Rohn et al. (1993). The method allows quantification of three distinct Mg^{2+}-dependent ATPase activities (Na^+/K^+-ATPase activity, Ca^{2+}-ATPase activity, and ouabain/Ca^{2+}-insensitive ATPase activity) in the same sample. Activities were measured in quadruplicate in covered 96 well microtiter plates at 37°C on a shaker. Ninety microliters of assay buffer (18 mM histidine, 18 mM imidazole, 80 mM NaCl, 15 mM KCl, 3 mM $MgCl_2$, and 0.1 mM EGTA, pH 7.1.) containing 2 μg of membrane protein was added to each well. The Na^+/K^+-ATPase activity was determined by subtracting the ouabain (0.2 mM) sensitive activity from the overall Mg^{2+}-ATPase activity level. The Ca^{2+}-ATPase activity was determined by subtracting activity measured in the presence of Ca^{2+} and ouabain from that determined in the absence of Ca^{2+} (no added Ca^{2+} plus 0.1 mM EGTA) and the presence of ouabain. The plate was preincubated at 37°C for 10 min, and the assay was started with the addition of 10 μl of ATP (final concentration 3 mM) making the final reaction volume 100 μl. After 60 min, the reaction was terminated by the addition of 25 μl of 5% SDS. The level of inorganic phosphate present, quantified using the colorimetric method of Fiske and Subbarow (1925), was used as a measure of ATPase activity. The plates were read on the Bio-tek EL-340 plate reader at 630 nm. The absorbance values obtained were converted to activity values by linear regression using a standard curve of sodium monobasic phosphate that was included in the assay procedure. Values reported represent the mean and SD of at least three separate experiments.

Na^+/Ca^{2+} exchange assay. The activity of the Na^+/Ca^{2+} exchanger was measured by the method of Michaelis et al. (1992). Following treatment, cells were washed with PBS then scraped in a sodium phosphate (150 mM) solution, transferred to 15 ml conical tubes and stored at 4°C for 12–16 hr. This allowed the membranes to vesicularize and to load with Na^+. To begin the reaction, 50 μg of vesicle protein was added to a tube containing 1 ml of either gradient or nongradient solution prewarmed to 37°C in a water bath. The gradient solution consisted of 160 M KCl, 10 M NH_4OH, 10 μM $CaCl_2$, and 100,000 cpm $^{45}Ca^{2+}$ (pH 9.5). The nongradient solution consisted of 160 M NaCl, 10 M NH_4OH, $CaCl_2$ (10 μM), and 100,000 cpm $^{45}Ca^{2+}$ (pH 9.5). The assay was allowed to procede for 5–60 sec and was stopped with 2 ml of ice cold stop solution which consisted of 160 M KCl, 1 M EDTA, and 10 M Tris (pH 7.4). The mixture was filtered through a Whatman GF/C filter and washed with an additional 2 ml of stop solution. The filter was air dried and counted in a scintillation counter. Gradient and nongradient samples were done in triplicate. All chemicals were purchased from Sigma (St. Louis). ^{45}Ca was purchased from New England Nuclear.

Measurement of intracellular free calcium levels. Ratiometric imaging with the calcium indicator dye fura-2 AM (from Molecular Probes) was performed as previously described (Mattson et al., 1992, 1995b). Cells were incubated at 37°C for 30 min with 2μM fura-2 AM, followed by two washes with culture medium. Cells were then incubated in the normal culture medium containing experimental treatments (for up to 3 hr), and then switched to Hank's Balanced Salt Solution supplemented with 10 M glucose and 10 M Hepes (pH 7.2) and were imaged within 20 min. Parallel control cultures (receiving vehicle) were imaged at the 30 min and 3 hr time points following loading with fura-2 and exhibited no change in rest $[Ca^{2+}]_i$ during this time period. In additional preliminary experiments we found that basal $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ responses to glutamate were unchanged in neurons incubated in HBSS for at least 3 hr. In contrast to other cell types which can lose considerable fura-2 over periods of several hr (e.g., neuroblastoma cells and astrocytes) primary hippocampal neurons retain fura-2 for at least 6 hr and calibrations were unchanged during this time period. Cells were imaged using a Zeiss Attoflour system which included a Zeiss Axiovert microscope with a 40× oil objective, and an Attofluor intensified CCD camera. The dye was excited at 334 nm and 380 nm, and images were taken of the emitted fluorescence after excitation at each wavelength. The method of Grynkiewicz et al. (1985) was used to calibrate the system and quantify $[Ca^{2+}]_i$.

Preparation of synaptosomes from postmortem human hippocampus. Synaptosomes were prepared as describe previously (Butterfield et al., 1994). Briefly, hippocampal slices were obtained from four neurologically normal individuals (age range of 79–85 years) with postmortem intervals from 2 to 5 hr. Tissue was homogenized in a 0.32 M sucrose solution and synaptosomes were isolated by ultracentrifugation through a sucrose gradient. Purified synaptosomes were either assayed immediately for ATPase activity, or incubated for 8 hr in a 150 M sodium phosphate solution for Na^+/Ca^{2+} exchange activity assay.

Methods for evaluation of nuclear condensation and DNA fragmentation. For staining with Hoescht dye (Hoescht 33342; Molecular Probes), cells were fixed for 30 min in a solution containing 4% paraformaldehyde in PBS. Cells were then exposed to 1 μg/ml of the dye for 30 min at 37°C. Cultures were washed twice with PBS and twice with water and covered with Vectashield (Vector Labs, Bloomfield,

CA). Fluorescence was imaged with a 40× oil immersion lens using an inverted microscope (Nikon). The dye was excited at 340 nm and emission was filtered with a 510 nm barrier filter. Photomicrographs were taken using a 35 mm Nikon camera and Kodak T-Max film. For staining with ethidium bromide homodimer (Molecular Probes), cells were incubated for 50 min in the presence of 2 μM of the dye. The cells were then washed three times in HBSS and imaged on a confocal laser scanning microscope (Molecular Dynamics, Sarastro 2000) coupled to an inverted microscope (Nikon). The dye was excited at 488 nm and emission was filtered with a 510 nm barrier filter.

Results

Aβ is neurotoxic and selectively impairs Na^+/K^+-ATPase activity

Cultures were exposed to 50 μM Aβ25-35 or 20 μM Aβ1-40 and neuronal survival was monitored during a 6 d exposure period (Fig. 1*A*). Each Aβ caused a progressive reduction in neuronal survival. Neuronal survival was reduced to approximately 50% of control levels within 4 d of exposure to Aβ25-35 and Aβ1-40. Neurons were killed more rapidly by Aβ25-35 (significant reduction in survival within 12 hr of exposure) compared to Aβ1-40 (significant reduction in neuronal survival within 24–48 hr of exposure) consistent with more rapid aggregation (Pike et al., 1993) and free radical-generating (Hensley et al., 1994) kinetics of Aβ25-35. These results are in agreement with previously published data in cultured embryonic rat hippocampal and neocortical neurons (Yankner et al., 1990; Pike et al., 1991; Mattson et al., 1993b; Pike et al., 1993).

In preliminary studies we found that cultured embryonic hippocampal cells exhibit a relatively high level of Na^+/K^+-ATPase activity as well as ouabain- and Ca^{2+}-insensitive Mg^{2+}-dependent ATPase activities. However, the basal level of Ca^{2+}-ATPase activity was quite low (typically less than 10% of the total Mg^{2+}-dependent ATPase activity) and we could therefore not reliably quantify its activity. In the cultured cells, we therefore focused on examining the effects of Aβ on Na^+/K^+-ATPase and Na^+/Ca^{2+} exchange activities. We were, however, able to examine the effects of Aβ on Ca^{2+}-ATPase activity in synaptosomes from adult human hippocampus (see below). Measurement of Na^+/K^+-ATPase activities in cultures exposed to Aβ25-35 or Aβ1-40 revealed a relatively rapid and progressive reduction in Na^+/K^+-ATPase activity (Fig. 1*B*). The basal level of Na^+/K^+-ATPase activity was consistently between 30 and 35 nmol inorganic phosphate released/mg protein/min. The Na^+/K^+-ATPase activity was reduced to less than 80% of basal levels within 30 min of exposure to Aβ25-35, with a further reduction to less than 50% of basal levels during a 3 hr exposure period. Longer exposures to Aβ25-35 (6–10 hr) resulted in a leveling off of Na^+/K^+-ATPase activity at aproximately 45% of basal levels. Exposure of cells to Aβ1-40 resulted in a decrease in Na^+/K^+-ATPase activity to approximately 73% of basal levels within 12 hr of exposure (Fig. 1*B*). The rate of decline in Na^+/K^+-ATPase activity was considerably slower in cultures exposed to Aβ1-40 compared to that in cultures exposed to Aβ25-35. The impairment of Na^+/K^+-ATPase activity clearly preceded neuronal degeneration by many hours to days (compare time courses shown in Fig. 1*A* and 1*B*). The slower time course of inactivation of Na^+/K^+-ATPase by Aβ1-40 compared to Aβ25-35 is consistent with the somewhat slower time course of neurotoxicity of Aβ1-40 (Fig. 1*A*). A control peptide (50 μM) with the same amino acid composition as Aβ25-35, but with a scrambled sequence (NH_2-IMLKGNGASIG-COOH; see Mattson et al., 1992), had no significant effect on Na^+/K^+-ATPase activity during 4 and 10 hr exposure periods (Fig. 1*B*). Another control peptide with an amino acid sequence that is the reverse of Aβ1-40 (Aβ40-1), at concentrations of 20–50 μM, did not affect either Na^+/K^+-ATPase activity or cell survival (data not shown; cf. Goodman and Mattson, 1994a).

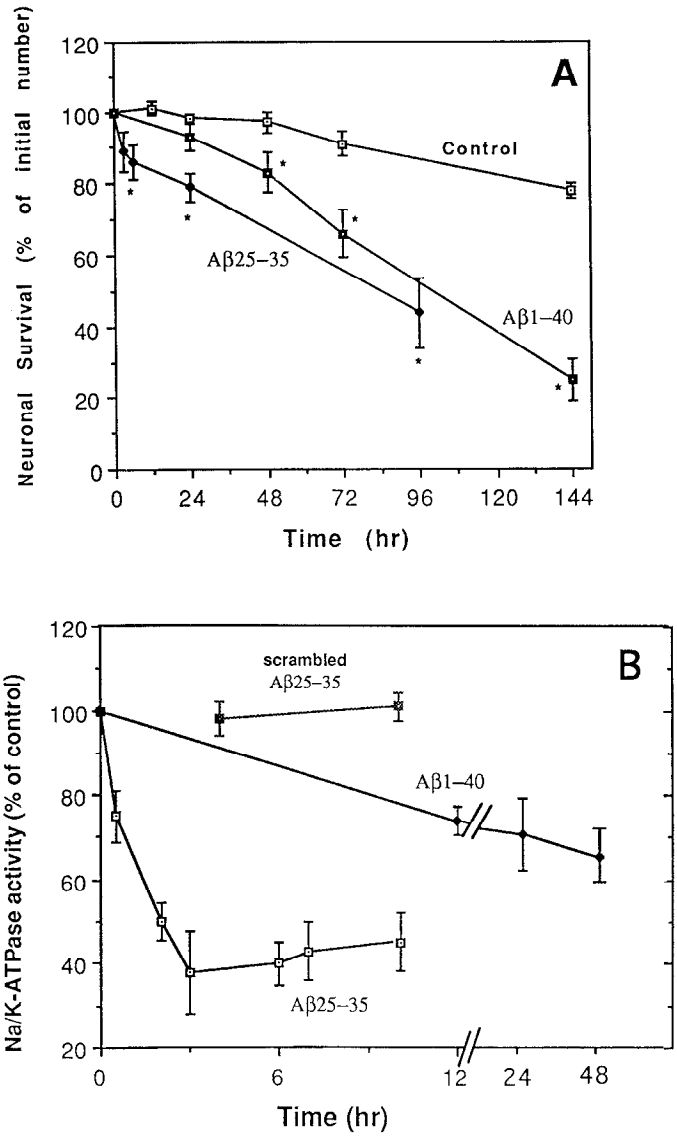


Figure 1. Aβ induces an impairment of Na^+/K^+-ATPase activity which precedes neuronal degeneration. *A*, Cultures were exposed to vehicle (*Control*), 50 μM Aβ25-35 or 20 μM Aβ1-40 and neuronal survival was determined at the indicated time points. Values represent the mean ± SD of determinations made in four separate cultures with 200–300 cells counted per culture. *, $p < 0.01$ compared to corresponding values in Control cultures. (ANOVA with Scheffe's post-hoc test for pairwise comparisons). *B*, Cultures were exposed to vehicle (Control), 50 μM Aβ25-35, 50 μM scrambled Aβ25-35, or 20 μM Aβ1-40, and Na^+/K^+-ATPase activity was determined at the indicated time points. Values represent the mean ± SD of determinations made in three separate experiments. Control values were 32–35 nmol Pi liberated/mg protein/minute. Values in Aβ-treated cultures were significantly less than the control value at the 0.5, 2, 3, 6, 7, and 10 hr time points for Aβ25-35 ($p < 0.01$) and at the 12, 24, and 48 hr time points for Aβ1-40 ($p < 0.01$).

The effects of Aβ on Na^+/K^+-ATPase activity were concentration dependent. With increasing concentrations of Aβ25-35 (5 μM, 50 μM, and 200 μM) Na^+/K^+-ATPase activity was progressively reduced to less than 50% of basal levels during a 3 hr

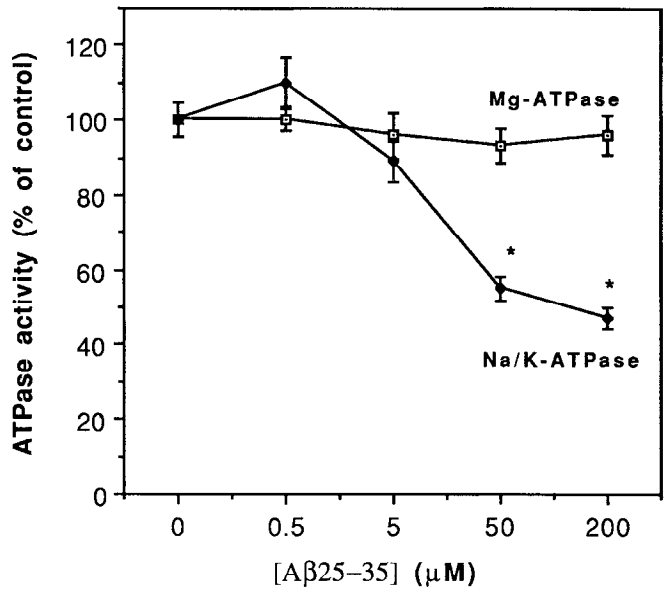
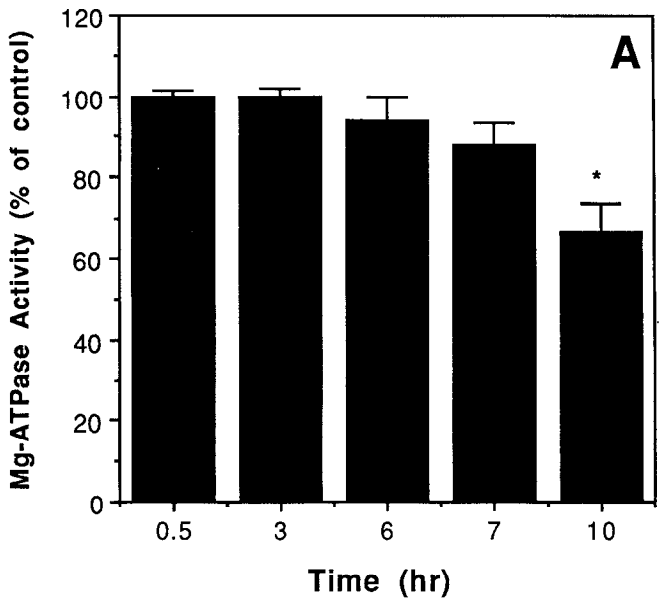


Figure 2. Aβ causes a concentration-dependent reduction in Na^+/K^+-ATPase activity. Cultures were exposed to the indicated concentrations of Aβ25-35 for 3 hr, at which time either Na^+/K^+-ATPase or ouabain-insensitive Mg^{2+}-ATPase (Mg^{2+}-ATPase) activities were determined. Values represent the mean ± SD of determinations made in three separate experiments. *, $p < 0.01$ compared to basal level of activity.

exposure period (Fig. 2). Exposure of cultures to 50 μM Aβ25-35 for up to 7 hr had no significant effect on ouabain-insensitive Mg^{2+}-ATPase activity (Figs. 2, 3*A*). However, more prolonged incubations in the presence of Aβ did lead to a reduction in ouabain-insensitive Mg^{2+}-ATPase activity which reached statistical significance at the 10 hr time point (Fig. 3*A*). Na^+/Ca^{2+} exchange activity was unchanged during 3 and 10 hr exposure periods to 50 μM Aβ25-35 (Fig. 3*B*). Taken together, these data indicate that the Na^+/K^+-ATPase is particularly sensitive to impairment in primary neuronal cultures exposed to Aβ.

Impairment of Na^+/K^+-ATPase activity is sufficient to induce loss of $[Ca^{2+}]_i$ homeostasis and neuronal death

We previously reported that Aβ25-35 and Aβ1-40 induce a progressive elevation of $[Ca^{2+}]_i$ which precedes, and is required for, neuronal death (Mattson et al., 1992, 1993b). If impairment of Na^+/K^+-ATPase activity was mechanistically involved in Aβ neurotoxicity, then selective impairment of the Na^+/K^+-ATPase activity should result in elevation of $[Ca^{2+}]_i$ and neuronal death. Ouabain is a specific inhibitor of the Na^+/K^+-ATPase (Canessa et al., 1992). In preliminary studies we found that Na^+/K^+-ATPase activity was completely blocked with 0.2 M ouabain (data not shown). In order to examine the effect of inhibition of Na^+/K^+-ATPase activity on neuronal survival, cultures were exposed to increasing concentrations of ouabain (10 nM to 1 M) and neuronal survival was assessed at 4, 8, 24, and 50 hr (Fig. 4*A*). Ouabain induced a concentration-dependent decrease in neuronal survival. Survival was reduced to approximately 80%, 47%, and 39% of control levels in cultures exposed for 24 hr to 1, 10, and 1000 μM ouabain, respectively, with a further reduction in neuronal survival with continued incubation. Ouabain at concentrations of 1–10 μM inhibited Na^+/K^+-ATPase activity by 40–60% (data not shown), concentrations which caused a level of neurodegeneration similar to that induced by 50 μM Aβ25-35 (compare Fig. 1*A* to Fig. 4*A*).



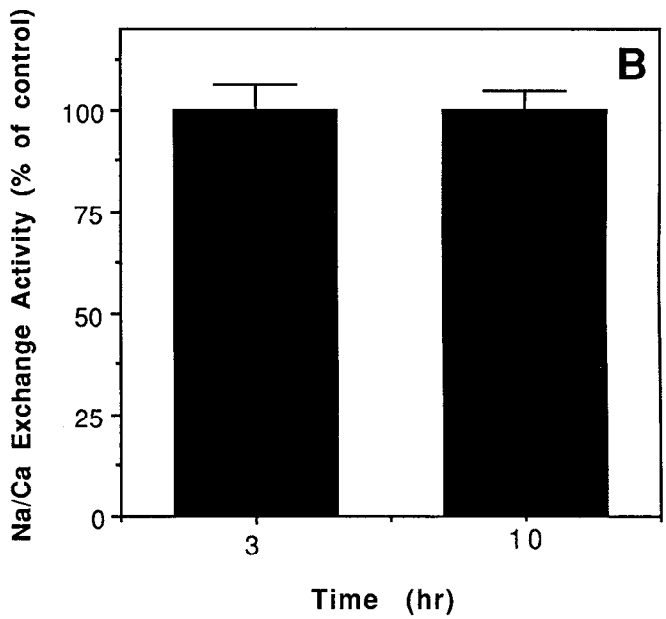
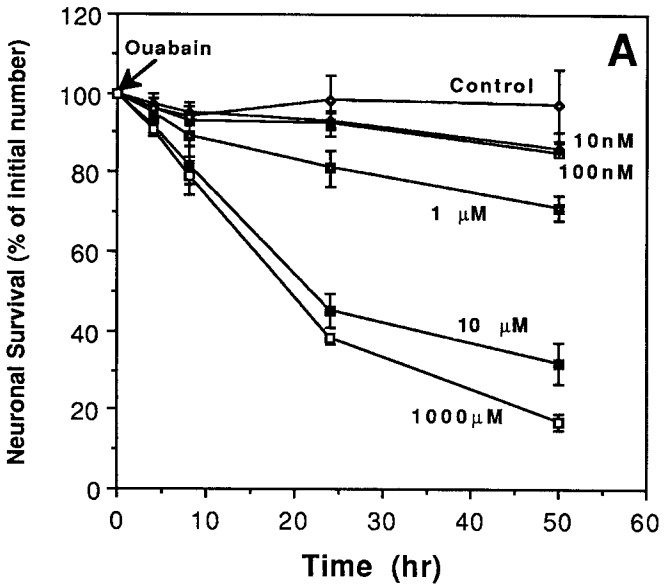


Figure 3. Lack of effect of neurotoxic concentrations of Aβ on ouabain-insensitive Mg^{2+}-ATPase activity and Na^+/Ca^{2+} exchange activity. Cultures were exposed to 50 μM Aβ25-35 for the indicated time periods, and ouabain-insensitive Mg^{2+}-ATPase activity (*A*) and Na^+/Ca^{2+} exchange activity (*B*) were quantified. Values represent the mean and SD of determinations made in three separate experiments. *, $p < 0.05$ compared to control value.

Measurement of $[Ca^{2+}]_i$ using the calcium indicator dye fura-2 revealed that ouabain caused a progressive elevation of $[Ca^{2+}]_i$ which preceded neuronal degeneration. Within 30 min of exposure to 0.2 M ouabain the $[Ca^{2+}]_i$ was elevated to 169% of control levels (Fig. 4*B*). Two approaches were employed to determine whether Na^+ influx was required for elevation of $[Ca^{2+}]_i$ induced by ouabain. One approach involved incubation in Na^+-deficient medium in which Na^+ was replaced with Li^+. Although Li^+ may influence certain inositol phospholipid signaling cascades (Jope and Williams, 1994), it can substitute for Na^+ in the



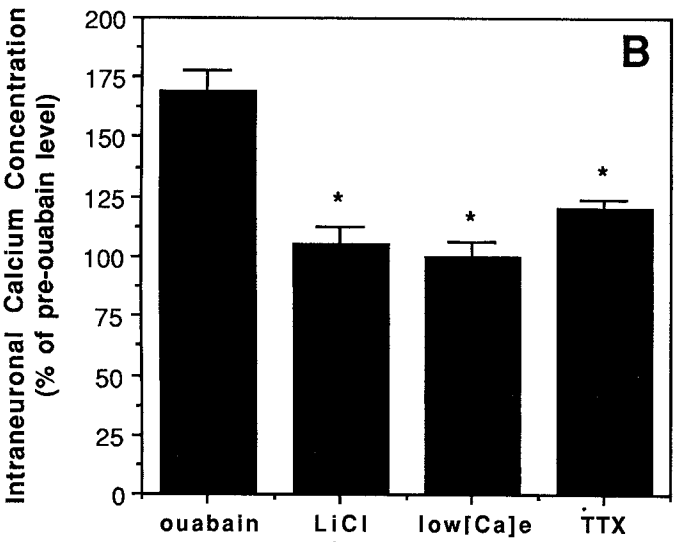


Figure 4. Ouabain is neurotoxic and induces a Na^+-dependent elevation of $[Ca^{2+}]_i$. *A*, Cultures were left untreated (*Control*) or were exposed to the indicated concentrations of ouabain. Neuronal survival was determined at 3, 6, 24, and 50 hr following exposure to ouabain. Values represent the mean ± SD of determinations made in four separate cultures. *B*, Cultures were incubated in the indicated conditions and then exposed to 0.2 mM ouabain. Ouabain, control medium containing 154 mM Na^+ and 2 mM Ca^{2+}; LiCl + ouabain, medium in which Na^+ was replaced with Li^+ (equimolar LiCl); low $[Ca]_e$ + ouabain, medium which lacked added Ca^{2+}; TTX + ouabain, medium containing 1 μM tetrodotoxin. Cells were exposed to LiCl medium, low $[Ca]_e$ medium, or TTX 30–60 min prior to calcium imaging. The $[Ca^{2+}]_i$ was determined immediately prior to, and 30 min following, exposure to ouabain. Values represent the mean and SD of three separate cultures (9–16 neurons examined/culture). The rest level of $[Ca^{2+}]_i$ (prior to exposure to ouabain) averaged 145 ± 6.4 nM.

Na^+/Ca^{2+} exchanger and therefore does not block that important mechanism of Ca^{2+} extrusion. The other approach involved exposing cultures to tetrodotoxin, a specific blocker of voltage-dependent Na^+ channels. Incubation in Na^+-deficient medium prevented ouabain-induced elevation of $[Ca^{2+}]_i$ as did 1 μM tetrodotoxin (Fig. 4*B*). Removal of extracellular calcium also significantly attenuated ouabain-induced elevation of $[Ca^{2+}]_i$, demonstrating that the involvement of influx of extracellular Ca^{2+} Taken together, the data indicate that ouabain-induced elevation of $[Ca^{2+}]_i$ was mediated by influx of Ca^{2+} which occurred secondary to Na^+ influx.

Evidence that influx of Na^+ is involved in loss of calcium homeostasis and neuronal degeneration resulting from Aβ-induced impairment of Na^+/K^+-ATPase activity

Exposure of cultures to Aβ25-35 resulted in a progressive elevation of $[Ca^{2+}]_i$ in neurons (Fig. 5*A*). The average rest $[Ca^{2+}]_i$ was approximately 210 nM and was essentially unchanged during a 30 min exposure period to 50 μM Aβ25-35. However, with continued exposure to Aβ25-35, the $[Ca^{2+}]_i$ rose to 450 nM and 640 nM by 1 and 3 hr, respectively. A significant reduction in Na^+/K^+-ATPase activity occurred within 30 min of exposure to Aβ25-35, a time point prior to elevation of $[Ca^{2+}]_i$ (Fig. 5*A*). In order to determine whether Na^+ influx was causally involved in Aβ toxicity, cultures were incubated in the presence of 1 μM tetrodotoxin. Neuronal survival was significantly reduced in cultures exposed to 50 μM Aβ25-35 for 72 hr (survival was 97 ± 4% in control cultures and 71 ± 5% in cultures exposed to Aβ; mean ± SD). Neuronal survival was significantly increased in cultures cotreated with 1 μM tetrodotoxin and 50 μM Aβ25-35 (88 ± 7% survival; $p < 0.05$ compared to cultures exposed to Aβ alone). Prolonged incubation (greater than 12 hr) in Na^+-deficient medium, or in medium lacking Ca^{2+}, resulted in progressive neuronal loss, and so the ability of these manipulations to modify Aβ toxicity could not be tested. However, the earlier elevation of $[Ca^{2+}]_i$ (3 hr following exposure to Aβ) was significantly attenuated in neurons incubated in Na^+-deficient medium or in the presence of 1 μM tetrodotoxin (Fig. 5*B*). Taken together, the data indicate that impairment of the sodium pump plays a role in the $[Ca^{2+}]_i$-destabilizing and neurotoxic actions of Aβ.

Ouabain induces nuclear condensation and DNA fragmentation

There have been several reports that Aβ induces an apoptotic form of death in neurons characterized by nuclear DNA fragmentation and condensation (Forloni et al., 1993; Loo et al., 1993; Cotman et al., 1994; Watt et al., 1994). To investigate whether impairment of the Na^+/K^+-ATPase was sufficient to induce apoptosis, we exposed hippocampal cultures to neurotoxic concentrations of ouabain and then visualized nuclear DNA using two different fluorescent probes. Confocal laser scanning microscope images of unfixed cells stained with ethidium bromide homodimer revealed nuclear condensation and DNA fragmentation in neurons damaged by ouabain (Fig. 6). In control cultures stained with Hoescht dye the neurons exhibited diffuse staining which filled the nucleus. In contrast, Hoescht staining in neurons exposed to ouabain revealed condensed and fragmented DNA (Fig. 6).

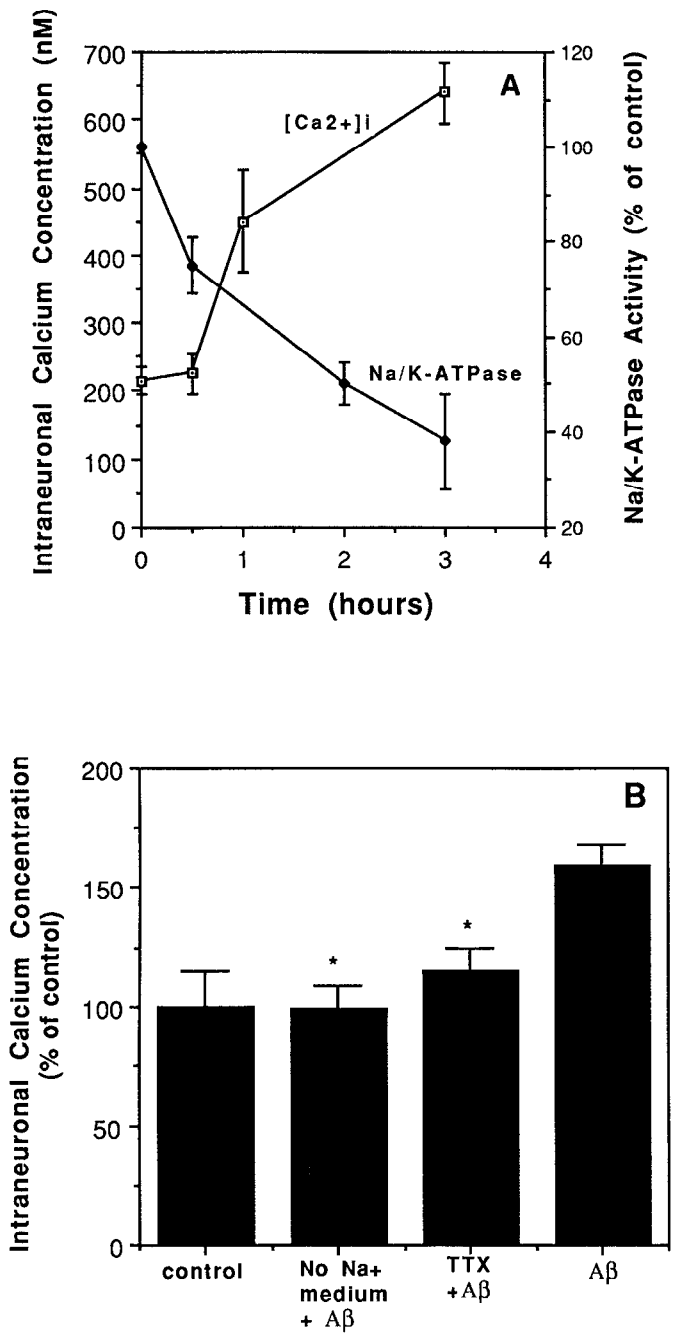


Figure 5. *A*, Impairment of Na^+/K^+-ATPase activity precedes elevation of $[Ca^{2+}]_i$ in neurons exposed to Aβ. Cultures were exposed to 50 μM Aβ25-35 and either Na^+/K^+-ATPase activity or $[Ca^{2+}]_i$ were quantified. For Na^+/K^+-ATPase activity, values represent the mean and SD of three separate experiments. For $[Ca^{2+}]_i$, values represent the mean and SD of determinations made from at least 60 neurons per time point. *B*, Sodium influx is necessary for the Aβ-induced rise in intracellular calcium. Cultures were pretreated with 1 μM TTX or were switched to medium in which Na^+ had been replaced with Li^+ prior to exposure to 50 μM Aβ25-35. $[Ca^{2+}]_i$ was quantified after 3 hr. Values represent the mean and SD of determinations made from four seperate cultures, measuring at least 60 neurons per culture. *, $p < 0.01$ compared to Aβ values.

Antioxidants attenuate Aβ-induced impairment of Na^+/K^+-ATPase activity, loss of calcium homeostasis and cell death

Previous studies of the mechanism of Aβ neurotoxicity suggested a role for ROS and membrane oxidation (Behl et al., 1994; Butterfield et al., 1994; Goodman and Mattson, 1994; Goodman et al., 1994). We therefore tested the hypothesis that ROS were involved in Aβ-induced impairment of Na^+/K^+-ATPase activity. Pretreatment of cultures with 50 μg/ml vitamin E or 5 μM propylgallate significantly attenuated the reduction in Na^+/K^+-ATPase activity caused by Aβ (Table 1). Vitamin E and propylgallate pretreatment also significantly attenuated Aβ-induced elevation of $[Ca^{2+}]_i$ and neurotoxicity (Table 1). The effects of Aβ on Na^+/K^+-ATPase activity, $[Ca^{2+}]_i$ and cell survival were also less pronounced in neurons pretreated with 50 μM of the spin-trapping compound *N*-tert-butyl-phenylnitrone (PBN) although the values did not reach statistical significance. While these data suggested that ROS are involved in the effects of Aβ on Na^+/K^+-ATPase activity, it was important to establish whether a better characterized oxidative insult would also impair Na^+/K^+-ATPase activity. To this end, cultures were exposed to iron (100 μM), an inducer of hydroxyl radical production (Zhang et al., 1993), for increasing time periods. Iron caused a rapid impairment of Na^+/K^+-ATPase activity with levels being reduced to 34% of basal levels within 1 hr of exposure. Ouabain-insensitive Mg^{2+}-ATPase activity was also severely impaired by iron during a 1 hr exposure period.

Aβ selectively impairs Na^+/K^+-ATPase and Ca^{2+}-ATPase activities in synaptosomes from adult human hippocampus

Although the data from the cell culture studies above indicated that Aβ can impair Na^+/K^+-ATPase activity, and that this action of Aβ contributed to elevation of $[Ca^{2+}]_i$ and neuronal death, it was important to establish whether Aβ also affect ion-motive ATPases in adult human brain. To this end, synaptosomes were prepared from hippocampus of four neurologically normal adults (see Materials and Methods). Exposure of synaptosomes to 50 μM Aβ25-35 for 1 hr significantly reduced Na^+/K^+-ATPase and Ca^{2+}-ATPase activities to approximately 70% and 35% of control levels, respectively (Fig. 7). In contrast, Aβ did not significantly alter ouabain/Ca^{2+}-insensitive Mg^{2+}-dependent ATPase activities or Na^+/Ca^{2+} exchange in the human hippocampal synaptosomes (Fig. 7). Activity levels of Na^+/K^+-ATPase, Ca^{2+}-ATPase and ouabain/Ca^{2+}-insensitive Mg^{2+}-dependent ATPase were all significantly reduced in synaptosomes exposed for 1 hr to 100 μM $FeSO_4$, whereas Na^+/Ca^{2+} exchange was not significantly affected by $FeSO_4$ (Fig. 7). Thus, neuronal ion-motive ATPases from adult human brain are impaired by Aβ.

Discussion

Sequence of events involved in Aβ-induced neuronal death

The present findings demonsrate that Aβ can selectively impair ion-motive ATPase activities in both primary neuronal cultures and synaptosomes from adult postmortem human hippocampus. When taken together with previous findings, the data suggest a specific sequence of events involved in the neurotoxic activity of Aβ. Aβ induces peroxide accumulation and lipid peroxidation (Behl et al., 1994; Butterfield et al., 1994; Goodman and Mattson, 1994) which results in impairment of Na^+/K^+-ATPase and Ca^{2+}-ATPase activities. Impaired Na^+/K^+-ATPase activity results in Na^+ influx, membrane depolarization, and Ca^{2+} influx through voltage-dependent channels, while impaired Ca^{2+}-ATPase activity reduces the ability of the cell to remove Ca^{2+}. Both

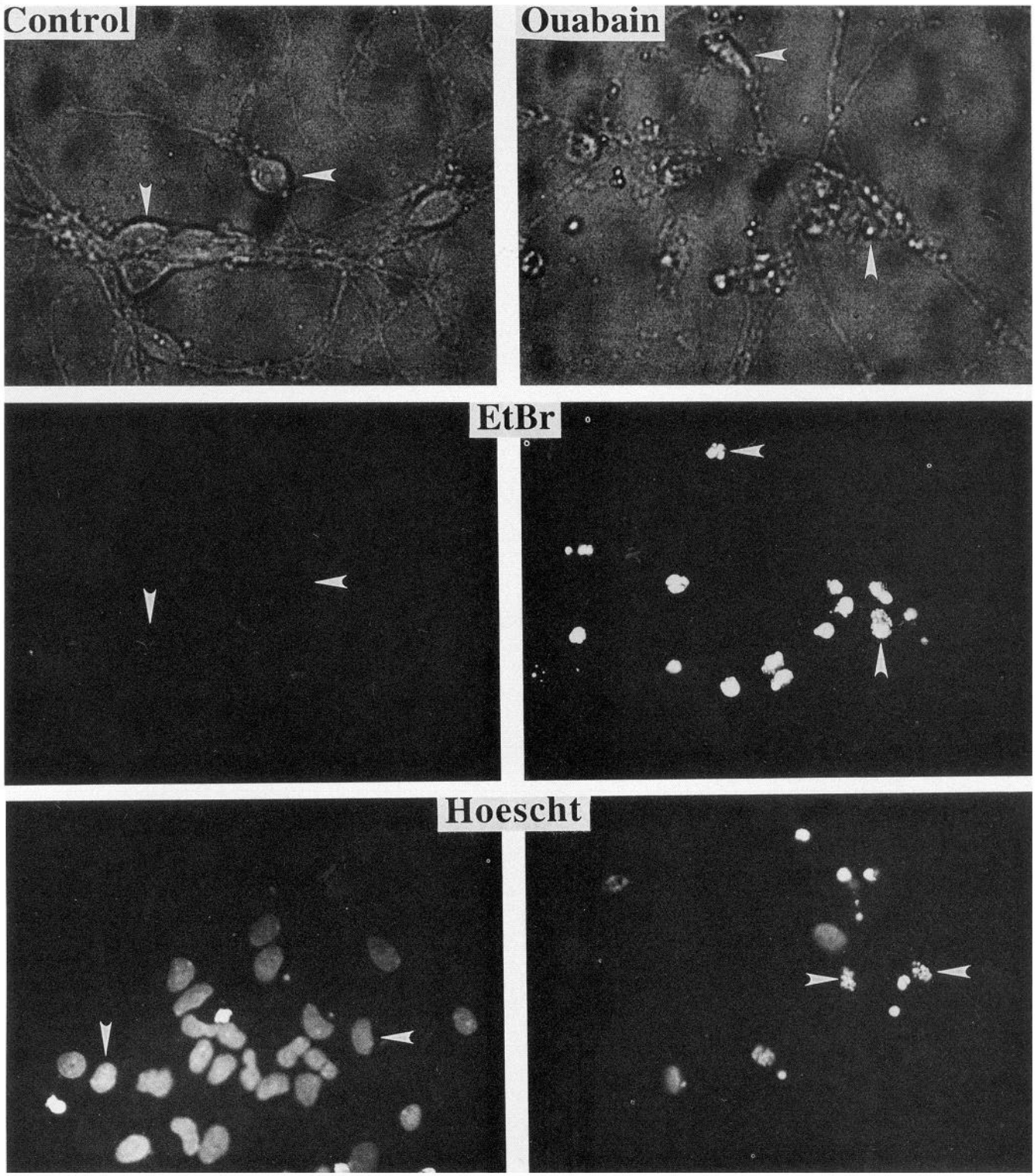


Figure 6. Ouabain induces nuclear DNA condensation and fragmentation in hippocampal neurons. *Left panels* are micrographs of neurons in untreated control cultures, and *right panels* are neurons in cultures exposed to 100 μM ouabain for 12 hr. *Upper panels*, Micrographs of cells scanned with visible light. Note that neurons in the control culture appear undamaged, while neurons in ouabain-treated culture exhibit extensive damage including apparent nuclear condensation (e.g., *arrowheads*). *Middle panels*, Fluorescence confocal laser scanning microscope images of neurons stained with ethidium bromide homodimer (*EtBr*); these are the same microscope fields as shown in the *upper panels*. Note that neurons in control cultures are unstained (EtBr only enters damaged cells), while the dye stains DNA which appears condensed and fragmented (e.g., *arrowheads*) in neurons damaged by ouabain. *Lower panels*, Fluorescence microscope images of neurons stained with Hoescht dye. Note that DNA staining in control control cultures appears diffuse and fills the nucleii (e.g., *arrowheads*), while DNA in neurons exposed to ouabain appears condensed and fragmented (e.g., *arrowheads*).

Table 1. Evidence for the involvement of reactive oxygen species in Aβ-induced impairment of Na^+/K^+-ATPase activity, elevation of $[Ca^{2+}]_i$ and neurotoxicity

	% Survival	Na^+/K^+-ATPase activity (nmol Pi/mg protein/min)	$[Ca^{2+}]_i$ (nM)
Control	92 ± 2.2	34.0 ± 1.1	130 ± 10
Aβ(25–35)	66 ± 11*	22.8 ± 1.8*	430 ± 31*
Aβ+PBN	78 ± 1.3	26.3 ± 4.9	250 ± 95
Aβ+PG	89 ± 5**	37.1 ± 2.5**	216 ± 28**
Aβ+VitE	85 ± 6**	33.6 ± 1.1**	136 ± 32**
PBN	92 ± 4.5	33.6 ± 1.1	143 ± 14
PG	93 ± 3	36.8 ± 3.2	127 ± 19
VitE	92 ± 5.6	35 ± 1.7	79 ± 22

Cultures were left untreated or were pretreated for 10 hr with 50 μg/ml vitamin E or for 15 minutes with 5 μM propylgallate or 50 μM PBN. Cultures were then exposed to 50 μM Aβ25-35. Na^+/K^+-ATPase activity, $[Ca^{2+}]_i$ and neuronal survival were assessed 10 hr, 8 hr, and 48 hr (respectively) following exposure to Aβ. Values for Na^+/K^+-ATPase activity represent the mean ± SD of determinations made in three separate experiments. Values for $[Ca^{2+}]_i$ represent the mean ± SD of determinations made in three separate experiments assaying about 50 neurons per culture. Values for neuronal survival represent the mean ± SD of determinations made in four separate cultures with 200–300 cells being counted per culture. *, $p < 0.01$ compared to control values; **, $p < 0.01$ compared to Aβ values (ANOVA with Scheffe's post hoc test for pairwise comparisons).

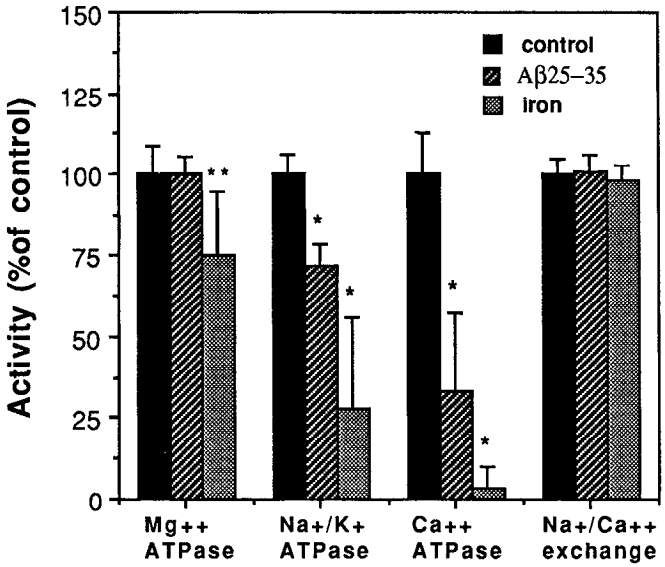


Figure 7. Aβ selectively impairs ion-motive ATPase activities in hippocampal synaptosomes from adult human postmortem brain. Synaptosomes were exposed to vehicle (*control*), 50 μM Aβ25-35, or 100 μM $FeSO_4$ for 1 hr and then levels of the indicated ATPase activities or Na^+/Ca^{2+} exchange were quantified. Values represent the mean and SD of determinations made in samples from four different neurologically normal patients (see Materials and Methods). Basal levels of the different activities were: Na^+/K^+-ATPase, 190 ± 12 nmol Pi/μg protein/min; Ca^{2+}-ATPase, 32± 2.8 nmol Pi/μg protein/min; ouabain/Ca^{2+}-insensitive ATPase, 330 ± 28 nmol Pi/μg protein/min; Na^+/Ca^{2+} exchange, 2300 cpm ^{45}Ca/50 μg protein. *, $p < 0.001$ compared to corresponding control value. **, $p < 0.01$ compared to corresponding control value. Student's *t* test with Bonferoni correction factor.

Ca^{2+} influx and ROS contribute to Aβ-induced neuronal degeneration because removal of extracellular calcium (Mattson et al., 1993b), Ca^{2+} channel blockers (Weiss et al., 1993), and antioxidants (Behl et al., 1994; Goodman and Mattson, 1994, Goodman et al., 1994; present study) protect neurons against Aβ toxicity. We found that impairment of Na^+/K^+ ATPase activity was sufficient to induce elevation of $[Ca^{2+}]_i$ and neuronal degeneration. Supporting a causal role for Na^+ influx in elevation of $[Ca^{2+}]_i$ and neurotoxicity of Aβ are the findings that: impairment of Na^+/K^+ ATPase activity preceded elevation of $[Ca^{2+}]_i$ and neuronal degeneration; tetrodotoxin, Na^+-deficient medium and Ca^{2+}-deficient medium attenuated ouabain-induced elevation of $[Ca^{2+}]$; Aβ-induced elevation of $[Ca^{2+}]_i$ was significantly attenuated by incubation in Na^+-deficient medium or the presence of tetrodotoxin; and Aβ neurotoxicity was significantly attenuated by tetrodotoxin. Although we were not able to reliably quantify Ca^{2+}-ATPase activity in the cultured embryonic hippocampal neurons, we found that Aβ markedly reduced Ca^{2+}-ATPase activity in hippocampal synaptosomes from adult human brain, an effect predicted to promote elevation of $[Ca^{2+}]_i$ and neurotoxicity.

We did not observe an effect of Aβ40-1 or scrambled Aβ25-35 on Na^+/K^+-ATPase activity or neuronal survival in the present study indicating sequence specificity for impairment of ion-motive ATPases by Aβ. Whereas Aβ1-40 and Aβ25-35 induced elevation of $[Ca^{2+}]_i$ and peroxide accumulation in cultured hippocampal neurons, scrambled Aβ25-35 and reverse Aβ1-40 were ineffective (Mattson et al., 1992, 1993b; Goodman and Mattson, 1994). Behl et al. (1994) found that a scrambled Aβ25-35 peptide did not induce peroxide accumulation in cultured neuroblastoma cells and cortical neurons, Butterfield et al. (1994) showed that Aβ25-35 induced lipid peroxidation in synaptosomes whereas Aβ35-25 was without effect (Butterfield et al., 1994), and Hensley et al. (1994) reported that Aβ25-35, but not a scrambled control peptide, induced oxygen-dependent inactivation of glutamine synthetase and creatine kinase, two enzymes known to be sensitive to oxidative injury. These data, taken together with additional neurotoxicity studies that employed various control peptides (e.g., Yankner et al., 1990; Kowall et al., 1991; Pike et al., 1993) clearly demonstrate the sequence specificity of the neurotoxic action of Aβ (but see Giordano et al., 1994).

Both ROS and calcium are implicated in the mechanism of "programmed cell death" termed apoptosis (see Steller, 1995; Thompson, 1995, for review). Because of the considerable evidence that ROS and calcium mediate the neurotoxicity of Aβ (see above), and because several laboratories have provided evidence that Aβ neurotoxicity is of an apoptotic form (Forloni et al., 1993; Loo et al., 1993), we determined whether neuronal death induced by ouabain also exhibited features of apoptosis. Staining of cultures with Hoescht dye and ethidium bromide homodimer revealed that ouabain induced nuclear condensation and DNA fragmentation. These observations further support the involvement of sodium pump impairment in the mechanism Aβ neurotoxicity.

Ion-motive ATPases and neuronal injury

Previous studies of both non-neural cells and neurons characterized the effects of ischemic and oxidative insults on Na^+/K^+ ATPase and Ca^{2+}-ATPase activities, and addressed the issues of the role of ROS in impairment of ion-motive ATPase activities and the role of impairment of ion-motive ATPases in cell injury (see Lees, 1991; Rohn et al., 1993; Silverman and Stein, 1994, for review). Neurons are particularly vulnerable to impairment of Na^+/K^+ ATPase activity and the Na^+/K^+ ATPase is vulner-

able to oxyradical-induced damage and lipid peroxidation (Lee, 1991). Ca^{2+}-ATPases are also vulnerable to damage by ischemia and oxyradicals and such damage plays a role in loss of cellular ion homeostasis and cell injury (Silverman and Stern, 1994). We found that neurotoxic concentrations of Aβ selectively impaired Na^+/K^+-ATPase and Ca^{2+}-ATPase activities without affecting ouabain/Ca^{2+}-insensitive Mg^{2+}-ATPase activity or Na^+/Ca^{2+} exchange. On the other hand ouabain/Ca^{2+}-insensitive Mg^{2+}-ATPase activity was severely impaired by iron, indicating that the mechanism(s) by which iron and Aβ impair enzymes differs. A similar difference was noted in lipoperoxidation studies induced by either Aβ25-35 or Fe^{2+}/H_2O_2 (Butterfield et al., 1994). Whereas Aβ25-35 derived free radicals selectively reduced the EPR signal intensity of a lipid-specific spin label deep within the synaptosomal membrane lipid bilayer, Fe^{2+}/H_2O_2 derived hydroxyl free radicals affected selectively lipid-specific spin labels at the lipid–water interface. The reason for these differences is not clear but may be related to the nature of the ROS induced and the severity of the oxidative insult. On the other hand, we have found that exposure of partially purified membranes to Aβ does not cause a reduction in either Na^+/K^+-ATPase or Ca^{2+}-ATPase activities (R.J.M. and M.P.M., unpublished data) indicating that the mechanism whereby Aβ impairs the ion-motive ATPases involves a cytosolic component(s).

The fact that Aβ-induced impairment of the Na^+/K^+-ATPase activity preceded cell death by many hours to days indicates that neurons can survive for considerable time periods with compromised pump activity. Impairment of ion-motive ATPase activities by Aβ may contribute to increased neuronal vulnerability to excitotoxic (Koh et al., 1990; Mattson et al., 1992), metabolic (Copani et al., 1992), and oxidative (Goodman and Mattson, 1994) insults documented in previous studies. Indeed, we previously showed that $[Ca^{2+}]_i$ responses to glutamate and membrane depolarization are markedly enhanced in cultured human cortical and rat hippocampal neurons pretreated with Aβ (Mattson et al., 1992, 1993b). The latter data are consistent with the action of Aβ on Na^+/K^+-ATPase activities documented in the present study in that Na^+ influx would lead to membrane depolarization and Ca^{2+} influx through the NMDA receptor channel and voltage-dependent channels (Mayer and Westbrook, 1987). In addition, reduced Na^+/K^+-ATPase activity would lead to increased levels of extracellular glutamate as the result of Ca^{2+}-induced glutamate release and compromise of glutamate transport (Brines and Robbins, 1992; Koyama et al., 1993). Finally, membrane depolarization and Na^+ influx can cause reversal of the Na^+/Ca^{2+} exchanger and thereby exacerbate Ca^{2+} influx (Stys et al., 1992). The fact that Aβ did not impair Na^+/Ca^{2+} exchanger activity leaves open the possibility that reverse Na^+/Ca^{2+} exchange contributes to loss of Ca^{2+} homeostasis and cell death induced by Aβ.

Previous studies have shown that ouabain can be neurotoxic at concentrations in the 0.1–1 M range (e.g., Garthwaite et al., 1986). We observed neurotoxicity with concentrations of ouabain (1–10 μM) that inhibit the α3 isoform of the Na^+/K^+-ATPase, but not the α1 isoform (Sweadner, 1989; Lees, 1991). This is in contrast to Murphy et al., (1988) who reported that a neuroblastoma/embryonic retinal cell hybrid cell line was not killed by 0.25 M ouabain. The resistance of the cell line may result from a different complement of Na^+/K^+-ATPase isozymes, a lack of NMDA receptors, and/or a less prominent Na^+/Ca^{2+} exchange mechanism.

Implications for the pathogenesis of Alzheimer's disease

In addition to deposition of Aβ, two prominent alterations in the AD brain are reduced glucose availability to neurons and increased protein oxidation (see Mattson, 1994, for review). Reduced glucose availability could lead to reduced neuronal ATP levels and, because the Na^+/K^+-ATPase utilizes up to 50% of cellular ATP, Na^+/K^+-ATPase activity may be compromised by such a reduction in glucose availability. Metabolic impairment has been shown to increase neuronal vulnerability to excitotoxicity (Novelli et al., 1988) and Aβ toxicity (Copani et al., 1991). Increased oxidation would impair Na^+/K^+-ATPase activity as indicated by the present findings and previous data discussed above. Previous *in vitro* and *in vivo* studies have shown that excitatory amino acids, energy impairment and Aβ can induce, in a cooperative manner, antigenic and biochemical alterations in the neuronal cytoskeleton similar to those seen in the neurofibrillary tangles of AD (Mattson, 1990; Cheng and Mattson, 1992; Mattson et al., 1992; Elliott et al., 1993; Stein-Behrens et al., 1994). Such data suggest that the different alterations believed to contribute to the pathogenesis of neuronal injury in AD, including amyloid deposition, metabolic impairment, loss of calcium homeostasis, and oxidative processes (see Beal, 1992; Hoyer, 1993; Selkoe, 1993; Mattson, 1994; for reviews), may each contribute to a common pathway of cell injury involving generation of ROS and impairment of ion-regulating systems.

If the hypothesis that Aβ contributes to the pathogenesis of AD by a mechanism involving impairment of ion-motive ATPase activities is correct, then it would be predicted that levels of activity of such ion-motive ATPases would be reduced in vulnerable regions of AD brain. Only limited information on these important parameters has been obtained. Liguri et al. (1990) reported that levels of Na^+/K^+-ATPase activity were reduced in some vulnerable regions of AD brain including the nucleus basalis, whereas Ca^{2+}-ATPase activity appeared to be relatively unaffected in AD brain. Studies of synaptosomes from aged rats indicate there are modest but significant reductions in activity of the Ca^{2+}-ATPase and Na^+/Ca^{2+} exchanger and elevation of $[Ca^{2+}]_i$ (see Michaelis, 1994 for review). The latter findings are of interest because age is a major risk factor for AD, and the relative contributions of the normal aging process and disease-specific alterations to the neurodegenerative process are unclear. However, it is reasonable to consider that the marked impairment of human hippocampal synaptosome Na^+/K^+-ATPase and Ca^{2+}-ATPase activities by Aβ documented in the present study would exacerbate the age-related decrement in function of such ion-motive ATPases. Regarding Na^+/Ca^{2+} exchange activity, Colvin et al. (1991) reported that levels of Na^+/Ca^{2+} exchange activity were not reduced, but rather were increased, in AD brain tissue. Those data are consistent with our observation that neurotoxic levels of Aβ did not impair Na^+/Ca^{2+} exchange activity in cultured rat hippocampal neurons or synaptosomes from postmortem human hippocampus.

Our studies of human hippocampal synaptosomes are of particular relevance to Alzheimer's disease because they show that the effects of Aβ on ion-motive ATPase activities are not peculiar to embryonic rodent cells in culture, and also occur in adult human brain tissue. Because rodents do not develop Alzheimer-like pathology it was important to establish that Aβ impairs Na^+/K^+-ATPase and Ca^{2+}-ATPase activities in human neuronal tissue. Interestingly, Games et al. (1995) recently reported

that a transgenic mouse expressing the human APP717 mutation exhibited Aβ accumulation and synapse loss, findings consistent with a role for Aβ in damage to synapses *in vivo*. Moreover, synapse loss appears to correlate more strongly with dementia than do plaques and tangles (Scheff and Price, 1993; Terry, 1994) and our data are therefore consistent with the possibility that by disrupting ion homeostasis in synaptic membranes, Aβ could contribute to synapse loss in Alzheimer's disease.

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