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Expression of type VI adenylyl cyclase in the central nervous system: implication for a potential regulator of multiple signals in different neurotransmitter systems

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Abstract The aim of this study was to investigate the distribution of a calcium-inhibitable adenylyl cyclase type VI (type VI AC) in the central nervous system using an antiserum directed against the N-terminus of type VI AC. Our results indicate that type VI AC immunoreactivity is generally expressed in many brain regions with different levels of intensity. Most interestingly, the majority of the detected type VI AC immunoreactivity is present in cells of neuronal phenotype. Double immunostaining of type VI AC and markers of various neurotransmitter systems suggest that type VI AC might participate in regulation of the classical neurotransmitter systems and therefore appeared to play a very important role in the central nervous system.

Key words: Adenylyl cyclase; Calcium; Immunocytochemistry; cAMP; Brain; Protein kinase C

1. Introduction

Genes of at least 9 distinct mammalian adenylyl cyclases (AC), which can be further divided into five subfamilies, have been reported [1]. These enzymes can all be activated by the α subunit of Gs protein to synthesize cAMP. Nevertheless, Gsα protein stimulates each AC in a fundamentally distinct manner [2]. Likewise, individual AC isozyme exhibits different susceptibility to Giα-mediated inhibition [3]. Intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) also play a critical regulatory role in modulating AC activity. Some AC isozymes (type I AC and type VIII AC) can be activated by Ca^{2+} in the presence of calmodulin [4], while type V AC and type VI AC are directly inhibited by sub-micromolar concentrations of calcium. In addition, protein kinase C (PKC) has been implicated in modulating activities of some ACs positively (types II and V adenylyl cyclases), but that of type VI negatively [5–7]. This heterogenous multigene AC family thus appears to play a very critical role in integrating multiple signals. For example, Marjamaki and colleagues [8] recently suggested that regulation of the cellular cAMP levels by G protein-coupled receptors might depend on the isoforms of AC expressed in the cells and the phosphorylation states of these ACs.

At present, all of the cloned mammalian ACs were found to be present in brain [8], suggesting a unique and critical role for ACs in mediating the functions of the central nervous system (CNS). For example, cyclic AMP-dependent mechanisms have been implicated in release of neurotransmitter [9] and long-term memory [10]. The most abundant ACs in CNS are types I, II, and V ACs [4]. In contrast, no significant level of type VI AC transcript was observed by the in situ hybridization technique in any discrete brain region, although the expression of type VI AC mRNA could be detected in the brain using Northern blot analysis or the RT-PCR technique [4]. Since type VI AC can be inhibited by Ca^{2+}, by protein kinase A (PKA), or by PKC [4,7,11], type VI AC might integrate multiple diverse signals which occur constantly in the nervous system. To understand the neuronal significance of this Ca^{2+}-inhibitable AC, we have examined the protein expression patterns of type VI AC in rat brain by immunocytochemical staining using a subtype-specific antiserum. Our data suggest that type VI AC exists predominantly in neurons, and might play a critical role in multiple signal integration.

2. Materials and methods

2.1. Preparation of antibody

Oligopeptides corresponding to amino acids 1–19 of rat type VI AC were purchased (Genosys; TX, USA) and conjugated to bovine serum albumin (BSA) using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester [12]. The antiserum was generated by injecting male New Zealand white rabbits with the BSA-conjugated peptide using standard procedures [12]. This polyclonal anti-peptide antiserum is designated AC6N. To remove the potential existing anti-BSA IgG, this anti-type VI AC antiserum was preabsorbed with 0.5% BSA in PBS.

2.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Coding regions of rat type II AC [13], dog type V AC [14], and rat type VI AC [15] were subcloned into a baculovirus expression vector (pVL1393). Expressions of types II, V, and VI ACs were carried out in a recombinant baculovirus-driven Sf21 cell system following the manufacturer's protocol (Pharmingen, San Diego, CA, USA). Membrane fractions were prepared as described earlier [7]. SDS-PAGE of membrane fractions were carried out according to the method of Laemmli [16]. Western blot analysis was performed as described elsewhere [7]. Typically, we used a 1:500 dilution for AC6N antiserum and 1:1000 for R32, an antiserum broadly reactive with the AC family members (Santa Cruz Biotech., Santa Cruz, CA, USA), unless stated otherwise. The immunoreactive bands were stained using a light emitting non-radioactive method (ECL, Amersham, UK).

2.3. Immunohistochemistry

Immunostaining of type VI AC using AC6N antiserum (1:500) was

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carried out by the avidin-biotin-peroxidase complex method (ABC) as previously described [17]. Controls for the specificity of AC6N were performed by incubating sections with AC6N preadsorbed with the immunizing peptide (designated as the AC6n peptide; 0.5 mg/ml) and by incubating sections with the preimmune serum. Type VI AC immunostaining was abolished almost completely with the preadsorption treatments. Double immunofluorescence staining of AC6N antiserum (1:500) and MAP-2 (1:2000; Boehringer Mannheim Biochemicals, Indianapolis, IN, USA), or AC6N and tyrosine hydroxylase (1:5000; Incstar, Stillwater, MN, USA) was carried out as previously described [18]. For double staining of type VI AC and choline acetyltransferase (ChAT), sections were first immunostained with type VI AC using DAB as the substrate, then were stained by the immunofluorescent method with a polyclonal goat anti-ChAT antiserum (1:500; Chemicon, Temecula, CA, USA). The pattern of double immunostaining was studied with the aid of a laser confocal microscope (Bio-Rad, MRC-1000, Hercules, CA, USA). Controls for the specificity of immunofluorescence were determined by omission of the secondary antibody in the staining process. Elimination of the secondary antibody resulted in a loss of immunofluorescence staining.

2.4. Polymerase chain reaction (PCR) and Southern blot analysis

To amplify the type VI AC fragment, total RNA was purified from the indicated rat tissue using silica-gel-based chromatography (RNeasy Midi, Qiagen, Hilden, Germany). Production of cDNA was carried out using Superscript* II reverse transcriptase (Gibco-BRL) following the manufacturer's protocol from 3.6 μg of the indicated total RNA. DNA amplification was performed as described

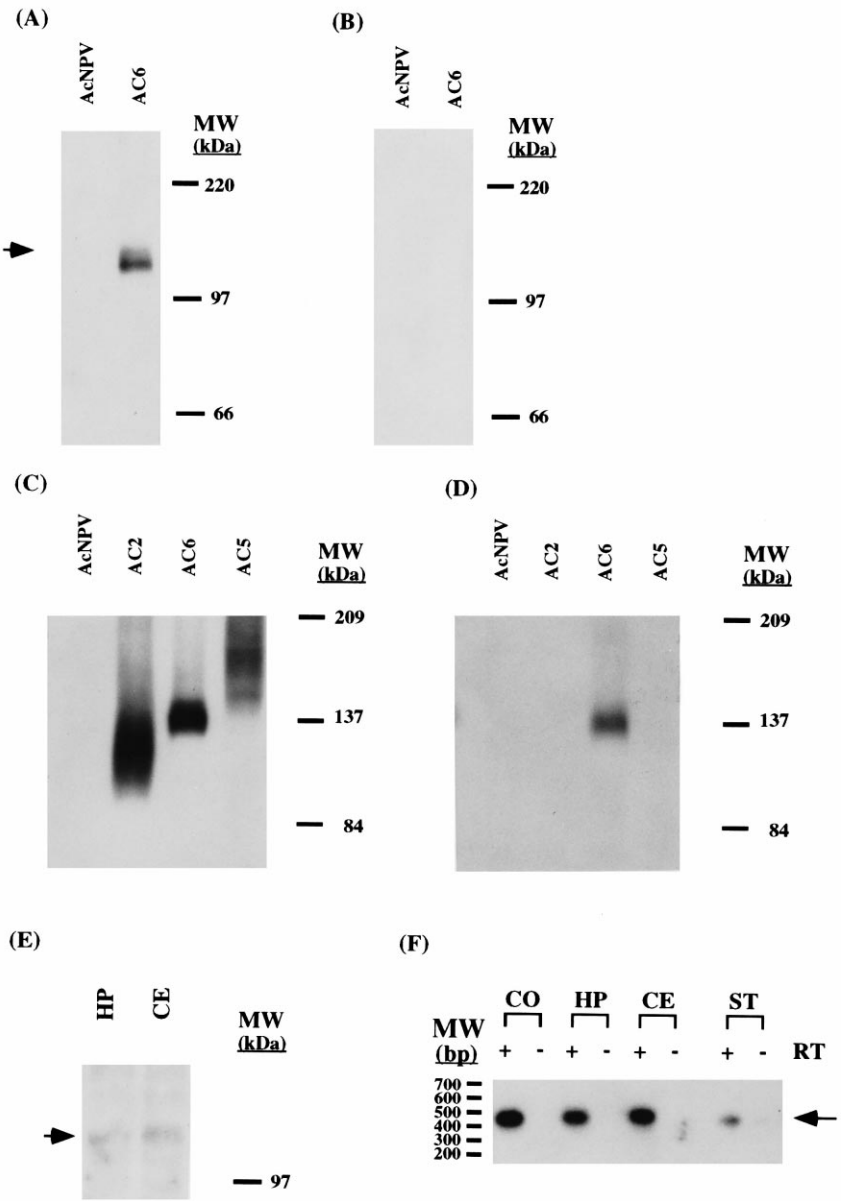

Fig. 1. Expression of type VI AC in the central nervous system. A–D: Characterization of an anti-AC VI antibody, AC6N. Immunoblots of the indicated recombinant proteins (20 μg per lane) using AC6N (A and D; 1/500 dilution), or AC6N pre-absorbed with AC6n peptide (B; 1/500 dilution), or a common antibody against adenylyl cyclases, R32 (C, 1/1000). AcNPV: *Autographa californica* nuclear polyhedrosis virus; AC2: type II AC; AC5: type V AC; AC6: type VI AC. E: An equal amount (100 μg) of protein from the hippocampus (HP) or the cerebellum (CE) was loaded to each lane for Western blot analysis using AC6N. The arrow indicates the type VI immunoreactive band. F: The type VI AC DNA fragments were amplified from the indicated cDNA prepared in the presence (+) or absence (−) of reverse transcriptase (RT), analyzed by ethidium bromide/agarose (1%) electrophoresis, and transblotted for Southern blot analysis to verify the specificity. The predicted size of the AC VI DNA fragment amplified was 480 bp. MW: molecular marker; CO: cortex; HP: hippocampus; CE: cerebellum; ST: striatum.

Table 1
Immunostaining patterns of AC6N in the central nervous system of rats

Regions	Expression level
Molecular layer	+
Granular cell layer	+/−
Purkinje cells	+
Interpose nu. (Int)	++
Medulla and pons	
Spinal trigeminal nu. (SP5O, SP5I, SP5C)	+
Spinal trigeminal tract (sp5)	+
Nu. lateral lemniscus (DLL, VLL)	+
Facial nu. (VII)	+
Lateral reticular nu. (LRt)	+
Trapezoid body (tz)	++
Inferior olive (IO)	++
Pontine nu. (Pn)	++
Dorsal cochlear nu. (DC)	+
Mesencephalon	
Superior colliculus (SC)	+
Inferior colliculus (IC)	+
Red nu. (R)	+
Ventral tegmental area (VTA)	+
Substantia nigra, compacta (SNc)	+
Substantia nigra, reticular (SNr)	+
Mesencephalic trigeminal nu. (Me5)	+
Diencephalon	
Thalamus	
Reticular nu. (Rt)	++
Anterodorsal nu. (AD)	++
Anteromedial nu. (AM)	++
Anteroventral nu. (AV)	++
Mediodorsal nu. (MD)	+
Laterodorsal nu. (LD)	++
Lateroposterior nu. (LP)	++
Ventrolateral nu. (VL)	++
Ventral posterolateral nu. (VPL)	++
Ventral posteromedial nu. (VPM)	++
Posterior nu. (Po)	++
Subthalamus	++
Hypothalamus	
Arcuate nu. (Arc)	++
Lateral hypothalamus (LH)	+
Ventromedial nu. (VMH)	+
Geniculate	
Medial (MGN)	++
Lateral (LGN)	++
Zona incerta	++
Supraoptic nu. (SO)	++
Habenular nu. (Hb)	++
Telencephalon	
Basal forebrain	
Lateral and medial septal nu. (LS, MS)	++
Horizontal limb of diagonal band (HDB)	+
Vertical limb of diagonal band (VDB)	++
Nu. basalis (B)	+
Substantia innominata (SI)	+
Ventral pallidum (VP)	++
Amygdaloid nuclei	
Anterior (AA)	++
Basolateral (BLA)	++
Basomedial (BMA)	++
Bed nu. striata terminalis (BST)	++
Globus pallidus (GP)	++
Caudoputamen (CPu)	++

Table 1 (*continued*).
Immunostaining patterns of AC6N in the central nervous system of rats

Regions	Expression level
Nu. accumbens (Acb)	++
Hippocampus	
Dentate gyrus (DG)	++
CA1	+
CA2	++
CA3	++
Subiculum (S)	++
Claustrum (Cl)	++
Cerebral cortex	++
Olfactory tubercle (OTu)	+/−

++: intense staining; +: moderate staining; +/−: weak staining.

earlier [19]. Primers for rat type VI AC gene [15] were as follows: 5′-ATGCCCCTGCCCGTGGCC-3′ and 5′-GTTCATCTGGAAGAAGTA-3′. These two primers amplify a DNA fragment of rat VI AC from bases 14–493 [15]. Expression of type VI AC was verified by hybridization with a ^{32}P-labeled, type VI AC-specific primer (5′-CCGTCCTGCTGGCACCGG-3′, corresponding to rat type VI AC from bases 395–412).

3. Results and discussion

In order to determine the regional expression of type VI AC in the central nervous system (CNS), an oligopeptide corresponding to the hypervariable N-terminal region (amino acids 1–19) of rat type VI AC was used to prepare the anti-type VI AC antiserum, designated as AC6N, in rabbits. There is no sequence homology between the amino acids 1–19 of type VI AC and those of other AC isoforms. Recombinant types II, V, and VI adenylyl cyclase proteins were used as controls to characterize the specificity of AC6N antiserum. Expression levels of the indicated recombinant AC protein were visualized using a common antibody, R32, which recognizes all ACs tested (Fig. 1C). As demonstrated in Fig. 1, the AC6N antiserum recognized only recombinant type VI AC, but not type V AC (the closest cousin of type VI AC in the AC family). Nor did the AC6N antiserum cross-react with type II AC. Typically, two type VI AC-immunoreactive bands (141 and 150 kDa) were observed. Blocking glycosylation using tunicamycin (1 mg/ml) effectively removed the higher type VI AC-immunoreactive band (data not shown), suggesting that type VI AC was partially glycosylated in Sf21 cells. Addition of excess AC6n peptide (amino acids 1∼19 of type VI AC) resulted in the complete disappearance of the two type VI AC-immunoreactive bands (Fig. 1B). In addition, immunoreactivities of type VI AC were detected in the hippocampus and the cerebellum of rat brain (Fig. 1E). These data demonstrate that AC6N recognized type VI AC specifically, and was suitable for the subsequent immunohistochemical analysis of type VI AC in CNS.

The existence of type VI AC in CNS was also verified by a PCR-based detection of the type VI AC transcripts. As shown in Fig. 1F, a 480-bp DNA fragment of type VI AC was amplified from mRNAs of the indicated tissue by a RT-PCR technique. The specificity of the amplified DNA fragment was confirmed by Southern blot analysis using an internal primer as a radio-labeled probe. No signal was detected when the reverse transcriptase was omitted in the cDNA synthesis reactions. Our data is consistent with a previous report by Premont et al. [15] which showed that transcripts of type VI existed in the rat brain.

We next carried out immunocytochemical analysis of adult rat brains using the anti-type VI AC antiserum, AC6N. Detailed analysis suggest that the type VI AC immunoreactivity was generally expressed in many brain regions with different levels of intensity (Table 1). The specificity of type VI AC immunostaining was demonstrated by preadsorption of AC6N antiserum with AC6n peptides (the immunizing peptides), which abolished nearly all immunostaining except for some weak staining still present in Purkinje cells of the cerebellum (data not shown). This residual staining appeared to be derived from non-specific antibodies present in the antiserum, as Purkinje neurons were weakly stained with the preimmune serum.

In the hippocampus, a striking pattern of heterogenous expression of type VI AC immunostaining was observed. Strong type VI AC immunoreactivity was present in the pyramidal layers of CA2 and CA3, but not in CA1 (Fig. 2A). The cellular staining indicated that type VI AC was primarily expressed in the perikarya of pyramidal neurons (Fig. 2B). High levels of type VI AC immunoreactivity was also present in the dentate gyrus (Fig. 2C). In contrast to the strong staining in CA2, CA3 and the dentate gyrus, type VI AC was expressed at very low levels in the CA1 region (Fig. 2A). The dramatic shift from the low level of type VI AC expression in the CA1 region to the high level of expression in the CA2 region led to a sharp demarcation between these two regions in the hippocampus (Fig. 2A). There were also type VI AC-positive cells scattered throughout the hippocampus. Some of these cells were apparently interneurons in the hippocampus (Fig. 2B,C).

ACs have been implicated in several important functions, including long-term potentiation (LTP) and neurotransmitter release, of the hippocampus [9,10]. Among all ACs, Ca^{2+}-stimulated ACs have become the focus of study lately since LTP-inducing tetanic stimulation in the hippocampal area leads to a Ca^{2+}/calmodulin-dependent increase in the cellular cAMP levels [20]. Such Ca^{2+}/calmodulin-mediated stimulation of postsynaptic AC appears to be generated by activation of Ca^{2+}-stimulated ACs, such as type I AC and type VIII AC [21,22]. Using a common antibody against all ACs, Mons et al. [23] previously reported that the densest staining in the hippocampus was seen in the dentate gyrus with all granular

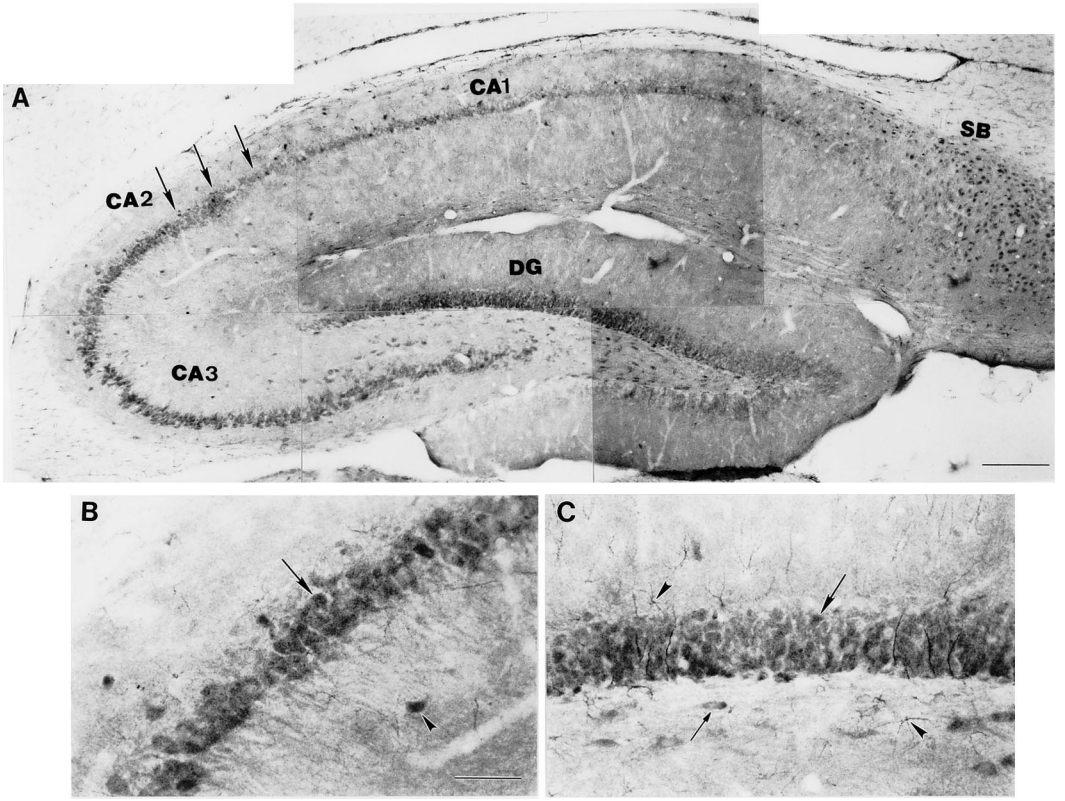

Fig. 2. Photomicrographs illustrating type VI AC immunoreactivity in the hippocampus. A: Strong type VI AC-immunoreactive cells appear in CA2, CA3, the dentate gyrus (DG), and subiculum (SB). By contrast, very low levels of type VI AC immunoreactivity are present in CA1. The arrows point to the transition zone from type VI AC-poor CA1 to type VI AC-rich CA2. B: High magnification of CA2 region. The pyramidal neurons of CA2 region are highly immunoreactive for type VI AC (arrow). A few scattered interneurons are also type VI AC-positive (arrowhead). C: High magnification of the dentate gyrus. Many type VI AC-positive granular cells (large arrow) are present in the dentate gyrus. A few interneurons (small arrow) and small glia-like cells (arrowheads) also appear in this area. Scale bars in A indicate 200 μm, in B (for B and C) indicate 50 μm.

cells strongly labeled. Transcripts of both type I AC and type VIII AC were found to be expressed at relatively high levels in the granule cell layers of the dentate gyrus and pyramidal cells of the CA2 region in the hippocampus [22]. Moreover, high levels of the type VIII AC transcript were observed in all pyramidal cells of CA1–CA3 fields [21,24]. Most interestingly, both LTP in the CA1 region of the hippocampus and spatial memory were impaired in mice lacking type I AC [10]. Type I AC was therefore suggested to play an important role in learning and memory processes. Our data suggest that a Ca^{2+}-inhibitable AC (type VI AC) might be co-expressed with two Ca^{2+}-stimulated ACs (types I and VIII ACs) by neurons in the hippocampus. Enzymatic analyses showed that EC_{50} values of $[Ca^{2+}]_i$ for stimulation of type I AC and type VIII AC are 100 and 800 nM, respectively [22], while type VI AC is significantly suppressed by submicromolar concentrations of $[Ca^{2+}]_i$ [25]. Impacts of these Ca^{2+}-sensitive ACs on the functions of the hippocampus may be determined by their different activation thresholds to $[Ca^{2+}]$, by their other enzymatic properties (see below) and by their subcellular spatial localizations in synaptic sites.

Type VI AC immunoreactivity was also detected throughout the cerebral cortex. Strong type VI AC-positive cells were observed in the deep layers (layers V and VI), whereas lightly stained cells were found in the upper layers of the cortex (data not shown). In the basal ganglia, type VI AC immunoreactivity was detected in the striatum, the nucleus accumbens, the globus pallidus and the substantia nigra (Table 1). In the cerebellum, type VI AC immunoreactivity was found in the interneurons of the molecular layer, Purkinje cells, and granular cell layer (Table 1). Type VI AC immunoreactivity was also present in many regions of the hindbrain (Table 1).

To examine whether the type VI AC-immunoreactive cells were neurons, we carried out double immunostaining for type VI AC and microtubule-associated protein 2 (MAP-2), which is a marker for neurons [26]. The results show that type VI AC-positive cells in most areas examined co-express MAP-2 (Fig. 3A,B). Thus, the detected type VI AC immunoreactivity was present in cells with neuronal phenotype. We and other laboratories have previously reported that this type VI AC can be suppressed by physiologically relevant concentrations of $[Ca^{2+}]_i$ [25], by PKC [7] and by PKA [11]. Since cAMP/PKA, calcium, and PKC are three very important signal messengers generally used in the nervous system, type VI AC appears to integrate multiple signals and might provide an exquisite mechanism for the fine-tuning of cAMP synthesis by Ca^{2+}, PKA, and PKC in neurons. In contrast, most of the astrocytes identified by positive immunoreactivity of glial fibrillary acidic protein (a marker of astrocytes) were negative for type VI AC immunoreactivity, except some astrocytes in specific areas including the hippocampus and the cerebellum (data not shown). These data suggest that type VI AC protein might be more important in regulating the physiological functions in neurons than those in astrocytes.

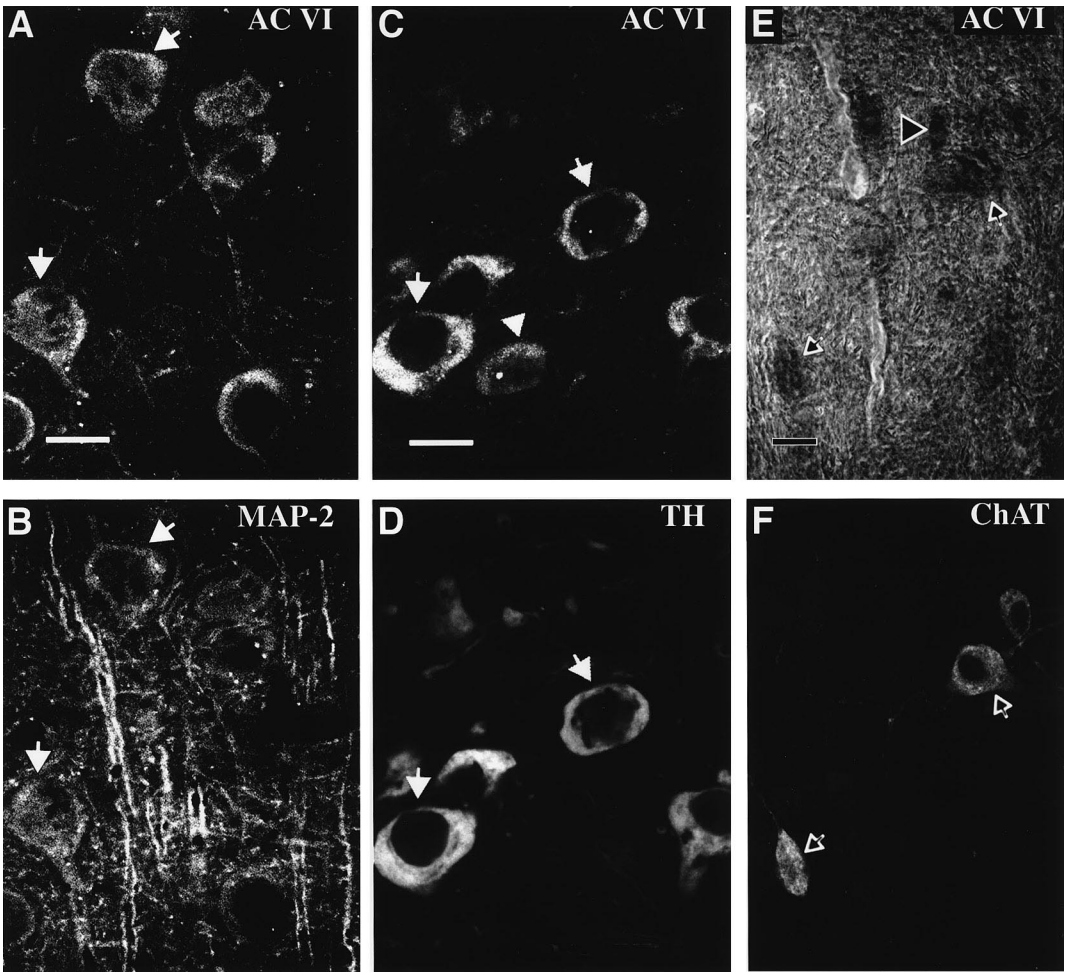

Fig. 3. Existence of type VI AC immunoreactivities in different neurotransmitter systems. A, B: Confocal images illustrating coexpression of type VI AC (A) and MAP-2 (B) in the cerebral cortex (arrows). C, D: Confocal images of double immunofluorescent staining of type VI AC (C) and TH (D) in the same field of the substantia nigra pars compacta. All TH-positive dopaminergic neurons co-express type VI AC (arrows). A few type VI AC-positive cells, however, do not express TH (arrowhead). E, F: Photomicrographs of double immunostaining of type VI AC (E) and ChAT (F) in the same field of the basal forebrain. Type VI AC-positive cells appear black in the lighter background in E, whereas ChAT-positive cells appear as white cells in F. ChAT is expressed in some but not all type VI AC-positive cells (arrows). ACVI: type VI AC. Scale bars in A, C, and E indicate 20 μm.

The differential expression of type VI AC protein within a brain region suggests that type VI AC molecules may be coupled to specific signal transduction pathways in different neuronal populations. We tested this possibility by performing double immunostaining of type VI AC and markers of different neurotransmitter systems, including dopaminergic and cholinergic systems. For the dopaminergic system, double immunofluorescence of type VI AC and tyrosine hydroxylase (TH, a synthetic enzyme for catecholamine) showed that TH-positive dopaminergic neurons in the pars compacta of substantia nigra co-expressed type VI AC (Fig. 3C,D). We also carried out double immunostaining analysis of type VI AC and DARPP-32 (dopamine- and adenosine 3′:5′-monophosphate-regulated phosphoprotein). DARPP-32 is a molecule known to be associated with D1 dopamine receptors [27]. Our results showed that type VI AC and DARPP-32 were co-localized in neurons of the caudoputamen (data not shown). Thus, type VI AC protein was expressed in the mesostriatal dopaminergic system. For the cholinergic system, double immunostaining of type VI AC and choline acetyltransferase (ChAT) demonstrated the co-expression of ChAT and type VI AC in the neurons of basal forebrain including the nucleus basalis of Meynert and substantia innominata (Fig. 3E,F). These results showed that type VI AC protein is expressed in dopaminergic and cholinergic neurons. In addition, type VI AC immunoreactivity was detected in neurons of several brain regions that are known to use glutamate and GABA as neurotransmitters, such as the hippocampus and the striatum (Table 1). Expression of type VI AC protein therefore is apparently associated with the glutamatergic and GABAergic systems. Collectively, type VI AC may participate in regulation of the classical neurotransmitter systems, including dopamine, acetylcholine, glutamate, and GABA.

In addition to their different sensitivities toward calcium, the enzymatic properties of type VI AC compared to other ACs expressing in CNS are also different. Harry et al. [2] have recently reported that stimulation of type VI AC by Gsα can be analyzed using a two-site model which is mechanistically very different from those of types I and II ACs. The susceptibility of each individual AC to inhibition by Giα is also different [3]. Such fundamentally different regulation of individual AC by Gα proteins might contribute to variable re-

sponses to cAMP-regulating hormones in different tissues. In addition, the basal activity of type VI AC is significantly lower than that of type II AC [28], suggesting that neurons dominantly expressing type VI AC might have a lower basal cAMP level than do those overbearingly expressing type II AC. The significance of the above finding is tremendous since the basal cAMP levels, which determine the basal protein kinase A activity, play a critical role in various important physiological processes [29,30].

In summary, we have demonstrated that type VI AC is widely expressed in CNS, mainly in neurons. This calcium-inhibitable AC is likely to integrate diverse signals (cAMP/PKA, calcium, and PKC) in neurons, and plays an important role in CNS.

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