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Human butyrylcholinesterase components differ in aryl acylamidase activity

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Abstract

Apart from its esterase activity, butyrylcholinesterase (BuChE) displays aryl acylamidase (AAA) activity able to hydrolyze *o*-nitroacetanilide (ONA) and its trifluoro-derivative (F-ONA). We report here that, despite amidase and esterase sites residing in the same protein, in human samples depleted of acetylcholinesterase the ratio of amidase to esterase activity varied depending on the source of BuChE. The much faster degradation of ONA and F-ONA by BuChE monomers (G_1) of colon and kidney than by the tetramers (G_4) suggests aggregation-driven differences in the AAA site between single and polymerized subunits. The similar ratio of F-ONA to butyrylthiocholine hydrolysis by serum G_1 and G_4 forms support structural differences in the amidase site according to the source of BuChE. The changing ratios of amidase to esterase activities in the human sources probably arise from post-translational modifications in BuChE subunits, the specific proportion of monomers and oligomers and the variable capacity of the tetramers for degrading ONA and F-ONA. The elevated amidase activity of BuChE monomers and the scant activity of the tetramers justify the occurrence of single BuChE subunits in cells as a means to sustain the AAA activity of BuChE which otherwise could be lost by tetramerization.

Keywords: blood serum; cholinesterases; colon; human; kidney.

Introduction

Butyrylcholinesterase (BuChE, EC 3.1.1.8) is a serine hydrolase structurally and functionally related to acetylcholinesterase (AChE, EC 3.1.1.7) (Massoulié, 2002; Cokugras, 2003; Darvesh et al., 2003a). BuChE is widely distributed in tissues and body fluids and, in spite of the initial perception perceiving the enzyme as unessential, its capacity to replace AChE in AChE-null mice (Li et al., 2000; Chatonnet et al., 2003) and its proposed roles in neurogenesis (Layer and Willbold, 1995; Mack and Robitzki, 2000), Alzheimer's disease (AD) (Geula and Mesulam, 1995; Greig et al., 2005; Diamant et al., 2006) and type 2 diabetes (Sridhar et al., 2006) have attracted the attention of investigators. From a toxicological point of view, BuChE in blood is important because of its ability to cleave or bind anti-cholinesterase agents (organophosphates and carbamates) (Saxena et al., 1997), drugs of abuse (cocaine) (Mattes et al., 1997) and pharmacologically important compounds, such as succinylcholine, a muscle relaxant used in anesthesia (Lockridge, 1990), aspirin, clinical antidepressants and other drugs (Cokugras, 2003).

AChE and BuChE show a complex molecular diversity, which arises at the transcriptional, post-transcriptional and post-translational levels (Massoulié et al., 2005). A single BuChE transcript has been identified so far, which contrasts with the occurrence of various AChE mRNAs (Meshorer and Soreq, 2006; Montenegro et al., 2006). The BuChE subunit can polymerize and yield globular monomers, dimers and tetramers (G_1, G_2 and G_4) that can exist as hydrophilic (G^H) or amphiphilic (G^A) species according to the particular folding of subunits (Altamirano and Lockridge, 1999) and the presence of the hydrophobic PRiMA subunit (Perrier et al., 2002). In addition to their esterase activity, AChE and BuChE exhibit aryl acylamidase (AAA) activity (Balasubramanian and Bhanumathy, 1993; Checler et al., 1994), and although it can reflect the promiscuity of BuChE in substrate acceptance, the high amidase activity displayed by ChEs at early brain development (Boopathy and Layer, 2004) and its possible role in AD pathogenesis (Darvesh et al., 2003a) make the AAA activity a matter of interest.

In our studies on ChEs in gut cancer (Montenegro et al., 2006), we observed that, although both BuChE and its amidase activity fell in malignant pieces, there were differences in the ratio of amidase to esterase activities between samples comprising each type (control or cancerous). In light of the proposed straightforward relationship between esterase and amidase activities of BuChE (Checler et al., 1994), variable ratios between amidase and esterase activities were fully unexpected, and this prompted us to search for a plausible explanation. We report here for the first time that the level of AAA activity in BuChE of human gut and kidney depends on the aggregation state of the enzyme subunits. The variable extent to which G_1 and G_4 BuChE of serum degrade *o*-nitroacetanilide (ONA) and its trifluoro-derivative N-(2-nitrophenyl)-trifluoroacetamide (F-ONA) (Figure 1) not only confirm the change in the microenvironment of the AAA site according to the oligomerization state, but it

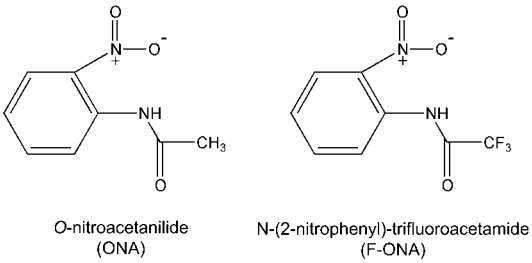

Figure 1 Structure of ONA and F-ONA.
The presence in F-ONA of three electron-withdrawing fluorine atoms on the carbon atom next to the carbonyl group leads to a faster hydrolysis of the amide group in F-ONA than in ONA.

also emphasizes how important the choice of the substrate is for measuring the amidase activity of BuChE.

Results

Assessment of the relationship between aryl acylamidase activity and BuChE

Taking advantage of the great affinity of serum BuChE for procainamide-sepharose (Lockridge et al., 2005), diluted serum was incubated with the gel and the bound and unbound fractions were tested for its BuChE and AAA activities. Both activities were fully bound by the procainamide gel confirming the BuChE origin of amidase and esterase activities. Moreover, the parallel and complete inhibition of gut BuChE and its associated AAA activity with increasing *Iso*-OMPA and Triton X-100, two well established inhibitors of BuChE (Figure 2A), ruled out a significant contribution of non-BuChE esterases to ONA hydrolysis. In addition, the great extent (80–100%) to which esterase and ONA-degrading activities of gut and blood serum BuChE bound to PGA-immobilized anti-BuChE antibodies (Figure 2B) confirmed the BuChE origin of the amidase activity. Sedimentation analysis (not shown) revealed that BuChE tetramers (G_4) were more easily immunoprecipitated than monomers (G_1). Consequently, the weaker binding of anti-BuChE antibodies to G_1 than G_4 species and the greater ONA-hydrolyzing activity of the monomers compared with the tetramers (see later) explain the unequal adsorption of esterase and amidase activities to the antibodies.

Variation between human sources in the ratio of amidase to esterase activities of BuChE

Mean values for BuChE and AAA activities in AChE-depleted extracts of gut and kidney and in blood serum

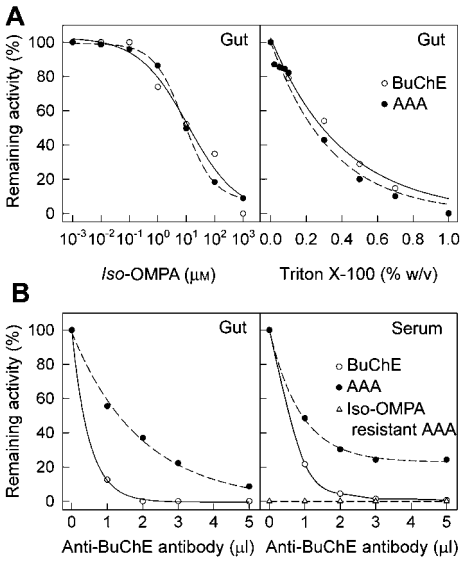

Figure 2 Inhibition and immunoprecipitation assays confirming the BuChE origin of the amidase activity.
(A) Effects of increasing *Iso*-OMPA or Triton X-100 (two BuChE inhibitors) on esterase (○) and amidase (●) activities of AChE-depleted gut samples assayed with BuTCh and ONA. (B) Adsorption of esterase (○) and amidase (●) activities of gut and serum to agarose-bound anti-BuChE antibodies. The lower binding of amidase than esterase reflects the lower contribution of G_4 than G_1 BuChE to ONA hydrolysis (see Figure 3) and the weaker affinity of the antibodies for the monomers. As an exception, the *Iso*-OMPA-resistant activity was not subtracted in these experiments. Empty triangles show ONA hydrolysis in the presence of 100 μM *Iso*-OMPA.

are shown in Table 1. The results agree with recent observations regarding the faster hydrolysis of F-ONA compared with that of ONA by BuChE (Darvesh et al., 2006). A great variation in the ratio of amidase to esterase activities in samples of gut and kidney when compared to serum was observed. In fact, the samples with high BuChE activity and those with much *Iso*-OMPA-sensitive amidase activity not always coincided, and this was puzzling considering the BuChE origin of both esterase and amidase activities (Checler et al., 1994). In addition, we realized that although the decrease in specific activity of BuChE in cancerous gut (60% on average) (Montenegro et al., 2006) agreed with the fall in AAA activity with F-ONA as the substrate, it dropped even more with ONA (Table 1). The much lower ratio of AAA to BuChE activities in serum than in tissues both when using ONA and F-ONA definitively discarded a straightforward relationship between BuChE and AAA activities in human peripheral tissues and blood.

Table 1 Variation in the ratio of AAA to BuChE activities in human tissues and blood serum.

Sample	n	BuChE (mU/mg)	AAA (ONA) (mU/mg)	AAA/BuChE (ONA)	AAA (F-ONA) (mU/mg)	AAA/BuChE (F-ONA)	F-ONA/ONA
Healthy gut	6	3.0±0.8	0.18±0.18	0.07±0.09	5.5±4.6	2.2±2.1	45±38
Cancerous gut	4	1.1±0.4	0.02±0.01	0.06±0.09	2.4±1.9	1.7±1.3	109±16
Kidney	4	2.6±0.3	0.18±0.04	0.07±0.02	7.8±1.6	2.5±0.9	40±4
Blood serum	12	29.1±5.8	0.07±0.02	<0.004	0.6±0.1	<0.02	8±1

BuChE-associated esterase activity was measured using butyrylthiocholine (BuTCh) as the substrate, and amidase activity with *o*-nitroacetanilide (ONA) and N-(2-nitrophenyl)-trifluoroacetamide (F-ONA). One milliunit (mU) refers to the hydrolysis of one nmol of the substrate per min. The activities in gut and kidney samples were determined using mixtures of the S1 (detergent-free) and S2 (detergent-containing) supernatants.

Differences between BuChE components in their expression of amidase activity

Considering the distinct composition of BuChE species in human gut (Montenegro et al., 2006) and blood serum (Altamirano and Lockridge, 1999), the varying ratio of AAA to BuChE activities could be related to the molecular polymorphism of the enzyme. This issue was studied by testing possible differences between BuChE components in their efficiency to hydrolyze ONA and F-ONA.

Centrifugation analysis of healthy and cancerous gut allowed us to identify BuChE forms with sedimentation coefficients of 11.9S, 9.9S and 4.5S (Figure 3) which, according to our previous data (Montenegro et al., 2006), were assigned to hydrophilic tetramers (G_4^H), amphiphilic tetramers (G_4^A) and hydrophilic monomers (G_1^H). While the esterase activity was distributed between various components, the *Iso*-OMPA-sensitive AAA activity peaked at 4.3S irrespective of the use of ONA or F-ONA in assays (Figure 3). The very small peak at 10–12S corroborated the poor amidase activity developed by BuChE tetramers. The free elution of the 4.3S species from phenyl-agarose (elution profile not shown) indicated that they corresponded to hydrophilic G_1^H forms. The amidase activity exhibited by BuChE monomers and its lack (or great reduction) from the tetramers strongly supported the relationship between the molecular polymorphism of BuChE and the varying AAA activity in samples.

For testing whether the lack of AAA activity in BuChE tetramers was restricted to gut or extended to other sources, the expression of AAA activity by BuChE species of kidney and serum was studied. As for the gut tetramers, the renal isoforms displayed very minor AAA activity (Figure 3); again, ONA and F-ONA hydrolysis arose principally from G_1^H BuChE. In blood serum, the amidase activity came mostly from the monomers too, providing that the activity was assayed with ONA. Unexpectedly, both the G_4^H and G_1^H species of serum were able to degrade F-ONA with similar AAA/BuChE activity ratios (Figure 3).

A close examination of profiles such as those depicted in Figure 3 revealed that in assays with ONA the ratio of AAA to BuChE activities was approximately the same for the G_1^H species of healthy colon and kidney, but much less for the serum isoforms (Table 2). With F-ONA, again the AAA/BuChE ratio was much lower for serum. The variable capacity of BuChE monomers and tetramers to degrade ONA and F-ONA in gut, kidney and serum pointed to structural changes in the amidase site between BuChE subunits according to their quaternary organization and the enzyme source.

Effect of proteolysis on the amidase activity of BuChE

Additional information on how a structural change may affect acetanilide hydrolysis was sought by studying the effect of trypsin on esterase and amidase activities of BuChE. In colon extracts exposed to mild trypsin (100 U/mg of protein), a maintenance of the esterase activity and a progressive increase in F-ONA hydrolysis with time was observed (Figure 4A). The same treatment applied to serum BuChE had little effect on esterase and amidase activities, but at higher trypsin (1000 U/mg) both were, to an important extent, lowered (Figure 4B).

Sedimentation profiles of colon samples treated or not with mild trypsin revealed that the protease produced a moderate drop in the amount of BuChE tetramers (Figure 4C), whilst that of monomers did not change. The enhanced capacity of the monomers to degrade F-ONA, in spite of their unchanged content, supported their involvement in the surplus of amidase activity observed in colon samples incubated with trypsin (Figure 4A) and the role of proteolysis in the development of AAA activity.

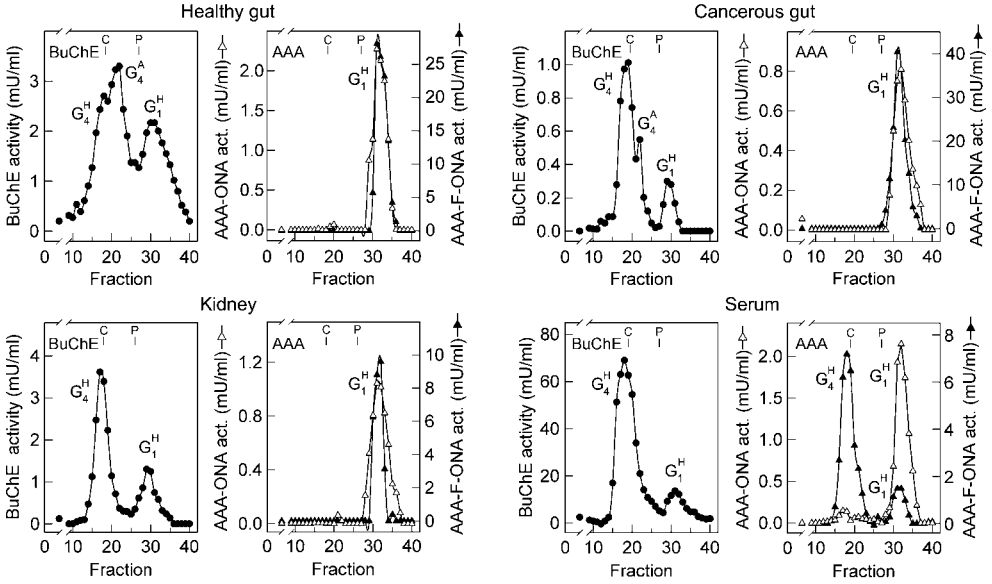

Figure 3 Difference in AAA activity of BuChE components.
After tissue extraction without (S1) and with (S2) detergent, the supernatants were mixed and BuChE molecules resolved by centrifugation in sucrose gradients containing Brij 96. Sedimentation profiles show the distribution of *Iso*-OMPA-sensitive esterase (●) and amidase activities in assays performed with ONA (△) and F-ONA (▲). Note the negligible amidase activity of BuChE tetramers in gut and kidney, regardless of the use of ONA or F-ONA as the substrates, whereas G_4^H BuChE of serum is able to hydrolyze F-ONA (but not ONA).

Table 2 Expression of AAA activity by BuChE species in human sources and ratios of AAA to BuChE activities.

Human source	Species	BuChE (mU)	AAA (ONA) (mU)	AAA/BuChE (ONA)	AAA (F-ONA) (mU)	AAA/BuChE (F-ONA)	F-ONA/ONA
Healthy gut	G1	4.7	2.4	0.5	25.5	5.5	10.5
	G4	7.2	0.0	–	0.0	–	–
Cancerous gut	G1	0.3	1.0	4.0	39.0	130	39
	G4	1.4	0.0	–	0.0	–	–
Kidney	G1	1.7	1.4	0.8	7.1	4.3	5.0
	G4	4.1	0.0	–	0.0	–	–
Blood serum	G1	22.2	2.2	0.1	1.5	0.07	0.7
	G4	105.4	0.2	<0.01	7.7	0.07	38.5

Data taken from the sedimentation profiles depicted in Figure 3. Although the values obtained from different samples varied, the scant amidase activity with both ONA and F-ONA of gut and kidney BuChE tetramers was always observed, as well as the distinct behavior of the tetramers and monomers from serum.

As expected, when serum was exposed to 100 U/mg trypsin sedimentation profiles were unchanged (Figure 4D, left), whereas at 1000 U/mg trypsin both esterase and amidase activities increased in the sucrose fractions where the G_1^H forms peaked (Figure 4D, right). The decrease in activity of G_4^H species agreed with previous data regarding the conversion of BuChE tetramers into monomers by trypsin (Lockridge and La Du, 1982).

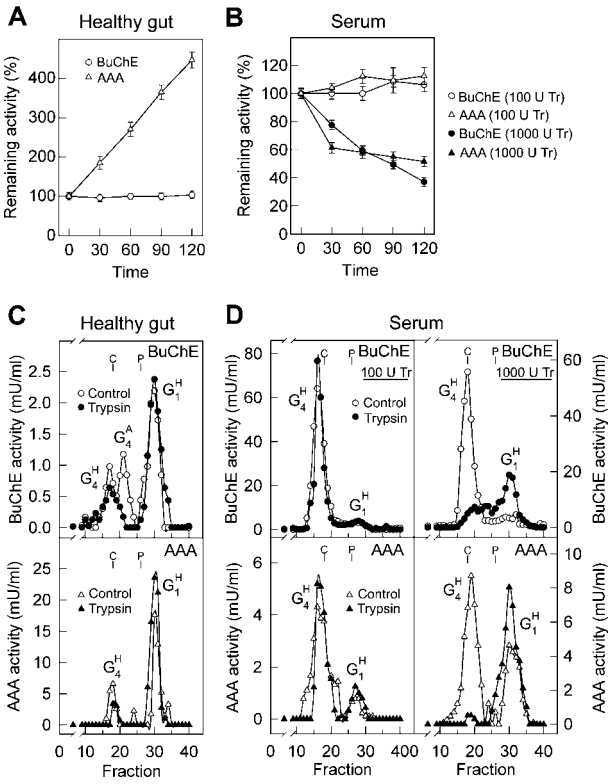

Figure 4 Effect of proteolysis on esterase and amidase activities of BuChE in gut and serum.
(A) Gut extracts were incubated with 100 units of trypsin/mg of protein and assayed for esterase (○) and amidase (△) activities with BuTCh and F-ONA. (B) Progression curves outlining the effect of 100 and 1000 units of trypsin/mg of protein on the esterase (○, ●) and F-ONA hydrolyzing activities (△, ▲) of human serum. (C) Sedimentation profiles with esterase (circles) and F-ONA hydrolyzing (triangles) activities of gut BuChE forms before (○, △) and after (●, ▲) incubating with 100 units of trypsin/mg of protein for 1 h. (D) Sedimentation patterns of serum incubated 1 h without (empty symbols) and with (filled symbols) 100 (left) or 1000 (right) units of trypsin/mg. Note how high trypsin increases both esterase and F-ONA hydrolyzing activities in the G_1 peak of serum at the expense of the G_4 peak.

Discussion

Despite the early identification of esterase and amidase activities of ChEs (Fujimoto, 1976), current information on the AAA activity of human BuChE is scarce (George and Balasubramanian, 1981). Moreover, in spite of the BuChE origin of both activities no convincing explanation has been provided yet to the discrepant activity ratios depending on BuChE sources and animal species (Weitnauer et al., 1998). This work focused on exploring possible changes between human sources in the ratios of amidase to esterase activities of BuChE and, if they existed, to provide a rational explanation for them. Our estimation of amidase activity (measured with ONA) in human serum (3.3±1.2 mU/ml, 0.07±0.02 mU/mg; Table 1) yields a lower value than the one reported by others [0.5 μmol/(ml×h); 8 mU/ml according to our definition] (Oommen and Balasubramanian, 1979). Inter-individual changes in BuChE activity of serum (Kálman et al., 2004) and/or overestimation of amidase activity due to proteins other than BuChE able to hydrolyze ONA (Manoharan and Boopathy, 2006; Masson et al., 2007a) may account for the difference.

As regards the substrate preference in the amidase site, if BuChE uses the same mechanism for hydrolyzing BuTCh and anilides (Nicolet et al., 2003), it is expected that when ONA binds at the catalytic site its C=O bond becomes polarized, so that the partially positive C atom undergoes the nucleophilic attack of the O atom of the active site Ser. The electron-attracting F atoms in F-ONA (Figure 1), which ONA lacks, would increase the electron deficiency at the C atom facilitating the nucleophilic attack and, therefore, improving F-ONA hydrolysis by lowering the energy barrier for forming the tetrahedral intermediate. Accordingly, compared with ONA the rate of F-ONA hydrolysis by gut, kidney and serum BuChE was approximately 40-, 40- and 8-fold faster (Table 1), which differs from the reported 100-fold faster degradation of F-ONA by human serum BuChE (Darvesh et al., 2006). Although methodological changes can explain the discrepancy, the crucial point is the changing ratios of F-ONA/ONA hydrolysis displayed by BuChE and, therefore, the varying ratios of amidase/esterase activities for

both substrates (Table 1). The usually much faster hydrolysis of F-ONA compared with ONA argues in favor of the former for measuring the amidase activity of ChEs, but the distinct behavior of separated BuChE species on ONA and F-ONA (Table 2) makes it advisable to compare their amidase activity with both acetanilides.

The unequal ratios of amidase to esterase activities according to the human sample (Table 1) agree with the discrepant ratios for BuChE of various animal sources (Weitnauer et al., 1998) and for AChE of several anatomical areas and subcellular fractions of monkey brain (Oommen and Balasubramanian, 1979). Because both AChE and AAA activities reside in the same protein, their varying ratios have been attributed to: (i) endogenous factors which affect more AChE than AAA activity (Oommen and Balasubramanian, 1979); (ii) variations in the tertiary/quaternary structure due to post-translational changes (Boopathy and Layer, 2004); and (iii) differences between AChE species in their expression of AAA activity (Oommen and Balasubramanian, 1979; Russ et al., 1998). Evidence favoring any alternative is still lacking, but the similar protein fold of AChE and BuChE (Loewenstein et al., 1993; Darvesh et al., 2003a; Suarez and Field, 2005), the higher conformational freedom of enzyme subunits in monomers than oligomers, as judged by the thermosensitivity of G_1 AChE (Yagüe-Guirao et al., 1986), and the subtle structural change associated with oligomerization (Shin et al., 2002) pointed to a relationship between the extent of oligomerization of ChE subunits and their expression of amidase activity.

In our attempts to investigate the issue, BuChE forms were resolved by centrifugation and examined for their esterase and amidase activities (Figure 3). The assay of amidase activity of BuChE species allowed us to uncover: (i) the low capacity of G_4 BuChE of gut and kidney for splitting ONA and F-ONA (Figure 3 and Table 2) and the much greater capacity of the G_1 species; (ii) the fact that only the tetramers of serum, and not those of colon and kidney, degrade F-ONA significantly; and (iii) the comparable efficiency to which G_4^H and G_1^H BuChE of serum degrade esterase (BuTCh) and amidase (F-ONA) substrates, a finding that agrees with recent data (Masson et al., 2007b). The last point has been taken as a proof of the lack of relationship between the amidase activity of BuChE and the oligomerization state (Masson et al., 2007b). Nevertheless, the data depicted in Table 2 and Figure 3, the results of Layer's group (Boopathy and Layer, 2004) and our data concerning the amidase activity of AChE components (unpublished) firmly support the importance of BuChE and AChE polymerization in the expression of amidase activity.

As regards the origin of BuChE activity in tissues, although blood serum may provide some activity, the observation of BuChE-mRNA in human gut (Montenegro et al., 2006) and kidney (authors' unpublished results), and the presence of G_4 (85–70%) and G_1 BuChE (15–30%) in gut, breast (Ruiz-Espejo et al., 2002), lymph node (Ruiz-Espejo et al., 2003), meningioma (Sáez-Valero and Vidal, 1996) and kidney (Figure 3) support an ability of these tissues to make monomeric and tetrameric BuChE. But why do single subunits elude polymerization? The keeping of G_1 BuChE in tissues, along with the predominant G_4 species, suggests that the monomers might play a role not played by the tetramers. If the amidase activity has a function, BuChE monomers, instead of being only precursors of the G_4 species, could maintain their individuality for developing amidase activity.

As regards the relationship between esterase and amidase sites of BuChE, competition of BuTCh with ONA (Darvesh et al., 2006) and the lack of AAA activity in the silent variant of BuChE, unable to express esterase activity (Manoharan and Boopathy, 2006), support overlapping, if not identical, sites. The distinct effect of benzalkonium (Jaganathan and Boopathy, 2000) and huperzine A enantiomers (Darvesh et al., 2003b) on the esterase and amidase activities of BuChE does not necessarily imply two separate sites; it can be attributed to the particular action of a ligand on the hydrolysis of a positive substrate (BuTCh) or a neutral substrate (ONA). In addition, the observed difference between ONA and F-ONA hydrolysis indicates that the action of BuChE depends on other features besides the type of bond, ester or amide, in the substrate. The unequal capacity of G_4 and G_1 BuChE for degrading acetanilides (Figure 3) agrees with the proposed oligomerization-driven polypeptide rearrangement that takes place when free BuChE subunits are packed into tetramers (Giles, 1997; Shin et al., 2002; Nicolet et al., 2003). The scarce amidase activity of BuChE tetramers of gut and kidney, compared to monomers, regardless of the use of ONA or F-ONA, could be attributed to a lower flexibility of the subunit in oligomers, which would decrease its capacity to accommodate substrates. Moreover, the incapacity of the intestinal and renal BuChE tetramers to degrade F-ONA and its hydrolysis by the serum isoforms may reflect small structural changes between intracellular and secreted tetramers. The differences in both size (Treskatis et al., 1992) and lectin binding between BuChE tetramers in tissues and blood (Tornel et al., 1992) support changes in their oligoglycan processing and a possible implication of sugars in the structural variation. Thus, amino acid sequence, quaternary structure and oligosaccharidic moieties can contribute to structural differences in the amidase site microenvironment of the BuChE subunit and, therefore, to the changing hydrolysis rates of *o*-nitroacetanilides and varying ratios of amidase to esterase activities in different BuChE components, in isoforms of distinct organs of a selected animal and in isoforms of the same organ from distinct animal species (Weitnauer et al., 1998; Boopathy and Layer, 2004).

If tetramerization alters the acetanilide-degrading capacity of BuChE, could it be restored by monomerization of the tetramers? In the case of gut, although the activating action of trypsin on the amidase activity (Figure 4A) apparently favored the contention, sedimentation profiles (Figure 4C) suggested that the rise in activity comes from the production of trimmed G_1 BuChE (lacking the C-terminal segment) (Lockridge and La Du, 1982) rather than from changes in the quaternary structure of BuChE leading to an increase in the number of non-lytic monomers.

The activating action of trypsin on the amidase activity of gut (Figure 4A) supported a link between proteolysis and the level of amidase activity. This does neither make

proteolysis a condition for the expression of AAA activity nor does it mean that proteolysis does not occur inside the cell. The hydrolysis of ONA and F-ONA by GPI-anchored AChE of human erythrocytes (authors' unpublished results) argues against the necessity of protein degradation for the expression of amidase activity. Nevertheless, in view of the impact of proteolysis in the content of BuChE monomers and, therefore, in the level of AAA activity, fresh samples should be used if possible, and if not, they must be stored carefully when testing the possible use of BuChE-associated amidase activity for diagnostic purposes, e.g., in liver pathologies (Boopathy et al., 2007).

In summary, our results show that, despite their BuChE origin, human gut, kidney and serum differ in the ratio of amidase to esterase activities. The level of amidase activity exhibited by human BuChE subunits seems to depend on their polymerization extent and attached oligosaccharides, so that the difference between BuChE monomers and tetramers in acetanilide hydrolysis may reflect spatial changes in the amidase site. The action of trypsin on the amidase activity confirms the relationship with the structure of BuChE subunit and its level of activity. Thus, the changing ratio of AAA to BuChE activities in human sources probably arises from post-translational modifications and the particular distribution of BuChE components in them. Whether the above features are specific of human BuChE or they extend to AChE or ChEs in animal species is currently unknown.

Materials and methods

Materials

Aprotinin, bacitracin, benzamidine, leupeptin, pepstatin, trypsin (type XI), soybean trypsin inhibitor (STI) (type II-S), polyoxyethylene$_{10}$-oleyl ether (Brij 96), Triton X-100, butyrylthiocholine iodide (BuTCh), tetraisopropyl pyrophosphoramide (*Iso*-OMPA), 1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide (BW284c51, BW), 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), N-(2-nitrophenyl)-trifluoroacetamide [also called 2,2,2-trifluoro-N-(2-nitrophenyl)acetamide, F-ONA], protein markers for sedimentation analysis (beef liver catalase and intestine alkaline phosphatase) and phenyl-agarose were all purchased from Sigma (St. Louis, MO, USA). The chemicals *o*-nitroaniline and *o*-nitroacetanilide (ONA) were obtained from Merck (Darmstadt, Germany), fasciculin-2 (Fas2) from Latoxan (Valence, France), protein G-agarose (PGA) from Boehringer Mannheim (Germany), HR2 antibodies against human cerebellum AChE from Affinity Bioreagents (Golden, CO, USA) and a rabbit IgG fraction against human BuChE from Cortex Biochem (San Leandro, CA, USA). Procainamide-sepharose 4B was kindly provided by Prof. O. Lockridge at The Eppley Institute, University of Nebraska Medical Center (Omaha, NE, USA). The protein assay reagent was from Bio-Rad (Hercules, CA, USA).

Extraction of ChEs

Healthy and cancerous human colorectal pieces were kindly provided by Dr. M. Butrón from the Hospital Xeral of Vigo (Spain). Human kidney and serum samples were donated by Drs. J.E. Hernández-Barceló and F. Ruiz-Espejo, both from the Hospital Virgen de la Arrixaca of Murcia (Spain). Cholinesterases (ChEs) were extracted from tissues as reported before (Montenegro et al., 2006). Briefly, intestinal and renal specimens (0.05-2 g) were homogenized (10 ml/g of tissue) with a Tris-saline buffer (TSB; 1 M NaCl, 50 mM $MgCl_2$, 10 mM Tris, pH 7.4) containing antiproteinases. The suspensions were centrifuged and soluble and loosely bound ChEs were saved in the S1 supernatant. The pellet was re-extracted with TSB plus antiproteinases and 1% Brij 96 and, after centrifugation, membrane-bound ChEs were recovered in S2. Finally, AChE and BuChE activities and the protein content were determined. In gut and kidney, the proportions of BuChE activity in the S1 and S2 supernatants were approximately 2 to 1. The AAA activity expressed by BuChE was always studied using mixtures of S1+S2.

Amidase to esterase relationship

Because both BuChE and AChE express AAA activity, depletion of AChE was required before estimating the amidase activity of BuChE. For this, S1 and S2 supernatants (0.5 ml each) were mixed and added to anti-AChE antibodies (HR2) bound to PGA (5 μl of antibody per 50 μl of agarose). After incubation overnight at 4°C, agarose-bound AChE was discarded. Then, BuChE (and AChE) activity was measured in the supernatant, and the molecular distribution of BuChE and AAA activities assessed by sedimentation analysis. The fact that BuChE is the source of the amidase activity was corroborated by binding to anti-BuChE antibodies. For this, AChE-depleted extracts of colon and kidney, or blood serum (1:10 diluted in TSB) were mixed with agarose bound anti-BuChE antibodies raised against the human plasma enzyme (5 μl of antibodies per 50 μl of PGA) and incubated as above before determining esterase and amidase activities in the unbound fraction.

Enzymatic assays

BuChE was assayed in the presence of 10 μM BW as reported elsewhere (Ruiz-Espejo et al., 2002), one milliunit (mU) of activity meaning the hydrolysis of one nmol of BuTCh per min at room temperature (20–25°C). True BuChE activity was estimated by subtracting the hydrolysis of BuTCh due to non-BuChE esterases in assays where BuChE activity was suppressed by prior incubation with 100 μM *Iso*-OMPA. In sedimentation analysis, BuChE activity was measured by a microtiter assay (Moral-Naranjo et al., 1999), but taking into account the volume of sample loaded onto the gradient for calculating BuChE activity. The protein content was measured with the Bio-Rad reagent. The use of BW for measuring BuChE in samples depleted of AChE allowed us to determine BuChE (and esterases other than ChEs) in the conditions used for measuring samples with AChE and BuChE activities.

The amidase activity of BuChE was firstly measured with ONA and later with F-ONA (Darvesh et al., 2006). The low solubility of ONA in water made it necessary to try various organic solvents for improving its dissolution. Thus, we tested ONA dissolved at 15 mM in amidase assay buffer (AAB; 50 mM phosphate buffer, pH 8.0) without and with 2% ethanol, dimethylformamide or dimethylsulfoxide and used at 10 mM final concentration. Dimethylsulfoxide was selected for its capacity to improve the solubility of ONA without decreasing the amidase activity of BuChE.

Amidase activity of BuChE was measured by adding to microtiter plates the sample (25–75 μl), 10 mM ONA (150 μl of a 15-mM solution made in 2% dimethylsulfoxide in AAB) and amidase activity buffer to complete 225 μl. Parallel assays containing 100 μM *Iso*-OMPA allowed us to evaluate the hydrolysis of ONA by esterases other than BuChE, referred to as the *Iso*-OMPA-resistant amidase activity. The mixture was incubated for 1–3 h at 37°C in an oven and the release of *o*-nitroaniline was recorded

every 30 min (as A_{415}–A_{630}) in a microplate reader (Bio-Rad, Mod. 680). The amidase activity was evaluated by reference to a calibration curve made with 1–200 μM *o*-nitroaniline and the true activity (the *Iso*-OMPA-sensitive amidase activity) was calculated by subtracting the fraction of *Iso*-OMPA-resistant amidase activity. AAA activity is given in nmol of substrate (ONA or F-ONA, see later) hydrolyzed per min (mU). The hydrolysis of ONA by non-BuChE esterases in serum (Masson et al., 2007a), gut and kidney was less than 5% of the total. In experiments for comparing the effect of BuChE inhibitors on its esterase and amidase activities, samples were incubated with *Iso*-OMPA (final concentration 0.001–1000 μM) or with Triton X-100 (0–1%) for 10 min before adding BuTCh or ONA to wells.

In assays with F-ONA, sample (10–20 μl), 0.3 mM F-ONA (10 μl of a 7.5-mM solution made in acetonitrile) and AAB to complete 250 μl, were mixed. The release of *o*-nitroaniline was recorded at 25°C every 2 min for up to 1 h. Parallel assays with completely inhibited BuChE (by prior incubation with 100 μM *Iso*-OMPA) were made to estimate the *Iso*-OMPA-resistant amidase activity, which was always subtracted for estimating true AAA activity of BuChE. The fact that the ONA and F-ONA-hydrolyzing activity of human albumin is not inhibited by 100 μM *Iso*-OMPA (authors' observation) discarded its contribution to the *Iso*-OMPA sensitive amidase activity (Masson et al., 2007a,b). The contribution of esterases other than ChEs to F-ONA hydrolysis amounted to 20–30% in healthy colon (n=6), 30–40% in cancerous colon (n=4), 20–30% in kidney (n=4) and 40–50% in serum (n=12).

Velocity sedimentation analysis

BuChE components in human gut, kidney and serum were separated and identified by their sedimentation coefficients. Moreover, their expression of AAA activity was examined by using ONA and F-ONA as the substrates. With this aim, samples (0.2–0.3 ml) and internal markers, catalase (11.4S) and alkaline phosphatase (6.1S), were centrifuged on 5–20% sucrose gradients containing 0.5% Brij 96 (Ruiz-Espejo et al., 2002). Sedimentation profiles allowed us to compare both the extent of ONA and F-ONA hydrolysis by BuChE oligomers and monomers and their ratio of amidase to esterase activities.

Phenyl-agarose chromatography

The amphiphilic or hydrophilic behavior of BuChE monomers in colon, kidney and serum was assessed by their retention or free elution from phenyl-agarose. For this, 2 ml of AChE-free extracts of colon or kidney, or 5 ml of serum (1:5 diluted with TSB), was loaded onto a column filled with 6 ml of gel and pre-equilibrated with TSB. During elution with TSB (40 ml at a flow rate of 20 ml/h), fractions (1 ml) were collected and assayed for BuChE activity.

Effect of trypsin on esterase and amidase activities of BuChE

Trypsin digestion was attempted as a means to explore whether the variable expression of AAA activity arose from changes in the conformation adopted by the BuChE subunit. For this, 2 ml of AChE-free extracts of healthy colon were incubated for up to 2 h at 37°C without and with trypsin added (100 U/mg of protein). Samples of serum (1:10 diluted) without and with 100 U or 1000 U of trypsin/mg of protein were incubated in parallel. After adding STI (10 mg/mg of trypsin), the variation in BuChE and AAA activities was assessed. BuChE components in colon and serum samples incubated 1 h with trypsin were resolved by centrifugation in Brij 96-containing sucrose gradients.

Trypsin showed a very small capacity to hydrolyze ONA and F-ONA, each trypsin unit splitting less than 0.0005 nmol of ONA and less than 0.005 nmol of F-ONA per min. Nevertheless, as this trypsin activity is not inhibited by *Iso*-OMPA, the given data, which are of *Iso*-OMPA-sensitive BuChE and AAA activities, have subtracted the trypsin contribution to substrate hydrolysis. As expected, ONA and F-ONA were not hydrolyzed at all by the mixture of trypsin and STI.

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