Hippocampal level of neural specific adenylyl cyclase type I is decreased in Alzheimer’s disease

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Abstract

Previous studies reported disruption of adenylyl cyclase (AC)-cyclic AMP (cAMP) signal transduction in brain of Alzheimer’s disease (AD). We also demonstrated that basal and stimulated AC activities in the presence of calcium and calmodulin (Ca²⁺/CaM) were significantly decreased in AD parietal cortex. In the present study, we examined the amounts of Ca²⁺/CaM-sensitive types I and VIII AC, and Ca²⁺/CaM-insensitive type VII AC in the postmortem hippocampi from AD patients and age-matched controls using immunoblotting. The specificities of the anti-type VII and VIII AC antibodies were confirmed by preabsorption with their specific blocking peptides. We observed a significant decrease in the level of type I AC and a tendency to decrease in the level of type VIII AC in AD hippocampus. On the other hand, the level of type VII AC showed no alteration between AD and controls. A body of evidence from the studies with invertebrates and vertebrates suggests that types I and VIII AC may play an essential role in learning and memory. Our finding thus firstly demonstrated that a specific disruption of the Ca²⁺/CaM-sensitive AC isoforms is likely involved in the pathophysiology in AD hippocampus. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

The intact intracellular signal transduction from postsynaptic receptor to an effector system is required for the maintenance of neuronal activity and efficient interneuronal communication. The response of the effector system depends on the type of neurotransmitter receptor activated and can result in the opening of ion channels or the modulation of effector enzymes, such as adenylyl cyclase (AC).

Disruptions in the AC complex are well recognized to exist in Alzheimer’s disease (AD) [1]. It has been reported that G protein-mediated activation of AC is decreased in the neocortex and cerebellum in AD subjects [2]. Reduced basal and stimulated AC activities have also been observed in the AD hippocampus and cerebellum [3,4].
AC catalyzes the formation of cyclic AMP (cAMP), an important second messenger for protein phosphorylation and ion-channel gating [5]. This enzyme is regulated by a wide range of neurotransmitter receptors, as well as by intracellular free calcium and its binding protein, calmodulin. To date, nine mammalian AC subtypes (types I–IX AC) have been cloned, and they show different biochemical features and tissue distributions [6–8] (Table 1). Although all of the isoforms are expressed in neural tissue, types I and VIII AC are expressed exclusively in brain, whereas the other isoforms are expressed also in non-neural tissues [6,7,9]. Type I, type III, and type VIII AC can be stimulated by calcium and calmodulin (Ca$^{2+}$/CaM) [6,10,11], but the other types are inhibited by this combination (types V and VI AC) [12] or insensitive to it (types II, IV, and VII) [13].

Our previous studies reported decreased levels of types I and II AC in AD parietal cortex and also showed decreased basal and stimulated ( forskolin and manganese) AC activities in the presence of Ca$^{2+}$/CaM in the same brain region of AD [14,15]. These findings indicate that specific changes in the catalytic subunits of Ca$^{2+}$/CaM-sensitive AC isoforms may be involved in pathophysiology of AD. There is now growing evidence that types I and VIII AC likely play an important role in construction of some aspects of learning and memory in mammals [16,17]. Moreover, we have also reported decreased immunoreactivity of the phosphorylated form of cAMP response element binding protein (CREB) in AD hippocampus [18], suggesting the possibility that a widely impaired cAMP signal transduction including dysregulation of gene transcription might occur in AD brain.

The aim of the present study is to determine whether Ca$^{2+}$/CaM-sensitive type I and type VIII AC are impaired in AD hippocampus, which shows severe AD pathology, and to provide additional evidence on the role of cAMP as a functional molecule in human brain. We have thus investigated immunoreactivities of types I and VIII AC in hippocampal membranes from AD patients and age-matched controls using immunoblotting. Moreover, Ca$^{2+}$/CaM-insensitive type VII AC isoform was also examined.
to compare with the two Ca\(^{2+}\)/CaM-sensitive AC subtypes. Reliabilities of polyclonal antibodies against types VII and VIII AC were confirmed by preabsorption with blocking peptides as we previously performed with anti-type I AC antibody.

2. Materials and methods

2.1. Human postmortem brain tissues

Hippocampal membranes were obtained from 11 AD patients and 11 age-matched controls with no neuropsychiatric disorders. AD was diagnosed in accordance with NINCDS-ADRDA criteria [19]. Detailed characteristics of patients and controls are presented in Table 2. The procedure used for acquisition, clinical diagnosis, dissection, storage, and distribution of brain materials in our brain bank system was previously described in detail [20].

2.2. Preparation of membranes and protein measurement

The brain tissues were homogenized in a buffer containing 20 mM Hepes, 0.25 M sucrose, 0.3 mM PMSF, 1 mM DTT, 1 mM EGTA, and 1 mM MgCl\(_2\) (pH 7.4), and centrifuged at 600 × g for 10 min. The supernatants were centrifuged at 48,000 × g for 20 min. The pellets were then resuspended in a buffer containing 20 mM Hepes, 0.3 mM PMSF, and 1 mM DTT (pH 7.4), and centrifuged in the same way. The membrane-enriched pellets were resuspended in the same buffer and stored at −80°C until use.

Protein concentrations were determined by the Coomassie blue binding method using bovine serum albumin (BSA) as a standard [21].

2.3. Gel electrophoresis and immunoblotting of AC isoforms

The hippocampal membranes were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose membranes for subsequent immunoblotting as previously described [14,15]. For detection of type I AC, 20 μg of protein was used and for detection of types VII and VIII AC, 30 μg of protein was needed. In brief, membranes were subjected to SDS-PAGE with 4–12% polyacrylamide gels at 125 V for 2 h. Proteins were transferred to nitrocellulose membranes at 30 V for 75 min at room temperature. The membranes were blocked in TBS-T buffer (10 mM Tris, 500 mM NaCl, 0.1% Tween-20, pH 7.5) containing 5% BSA for 1 h at room temperature and then incubated in TBS-T buffer containing 3% BSA overnight at 4°C with primary antibodies diluted 1:1000 in anti-type I AC: sc-586 and anti-VIII AC: sc-1967, and 1:2500 in anti-type VII AC: sc-1966 (Santa Cruz Biotechnology, CA, USA). Membranes were washed and incubated with secondary antibodies, anti-rabbit Ig HRP-linked F(ab‘)\(_2\) (Amersham Life Science, UK) diluted to 1:2500 for type I AC and HRP-conjugated anti-goat IgG (H and L) (Rockland, PA, USA) diluted to 1:4000 for types VII and VIII AC, in 3% BSA/TBS buffer for 1 h at room temperature.

2.4. Preabsorption of type VII and type VIII AC antibodies

Specific blocking peptides against anti-type VII and VIII AC antibody (sc-1966P and sc-1967P; Santa Cruz Biotechnology) were added to primary antibody solution in concentrations of 0.01–1 μg peptide μl\(^{-1}\), respectively [14]. The mixture was incubated overnight at 4°C with rocking, then centrifuged at 3000 rpm for 30 min at room temperature. Immunoblotting was then performed with the recovered supernatant as the primary antibody solution.

2.5. Densitometric measurement of immunoreactivities and statistical analysis

Immunoreactive bands were detected with the enhanced chemiluminescence (ECL) system (Amersham) and analyzed by laser densitometry (NIH 1.55 image analysis system).

Results are given as the means ± S.E.M. The data were analyzed by Welch’s t-test and values of \(P < 0.01\) were taken to indicate statistical significance of difference between groups. The effects of age and postmortem interval on each immunoreactivity were determined by Spearman’s rank order correlation analysis.
3. Results

3.1. Linearity of immunoblots of types I, VII, and VIII AC in human hippocampal membrane

Linearity of our immunoblots was tested using control human hippocampal membrane. In the immunoblots using this standard membrane, the densitometric values were in proportion to protein amounts loaded on to the gel within the limits 5–50 µg for type I AC and 5–75 µg for types VII and VIII AC (data not shown). Therefore, we decided that the optimal protein amounts for immunoblottings were 20 µg for type I AC and 30 µg for types VII and VIII AC, respectively, and the identical control membrane preparation was routinely used as a standard to ensure that the analysis of the samples was performed within the linear range of the method.

3.2. Effects of age and postmortem delay time on AC subtypes in human hippocampus

We previously demonstrated that age and post-mortem delay time (PMDT) did not influence the immunoreactivity of type I AC in human cortical membrane [14]. Similarly, we examined the effects of age and PMDT on immunoreactivities of types I, VII, and VIII AC in human hippocampal membranes with Spearman’s rank order analysis using all

![Fig. 1. Results of immunoblots of types VII and VIII AC with anti-type VII and VIII AC antibodies and after preabsorption of the antibodies with each specific blocking peptide. Lane 1: no peptide was applied. Lanes 2-5: 0.01, 0.1, 0.5, and 1.0 µg peptide µl⁻¹ of primary antibody solution, respectively. Molecular mass markers are indicated at the left margin of each panel.](image)

![Fig. 2. Effects of the dose of specific blocking peptides on the immunoreactivities of anti-type VII AC (a) and type VIII AC (b) antibodies in the preabsorption study. An equal amount of total protein (30 µg) of control human hippocampal membrane was used for each immunoblot. The immunoreactivity and the amount of applied peptide shows a biphasic linear relation.](image)
subjects. Neither age nor PMDT had a significant effect on the measured immunoreactivities of the AC isoforms in controls (\( P > 0.10 \) in all cases). There were also no significant correlations between age or PMDT and immunoreactivities of the three subtypes in AD subjects (\( P > 0.14 \) in all cases).

### 3.3. Effect of preabsorption of the type VII and VIII AC antibodies on the immunoreactivity

Fig. 1 shows the preabsorption study of anti-type VII AC antibody and anti-type VIII AC antibody using control human standard membrane. Preabsorption of the antibodies with their specific blocking peptides diminished or completely abolished the immunoreactivities of each AC isoform and the eliminations occurred in a biphasic linear relation, with one phase between 0 and 0.01 \( \mu \text{g}/\mu\text{l} \) of added peptide and a second one between 0.01 and 1.0 \( \mu \text{g}/\mu\text{l} \) of competing peptide (Fig. 2).

### 3.4. Protein levels of AC isoforms in the postmortem hippocampus of AD patients

Representative immunoblots of the type I, VII, and VIII AC isoforms in hippocampus from AD subjects and controls are presented in Fig. 3. Immunoreactive bands of each AC subtype migrated at the expected molecular mass range (120–130 kDa) [5]. The anti-type I AC antibody reacted with the protein band that was detected at an apparent molecular mass of 120 kDa. We have previously demonstrated by immunoblotting and preabsorption of the antibody with the specific blocking peptide that the 120 kDa protein is type I AC [14,22].

Fig. 4 depicts data obtained by densitometric analysis of all results obtained from 11 AD patients and 11 controls. Immunoreactivity of \( \text{Ca}^{2+}/\text{CaM-sensi}- \\
\text{tive type I AC was significantly decreased to 40.1\% of control levels (} P < 0.01 \) in the AD hippocampus. A moderate reduction of \( \text{Ca}^{2+}/\text{CaM-sensi}- \\
tive type VIII AC immunoreactivity was observed in AD subjects; however, that did not reach significance. In contrast, immunoreactivity of type VII AC, which is a \( \text{Ca}^{2+}/\text{CaM-insensi}- \\
tive subtype, was not altered between AD and controls.

### 4. Discussion

As factors which might affect the detection of immunoreactivities of AC isoforms, we examined the
effects of age and PMDT on each AC subtype. The absence of correlations between the immunoreactivity of each AC isoform and age or PMDT in the present study argues against a confounding influence of aging and postmortem effect on the obtained observations.

In the preabsorption study of anti-type VII AC antibody and anti-type VIII AC antibody with their respective complementary peptides, each antibody reacted with its specific blocking peptide and the immune complex formed was removed as a pellet by centrifugation. The reaction between antibody and its blocking peptide occurred in a biphasic linear manner and thus the specific immunoreactive band completely disappeared when a high dose of the peptide was applied. These results confirmed the specificity and reliability of the antibodies used.

Impaired signal transduction could occur as a result of alterations in neurotransmitter receptor levels, receptor/G-protein coupling, G-protein levels, G-protein effector enzyme coupling, effector enzyme levels, or due to actions of intracellular second messengers. Previous studies demonstrated that the AC signal transduction pathway is disrupted at a number of these components in AD brain.

It was reported that Gs-protein-stimulated AC activity is decreased in AD frontal, temporal, and occipital cortices, as well as angular gyrus and cerebellum, while basal and forskolin-stimulated activities showed no alteration [2]. Another study from the same group also showed a specific impairment of Gs-protein-stimulated AC activity in AD hippocampus [23]. These findings suggest that there is a specific lesion in AD brain at the level of Gs-protein–AC interactions. On the other hand, it was shown that basal, forskolin-stimulated AC activities, as well as Gs-protein-stimulated activity, are decreased in AD hippocampus and cerebellum [3,4], indicating that both the Gs protein and catalytic subunit of AC are impaired in AD brain. The discrepancy between these studies may be reflected by differences in the AC assay conditions, i.e., presence or absence of Ca²⁺/CaM. In this respect, we have previously measured AC activity in both the presence and absence of Ca²⁺/CaM and reported that basal, forskolin-, and Mn²⁺-stimulated AC activities in the presence of Ca²⁺/CaM are decreased in AD parietal cortex, whilst AC activities assayed without Ca²⁺/CaM show no significant alteration in the disease [15]. This suggests that Ca²⁺/CaM-sensitive AC isoforms may play a critical role in altered AC signal transduction in AD brain. Indeed, we have seen that Ca²⁺/CaM-sensitive and neuronal specific type I AC is significantly reduced in AD parietal cortex [14,15].

In the present study, we examined protein levels of type I, VII, and VIII AC isoforms in the hippocampus of 11 AD patients and 11 control subjects using immunoblotting and observed a significant decrease in the immunoreactivity of type I AC in AD patients. Accordingly, we assume this alteration to be a common lesion in the brain affected by the disease. On the other hand, we observed a tendency to decreased immunoreactivity of type VIII AC and no significant alteration in the level of type VII AC in AD hippocampus. Thus, immunoreactivities of the three AC isoforms were not consistently altered, indicating that expression of each AC subtype might be differently regulated in AD hippocampus. The alterations of types I and VIII AC observed in the present study might have been a consequence of the neuronal loss seen in AD affected brain. However, we cannot simply explain the decrease and tendency to decrease of these AC isoforms by the effect of change in neuronal density in this brain region, since the amount of type VII AC, which is also contained in synaptic membranes, was not altered.

Mammalian AC isoforms are expressed in all tissues, but at very low levels, approximately 0.01–0.001% of membrane protein [7]. Types I and VIII AC show prominent expression in neurons, particularly in regions of the brain associated with learning and memory [9,24]. In rat brains, the strongest mRNA signals of these subtypes are found in the hippocampus, and moderate levels of both mRNAs are detected in the neocortex and other regions. Type VII AC is distributed among a number of tissues, including brain, heart, kidney, and liver. In brain, type VII mRNA is primarily expressed in the cerebellar granule cell layer [25]. Interestingly, we have found changes only in the levels of the type I and VIII AC isoforms in AD which show dominant localizations within the hippocampus, indicating that these two subtypes may be important molecules in mediating the functions of hippocampal neurons. Future investigations concerning mRNA expressions of AC isoforms in human brain regions including AD.
brain will be strongly expected to address the molecular mechanism of the alteration observed.

We have previously demonstrated decrease in the level of phosphorylated form of cAMP-response element binding protein (CREB) in AD hippocampus [18], which is a transcription factor in the downstream AC–cAMP signaling system [26]. The present study includes seven AD and seven control cases, which are same subjects used in our previous study on CREB proteins [18]. Concerning these samples, we have examined correlations between the reported immunoreactivity of phosphorylated or total CREB [18] and the present immunoreactivity of each AC isoform, respectively. There were no significant correlations between the phosphorylated or total CREB immunoreactivity and the immunoreactivities of three AC subtypes in AD subjects, as well as in controls (data not shown). The lack of significant correlation between the phosphorylated CREB and the type I and type VIII AC suggests that the decrease in the level of phosphorylated CREB reported in AD hippocampus may not be induced directly by the disruption of these AC subtypes. Phosphorylation of Ser133 is crucial for transcriptional activation of CREB and this is catalyzed not only by protein kinase A (PKA) but also by Ca\(^{2+}\)-activated calmodulin kinases, ribosomal S6 kinase 2, and mitogen-activated protein kinase-activated protein kinase 2 [27]. It might be possible that disruptions of these other enzymes occur in AD hippocampus and reveal the decreased amount of the phosphorylated CREB.

Evidence from the studies with neuronal systems of invertebrate, as well as with mammalian brain, suggest that Ca\(^{2+}\)/CaM-sensitive AC isoforms may be important for learning and memory [17,28]. Furthermore, activation of the AC–cAMP signaling system, including PKA and CREB, may play an essential role in regulation of gene transcription and synthesis of specific proteins required for long-term synaptic changes [29,30]. It was observed that some forms of long-term potentiation (LTP), which is at present supposed to be a model for long-term memory formation in the brain, elevate cAMP levels in the hippocampus [31]. Animal studies have demonstrated that the functional knock-out of type I AC or CREB by transgenic techniques or specific antisense oligodeoxynucleotides results in impaired formation of long-term memory, whereas enhancement of the AC subtype or the transcription factor accelerates the process [16,29,32,33]. AC–cAMP signaling and CREB-dependent transcription thus appear essential for long-term memory consolidation in animals. However, there was little information to address the molecular mechanisms of learning and memory in the human brain. Our previous and present observations provide the first evidence that the Ca\(^{2+}\)/CaM-sensitive AC isoforms, especially type I AC, may contribute to formation of long-term memory in the human brain.

In most cases of AD, the brain regions including the cholinergic basal forebrain complex, the locus ceruleus, the median raphe nuclei, and hippocampus show severe neurodegenerative changes [34,35]. Therefore, it is strongly suggested that the specific disruptions of the Ca\(^{2+}\)/CaM-sensitive AC subtypes found in the present study in AD hippocampi may be involved in the pathophysiology of AD, and possibly become manifest as the impaired memory consolidation observed in the disease.

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