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Acetylcholinesterase in Human Red Blood Cells

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Abstract

Acetylcholineasterase (AChE) is present in sensory innervated tissues and it functions in the central and peripheral neuron system to terminate nerve signal transduction at the neuromuscular junction by rapid hydrolysis of the acetylcholine (ACh) receptor released into the cholinergic synaptic cleft. AChE is also found in human red blood cells (RBCs) (Brauer and Root, 1947), and it is one of the typical extrinsic membrane bound enzymes (Heller and Hanahan, 1972). Although the physiological functions of AChE in RBC remain obscure, changes in activity associated with pathologic conditions are found regularly only with AChE. Recent studies have disclosed much of the primary structure of AChE and the membrane anchor structure. However, the location of this enzyme at or near the cell surface gives it special significance in studies of cellular membranes and the activity alterations seen in several hemolytic disorders may be of importance in understanding certain basic disease process. The enzyme may be regarded as a model of AChE in the nervous system. AChE inhibition has been used as a peripheral surrogate biomarker for the activity of centrally acting AChE inhibitors (AChEIs) in the treatment of Alzheimer's disease (AD). AChE inhibition in RBC should reflect the central pharmacodynamic activity of the compound and the degree of inhibition should correlate to yielding maximum cognitive or global improvement in patients with AD. AChEI is also a useful clinical tool in dose optimization!

Keywords: Acetylcholinesterase, butyrylcholinesterase, membrane trafficking, Alzheimer's disease, DTNB: 5,5'-dithiobis-2-nitrobenzoic acid, neuromuscular transmission, human poisoning, anti-inflammation, acetylecholinesterase inhibitors, nerve agent, DAF; decay accelerating factor.

Introduction

Cholinesterases (Galehr and Plattner, 1927; Rappaport et al., 1959) are serine hydrolases which are traditionally classified into two main types, corresponding to two distinct genes: "true" or "specific" acetylcholine esterase [E.C. 3.1.1.7], and "pseudo" or "nonspecific" butyrylcholinesterase or pseudocholinesterase [E.C. 3.1.1.8] (Augustinsson, 1948; Bauer, et al, 1974). The major role of AChE is to catalyze the hydrolysis of ACh in cholinergic synapses, whereas the function of butyrylcholinesterase (BChE) is less clearly defined though it can partially compensate for the absence of AChE in the nervous system and it has the power of splitting ACh (Goedde and Altland, 1968; Xie et al., 2000; Duysen et al., 2001). This distinction has been made on the basis of substrate preference,

inhibitor specificity, and of antibody recognition (Mendel and Rudney, 1943; Austin and Berry, 1953; Brimjoin and Hammond, 1988) (Table 1). The human plasma is a rich source of cholinesterase whereas human red blood cell membranes are a source of acetylcholinesterase (Skater, 1973).

Table 1: Characteristics of cholinesterase
Adopted from: Augustinsson, K.B.(1948)"Cholinesterases: a study in comparative enzymology", Acta Physiol. Scand., vol. 15 (Suppl.52), pp.1-182; Yamada,M.,Marui,Y.,Hayashi,C.,Miki,Y and Takemura,S.(2001)"New thiocholine ester substrate for the assay of human serum cholinesterase", Clinical Chem.,vol.47(11), pp.1962-1966..

	Acetyl-cholinesterase	**Butyryl-cholinesterase**
Optimum pH	7.5-8.0	8.0-8.5
Optimum temperature (^oC)	25	37
Self inhibition, [S]↑	+	-
Active site cation-π interaction	Trp84	Trp82
Hydrolysis:		
Acetyl-β- methylcholine	+	-
Benzoylcholine	-	+
Tributyrine	-	+
Inhibition:		
Diisopropylphosphofluridate	++	++++
Esirine	++++	++++
Quinidine	+	++++

Therefore, BChE is important for hydrolyzing ACh in the circulation. The elucidation of the three-dimensional structure of AChE (Sussman et al., 1991) and of BChE (Nicolet et al., 2003) showed a general similarity between them, but indicated differences in the composition of the amino acids lining a deep, narrow gorge at the bottom of which resides a catalytic site. In human BChE, 6 of the 14 aromatic amino acids that line the gorge of AChE are replaced by aliphatic ones (Lockridge et al., 1987; Harel et al., 1992). Hence, it lacks the peripheral site found in AChEs, and its acyl pocket is larger (Radić et al., 1993; Saxena et al., 1997).

The enzymes physiological task is hydrolytic destruction of the cationic neurotransmitter ACh. Acetylcholinesterase is an extrinsic membrane bound enzyme that projects into the synapse. Acetylcholine is the neurotransmitter molecule responsible for "ferrying" the neuronal action potential across the synapse and initiating a new action potential on the muscle fiber sarcolemma, the membrane covering a striated muscle fiber (Fig.1). An action potential is a very rapid change in membrane potential that occurs when a nerve cell membrane is stimulated. The membrane potential goes from resting potential (-70 mV) to some positive value (~ +30 mV) in just few milliseconds period of time. Action potential is therefore a wave of membrane depolarization, a change in the electrical charge of a cell membrane from a negative resting membrane potential to a brief positive membrane potential. This opens more voltage-dependent ion channels in the adjacent membrane, and so a wave of depolarization courses along the cell. As the action potential nears its peak, the sodium gates close, and potassium gates open, allowing ions to flow out of the cell to restore potential of the membrane (Gutkin and Ermentrout, 2006)

The terminal button has numerous neurotransmitter vesicles that contain acetylcholine. The vesicles aggregate at active zones. The terminal button does not touch its target muscle cell; it is separated from the muscle fiber by the synaptic cleft. The muscle cell membrane, or sarcolemma, apposed to the terminal button has numerous synaptic folds where acetylcholine receptors are present in high density. The segment of the sarcolemma with such synaptic folds is known as the motor end plate.

The magnitude of the membrane potential at any given time depends, of course, upon the distribution of Na^+ , K^+ , and Cl^- , and the permeability of the membrane to each of these ions. An

equation that describes this relationship with considerable accuracy is the Goldman constant-field equation:

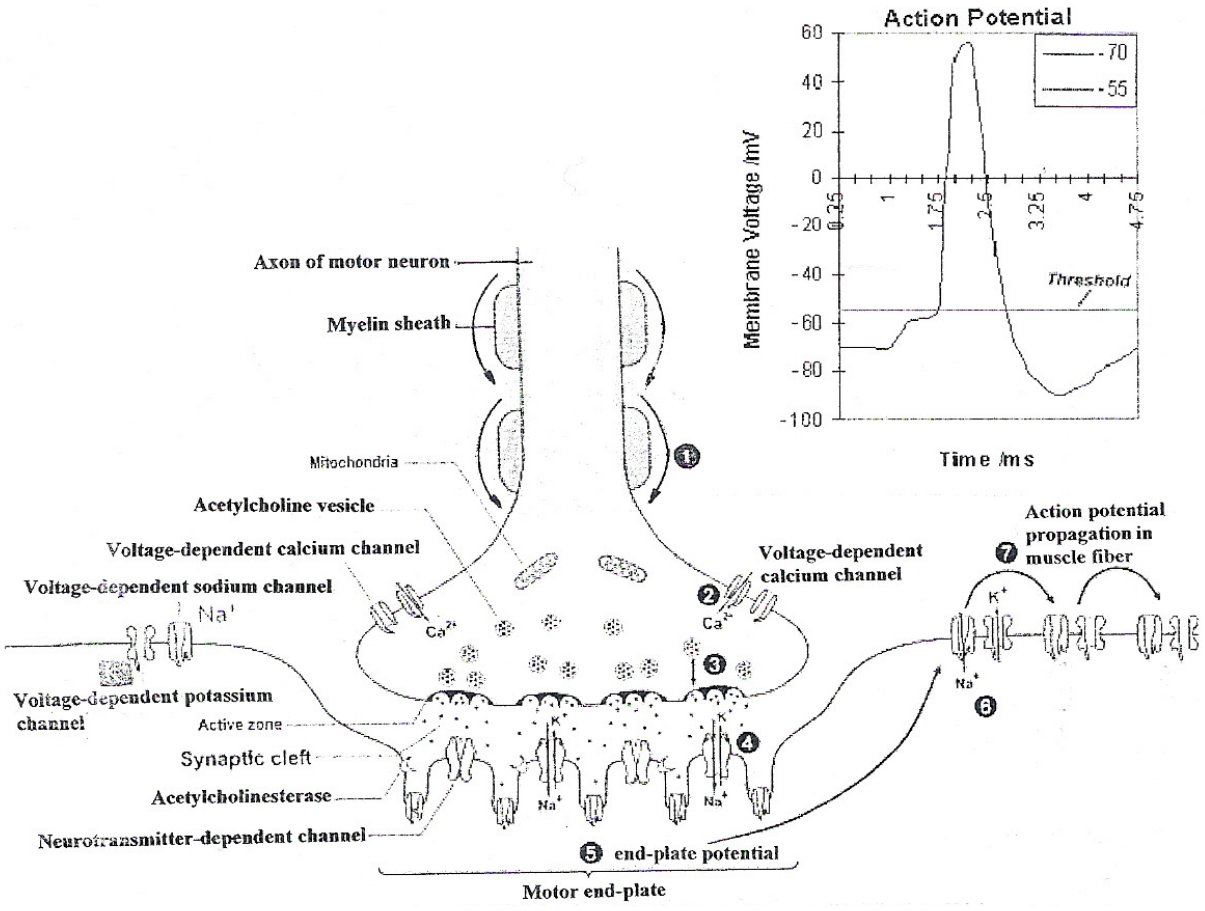
$$V = \frac{RT}{F} \ln\left(\frac{p_{K^+}[K_o^+] + P_{Na^+}[Na_o^+] + P_{Cl^-}[Cl_i^-]}{p_{K^+}[K_i^+] + P_{Na^+}[Na_i^+] + P_{Cl^-}[Cl_o^-]}\right)$$

where V is the membrane potential , R the gas content, T the absolute temperature, F the Faraday, P_{K+} , P_{Na+} , and P_{Cl-} the permeability of the membrane to K^+, Na^+, and Cl^- respectively. The brackets signify concentration, and I and o refer to inside and outside of the cell.

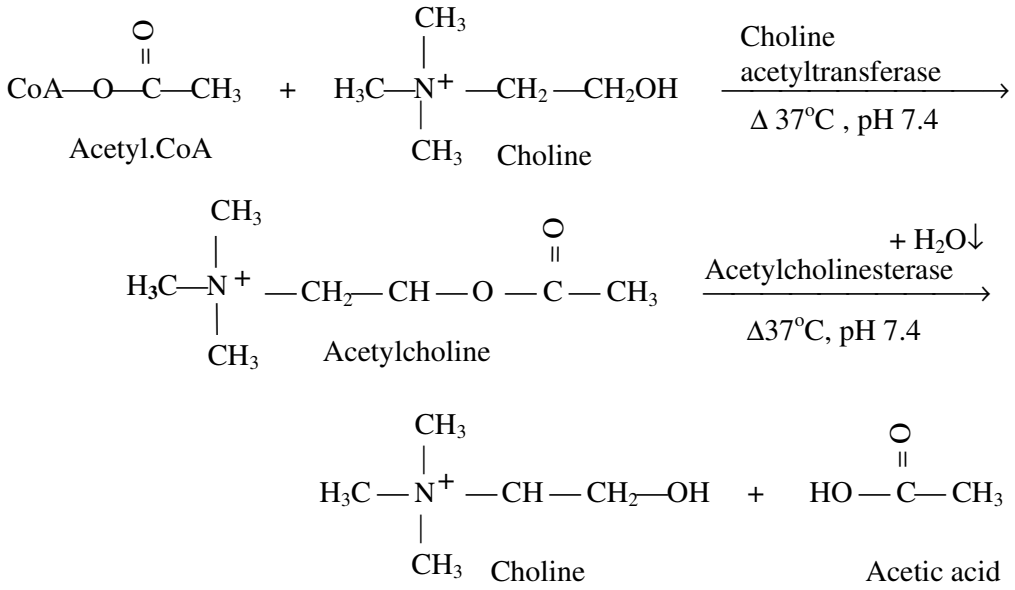
Inset: The activation of a nerve cell requires the following five components: a stimulus, one or more receptors for that stimulus, an intact plasma membrane, ion channels, and ion gradients. The effects are seen in the oscilloscope recording as full action potential is attained. Action potentials occur only when the membrane is stimulated (depolarized) enough, so that Na^+ channels open completely only briefly (0.5 millisecond). The Na^+ channels then close again suddenly and enter an inactive state; at the same time, K^+ leaks outward through its channels, thereby lowering the potential below the original resting-potential level. The refractory period terminates as the potential returns to the resting level and the Na^+ channels revert to the closed, but active state. The minimum stimulus needed to achieve an action potential is called threshold stimulus. Action potentials occur maximally or not at all (all-or-none law).

Adapted from: Sanes, J.R. and Lichtman, J.W. (2001) "Induction, assembly, maturation and maintenance of a postsynaptic apparatus", Nat. Rev.Neurosci., vol.2 (11), pp.791-805; Sheng, M. and Kim, M.J. (2001)"Postsynaptic signaling and plasticity mechanism", Science, vol.298, pp.776-780.

Figure 1: The neuromuscular junction.



Neurons produce ACh by transferring an acetyl group from acetyl-CoA to a molecule of choline. Acetylcholine is stored in the synaptic vesicles of motor neurons. When an action potential arrives at a motor neuron terminal, the transient increase in the membrane conductance of Na^+ and Ca^{2+} leads to an influx of Na^+ and Ca^{2+} down their respective electrochemical gradients that causes vesicle fusion with the nerve terminal membrane and the release of ACh into the synaptic cleft; 60 vesicles or so release their contents for each action potential (Ceccarelli et al., 1973; Heuser and Reese, 1973; Fesce et al, 1994). Each synaptic vesicle of a motor neuron terminal contains ~ 10,000 molecules of ACh. Once released from the presynaptic neuron, ACh diffuses across the synapse and two molecules of ACh bind to each ACh receptor, resulting in transformation of the neuronal action potential into a muscle fiber action potential by way of chemical signal. Acetylcholine soon diffuses from the receptor binding sites and is cleaved by synaptic AChE into acetate and choline (Eq.1), thus inactivating the neurotransmitter signal. The nerve terminal takes ~ 50% of the choline produced and recycled into new ACh.



(1)

The binding of two molecules of ACh to an ACh receptor leads to a conformational shift in the ACh receptor, with the opening of the ion channel and an increase in the sarcolemma permeability and conductance for both Na^+ and K^+ . Thus, the ACh receptor is a ligand-gated ion channel. As the ACh receptor cation channel is open, Na^+ enters the muscle fiber down its electrochemical gradient, and the sarcolemma is depolarized. The action potential propagates along the sacolemma and via T tubules into the muscle fiber. Depolarization is transmitted from the T tubules to the membrane of sarcoplasmic reticulum, which has numerous voltage-gated Ca^{2+} channel proteins on its surface. These include the L-type calcium channel, involved in slowly activated sustained conductance, and the dihydropyridine receptor that acts as a voltage sensor, relays the stimulus to release calcium from the sarcoplamic reticulum**.** With depolarization, the sarcoplamic reticulum channel opens, releasing Ca^{2+} from the sarcoplamic reticulum. Calcium ions thus diffuse into the cytoplasm. Calcium release from the sarcoplasmic reticulum stores is mediated by membrane protein receptors known as ryanodine-sensitive calcium release channels. These channels trigger the calcium-activated physiology pathways, not only in the skeletal and smooth muscle, but also in the heart and brain (Quinn, 1987). The ryanodine-sensitive calcium release channel is one of two types of calcium-release channels important in excitation-contraction coupling. The other channel, more abundant in smooth muscle, is the inositol 1,4,5-triphosphate-stimulated channel (Roberts et al., 1988).

Numerous reports have suggested that AChE plays other non-classical roles in addition to its 'classical' function in terminating impulse transmission at cholinergic synapses (Fig.1). The hydrolysis of ACh in a non-synaptic context is attributed to such non-classical functions. AChE displays sequence and putative structural homology to a group of neuronal adhesion proteins (Scholl and Scheiffele,

2003). AChE may be involved in synaptic development and maintenance; Laumonnier et al. (2004) have reported that mutations in neuroligin genes are associated with mental retardation and autism, Asperger's syndrome which is a developmental disorder that affects a person's ability to socialize and communicate effectively. Also, Genever et al. (1999) have reported an intriguing finding to implicate AChE as bone matrix protein , and it has been shown to interact with the noncollagen basement membrane glycoprotein laminin (Paraoanu and Layer, 2004). Sharma et al (2001) have reported the involvement of AChE in spatiotemporal definition of neurite outgrowth, refinement and retraction in the developing cochlea. Although the effects of various antiChE agents on neurite outgrowth are not easy to interpret, a 'peripheral' site rather than the active site catalytically inactive AChE stimulated strongly neurite outgrowth (Small et al., 1995; Whyte and Greenfield, 2003; Flaskos et al., 2011)

Acetylcholinesterase inhibitors are targets for clinical use of many Alzheimer's dementia drugs(Wilkinson et al., 2004; Birk, 2006),demecarium (humorsol) (Ellis, 1971; Dart, 2006), echthiophate (Phospholine iodide), edrophonium (Sakuma et al., 1992; Morgan et al., 2006), neostigmine (Sakuma et al., 1992; Morgan et al., 2006), physostigmine (Buccafusco et al., 1980), methyldopa (L-α-Methyl-3,4-dihydroxyphenylalanine: aldomet, aldoril, dopamet, dopegyt) (Wilson et al.,1962; Dart, 2006) , organophosphorus nerve gases (e.g. tabun, sarin, soman cyclosarin and VX are among the most toxic known) (Metcalf and Holmes, 1969; Duffy et al., 1979; Worek et al., 2007; Worek and Thiermann, 2011), and insecticides (e.g.carbaryl:1-naphthyl methylcarbamate) (Nostrandt et al., 1993; Petrie and Watson, 1999). These poisoning agents are known as ChE inhibitors block the function of acetylcholinesterase, and thus cause excessive accumulation of ACh in the synaptic cleft (Ganong, 2003). The excess of ACh and uncontrolled activation of cholinergic receptors cause cholinergic crisis of neuromuscular paralysis which is an interminable muscle contractions throughout the entire body, leading to death by asphyxiation (Feyersisen, 1995; Bajgar, 2004). On the other hand, controlled treatment with ChE inhibitors are used in therapeutics for patients suffering from myasthenia gravis in which antibodies block, alter or destroy the receptors for Ach at the neuromuscular junction which prevents the muscle contraction from occurring (Brenner et al., 2008; Mehndiratta et al., 2011), Alzheimer's disease (Brenner et al., 2008; Stone et al., 2011), and Parkinson's disease (Hutchinson and Fazzini, 1996; Emre et al., 2004). Some inhibitors of AChE such as physostigmine, dyflos and ecothiopate are made into eye drops for treatment of glaucoma (Rang, 2003).

Increasing evidence indicates that free radical formation and oxidative stress play a significant role in Alzheimer's disease though its etiology is not known as yet (Ellis, 2005). Introduction of drugs (commonly used medications: Aricept, Exelon, Namenda, and Razadyne) that can either reduce the formation of free radicals or protect cells from their damaging effects, may slow the progression of neurodegeneration in Alzheimer's disease (Cole et al., 2005; Liu and Ames, 2005). Therefore, effective therapy for Alzheimer's disease is more likely to be achieved by drugs that incorporate both antioxidant and ChE inhibitory activity within the same molecule (Tabet, 2006).

Studies have also shown that levels of AChE in seminal plasma have an inverse relationship with sperm motility, indicating that acetylcholine has a role in sperm motility (Mor et al., 2001). Artificial insemination increases the uterine motility resulting from the reflex activation of cholinergic nerve fibers, which was most prominent at 5-10min after intrauterine insemination and are sustained for 30 min (Raynal and Houdeau, 2004).Intramuscular injection of Ach immediately after natural mating significantly increased the frequency and amplitude of uterine contraction leading to an increased number of sperms recovered from the vagina after 1 hour (Hawk et al., 1982). The levels of AChE in red blood cells were significantly elevated during pregnancy and returned to normal pre-pregnancy levels at 6^{th} post-partum week, probably protecting the pregnancy (De Peyster et al., 1994).

Furthermore, microRNAs (miRNAs) are small, non-coding RNAs that negatively regulate gene expression. The miR-132 plays a key role in differentiation of dopamine (Lee et al, 1993). The miR-132-AChE interaction may also regulate neuron morphogenesis, as AChE is involved in neurite outgrowth and extension (Grisaru et al., 1999; Sklan et al., 2006). A regulation of AChE by miR-132 in

neuronal cells would also confer an indirect role for this miRNA in the regulation of cholinergic anti-inflammatory effects attributed to AChE (Rosas-Ballina, 2011). The miR-132 is also induced in lipopolysaccharide-stimulated primary human macrophages, where it represses the AChE expression (Shaked et al., 2009). By repressing the expression of AChE, an enzyme hydrolyzing the acetylcholine, considered as an important inhibitor of peripheral inflammation, miR-132 would play a role in the brain-to-body resolution of inflammation. The regulation of AChE by miR-132 occurs in the brain and plays a role in circadian rhythms (Shaked et al., 2009).

The density of RBC from whole blood increases *in vivo* with age of cell (Key 1921). This principle was exploited to separate RBC into 'younger' and 'older' cells by simple centrifugation; the activity of AChE decreases in older RBC (Sabine, 1955; Galbraith and Watt, 1981; Prall et al., 1998; Arrieta et al., 2009).

Biochemistry

In recent years the erythrocyte membrane has received considerable attention by many investigators (Bernhardt and Ellory, 2003**;** Deziel-Silva et al., 2010**;** Hajjawi, 2011**;** Kaestner, 2011).The basis for biochemical and histochemical assays is that AChE hydrolyzes acetylthiocholine (Oakes et al., 2003; Zhang et al., 2007). In the biochemical assay, this leads to the formation of sulfohydryl groups that can react with DTBN or Ellman reagent to give a yellow color, the intensity of which can be measured at 410nm (Ellman et al., 1961). Histochemical assays localize AChE in cells, in which Karnovsky and Roots (1964) have developed modification of a "direct coloring" thiocholine assay. A histochemically stained tissue containing known amounts of a highly purified AChE preparation are sectioned and incubated under the same conditions as experimental sections; a computerized image analyzer is used to convert the optical density of the stain to units of enzyme content via the standard curve (Biegon and Wolff, 1986). A clinical assay is based on the premise that AChE hydrolyzes ACh to form Ch and acetic acid; the change in pH due to the liberation of acetic acid is measured using metanitrophenol as indicator (Rappaport et al, 1959; Bauer et al, 1974). The indicator is yellow in alkaline solution and colourless in acid, i.e. the amount of yellow colour decreases as acetic acid concentration is increased.

Assays to quantitate AChE protein require a different approach. One stimulus for the development of true AChE immunoassays has been the need to characterize the deficit of AChE activity in red blood cells of patients with paroxysmal nocturnal haemoglobinturia. Chow et al (1985) measure the binding of ^{125}I-labeled anti-mouse IgG to red blood cells treated with saturating concentrations of specific monoclonal antibody against human AChE. A striking deficit was noted in the abnormal samples, which correlate with the percentage of complement-sensitive (i.e. defective) cells. In addition, it was shown that antibody-induced precipitation of radioactivity from extracts of radioiodinated red blood cells was reduced or absent in the case of patients samples. Both these methods may be viewed as primitive AChE immunoassays, and they clearly demonstrate the loss of immunoreactive protein in the abnormal samples.

A sophisticated immunodisplacement assay was developed using a solid-phase monoclonal antibody to AChE (Brimijoin et al., 1986). This method has been sensitive to minute amounts (≤5ng) of AChE. Also, the labeling of antibody with flurochrome simplifies sorting populations of red blood cells for varying levels of AChE, as in paroxysmal nocturnal haemoglobinuria.

The basic functional form of AChE is a dimmer amphipathic protein of molecular weight 160K, containing a single intersubunit disulfide bond (Rosenberg and Scoggin, 1984). Monomeric forms can be obtained in vitro by treatment with 2-mercaptoethanol (Ott et al., 1983) and they are fully active and it has been suggested that dimeric form is not essential (Ott and Brodbeck., 1984). Isoelectric focusing in Triton X resolved AChE into five molecular forms (4.55, 4.68, 4.81, 5.18), and density gradient centrifugation of detergent-depleted enzyme at high ionic strength revealed five molecular forms of one peak of AChE activity (6.3S, 10.2S, 12.2S, 14.2S, 16.3S), suggesting that

solubilized human AChE exists in various forms of net charge but of apparently similar molecular dimensions (Ott et al., 1975; Rosenberg and Scoggin, 1984).

As required by its biological function, AChE is a very efficient enzyme, with a turnover of 10^3–10^4 s^{-1}, operating at a speed near the diffusion limit (Quinn, 1987).This implies that the substrate is hydrolyzed so rapidly in which the concentration around the enzyme is depleted in relation to its concentration in the bulk solution (Quinn, 1987).

AChE is inhibited at high concentrations of substrate (Alles and Hawes, 1940; Marcel et al., 1998; Johnson et al, 2003) as well as BChE (Erikson and Augustinsson, 1979; Nicolet et al., 2003), rather than displaying Michaelis–Menten kinetics for nonallosteric enzymes. All current kinetic models for AChE propose the existence of at least two substrate-binding sites, the active site, near the bottom of the active-site gorge ~20 Å deep, and the peripheral anionic site, near its entrance (Bergmann et al., 1950; Changeux, 1966; Roufogalis and Quist, 1972; Taylor and Lappi, 1975; Nicolet et al., 2003). The active site is composed of two subsites. In the catalytic anionic subsite, it has been proposed that the choline moiety of ACh is stabilized principally via a cation-π interaction with Trp84, and also interacts with Glu199 and Phe330 (Sussman et al., 1991). The esteratic subsite contains a typical serine-hydrolase catalytic triad (Ser200-His440-Glu327) to accept the proton released during the enzyme reaction (Colletier et al., 2006). A substantial contribution to ACh binding within the active site also arises from stabilization of the carbonyl oxygen within the oxyanion hole, Gly118, Gly119 and Ala201, and of the acetyl group in the 'acyl-pocket', Trp233, Phe288, Phe290 and Phe331 (Harel et al., 1996). However, despite of the extensive kinetic studies on AChE, no direct structural evidence has been reported for the mode of its binding to a substrate (Pang et al., 2009; Carletti et al., 2010)

Conclusions

Acetylecholinesterase plays a pivotal role in cholinergic neurotransmission. The enzyme has been known to exist in human RBC membranes for many years. There is no evidence that its depletion during the life span of 120 days and of 280 km of continuous travel has pathogenic significance, especially in patients of paroxysmal nocturnal haemoglobinurea, in which AChE is usually absent (Pangburn et al., 1983). Although the physiological functions of this enzyme in the RBC remain obscure, the biochemical properties are better understood. RBC has provided biochemists with a convenient source of AChE for the assembly of AChE in interesting models to study numerous biological processes, such as protein-protein interactions, membrane trafficking of different states of AChEs, membrane protein degradation, post-translational modification, cell-cell interactions and its regulatory role haemopoiesis. Also, location of AChE at or near the outer cell membrane surface might have relevance for basic disease processes at the cellular level, and alterations in RBC activity associated with pathogenic conditions are found regularly with AChE.

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