Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 5191–5193, June 1993
Neurobiology

Release of amyloid β-protein precursor derivatives by electrical depolarization of rat hippocampal slices

(Alzheimer disease/electrical stimulation/neurotransmission)

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Communicated by Emilio Bizzi, February 5, 1993

ABSTRACT **Proteolytic processing of the β-amyloid protein precursor (APP) is regulated by cell-surface receptors. To determine whether neurotransmitter release in response to neuronal activation regulates APP processing in brain, we electrically depolarized superfused rat hippocampal slices and measured soluble APP derivatives released into the superfusate. Electrical depolarization caused a rapid increase in the release of both neurotransmitters and amino-terminal APP cleavage products. These derivatives lacked the APP carboxyl terminus and were similar to those found in both cell culture media and human cerebrospinal fluid. Superfusate proteins including lactate dehydrogenase were not changed by electrical depolarization. The release of amino-terminal APP derivatives increased with increasing stimulation frequencies from 0 to 30 Hz. The increased release was inhibited by the sodium-channel antagonist tetrodotoxin, suggesting that action-potential formation mediates the release of large amino-terminal APP derivatives. These results suggest that neuronal activity regulates APP processing in the mammalian brain.**

Amyloid deposits in the brains of Alzheimer disease patients consist of aggregates of βA4 peptides (also Aβ), which are 39- to 43-amino acid proteolytic derivatives of the amyloid β-protein precursors (APP) (for review, see ref. 1). APP are members of a large family of integral transmembrane glycoproteins existing in various forms derived from alternative mRNA splicing (for review, see ref. 1). Mature APP can be processed by several alternative proteolytic pathways that generate different breakdown products. A secretory pathway generates nonamyloidogenic soluble amino-terminal derivatives following cleavage within the βA4 segment (2, 3). Alternatively, endosomal–lysosomal pathways yield carboxyl-terminal derivatives, some of which contain intact βA4 sequences and, thus, are potentially amyloidogenic (4–6). An additional pathway results in the secretion of soluble βA4 fragments (7–9). In cell culture, the release of soluble APP derivatives is rapidly enhanced by stimulation of muscarinic receptor subtypes m1 and m3 (10, 11), which transiently activate protein kinase C (12). The biochemical mechanisms regulating APP processing pathways in the brain are unknown.

To determine whether APP processing is regulated by neurotransmission in intact brain samples, we electrically stimulated superfused rat hippocampal slices and measured the release of soluble APP derivatives by using immunoblotting (Western blotting) and densitometry.

MATERIALS AND METHODS

Animals and Hippocampal Slice Preparation. Male Sprague–Dawley rats (9–11 months) were anesthetized with ketamine (85 mg/kg of body weight i.m.) and were decapitated in a cold room at 4°C. Brains were rapidly removed and placed into chilled (4°C) oxygenated Krebs–Ringer buffer (see below) containing 1 mM ketamine. After removal of remaining meninges and chorioid plexus, hippocampal slices (300 μm) were quickly prepared with a McIllwain tissue chopper, washed three times, and placed into custom-made superfusion chambers [Warner Instrument, Hamden, CT; a modified version of those introduced by Maire and Wurtman (13)].

Superfusion and Electrical Stimulation. Slices were equilibrated for 50 min at 37°C by superfusing the chambers with oxygenated Krebs–Ringer buffer at a flow rate of 0.8 ml/min. Superfusion chambers contained two opposing silver mash electrodes that were connected to an electrical stimulator (model S88; Grass Instruments). A custom-made polarity reversal device was used to prevent chamber polarization and also to monitor both the current and the voltage 50 μs after the onset of each pulse to ensure uniform chamber resistance. After the equilibration period, slices in the presence or absence of 1 μM tetrodotoxin were stimulated electrically with 1-ms pulses at frequencies of 0–30 Hz and a current density of 4.95 mA/mm^2. The typical voltage of a pulse was 40 V at a current of 120 mA. Control groups were analyzed in parallel under identical conditions but were not electrically stimulated.

Immunoblot Analysis. Superfusates were collected for 50 min in chilled tubes (4°C) in the presence of 250 μM phenylmethylsulfonyl fluoride, centrifuged for 30 min at 10,000 × *g* to remove debris, and subjected to ultrafiltration against water at 4°C using cellulose dialysis tubing. The product of this ultrafiltration protocol (final volume, 4 ml) was lyophylized and reconstituted in SDS-containing gel loading buffer and boiled for 5 min. Total slice-protein levels were measured using the bicinchoninic acid assay (Pierce) and were constant among all groups. Reconstituted superfusate proteins equivalent to 600 μg of slice protein were electrophoresed on linear SDS/12% polyacrylamide minigels and electroblotted onto polyvinyl difluoride membranes (Immobilon; Waters), which subsequently were probed with either the monoclonal antibody 22C11 (Boehringer Mannheim) or the polyclonal antiserum anti-C_8. The secondary horseradish peroxidase-linked antibodies were visualized by enhanced chemiluminescence (ECL; Amersham) using linear (preflashed) films. Immunoreactive bands were compared densitometrically using a laser scanner (Pharmacia LKB UltroScan XL) set at 40-μm vertical interval size and 2.4-mm horizontal slit width. All densitometric measurements were performed in the linear range as determined by standard dilution curves of rat brain protein extracts. Groups used for statistical comparisons were always determined within the same Western blot. Results were normalized to the control values set to 100% and

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Abbreviations: APP, amyloid β-protein precursor(s); LDH, lactate dehydrogenase.

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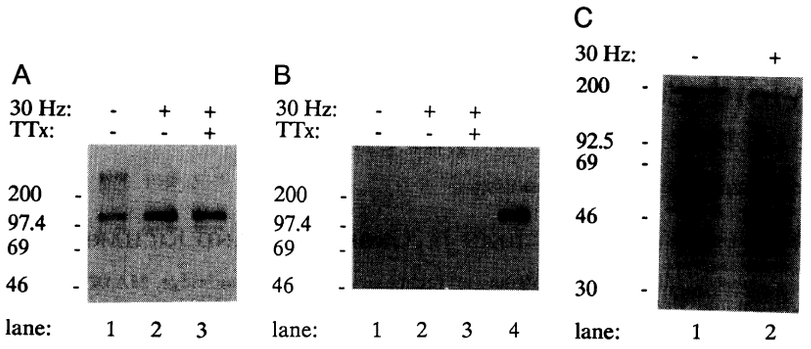

FIG. 1. (*A* and *B*) Western blots of soluble amino-terminal APP derivatives released from hippocampal slices *in vitro*. The release of hippocampal APP derivatives detected with the monoclonal antibody 22C11 (*A*) is increased by electrical depolarization (stimulation with 30 Hz for 50 min) (*A*, lane 2) and is blocked by 1 μM tetrodotoxin (TTx) (*A*, lane 3). APP derivatives released from superfused hippocampal slices *in vitro* lack the carboxyl terminus (*B*). Western blotting with the polyclonal antiserum anti-C_8 against the last 20 carboxyl-terminal amino acids of APP (*B*) did not react with the proteins detected by antibody 22C11, which was raised against a full-length APP fusion protein (*A*). Lanes: 1–3, as in *A*; 4, protein extract from hippocampal slices used in the same experiment indicates the presence of full-length APP within the tissue. (*C*) Coomassie blue staining of total proteins released into the superfusate. Lanes: 1, control; 2, electrical depolarization. Sizes are shown in kDa.

compared statistically by using analysis of variance and post hoc Newman–Keuls tests.

Neurotransmitter and Lactate Dehydrogenase (LDH) Assays. Acetylcholine in the superfusates was determined by HPLC with electrochemical detection (Coulochem II; ESA, Bedford, MA) by using a platinum electrode (300 mV) and a postcolumn reactor containing immobilized acetylcholinesterase and choline oxidase (ESA). LDH activity in 1-ml superfusates was assayed by using a modified version of a commercially available assay kit (Sigma no. 500).

RESULTS AND DISCUSSION

Immunoreactive material with apparent molecular masses ranging from 98 kDa to 130 kDa was detected in the superfusates with the monoclonal antibody 22C11 raised against a full-length APP fusion protein (14). The major band migrated at 117 kDa (Fig. 1*A*). These molecular masses are identical to those of secreted amino-terminal APP derivatives described in human cerebrospinal fluid and in conditioned cell culture media (14, 15). Western blot analysis of the secreted proteins using an antibody against the carboxyl-terminal 20 amino acids of full-length APP (anti-C_8) failed to detect immunoreactive material in the same superfusates, while protein extracts from the hippocampal slices clearly showed immunoreactivity of the expected molecular mass (16) (Fig. 1*B*). These data suggest that hippocampal slices *in vitro* contain intact, full-length APP and release soluble amino-terminal APP derivatives that lack the APP carboxyl terminus.

Electrical field stimulation increased the release of these APP derivatives within 50 min (Fig. 1*A*). Densitometric quantitation of this release indicated a significant 2-fold increase ($P < 0.01$) (Fig. 2*A*). Similar increases in the release of soluble amino-terminal APP derivatives were observed after stimulation with receptor agonists in cell culture (10, 11).

The enhanced release of these APP derivatives evoked by electrical depolarization was prevented ($P < 0.05$) by the addition of tetrodotoxin (Figs. 1*A* and 2*A*), which selectively blocks voltage-sensitive sodium channels necessary for the generation of action potentials (17). This finding indicates that neuronal activity can regulate APP processing in mammalian brain.

To ensure that interventions used in our study reliably modified neuronal activity, we monitored the efficacy of both electrical depolarization and tetrodotoxin by measuring the release of the neurotransmitter acetylcholine. Electrical stimulation increased acetylcholine release 4-fold [72.1 ± 19.9 to 294.8 ± 44.0 pmol/hr per mg of protein (means ± SEM; n = 5) per group; $P < 0.01$, t test], and this effect was blocked by tetrodotoxin (121.9 ± 12.2 pmol/hr per mg of protein; $P <$ 0.01).

To demonstrate cell viability and to control for the specificity of the release of APP derivatives, we measured the

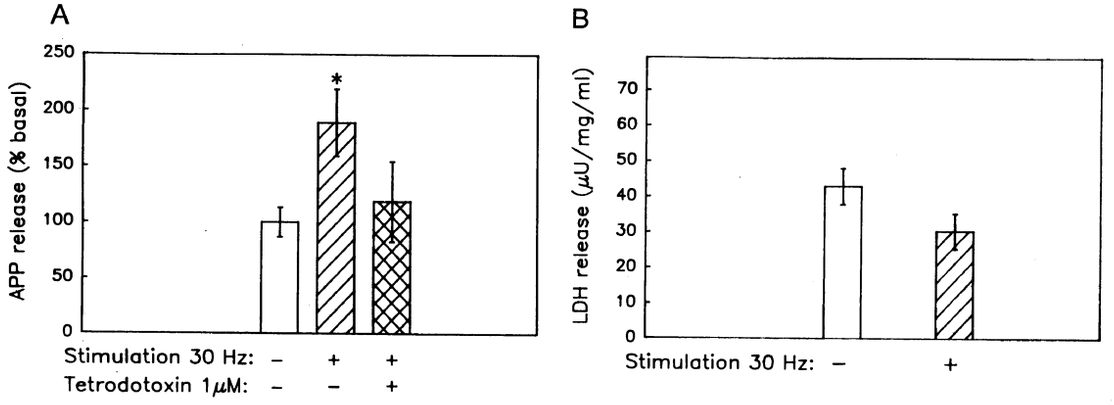

FIG. 2. (*A*) Densitometric quantitation of released soluble amino-terminal APP derivatives from hippocampal slices. Bars indicate means ± SEM (n = four animals per group), and the asterisk indicates $P < 0.01$ vs. unstimulated controls and $P < 0.05$ vs. tetrodotoxin-treated group (ANOVA and post hoc Neuman–Keuls test). (*B*) LDH released from hippocampal slices in response to electrical stimulation. Data are expressed in microunits (μU) of LDH per mg of slice protein per ml of superfusate. Bars indicate means ± SEM (n = four animals). No statistical significance was found between the groups.

release of LDH (18) into the superfusate during electrical stimulation. The basal amount of LDH release was low and was not increased by stimulation. These findings indicate that cells within the slices were intact during the experiment and that electrical stimulation did not affect viability (Fig. 2*B*). Moreover, stimulation did not change total release of protein into the superfusate: Coomassie blue staining of released proteins separated on linear SDS/12% polyacrylamide gels showed that the overwhelming majority of bands were unaffected by electrical depolarization (Fig. 1*C*).

Electrical depolarization via opening of voltage-gated sodium channels increases intracellular Ca^{2+} concentrations, which induces neurotransmitter release. It is unclear whether Ca^{2+} is directly involved in APP cleavage and release. In cell culture, however, Ca^{2+} was not necessary to stimulate the release of amino-terminal APP derivatives, nor were increases in intracellular Ca^{2+} sufficient to alter this release (10), although there was a synergistic effect of Ca^{2+} and activation of protein kinase C (R.M.N., unpublished observations). In hippocampal slices, the role of Ca^{2+} cannot be addressed because the absence of Ca^{2+} in the superfusate causes severe tissue damage leading to nonspecific release of proteins including LDH (19). Pharmacological blockade of Ca^{2+} channels inhibits neurotransmitter release and thus also is not suited to address the role of Ca^{2+} in regulating APP processing.

The increase in the release of soluble amino-terminal APP derivatives occurred within the first 50 min of stimulation, suggesting that these derivatives are likely to result from accelerated processing of preexisting proteins and not from increased APP gene expression. This notion is compatible with results in cell culture that show that derivatives of newly synthesized APP do not appear in the culture medium until at least 50 min after synthesis (14). The present study did not address the possibility that APP gene expression may also be affected by neuronal depolarization.

The release of soluble amino-terminal APP derivatives induced by neuronal depolarization increased with increasing frequencies in the range of 0–30 Hz. The half-maximal response was reached at 17 Hz, and the maximum was attained at 30 Hz (Fig. 3). Electrophysiological evidence from intracellular recording experiments indicates that large hippocampal pyramidal cells fire in response to activation with frequencies ranging from 8 to 18 Hz and can burst up to 150 Hz (20). Thus, the frequency–response curve for the release of soluble amino-terminal APP derivatives is consistent with the known electrophysiological properties of hippocampal neurons.

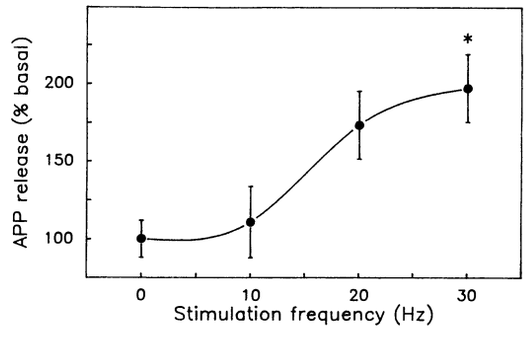

FIG. 3. Electrically stimulated release of soluble amino-terminal APP derivatives from hippocampal slices is frequency dependent. Values are normalized to basal, unstimulated release by using the monoclonal antibody 22C11. Symbols represent means ± SEM (n = three to five animals per group), and the asterisk indicates $P < 0.01$ vs. 0 Hz (ANOVA and post hoc Neuman–Keuls test).

The cellular source of APP derivatives released from hippocampal slices is unclear and could include neurons, glia, and cells from the cerebral vasculature. Unlikely sources are meninges, meningeal blood vessels, and circulating blood because the meninges were carefully removed, and slices were washed three times and superfused for 50 min prior to the collection and stimulation period.

This study demonstrates that hippocampal slices *in vitro* release soluble amino-terminal APP derivatives similar to those found in human cerebrospinal fluid and indicates that neuronal depolarization modulates this release. The functional significance of coupling APP processing to neurotransmission awaits discovery.

We thank Cindy Kim, Alice Wei, and Rajivi Pothiraj for excellent assistance as well as Dennis J. Selkoe and Kurt Naujocks (Boehringer Mannheim) for providing antibodies. This work was supported by the National Institute of Mental Health, the National Institute on Aging, and the Center for Brain Science and Metabolism Charitable Trust. R.M.N. is the Hoffman Fellow in Alzheimer disease at Massachusetts General Hospital.

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