

Noncholinergic functions of cholinesterases

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ABSTRACT Cholinesterases (acetylcholinesterase and butyrylcholinesterase) exhibit additional catalytic activities apart from their well-known action in hydrolyzing choline esters. An amine-sensitive aryl acylamidase activity is exhibited by both acetyl- and butyrylcholinesterases. A metallocarboxypeptidase-like activity is found associated with both acetyl- and butyrylcholinesterases. The peptidase activity exhibited by butyrylcholinesterase was located in a 50-kDa COOH-terminal fragment. Acetylcholinesterase is implicated in noncholinergic functions in the substantia nigra. A relationship between tumorigenesis, cell differentiation, and cholinesterases has been speculated. The sequence similarities between different esterases, lipases, thyroglobulin, cell adhesion proteins, and cholinesterases would make it appear that cholinesterases are capable of exhibiting more than one biological activity and their functions are wider than what is hitherto known.—Balasubramanian, A. S., Bhanumathy, C. D. *Noncholinergic functions of cholinesterases. FASEB J.* 7: 1354-1358; 1993.

Key Words: acetylcholinesterase • butyrylcholinesterase • aryl acylamidase • metallocarboxypeptidase • neural cell differentiation

ACETYLCHOLINESTERASE (AChE) IS WELL KNOWN for its role in terminating the action of acetyl choline at the cholinergic synapses. However, observations made by different investigators suggested that it may have additional noncholinergic functions (1, 2). AChE occurs in areas of the brain and in erythrocyte membrane where no cholinergic functions are known to take place. Moreover, the enzyme exists in polymorphic forms. This diversity in distribution, form, and cellular localization gave the initial impetus to study the possible additional functions of AChE.

Butyrylcholinesterase (BChE), which is closely related to AChE, occurs abundantly in human serum and in lesser amounts in various tissues (3). Cloning and sequencing have revealed striking sequence homology between the two enzymes (4, 5). BChE has 53% amino acid sequence identity to AChE, shows similar response to a number of classical inhibitors of AChE, and differs from AChE in its substrate preference. No well-established function of BChE is known.

The amino acid sequence at the active site of both AChE and BChE is well conserved. Besides, an active site serine residue, a histidine, and a glutamate residue are considered important for the catalytic activity of both AChE and BChE (6, 7). The near identity of the two types of cholinesterases is also reflected in several other properties exhibited by both of them as described below. The proposed biological activities of AChE and BChE are given in Table 1.

AN AMINE-SENSITIVE ARYL ACYLAMIDASE (AAA) ACTIVITY IN AChE AND BChE

Both AChE and BChE exhibit an aryl acylamidase activity capable of hydrolyzing the acyl amide bond in the substrate

o-nitroacetanilide (8-10). This AAA activity is strongly inhibited by classical cholinesterase inhibitors. In addition, AAA found in cholinesterase is susceptible to selective inhibition by 5-hydroxytryptamine (5HT) (8-10). The exhibition of a 5HT-sensitive AAA activity by AChE and BChE from several different sources has been demonstrated (9-14). Chemical modification studies have indicated overlapping active sites for AAA and AChE or BChE (15, 16). Yet another exclusive feature of the AAA activity in human serum BChE is its severalfold stimulation by tyramine (10, 14, 17). The serum BChE of monkey shows significant tyramine activation of AAA activity; the monkey is the only animal that shows immunological identity of its BChE to human BChE (17, 18). The sequence identity between Asn-68 to Leu-208 in serum BChE from human and monkey is 100%, and from other species nearly 91-97% (18). This would indicate that unique structural features or sequences exclusively present in the human and monkey sera BChE and absent in the serum BChE of other species are responsible for the tyramine stimulation of their AAA activity. In Table 2 the general characteristics of AAA associated with cholinesterases and AAA unassociated with cholinesterases are given. The human liver AAA, not associated with AChE or BChE, does not show any amine sensitivity and is unaffected by classical cholinesterase inhibitors.

The difference in the susceptibility to tyramine activation of AAA from AChE and BChE could be attributed to the sequence differences found in these two enzymes. A specific binding site for tyramine absent in AChE may be present in BChE. Alternately, BChE but not AChE may undergo a conformational change caused by tyramine binding. In this connection the recent modeling studies (19) that reveal the absence in BChE of six conserved aromatic residues that line the active-site gorge of AChE is noteworthy. A closer look into the sequence differences between AChE and BChE and the binding sites of tyramine will shed light on these possibilities.

A POSSIBLE ROLE OF THE AMINE-SENSITIVE AAA ACTIVITY

What could be the possible significance of the association of an AAA activity with cholinesterases and the sensitivity to amines of these enzymes? The acyl-amide bond cleaved by AAA is similar to those found in pain-relieving drugs such as paracetamol and phenacetin. If any such synthetic drug is cleaved by AAA, then 5HT, which inhibits AAA, may help prolong the life of such drugs in the circulation and improve their analgesic effects. 5HT is known to have an effect on pain relief (20). Attempts to demonstrate the deacetylation of phenacetin by AAA have been unsuccessful (21). However,

¹Abbreviations: AChE, acetylcholinesterase; BChE, butyrylcholinesterase; 5HT, 5-hydroxytryptamine; AAA, aryl acylamidase.

TABLE 1. *Proposed biological activities of cholinesterases*

Biological activity	Cholinesterases involved	Reference
Amine-sensitive aryl acylamidase activity	AChE, BChE	(8-14)
Metallocoarboxypeptidase-like activity	AChE, BChE	(23, 24, 28, 29, 31)
Cocaine hydrolysis	BChE	(57, 58)
Neural cell differentiation	AChE, BChE	(39-41)
Cell division and tumorigenesis	AChE, BChE	(46-48)
Functions in substantia nigra (potassium channel opening)	AChE	(42-43)
Cell-cell interaction	AChE, BChE	(59)

it is possible to envisage the existence of an unknown endogenous analgesic compound with an acyl-amide bond susceptible to cleavage by AAA and its modulation by acetylcholine and 5HT, suggesting a role for AChE in pain relief.

Migraine has been correlated with excessive tyramine. A defective tyramine conjugation has been suggested to be the reason for excessive tyramine (22). However, the exact relationship between tyramine and migraine is not clear. It may be speculated that tyramine promotes the degradation of an endogenous analgesic acyl-amide compound through stimulation of the AAA in circulation resulting in the derangement of a natural pain relief mechanism. A role for human serum BChE in the control of amine-sensitive pain mechanisms through its AAA modulated by both 5HT and tyramine remains a possibility.

THE PEPTIDASE ACTIVITY IN AChE AND BChE

Unlike the well-founded findings of AAA association with AChE and BChE, the presence of a peptidase activity in cholinesterases has been the subject of divergent observations from different laboratories. A peptidase activity in AChE that behaved like trypsin and like a carboxypeptidase (based on the substrates cleaved) was reported by Small et al. (23). The carboxypeptidase was also stimulated by divalent metal ions (24). Subsequently it was reported that the protease activity associated with purified AChE from human brain, fetal bovine serum, or electric eel could cleave the membrane-bound form of the amyloid protein precursor of Alzheimer's disease (25). Trypsin activity was suggested to be a contaminant in commercial electric eel AChE (26).

A dipeptidyl peptidase activity initially reported to be present in purified BChE was later demonstrated to be a contaminant and separable from BChE (27). Boopathy and Balasubramanian (28) observed that purified BChE of hu-

man serum exhibited a peptidase activity that could cleave amino acids from the COOH-terminus of several peptides. The peptidase and BChE activities were inseparable under several conditions of ion exchange, affinity, and molecular sieve chromatography and by electrophoresis. Further, both activities could be immunoprecipitated by monoclonal antibodies (29). As further evidence, Rao and Balasubramanian (30, 31) demonstrated that BChE could be cleaved by limited α -chymotrypsin digestion into enzymatically active fragments of 20 kDa and 50 kDa, the former exhibiting both BChE and AAA activity and the latter exhibiting peptidase activity alone. Supporting these findings was the observation that classical inhibitors of BChE that block the active site serine had no inhibitory effect on the peptidase activity (28, 31). The amino-terminal sequencing of these fragments showed that they arise by cleavage of Tyr (146)-Arg (147) and Phe (290)-Gly (291) bonds in the BChE molecule (30, 31). These results suggested that all three enzyme activities reside on the same polypeptide chain of BChE (Fig. 1). Further, they presented evidence that the peptidase behaves like a metallopeptidase stimulated by Zn^{2+} , Co^{2+} , and Mn^{2+} and that histidine residues were involved in metal ion stimulation (29). A His-X-X-Glu sequence suggested to be involved in metal ion binding (32, 33) in the peptidase domain of BChE supported these findings. A similar metal binding sequence is also found in AChE (Table 3).

Checler and Vincent and Checler et al. (34, 35) observed that protease activities that cleave Leu-enkephalin, substance P, and neurotensin in electric eel AChE and in human serum BChE preparations could not be immunoprecipitated by monoclonal antibodies raised against AChE and BChE, respectively. Apparently these were contaminating peptidases present in their cholinesterase preparations. This was also corroborated by the fact that the cleavage products from Leu-enkephalin in their studies was a tetrapeptide and leucine in contrast to observations by Rao and Balasubrama-

TABLE 2. *The comparative characteristics of aryl acylamidases (AAA) associated and unassociated with cholinesterases*

Property	AAA associated with AChE	AAA associated with BChE	AAA not associated with cholinesterases
Amine sensitivity	Inhibited by 5-hydroxytryptamine and unaffected by tyramine	Inhibited by 5-hydroxytryptamine and stimulated by tyramine	Unaffected by 5-hydroxytryptamine and tyramine
Sensitivity to choline esters	Inhibited by butyrylcholine and other choline esters	Inhibited by succinyl choline and other choline esters	Unaffected by choline esters
Sensitivity to cholinesterase inhibitors	Inhibited by eserine, neostygmine, and the AChE-specific inhibitor BW284C51	Inhibited by eserine, neostygmine, and the BChE-specific inhibitor tetraisopropyl pyrophosphoramidate	Not inhibited by the cholinesterase inhibitors

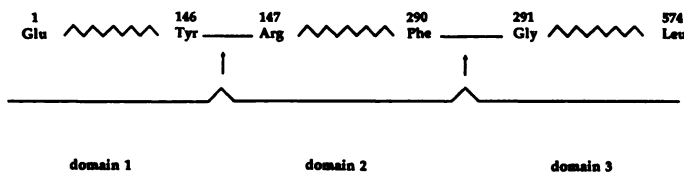


Figure 1. The three domains of human serum BChE isolated by limited α -chymotrypsin digestion. Domain 1 is an inactive fragment, domain 2 is a fragment exhibiting BChE and AAA activities, and domain 3 exhibits peptidase activity exclusively. Domain 2, which is devoid of galactose residues, can be separated from domain 3 by *Ricinus communis* agglutinin 120-Sepharose chromatography (30, 31).

nian (29), who found that Leu-enkephalin was cleaved to a tripeptide and two amino acids from the COOH-terminus under all conditions of assay. Although separation of contaminating peptidases from AChE and BChE does not mean that they have no peptidase activity, the detailed characteristics of the peptidases really associated with AChE and BChE need to be fully investigated. Purified BChE from the serum of monkey, the only animal that shows immunological cross-reactivity with human BChE antibody, also exhibited a metalcarboxypeptidase activity with characteristics similar to that of human BChE (C. D. Bhanumathy, R. V. Rao, and A. S. Balasubramanian, unpublished results).

The crystal structure of AChE that is closely related to BChE has revealed that wheat serine carboxypeptidase II shows a striking similarity to the overall structure of AChE (7). A great deal of similarity in the folds of wheat serine carboxypeptidase II and the zinc exopeptidase carboxypeptidase A has been pointed out by Liao and Remington (36). Their studies also suggested that the structural motif Gly-X-Ser-X-Gly found at the active site of several serine proteinases or esterases is fortuitous and might not have diverged from a common ancestor (37). Similarities in primary sequence between digestive and nondigestive carboxypeptidases are only 15–20% (38). In spite of these differences, sequence alignments clearly show that key residues of His and Glu for coordination of Zn^{2+} and Arg, Glu, and Tyr for catalysis are common to all these enzymes (38).

A ROLE FOR CHOLINESTERASES IN NEURODIFFERENTIATION

Long before any synaptogenesis occurs in the chicken neural tube, BChE is seen diffusely distributed along the ventricular layer whereas AChE is localized in cells along the mantle layer (39). The appearance of both enzymes correlates with

the end of cell proliferation in different tissues and cell types of embryonic chicken brain and retina. These observations on the expression of cholinesterases at critical times during early neurogenesis have suggested the possibility of their involvement in neurogenetic processes. A correlation between high proliferative rate and BChE expression has been observed in neuroblasts of the early neural tube, neural crest cells, retinal cells, and others (40). Layer and co-workers (41) consider BChE a transmittotic marker because high BChE activities are typical of cells in a transient state shortly before final mitosis and the beginning of cellular differentiation. The expression of AChE and BChE at early stages of embryogenesis before the formation of synapses also suggests a morphogenetic role for these enzymes at this stage.

NONCHOLINERGIC EFFECTS OF AChE IN THE SUBSTANTIA NIGRA AND CEREBELLUM

In the substantia nigra of brain, AChE appears to have a noncholinergic role. There is a disparity in the high levels of AChE relative to low levels of acetylcholine in this region. Moreover, behavioral and electrophysiological actions of AChE appeared to be unassociated with the hydrolysis of acetylcholine in substantia nigra (42).

Recent studies by Webb and Greenfield (43) have indicated that an ATP-sensitive potassium channel is operative in substantia nigra and that AChE has a role in opening this channel. The reversible hyperpolarizing action of AChE via an opening of potassium channels on a selective population of nigral neurons could be identified by an ability to generate bursts of action potentials. The AChE-induced hyperpolarization was seen with a highly purified preparation of AChE. These physiological effects of AChE were claimed not to result from any contaminants present in AChE (43). The effect of AChE was not attributed to its enzymatic action, as even a boiled preparation of AChE was efficacious. It was concluded from these studies that the tertiary structure of AChE was not needed for this noncholinergic action (43).

In another study (44, 45) a novel, modulatory effect of AChE in cerebellar Purkinje cells in increasing their ability to respond to excitatory input and fire Na^+ action potentials over an expanded range of frequency has been observed. Irreversible inhibition of AChE with soman did not prevent this action (indicating that acetylcholine hydrolysis was not involved) and BChE had no effect.

Secretory forms of AChE are implicated in these noncholinergic functions (45). Barring the presence of any contaminants in the AChE preparations that are responsible for the observed effects, the studies described above provide direct evidence for the noncholinergic physiological roles of AChE in the brain.

TABLE 3. Metal binding sequence (HXXE-H) found in some metallo-peptidases and their comparison to cholinesterases

Enzyme	Sequence*	Residues	Reference
Human insulin degrading enzyme	HFCEH	109–113	(33)
<i>Drosophila</i> insulin degrading enzyme	HFCEH	81–85	(33)
Bovine carboxy-peptidase A	HSRE (X) ₁₂₃ H	69–196	(32)
Bovine carboxy-peptidase B	HARE (X) ₁₂₃ H	69–196	(32)
Human BChE	HGYE (X) ₁₀₇ H	438–548	(5)
Torpedo AChE	HGYE (X) ₁₀₇ H	440–550	(4)

*X indicates any amino acid. Single-letter code for amino acids is used.

CHOLINESTERASES IN TUMORIGENESIS

Many tumor cells that display no detectable electrical response to acetylcholine have been shown to express cholinesterases and acetylcholine receptors (46). In many types of human tumors, cholinesterase genes are abnormally expressed (46). It is hypothesized that a defective cholinergic signaling may change the activities of cdc 2-like kinases with the potential to accelerate the rate of cell division and support tumor growth (47). The potential substrate proteins of cdc 2-related kinases as well as AChE and BChE contain the common peptide motif Ser/Thr-Pro-X-Z, where X is a polar amino acid and Z is a basic amino acid (48).

CONCLUSIONS AND FUTURE STUDIES

Partial sequences from several different proteins occurring in the polypeptide chain of one single protein increase the possibility of one protein exhibiting more than one biological activity. Sequence conservation analysis relates the cholinesterases to a superfamily of polypeptides including enzymes such as microsomal carboxylesterase, cholesterol esterase, lysophospholipase, *Geotrichum* lipase, and *Drosophila* esterase-6 as well as noncatalytic polypeptides such as the COOH-terminal domain of thyroglobulin, and the *Drosophila* cell adhesion proteins neurotactin and glutactin (49-56). Therefore the multiple activities of cholinesterases would not be altogether surprising. However, their true significance can be recognized only when their role within or outside the cell under in vivo conditions is evaluated. The amine-sensitive AAA and peptidase activities of AChE and BChE as well as the claims of Cocaine hydrolytic activity of BChE (57, 58) raise interesting possibilities as to the physiological functions of cholinesterases (Table 1). A linkage between cholinesterases and cell-cell interaction (59), cell division, neural cell differentiation, and neural development (46) has been strengthened by recent findings, and the proposed peptidase activity of cholinesterases may have a role to play in this process. An acetylcholine-sensitive hydrolysis of substance P in rabbit retina caused by a metallopeptidase, a proportionality between AChE activity and substance P hydrolysis (60), and the findings of carboxypeptidase inhibitors and indoleamines selectively inhibiting cholinesterases in histopathological structures of Alzheimer's disease (61) all point toward the relevance of the AAA and peptidase activities exhibited by cholinesterases. The existence of different forms of cholinesterases that differ in their catalytic functions and biological activities is a distinct possibility that remains to be explored. Future studies that help to fill in the gaps in our knowledge and understanding of the additional functions of cholinesterases would clarify the true significance of these findings. FJ

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