

RESEARCH ARTICLE

The cholesterol ester cycle regulates signalling complexes and synapse damage caused by amyloid- β

Ewan West, Craig Osborne and Clive Bate*

ABSTRACT

Cholesterol is required for the formation and function of some signalling platforms. In synaptosomes, amyloid- β ($A\beta$) oligomers, the causative agent in Alzheimer's disease, bind to cellular prion proteins (PrP^C) resulting in increased cholesterol concentrations, translocation of cytoplasmic phospholipase A₂ (cPLA₂, also known as PLA2G4A) to lipid rafts, and activation of cPLA₂. The formation of A β -PrP^C complexes is controlled by the cholesterol ester cycle. In this study, A β activated cholesterol ester hydrolases, which released cholesterol from stores of cholesterol esters and stabilised A β -PrP^C complexes, resulting in activated cPLA₂. Conversely, cholesterol esterification reduced cholesterol concentrations causing the dispersal of A β -PrP^C complexes. In cultured neurons, the cholesterol ester cycle regulated A β -induced synapse damage; cholesterol ester hydrolase inhibitors protected neurons, while inhibition of cholesterol esterification significantly increased A β -induced synapse damage. An understanding of the molecular mechanisms involved in the dispersal of signalling complexes is important as failure to deactivate signalling pathways can lead to pathology. This study demonstrates that esterification of cholesterol is a key factor in the dispersal of A β -induced signalling platforms involved in the activation of cPLA₂ and synapse degeneration.

KEY WORDS: Amyloid- β , Cholesterol, Cholesterol ester hydrolases, Phospholipase A₂, Prion, Rafts, Synapse

INTRODUCTION

The cellular prion protein (PrP^C) gained notoriety for its role in the transmissible spongiform encephalopathies after undergoing transformation to the disease-associated isoform (PrP^{Sc}). While the normal role of PrP^C remains unclear, reports that PrP^C is concentrated at synapses (Herms et al., 1999), and that transgenic mice in which the gene for PrP had been knocked-out [Prnp^(0/0)] showed synaptic and memory deficits (Maglio et al., 2006), suggest that it plays a role in neurotransmission. PrP^C is attached to cell membranes via a glycosylphosphatidylinositol (GPI) anchor (Stahl et al., 1987), which targets the protein to specific membrane microdomains called lipid rafts (Taraboulos et al., 1995). Many lipid rafts are enriched with signalling molecules and act as platforms in which GPI anchors interact with signalling proteins (Sharma et al., 2004; Suzuki et al., 2007). PrP^C is associated with numerous cell signalling pathways, including those of the tyrosine kinase Fyn (Mouillet-Richard et al., 2000), protein kinase A (Chiarini et al.,

2002) and cytoplasmic phospholipase A₂ (cPLA₂, also known as PLA2G4A) (Bate et al., 2010). The formation of signalling complexes can be triggered by the cross-linkage of lipid raft components, including the oligomerization of GPI-anchored proteins (Hammond et al., 2005; Lingwood et al., 2008). PrP^C acts as a scaffold protein that organises signalling complexes (Linden et al., 2012), and in neurons the clustering of specific GPIs attached to PrP^C caused aberrant cell signalling and synapse degeneration (Bate and Williams, 2012). Because GPI-anchored proteins are surrounded by a shell of membrane lipids (Anderson and Jacobson, 2002), the selective associative properties of cholesterol, sphingolipids and GPI-anchored proteins are capable of altering raft composition and function (Lingwood and Simons, 2010). The aggregation of GPIs attached to PrP^C is thought to alter the composition of the underlying cell membrane, leading to cell activation in a process similar to that reported in T cell signalling (Chen et al., 2006; Suzuki et al., 2007).

Synaptic abnormalities are caused by aggregated PrP^C (Chiesa et al., 2008) and by cross-linkage of PrP^C with monoclonal antibodies (mAbs) (Solforosi et al., 2004). More recently, PrP^C was identified as a receptor for amyloid- β ($A\beta$) oligomers (it is predominantly A β ₄₂ and A β ₄₀ peptides that are associated with disease) (Laurén et al., 2009) that are responsible for the synapse degeneration and cognitive decline in patients with Alzheimer's disease (AD) (Selkoe, 2002; Shankar et al., 2008). Cross-linkage of PrP^C by A β oligomers forms a signalling complex containing activated cPLA₂ (Bate and Williams, 2011) and leads to the production of platelet-activating factor (PAF) and prostaglandins (PGs). The observations that cPLA₂ is highly enriched at synapses (Moskowitz et al., 1983), and that PAF and PGs affect synaptic plasticity and memory formation (Chen and Bazan, 2005; Koch et al., 2010), suggest that controlled activation of this enzyme is a normal aspect of synapse function. However, aberrant activation of cPLA₂ is associated with synapse degeneration and clinical symptoms as the concentrations of PGE₂ and PAF are increased in the brains of AD patients when compared with non-demented controls (Montine et al., 1999; Ryan et al., 2009).

Cholesterol is highly enriched in synaptic membranes and the fine tuning of cholesterol dynamics is thought to underlie synapse plasticity and hence memory (Linetti et al., 2010). Given that the formation and function of lipid rafts depends upon cholesterol concentrations (Rajendran and Simons, 2005), it follows that fluctuations in cholesterol concentrations alter the functions of lipid rafts. The key finding in this study was that the addition of soluble A β oligomers, highly toxic forms of A β (Yang et al., 2017), increased synaptic cholesterol concentrations. Soluble A β oligomers isolated from brain extracts had effects at picomolar concentrations, concentrations similar to those in cerebrospinal fluid (Mc Donald et al., 2010; McLean et al., 1999). The increase in cholesterol was accompanied by a corresponding decrease in cholesterol esters and was blocked by selective cholesterol ester

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hydrolase (CEH) inhibitors. Pre-treatment of synaptosomes with CEH inhibitors also reduced the formation of A β -PrP^C complexes, the A β -induced translocation cPLA₂ to lipid rafts and activation of cPLA₂. In cultured neurons, CEH inhibitors reduced A β -induced synapse degeneration.

The dissociation of signalling platforms is thought to be a physiological process that limits the intensity of cell signalling. Consequently, conditions that prevent the dissociation of signalling platforms may lead to sustained activation, cell disruption and pathology. This study shows that in synaptosomes, esterification of cholesterol dispersed A β -PrP^C complexes and reduced cell signalling, and in neuronal cultures it reduced A β -induced synapse damage. Our results are consistent with the hypothesis that the esterification of cholesterol is a key component mediating the dissociation of A β -induced signalling platforms involved in synapse damage.

RESULTS

Soluble A β increases cholesterol concentrations in synapses

As soluble A β oligomers are too small to be identified via electron microscopy (Walsh et al., 2002), they were characterised using gel electrophoresis. Brain extracts contained A β monomers, dimers and trimers that were removed following immunodepletion with mAb 4G8 (reactive with epitopes 17–24 of A β) (Fig. 1A). The addition of brain extracts containing 1 nM A β ₄₂ significantly increased the concentrations of cholesterol within synaptosomes (Fig. 1B). Immunodepletion with mAb 4G8 reduced the concentrations of A β ₄₂ (1±0.07 nM compared with 0.03±0.015 nM, *n*=9, *P*<0.01) and A β ₄₀ (4.36±0.22 nM compared with 0.24±0.07 nM, *n*=9, *P*<0.01) in brain extracts, while immunodepletion with mAb 3F4 (reactive with human prion proteins, mock-depletion) had no significant effect on either A β ₄₂ (1±0.07 nM compared with 0.98±0.08 nM, *n*=9, *P*=0.38) or A β ₄₀ (4.36±0.22 nM compared with 4.15±0.28 nM, *n*=9, *P*=0.45). A β -depleted brain extracts did not alter synaptic cholesterol concentrations, indicating that the increase in cholesterol was triggered by A β . The addition of brain extract containing 1 nM A β ₄₂ to synaptosomes from neurons derived from Prnp knockout^(0/0) mice did not significantly alter cholesterol concentrations (0.83±0.07 μM compared with 0.87±0.07 μM, *P*=0.23, *n*=9).

The addition of A β oligomers caused a dose-dependent increase in synaptic cholesterol concentrations, whereas A β monomers had no significant effect (Fig. 1C). Pre-treatment with 1 μM squalenol, a squalene synthetase inhibitor (Baxter et al., 1992), did not significantly alter the A β -induced increase in synaptic cholesterol concentrations (Fig. 1D), indicating that A β did not stimulate cholesterol synthesis. The addition of brain extracts to synaptosomes reduced the concentrations of cholesterol esters, and there was a significant inverse correlation between the concentrations of cholesterol and cholesterol esters in synaptosomes incubated with brain extract containing 0.125–1 nM A β ₄₂ (Fig. 1E). However, the effects of A β on synaptic cholesterol were transient; changes in cholesterol/cholesterol ester concentrations were seen 1 and 2 h after the addition of 1 nM A β ₄₂ but had returned to normal after 4 h (Fig. 1F). Collectively, these findings suggest that the A β -induced increase in cholesterol was provided by the hydrolysis of cholesterol esters (Fig. 1G).

The cholesterol ester cycle controls the A β -induced increase in cholesterol

This hypothesis was tested using the CEH inhibitors diethylumbelliferyl phosphate (DEUP) (Gocze and Freeman,

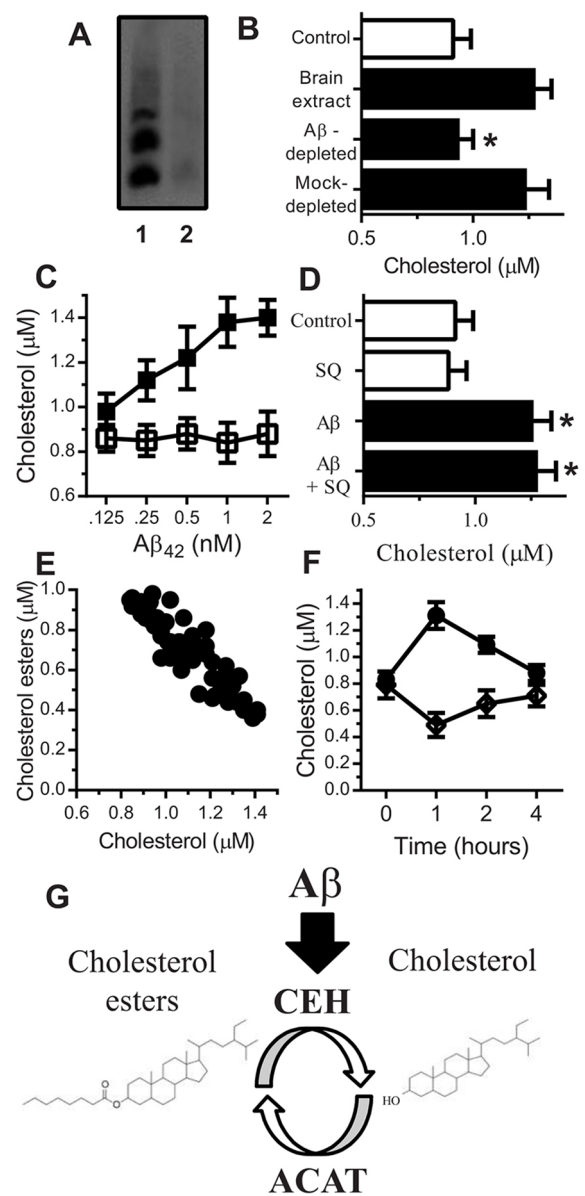


Fig. 1. The A β -induced increase in cholesterol is mediated by hydrolysis of cholesterol esters. (A) Immunoblot showing forms of A β in brain extract (1) and in A β -depleted brain extract (2). (B) The concentrations of cholesterol in synaptosomes incubated with control medium, brain extracts, and A β -depleted or mock-depleted brain extracts. Values are mean±s.d. from triplicate experiments performed three times (*n*=9). The asterisk indicates cholesterol concentrations significantly lower than in synaptosomes incubated with brain extract (*t*-test, *P*<0.01). (C) The concentrations of cholesterol in synaptosomes incubated with oligomer (■) or monomer (□) preparations containing A β ₄₂ as shown. Values are mean±s.d. from triplicate experiments performed three times (*n*=9). (D) The concentrations of cholesterol in synaptosomes treated with control medium or 1 μM squalenol (SQ) (□) or pre-treated with control medium or 1 μM SQ and incubated with brain extract containing 1 nM A β ₄₂ (■). Values are mean±s.d. from triplicate experiments performed three times; *n*=9. The asterisks indicate concentrations of cholesterol significantly higher than in control synaptosomes (*t*-test, *P*<0.01). (E) There was a significant inverse correlation between the concentrations of cholesterol and cholesterol esters in synaptosomes incubated with brain extract containing A β ₄₂ (0.125–1 nM; Pearson's coefficient=−0.93, *P*<0.01). (F) The concentrations of cholesterol (●) and cholesterol esters (◇) in synaptosomes incubated with brain extract containing 1 nM A β ₄₂ for the indicated time periods. Values are mean±s.d. from triplicate experiments performed three times; *n*=9. (G) Schematic of the proposed control of synaptic cholesterol concentrations via A β -induced activation of CEH and ACAT.

1992) and cholesteryl N-(2-dimethylaminoethyl) carbamate (Hosie et al., 1987). Pre-treatment of synaptosomes with either 20 μM DEUP or 5 μM cholesteryl N-(2-dimethylaminoethyl) carbamate inhibited both the $\text{A}\beta$ -induced increase in cholesterol concentrations (Fig. 2A) and the $\text{A}\beta$ -induced reduction in cholesterol esters (Fig. 2B). These drugs did not affect the concentrations of cholesterol or cholesterol esters in control synaptosomes.

To determine whether acetyl-coenzyme A acetyltransferase (ACAT), an enzyme that esterifies cholesterol in cell membranes (Chang et al., 2006), was involved in regulating $\text{A}\beta$ -induced

changes in membrane cholesterol concentrations, neurons were treated with the ACAT inhibitors (TMP-153) (Sugiyama et al., 1995) or YIC-C8-434 (Kaneko et al., 2001). First, the efficacy of ACAT inhibitors was determined by incubating neurons with squalene. The addition of 5 μM squalene, which was converted to cholesterol and subsequently esterified by ACAT, resulted in high concentrations of cholesterol esters in synaptosomes (Fig. 2C). Pre-treatment of neurons with either TMP-153 or YIC-C8-434 reduced the squalene-induced increase in cholesterol esters, indicating inhibition of ACAT (Fig. 2D). At concentrations of 100 nM, both TMP-153 and YIC-C8-434 fully blocked the squalene-induced increase in cholesterol ester concentrations. Subsequently, pre-treatment of synaptosomes with 100 nM TMP-153 or YIC-C8-434 significantly enhanced the $\text{A}\beta$ -induced increase in cholesterol concentrations (Fig. 2E), providing evidence that ACAT reverses the $\text{A}\beta$ -induced increase in cholesterol.

The cholesterol ester cycle controls PrP^C- $\text{A}\beta$ complexes

Cholesterol, but not cholesterol esters, affects membrane structure and function. For example, cholesterol concentrations can affect the expression of PrP^C (Gilch et al., 2006), which acts a receptor for $\text{A}\beta_{42}$ (Laurén et al., 2009). The addition of brain extract to synaptosomes caused the formation of PrP^C- $\text{A}\beta$ complexes (Fig. 3A). There was a significant correlation between the

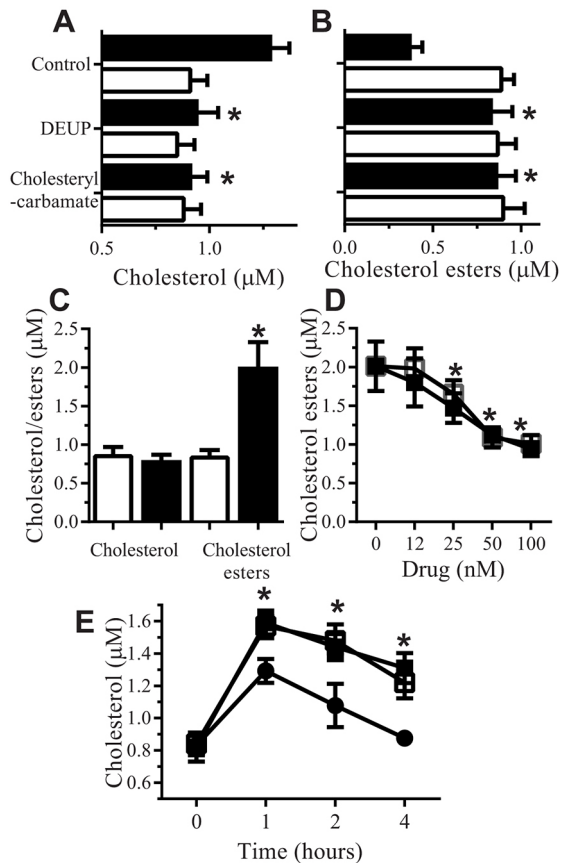


Fig. 2. CEH inhibitors block the $\text{A}\beta$ -induced increase in cholesterol.

(A,B) The concentrations of cholesterol (A) and cholesterol esters (B) in synaptosomes pre-treated with CEH inhibitors, 20 μM DEUP or 5 μM cholesteryl N-(2-dimethylaminoethyl) carbamate, and incubated with control medium (\square) or brain extract containing 1 nM $\text{A}\beta_{42}$ (\blacksquare). Values are mean \pm s.d. from triplicate experiments performed three times; $n=9$. The asterisks indicate concentrations of cholesterol/cholesterol esters significantly different to control synaptosomes incubated with $\text{A}\beta$ (t -test, $P<0.01$). (C) The concentrations of cholesterol and cholesterol esters in synaptosomes derived from neurons treated with control medium (\square) or 5 μM squalene (\blacksquare). Values are mean \pm s.d. from triplicate experiments performed three times; $n=9$. The asterisk indicates concentrations of cholesterol esters significantly higher than in control synaptosomes (t -test, $P<0.01$). (D) The concentrations of cholesterol esters in synaptosomes from neurons pre-treated with ACAT inhibitors, TMP-153 (\blacksquare) or YIC-C8-434 (\square), and incubated with 5 μM squalene. Values are mean \pm s.d. from triplicate experiments performed three times; $n=9$. The asterisks indicate concentrations of cholesterol esters significantly lower than in control synaptosomes incubated with squalene (t -test, $P<0.01$). (E) The concentrations of cholesterol in synaptosomes pre-treated with control medium (\bullet), 100 nM TMP-153 (\blacksquare) or 100 nM YIC-C8-434 (\square) and incubated with brain extract containing 1 nM $\text{A}\beta_{42}$ for the indicated time periods. Values are mean \pm s.d. from triplicate experiments performed three times; $n=9$. The asterisks indicate cholesterol concentrations significantly higher than in control synaptosomes incubated with $\text{A}\beta$, (t -test, $P<0.01$).

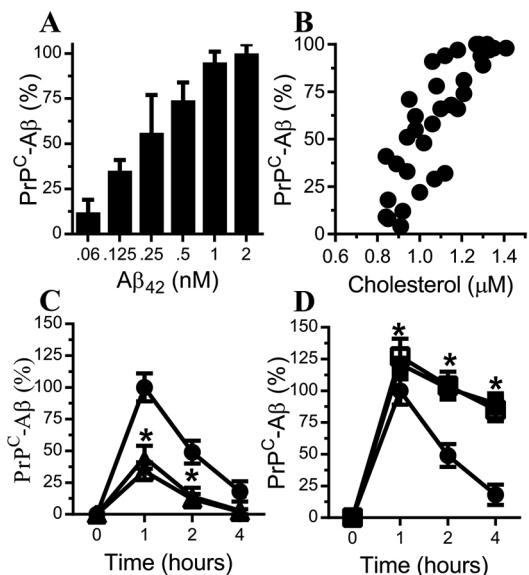


Fig. 3. The cholesterol ester cycle regulates PrP^C- $\text{A}\beta$ complexes. (A) The amounts of PrP^C- $\text{A}\beta$ complexes in synaptosomes incubated with brain extract containing $\text{A}\beta_{42}$ as shown. Values are mean \pm s.d. from triplicate experiments performed three times; $n=9$. (B) There was a significant correlation between the amounts of PrP^C- $\text{A}\beta$ complexes and concentrations of cholesterol following the addition of brain extract containing $\text{A}\beta_{42}$ (0.06–1 nM, Pearson's coefficient=0.85, $P<0.01$). (C) The amounts of PrP^C- $\text{A}\beta$ complexes in synaptosomes pre-treated with control medium (\bullet), 20 μM DEUP (\blacktriangle) or 5 μM cholesteryl N-(2-dimethylaminoethyl) carbamate (\triangle) and incubated with brain extract containing 1 nM $\text{A}\beta_{42}$ for the indicated time periods. Values are mean \pm s.d. from triplicate experiments performed three times; $n=9$. The asterisks indicate significantly less PrP^C- $\text{A}\beta$ complexes than in control synaptosomes incubated with $\text{A}\beta$ (t -test, $P<0.01$). (D) The amounts of PrP^C- $\text{A}\beta$ complexes in synaptosomes pre-treated with control medium (\bullet), 100 nM TMP-153 (\blacksquare) or 100 nM YIC-C8-434 (\square) and incubated with brain extract containing 1 nM $\text{A}\beta_{42}$ for the indicated time periods. Values are mean \pm s.d. from triplicate experiments performed three times; $n=9$. The asterisks indicate significantly more PrP^C- $\text{A}\beta$ complexes than in control synaptosomes incubated with $\text{A}\beta$ (t -test, $P<0.01$).

amounts of PrP^C-A β complexes and cholesterol concentrations in synaptosomes incubated with brain extracts containing between 0.06 and 1 nM A β ₄₂ (Pearson's coefficient= 0.85, P <0.01) (Fig. 3B). As both PrP^C (Naslavsky et al., 1997) and A β (Kawarabayashi et al., 2004; Williamson et al., 2008) are found within lipid rafts, the formation of which is cholesterol sensitive (Schroeder et al., 1994), we hypothesized that cross-linkage of PrP^C by A β oligomers stimulated the release of cholesterol that stabilised PrP^C-A β complexes. A time course study demonstrated that PrP^C-A β complexes were transient (Fig. 3C), as was the A β -induced increase in cholesterol (Fig. 1F), suggesting that PrP^C-A β complexes and cholesterol concentrations were linked. Pre-treatment of synaptosomes with CEH inhibitors [20 μ M DEUP or 5 μ M cholesteryl N-(2-dimethylaminoethyl) carbamate] reduced the formation of PrP^C-A β complexes (Fig. 3C). By contrast, pre-treatment of synaptosomes with ACAT inhibitors (100 nM TMP-153 or 100 nM YIC-C8-434) increased the amounts of PrP^C-A β complexes after 1, 2 and 4 h (Fig. 3D). Collectively, these results indicate that esterification of cholesterol is a factor in the dissociation of PrP^C-A β complexes.

A β activates synaptic cPLA₂

Synapse damage occurs in response to aberrant activation of cPLA₂ (Bate et al., 2010) and the addition of brain extract containing 1 nM A β ₄₂, but not A β -depleted brain extract, increased the amounts of activated (phosphorylated) cPLA₂ in synaptosomes (Fig. 4A,B). In synaptosomes incubated with brain extracts containing 0.125–1 nM A β ₄₂ there was a significant correlation between the amounts of PrP^C-A β complexes and activated cPLA₂ (Fig. 4C). The activation of cPLA₂ is the first step in the production of PGs including PGE₂, a bioactive lipid which causes synapse damage in cultured neurons (Bate et al., 2010). There was a significant correlation between the amounts of activated cPLA₂ and the concentrations of PGE₂ released by synaptosomes (Pearson's coefficient=0.89, P <0.01) (Fig. 4D). There were also significant correlations between the concentrations of cholesterol and the amounts of activated cPLA₂ (Pearson's coefficient=0.75, P <0.01) (Fig. 4E), and concentrations of PGE₂ (Pearson's coefficient=0.67, P <0.01) (Fig. 4F) in these synaptosomes.

The activation of cPLA₂ is associated with its migration to specific membranes by an N-terminal lipid-binding motif (Nalefski et al., 1994). Sucrose density gradients showed that in synaptosomes the addition of A β caused the migration of cPLA₂ to low density lipid rafts (Fig. 5A), without affecting the raft distribution of synaptophysin or ganglioside GM-1 (Fig. 5B). In synaptosomes, A β caused a dose-dependent translocation of cPLA₂ to rafts as defined by their resistant to cold triton X-100 (Fig. 5C). Immunoprecipitation studies showed that A β caused cPLA₂ to colocalize in PrP^C-containing rafts (Fig. 5D) (Bate and Williams, 2011). Following the addition of A β ₄₂ (0.125–1 nM), there were significant correlations between the amounts of cPLA₂ in rafts and the amounts of activated cPLA₂ (Pearson's coefficient=0.92, P <0.01) (Fig. 5E) and cholesterol concentrations (Pearson's coefficient=0.7, P <0.01) (Fig. 5F). These results support the hypothesis that the A β -induced increase in cholesterol stabilises raft-associated signalling complexes that attract and activate cPLA₂.

CEH inhibitors reduce the A β -induced activation of cPLA₂

The correlations between cholesterol concentrations, raft-associated and activated cPLA₂ suggested a causal relationship. In synaptosomes, the A β -induced increase in activated cPLA₂ was reduced by pre-treatment with CEH inhibitors [20 μ M DEUP or

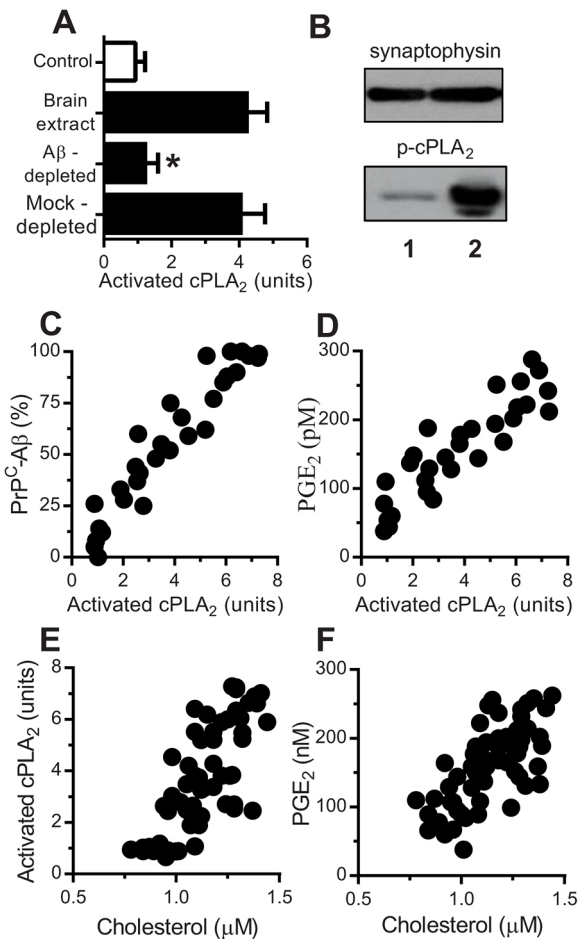


Fig. 4. A β -activated cPLA₂. (A) The amounts of activated cPLA₂ in synaptosomes incubated with control medium, brain extract, A β -depleted brain extract or mock-depleted brain extract. Values are mean \pm s.d. from triplicate experiments performed four times; n =12. The asterisk indicates significantly less activated cPLA₂ than in synaptosomes incubated with brain extract (t -test, P <0.01). (B) The amounts of synaptophysin and activated (phosphorylated) cPLA₂ in synaptosomes incubated with control medium (1) or with brain extract containing 1 nM A β ₄₂ (2). In synaptosomes incubated with brain extract containing A β ₄₂ (0.125–1 nM), there were significant correlations between the amounts of activated cPLA₂ and PrP^C-A β complexes (Pearson's coefficient=0.95, P <0.01) (C), and between the amounts of activated cPLA₂ and concentrations of PGE₂ (Pearson's coefficient=0.89, P <0.01) (D). There were also significant correlations between the concentrations of cholesterol and the amounts of activated cPLA₂ (Pearson's coefficient=0.75, P <0.01) (E), and between concentrations of cholesterol and PGE₂ (Pearson's coefficient=0.67, P <0.01) (F), in these synaptosomes.

5 μ M cholesteryl N-(2-dimethylaminoethyl) carbamate] (Fig. 6A). By contrast, pre-treatment of synaptosomes with ACAT inhibitors (100 nM TMP-153 or 100 nM YIC-C8-434) enhanced the A β -induced activation of cPLA₂ (Fig. 6B). Similarly, pre-treatment with CEH inhibitors reduced, while ACAT inhibitors increased, the A β -induced release of PGE₂ from synaptosomes (Fig. 6C). Neither CEH nor ACAT inhibitors affected the activation of cPLA₂ by phospholipase A₂-activating peptide (PLAP, also known as PLAA), indicating they did not have a direct effect on the enzyme (Fig. 6D). The A β -induced translocation of cPLA₂ to rafts was transient (Fig. 6E) and was reduced by pre-treatment with CEH inhibitors. In synaptosomes pre-treated with ACAT inhibitors (100 nM TMP-153 or 100 nM YIC-C8-434), cPLA₂ remained in rafts longer than in control synaptosomes following the addition of A β (Fig. 6F).

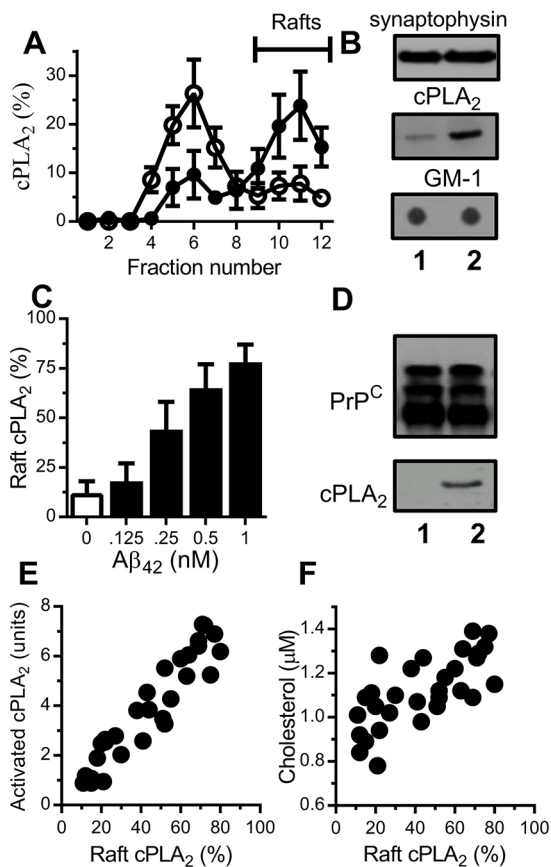


Fig. 5. A β causes the translocation of cPLA₂ to rafts. (A) The amounts of cPLA₂ in fractions from a sucrose density gradient derived from control synaptosomes (○) or from synaptosomes incubated with brain extract containing 1 nM A β ₄₂ (●). Values are mean \pm s.d. from triplicate experiments performed three times; $n=9$. (B) Immunoblots showing the amounts of synaptophysin, cPLA₂ and GM-1 in DRMs (rafts) from synaptosomes incubated with control medium (1) or with brain extract containing 1 nM A β ₄₂ (2). (C) The amounts of cPLA₂ in DRMs (rafts) derived from synaptosomes incubated with control medium (□) or with brain extract containing A β ₄₂ as shown (■). Values are mean \pm s.d., from triplicate experiments performed three times; $n=9$. (D) Immunoblots showing PrP^C and cPLA₂ in immunoprecipitates from synaptosomes incubated with control medium (1) or with brain extract containing 1 nM A β ₄₂ (2). There were significant correlations between the amounts of cPLA₂ in rafts and the amounts of activated cPLA₂ (Pearson's coefficient=0.92, $P<0.01$) (E) and the concentrations of cholesterol (Pearson's coefficient=0.7, $P<0.01$) (F) in synaptosomes incubated with brain extract containing A β ₄₂ (0.125–1 nM).

CEH inhibitors reduce A β -induced synapse damage

The effects of the cholesterol ester cycle on A β -induced synapse damage in cultured neurons were studied. Brain extracts caused the A β -dependent loss of synaptic proteins including synapsin-1, vesicle-associated membrane protein-1, synaptophysin and cysteine string protein (CSP, also known as DNAJC5) from cultured neurons in a tissue culture model of synapse degeneration (Osborne et al., 2016). Here, the addition of brain extracts reduced the amounts of synaptophysin (Fig. 7A) and CSP (Fig. 7B) in neurons, indicative of synapse degeneration. Pre-treatment with CEH inhibitors (20 μ M DEUP or 5 μ M cholesteryl N-(2-dimethylaminoethyl) carbamate) protected cultured neurons against the A β -induced loss of synaptophysin and CSP. By contrast, pre-treatment of neurons with 100 nM TMP-153 or 100 nM YIC-C8-434 increased the A β -induced loss of synaptophysin (Fig. 7C) and CSP (Fig. 7D). None of the drugs tested affected synapse

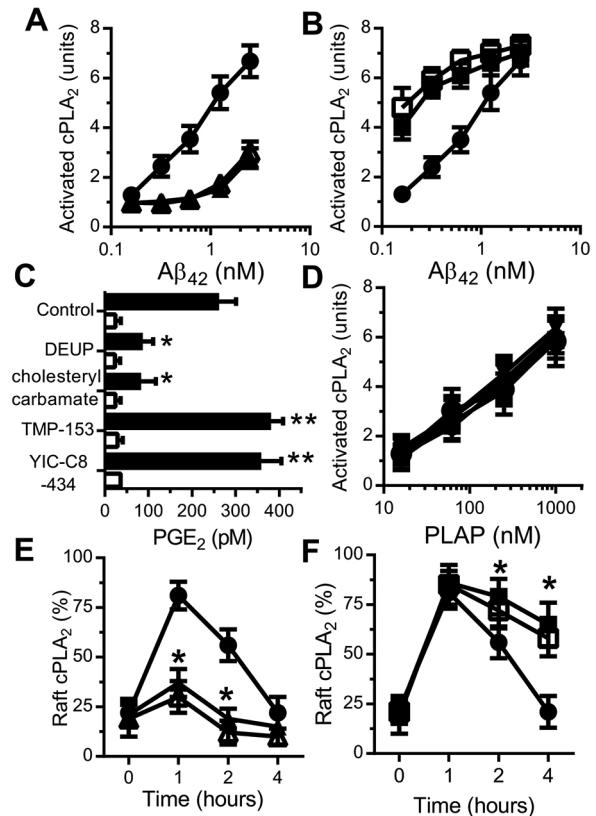


Fig. 6. CEH inhibitors reduce the A β -induced activation of cPLA₂. (A) The amounts of activated cPLA₂ in synaptosomes pre-treated with control medium (●), or the CEH inhibitors 20 μ M DEUP (△) or 5 μ M cholesteryl N-(2-dimethylaminoethyl) carbamate (▲), and incubated with brain extract containing A β ₄₂ as shown. Values are mean \pm s.d. from triplicate experiments performed four times; $n=12$. (B) The amounts of activated cPLA₂ in synaptosomes pre-treated with control medium (●), or the ACAT inhibitors 100 nM TMP-153 (■) or 100 nM YIC-C8-434 (□), and incubated with brain extract containing A β ₄₂ as shown. Values are mean \pm s.d. from triplicate experiments performed four times; $n=12$. (C) The concentrations of PGE₂ in synaptosomes treated with control medium, 20 μ M DEUP, 5 μ M cholesteryl N-(2-dimethylaminoethyl) carbamate, 100 nM TMP-153 or 100 nM YIC-C8-434, and incubated with control medium (□) or brain extract containing 1 nM A β ₄₂ (■). Values are mean \pm s.d. from triplicate experiments performed three times; $n=9$. Concentrations of PGE₂ significantly less (*) or more (**) than in control synaptosomes incubated with A β (t -test, $P<0.01$). (D) The amounts of activated cPLA₂ in synaptosomes pre-treated with control medium (●), 20 μ M DEUP (△), 5 μ M cholesteryl N-(2-dimethylaminoethyl) carbamate (▲), 100 nM TMP-153 (■) or 100 nM YIC-C8-434 (□), and incubated with PLAP as shown. Values are mean \pm s.d. from triplicate experiments performed four times; $n=12$. (E) The amounts of cPLA₂ in DRMs (rafts) from synaptosomes pre-treated with control medium (●), CEH inhibitors, 20 μ M DEUP (△) or 5 μ M cholesteryl N-(2-dimethylaminoethyl) carbamate (▲), and incubated brain extract containing 1 nM A β ₄₂ for the indicated time periods. Values are mean \pm s.d. from triplicate experiments performed three times; $n=9$. The asterisks indicate amounts of raft cPLA₂ significantly less than in control synaptosomes incubated with A β ₄₂ (t -test, $P<0.01$). (F) The amounts of cPLA₂ in DRMs (rafts) from synaptosomes pre-treated with control medium (●), ACAT inhibitors, 100 nM TMP-153 (■) or 100 nM YIC-C8-434 (□), and incubated brain extract containing 1 nM A β ₄₂ for the indicated time periods. Values are mean \pm s.d. from triplicate experiments performed three times; $n=9$. The asterisks indicate amounts of raft cPLA₂ significantly higher than in control synaptosomes incubated with A β ₄₂ (t -test, $P<0.01$).

damage caused by PLAP; there were no significant differences in the amounts of synaptophysin in neuronal cultures incubated with 250 nM PLAP after pre-treatment with control medium or 20 μ M

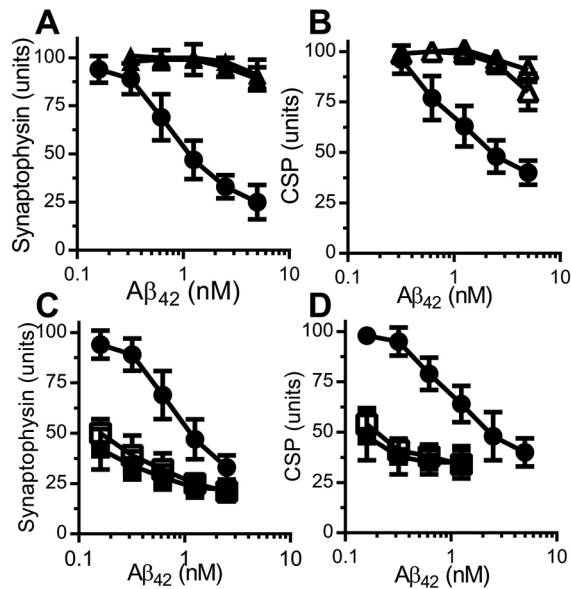


Fig. 7. CEH inhibitors protect neurons against A β -induced synapse damage. (A,B) The amounts of synaptophysin (A) and CSP (B) in neurons pre-treated with control medium (●), 20 μ M DEUP (▲) or 5 μ M cholesterolyl N-(2-dimethylaminoethyl) carbamate (Δ), and incubated with brain extract containing A β_{42} as shown. Values are mean \pm s.d. from triplicate experiments performed three times; $n=9$. (C,D) The amounts of synaptophysin (C) and CSP (D) in neurons pre-treated with control medium (●), 100 nM TMP-153 (■) or 100 nM YIC-C8-434 (□) and incubated with brain extract containing A β_{42} as shown. Values are mean \pm s.d. from triplicate experiments performed three times; $n=9$.

DEUP (31 ± 7 units compared with 36 ± 9 units, $P=0.13$, $n=9$) or 5 μ M cholesterolyl N-(2-dimethylaminoethyl) carbamate (31 ± 7 units compared with 31 ± 8 units, $P=0.73$, $n=9$), 100 nM TMP-153 (31 ± 7 units compared with 36 ± 7 units, $P=0.16$, $n=9$) or 100 nM YIC-C8-434 (31 ± 7 units compared with 33 ± 10 units, $P=0.71$, $n=9$).

Squalene increases A β -induced synapse damage

An altered cholesterol ester cycle, including the accumulation of cholesterol esters, has been reported in AD patients (Pani et al., 2009). To mimic this process, cultured neurons were fed squalene (0.6–5 μ M) for 24 h and synaptosomes were isolated. The addition of 5 μ M squalene did not affect concentrations of synaptophysin (98 ± 6 units compared with 100 ± 5 units, $n=6$, $P=0.25$) or CSP (99 ± 4 units compared with 100 ± 4 units, $n=6$, $P=0.61$), indicating that synaptic density was not altered. Squalene treatment increased the concentrations of cholesterol esters in synaptosomes (Fig. 8A). The addition of brain extract induced a significantly larger increase in cholesterol concentrations in synaptosomes from squalene-treated neurons than in synaptosomes from control neurons (Fig. 8B). There was a significant correlation between the cholesterol concentrations and activated cPLA₂ in synaptosomes derived from squalene-treated neurons incubated with brain extract containing 1 nM A β_{42} (Pearson's coefficient=0.9, $P<0.01$) (Fig. 8C). Synaptosomes from neurons treated with squalene produced higher amounts of activated cPLA₂ in response to A β than did synaptosomes from control neurons (Fig. 8D). Finally, pre-treatment of neurons with 5 μ M squalene significantly enhanced the A β -induced loss of synaptophysin (Fig. 8E) and CSP (Fig. 8F).

DISCUSSION

Here, we show a pivotal role for the cholesterol ester cycle in controlling the formation and dissociation of signalling complexes

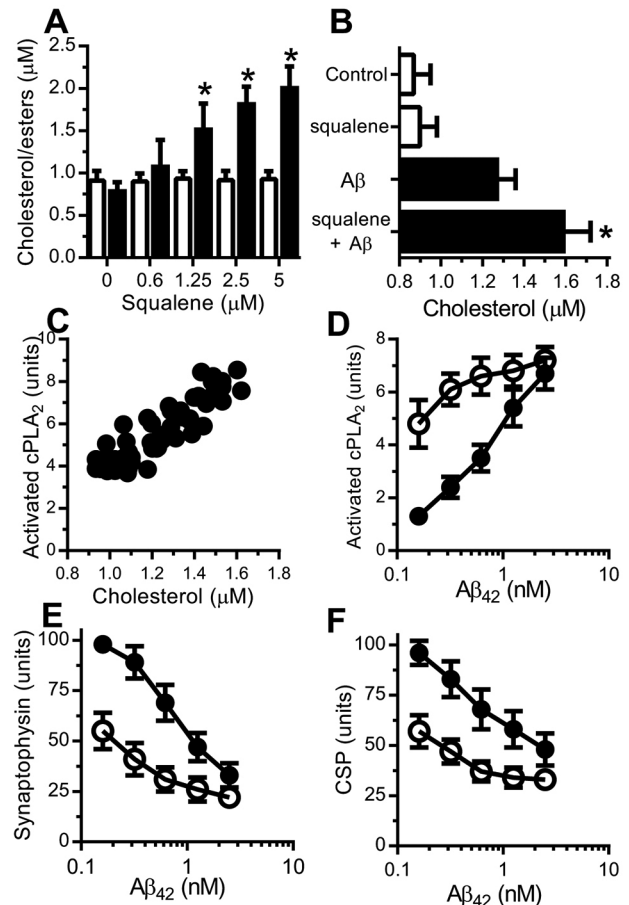


Fig. 8. Increased synaptic cholesterol esters are associated with increased A β -induced synapse damage. (A) The concentrations of cholesterol (□) and cholesterol esters (■) in synaptosomes derived from neurons treated with squalene. Values are mean \pm s.d. from triplicate experiments performed three times; $n=9$. The asterisks indicate concentrations of cholesterol esters significantly higher than in control synaptosomes (t -test, $P<0.01$). (B) The concentrations of cholesterol in synaptosomes derived from neurons treated with control medium or 5 μ M squalene (□), and in these synaptosomes incubated with brain extract containing 1 nM A β_{42} (■). Values are mean \pm s.d. from triplicate experiments performed three times $n=9$. The asterisk indicates concentrations of cholesterol significantly higher than in synaptosomes incubated with A β_{42} (t -test, $P<0.01$). (C) There was a significant correlation between the concentrations of cholesterol and activated cPLA₂ in synaptosomes derived from neurons treated with squalene (0.6 to 5 μ M) and incubated with brain extract containing 1 nM A β_{42} (Pearson's coefficient=0.9, $P<0.01$). (D) The amounts of activated cPLA₂ in synaptosomes from neurons treated with control medium (●) or 5 μ M squalene (○) and incubated with brain extract containing A β_{42} as shown. Values are mean \pm s.d. from triplicate experiments performed three times; $n=9$. The amounts of synaptophysin (E) and CSP (F) in neurons pre-treated with control medium (●) or 5 μ M squalene (○) and incubated with brain extract containing A β_{42} as shown. Values are mean \pm s.d. from triplicate experiments performed three times; $n=9$.

formed in synapses in response to A β . More specifically, these studies demonstrate that transient changes in cholesterol concentrations control the A β -induced activation of cPLA₂ at synapses. Thus, inhibition of CEHs reduced the A β -induced rise in cholesterol, reduced the formation of PrP^C-A β complexes and the activation of cPLA₂ in synapses, and reduced synapse damage in neuronal cultures. Conversely, inhibiting the esterification of cholesterol accentuated the A β -induced increase in cholesterol, stabilised PrP^C-A β complexes, increased activation of cPLA₂ and increased synapse damage.

It is widely believed that the concentration of cholesterol in cell membranes is a critical factor involved in neurodegeneration (Maxfield and Tabas, 2005). Here, we show that physiologically relevant concentrations of natural A β increased cholesterol concentrations within synaptosomes; an observation that is consistent with reports of increased cholesterol concentrations in A β -positive synapses in the cortex of AD patients (Gyls et al., 2007). Although the preparations used in these assays are likely to contain different fragments of the amyloid precursor protein, responses were similar when preparations were standardized according to their A β_{42} content. The A β -induced increase in synaptic cholesterol was not caused by cholesterol synthesis; rather it was controlled by the cholesterol ester cycle; the A β -induced increase in cholesterol was accompanied by a corresponding reduction in cholesterol esters, indicating the activation of a CEH. Inhibition of the A β -induced increase in synaptic cholesterol by two selective CEH inhibitors supported this conclusion. Time course studies demonstrated that the A β -induced increase in cholesterol/reduction in cholesterol esters was transient. These experiments could not be reliably extended beyond 4 h due to the degradation of isolated synaptosomes. CEH inhibitors did not affect the concentrations of cholesterol in the absence of A β , indicating that the cholesterol ester hydrolysis occurs in response to specific stimuli.

The concentration of cholesterol in the cell membranes is critical for the formation of lipid raft membrane platforms that concentrate molecules involved in cell signalling (Simons and Toomre, 2000). Specific stimuli cause individual rafts to coalesce to form a larger platform capable of cell activation (Lingwood et al., 2008; Lingwood and Simons, 2010). Raft formation is associated with the oligomerization of proteins (Hammond et al., 2005), including the aggregation of PrP^C by A β oligomers (Bate and Williams, 2011). Notably, the increase in synaptic cholesterol concentrations was associated with the toxic A β oligomers (McDonald et al., 2010; McLean et al., 1999) rather than nontoxic A β monomers (Giuffrida et al., 2009). The formation of PrP^C-A β complexes was also transient and mirrored the A β -induced changes in cholesterol; there was a close temporal association between concentrations of cholesterol and PrP^C-A β complexes. We hypothesise that A β stimulates the hydrolysis of cholesterol esters resulting in the release of cholesterol that stabilises PrP^C-A β complexes. Thus, the inhibition of CEHs reduced the A β -induced rise in cholesterol and consequently the formation of PrP^C-A β complexes.

The activation of cPLA₂ is the first step in the production of PGE₂. Since the concentrations of PGE₂ are raised in the cerebrospinal fluid of patients with AD (Montine et al., 1999) and PGE₂ caused synapse damage *in vitro* (Bate et al., 2010), we propose that concentrations of A β >1 nM cause aberrant activation of cPLA₂, leading to excess PGE₂ production and synapse degeneration. This hypothesis is supported by the demonstration of significant correlations between A β -induced increases in cholesterol concentrations, activated cPLA₂ and PGE₂ in synapses. Inhibition of CEHs reduced the A β -induced activation of cPLA₂ and PGE₂ production. As neither CEH nor ACAT inhibitors affected activation of cPLA₂ by PLAP, we concluded that these drugs did not have a direct effect upon the enzyme. The activation of cPLA₂ involves its translocation to lipid rafts (Gaudreault et al., 2004), specifically rafts containing PrP^C-A β complexes (Bate and Williams, 2011). There were significant correlations between concentrations of cholesterol, the amounts of cPLA₂ in lipid rafts, the amounts of activated cPLA₂ and concentrations of PGE₂ following the addition of A β . Furthermore,

time course studies demonstrated that the A β -induced rise in cholesterol, A β -PrP^C complexes and the translocation of cPLA₂ into rafts in synapses were transient and demonstrated a close temporal correlation. The inhibition of CEH in synaptosomes reduced both the amounts of cPLA₂ and the duration that cPLA₂ spent within rafts.

The dissociation of lipid raft platforms is a mechanism that limits cell signalling. Notably, the return of cholesterol/cholesterol ester concentrations to basal levels (indicating that cholesterol was being esterified) was closely associated with the dissociation of A β -PrP^C complexes and the return of cPLA₂ to the cytoplasm. Thus, the esterification of cholesterol limited the A β -induced increase of cholesterol in synapses as pharmacological inhibition of ACAT resulted in high cholesterol concentrations being maintained, increased A β -PrP^C complexes, increased time that cPLA₂ spent within rafts, increased activation of cPLA₂ and increased PGE₂ concentrations. Collectively, these results support the hypothesis that localised ACAT reduced synaptic cholesterol concentrations leading to the dissociation of PrP^C-A β complexes and hence the cessation of cell signalling. Conditions in which signalling platforms fail to dissociate may lead to sustained activation of signalling pathways, leading to cell disruption and disease. Here, we demonstrate that inhibition of ACAT increased A β -induced synapse damage. It is noteworthy that although this study focused upon cholesterol and cPLA₂, many other aspects of synaptic function are cholesterol-sensitive and may be influenced by the A β -induced increased cholesterol concentrations.

An altered cholesterol ester cycle in AD patients resulting in accumulation of cholesterol esters has been reported (Chan et al., 2012; Pani et al., 2009). The concentrations of cholesterol esters in synapses were increased by loading neurons with squalene. Synaptosomes from these neurons showed heightened responses to A β , increased concentrations of cholesterol and greater activation of cPLA₂. Of greater importance was the observation that A β caused greater synapse damage, as measured by the loss of synaptophysin and CSP, in squalene-loaded neurons when compared with control neurons. The role of ACAT in neurodegenerative diseases is complicated as it may affect different aspects of AD pathogenesis. ACAT inhibitors have been proposed as treatments for AD because they reduced the production of A β (Bryleva et al., 2010; Puglielli et al., 2001). However, in those studies ACAT inhibitors were used throughout the course of the experimental disease. The results presented here suggest that ACAT inhibitors might accelerate synapse damage in the presence of A β . Consequently, ACAT inhibitors might be able to prevent the development of AD but maybe contraindicated in the latter stages of AD, when concentrations of A β are already raised.

In summary, these results demonstrate the role of the cholesterol ester cycle in A β -induced cell signalling at synapses. The release of cholesterol from stores of cholesterol esters stabilises the complexes formed between PrP^C and A β that activate cPLA₂. Conversely, the esterification of cholesterol facilitates the dissociation of PrP^C-A β complexes and deactivation of cPLA₂.

MATERIALS AND METHODS

Primary neuronal cultures

Primary cortical neurons were prepared from the brains of mouse embryos (day 15.5) from Prnp wild-type^(+/+) and Prnp knockout^(0/0) mice after mechanical dissociation. Neuronal precursors were plated at 5×10^5 cells/well in 48-well plates in Hams F12 containing 5% foetal calf serum for 2 h. Cultures were shaken (600 rpm for 5 min) and non-adherent cells removed by two washes in PBS. Neurons were subsequently grown in neurobasal medium containing B27 components (Invitrogen) and nerve growth factor

(5 ng/ml) (Sigma-Aldrich) for 10 days. Immunohistochemistry revealed that ~95% of cells were neurofilament positive. Neurons were subsequently pre-treated with test compounds for either 24 h (squalene) or 1 h (CEH or ACAT inhibitors) before the addition of A β preparations or PLAP (Bachem). All experiments were performed in accordance with European regulations (European Community Council Directive, 1986, 56/609/EEC) and approved by the local authority veterinary service/ethical committee.

Cell extracts

Treated cells were washed twice in PBS and homogenised in an extraction buffer containing 10 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate and 0.2% SDS at 10⁶ cells/ml. Mixed protease inhibitors [4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride, Aprotinin, Leupeptin, Bestatin, Pepstatin A and E-46] (Sigma-Aldrich) and a phosphatase inhibitor cocktail including PP1, PP2A, microcystin LR, cantharidin and p-bromotetramisole (Sigma-Aldrich) were added, and nuclei and large fragments were removed by centrifugation (1000 g for 5 min).

Western blotting

Samples were mixed with Laemmli buffer containing β -mercaptoethanol, heated to 95°C for 5 min and proteins were separated by electrophoresis on 15% polyacrylamide gels (PAGE). Proteins were transferred onto a Hybond-P PVDF membrane by semi-dry blotting. Membranes were blocked using 10% milk powder; synaptophysin was detected with MAB368 (Millipore), PrP^C with mAb 4F2 (a gift from Professor Jaques Grassi, CEA, Saclay, France), cPLA₂ with goat polyclonal anti-cPLA₂ (Santa Cruz Biotechnology, sc-4049), phosphorylated-cPLA₂ with rabbit polyclonal anti-phospho-cPLA₂ (Cell Signaling Technology, 2831S) and GM-1 with biotinylated cholera toxin subunit B (Sigma-Aldrich, C9972) (all at 1 μ g/ml). These were visualised using a 1:1000 dilution of biotinylated anti-mouse/goat/rat/rabbit IgG (Sigma-Aldrich) followed by extravidin-peroxidase and enhanced chemiluminescence.

Synaptophysin ELISA

Maxisorb immunoplates (Nunc) were coated with an anti-synaptophysin mAb (MAB368, Millipore, 1 μ g/ml) and blocked with 5% milk powder. Samples were added for 1 h and bound synaptophysin was detected using rabbit polyclonal anti-synaptophysin (Abcam, ab53166, 1 μ g/ml) followed by a biotinylated anti-rabbit IgG (Sigma-Aldrich), extravidin-alkaline phosphatase and 1 mg/ml 4-nitrophenol phosphate solution (Sigma-Aldrich). Absorbance was measured at 405 nm. Samples were expressed as 'units synaptophysin', where 100 units was the amount of synaptophysin in 10⁶ untreated cells.

CSP ELISA

Maxisorb immunoplates were coated with 1 μ g/ml of a mouse mAb to CSP (sc-136468, Santa Cruz Biotechnology) and blocked with 5% milk powder. Samples were added for 1 h and bound CSP was detected using 1 μ g/ml rabbit polyclonal anti-CSP (sc-33154, Santa Cruz Biotechnology) followed by a biotinylated anti-rabbit IgG, extravidin-alkaline phosphatase and 1 mg/ml 4-nitrophenol phosphate solution. Absorbance was measured at 405 nm. Samples were expressed as 'units CSP', where 100 units was the amount of CSP in 10⁶ untreated cells.

Isolation of synaptosomes

Synaptosomes were prepared on a discontinuous Percoll gradient as described (Dunkley et al., 2008). Neurons were homogenised at 4°C in SED solution (0.32 M sucrose, 50 mM Tris-HCl pH 7.2, 1 mM EDTA, and 1 mM dithiothreitol) and centrifuged at 1000 g at 4°C for 10 min. The supernatant was transferred to a gradient of 3, 7, 15 and 23% filtered Percoll prepared in SED solution and centrifuged at 16,000 g for 30 min at 4°C. Synaptosomes were collected from the interface of the 15% and 23% Percoll steps, and washed (16,000 g for 5 min at 4°C) and suspended in neurobasal medium containing B27 components at a concentration equivalent to 5 \times 10⁶ neurons per ml. All synaptosomes were used on the same day of preparation.

After the test period, synaptosomes were homogenised in either extraction buffer (as above) or in the DRM extraction buffer (below). All synaptosomes preparations contained equal amounts of synaptophysin.

Isolation of DRMs

Detergent-resistant membranes (DRMs) were isolated by their insolubility in nonionic detergents as described (London and Brown, 2000). Briefly, synaptosomes were homogenised in an ice-cold buffer containing 1% Triton X-100, 10 mM Tris-HCl pH 7.2, 150 mM NaCl, 10 mM EDTA and mixed protease inhibitors, and nuclei and large fragments were removed by centrifugation (300 g for 5 min at 4°C). The post-nuclear supernatant was incubated on ice (4°C) for 1 h and centrifuged (16,000 g for 30 min at 4°C). The supernatant was reserved as the detergent-soluble membrane (DSM), while the insoluble pellet was homogenised in an extraction buffer containing 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.2% SDS and mixed protease inhibitors at 10⁶ cells/ml and centrifuged (10 min at 16,000 g), and the soluble material was reserved as the DRM fraction.

Sucrose density gradients

Synaptosomes were homogenised in a buffer containing 250 mM sucrose, 10 mM Tris-HCl pH 7.4, 1 mM EGTA, mixed protease inhibitors and 1 mM dithiothreitol. Particulate membrane fragments and nuclei were removed by centrifugation (1000 g for 5 min). Membranes were washed by centrifugation at 16,000 g for 10 min at 4°C and suspended in an ice-cold buffer containing 1% Triton X-100, 10 mM Tris-HCl pH 7.2, 150 mM NaCl, 10 mM EDTA. 5–40% sucrose solutions were prepared and layered to produce a gradient. Solubilised membranes were layered on top and centrifuged at 50,000 g for 18 h at 4°C. Serial 1 ml aliquots were collected from the bottom of gradients.

Cholesterol measurement

The concentrations of cholesterol in samples were measured using the Amplex Red cholesterol assay kit (Life Technologies) based upon Robinet et al. (2010). Briefly, control and treated synaptosomes were washed (400 g, 10 min) and lipids extracted by suspension in hexane:isopropanol (3:2, v/v) and disruption for 10 min in a cell disruptor (Disruptor Genie, Scientific Instruments). Samples were centrifuged (10,000 g, 1 min), and the supernatants collected and dried under liquid nitrogen. Lipids were dissolved in 500 μ l isopropanol:NP40 (9:1, v/v) and sonicated in a water bath (30 min). Samples were pre-treated with catalase before the enzyme cocktail of the Amplex Red kit [0.1 M potassium phosphate buffer pH 7.4, 0.25 M NaCl, 5 mM cholic acid, 0.1% Triton X-100, cholesterol oxidase (\pm cholesterol esterase), horse radish peroxidase and 0.4 mM 10-acetyl-3,7-dihydroxyphenoxazine] was added and incubated at 37°C for 30 min. Cholesterol is oxidised by cholesterol oxidase to yield hydrogen peroxide and ketones. The hydrogen peroxide reacts with 10-acetyl-3, 7-dihydroxyphenoxazine (Amplex Red reagent) to produce highly fluorescent resorufin, which is measured by excitation at 530 nm and emission detection at 590 nm. Each experiment contained cholesterol standards and solvent only controls. Cholesterol concentrations of samples were calculated by reference to the cholesterol standards.

cPLA₂ ELISA/activated cPLA₂/PGE₂ ELISA

The amounts of cPLA₂ in extracts was measured by ELISA as described (Bate and Williams, 2011). Maxisorb immunoplates were coated with 0.5 μ g/ml mouse mAb anti-cPLA₂ (clone CH-7, Upstate, 05-568) and blocked with 5% milk powder in PBS+0.1% Tween 20. Samples were incubated for 1 h and the amount of bound cPLA₂ was detected using 1 μ g/ml goat polyclonal anti-cPLA₂ (Santa Cruz Biotechnology, sc-4049) followed by biotinylated anti-goat IgG, extravidin-alkaline phosphatase and 1 mg/ml 4-nitrophenol phosphate solution. Absorbance was measured at 405 nm. The activation of cPLA₂ is accompanied by phosphorylation of the 505 serine residue, which creates a unique epitope and can be measured by ELISA (Bate et al., 2010). To measure the amount of activated cPLA₂, an ELISA using anti-cPLA₂ mAb (clone CH-7, Upstate, 05-568) combined with 1 μ g/ml rabbit polyclonal anti-phospho-cPLA₂ (Cell Signaling

Technology, 2831S), followed by biotinylated anti-rabbit IgG (Sigma-Aldrich), extravidin-alkaline phosphatase and 1 mg/ml 4-nitrophenol phosphate solution. Absorbance was measured on a microplate reader at 405 nm. Results were expressed as 'units activated cPLA₂' (1 unit=amount of activated cPLA₂ in control preparations). The amounts of PGE₂ in synaptosomes were determined using a competitive enzyme immunoassay kit (R&D Systems) according to the manufacturer's instructions.

Brain extracts

Samples of temporal lobes from three patients with a clinical and pathologically confirmed diagnosis of AD were supplied by Asterand, an international supplier of human tissue (informed consent was given to Asterand, and samples were collected according to the Declaration of Helsinki, 2000). Soluble extracts were prepared using methodology as previously described (Shankar et al., 2008). Briefly, brain tissue was cut into pieces of ~100 mg and added to 2 ml tubes containing lysing matrix D beads (Q-Bio). Ice-cold 20 mM Tris HCl pH 7.4 containing 150 mM NaCl was added to an equivalent of 100 mg brain tissue/ml and tubes were shaken for 10 min. This process was performed three times before tubes were centrifuged at 16,000 g for 10 min to remove particulate matter. Soluble material was prepared by passage through a 50 kDa filter (Sartorius) (16,000 g for 30 min to remove proteolytic enzymes, membrane-bound and plaque A β). The remaining material was then desalted (3 kDa filter, Sartorius) to eliminate bioactive small molecules and drugs, and the retained material collected (preparation contains molecules with molecular weights between 3 and 50 kDa). Monomers were prepared by passage through a 10 kDa filter (Sartorius) and oligomers were collected from the material that was retained (10–50 kDa). The concentrations of A β in each preparation were measured by ELISA (see below). Preparations were stored at –80°C. For cell experiments, preparations were diluted in neurobasal medium containing B27 components. For immunoblots, preparations were mixed with an equal volume of 0.5% NP-40, 5 mM CHAPS, 50 mM Tris pH 7.4, and separated by PAGE. Proteins were transferred onto a Hybond-P PVDF membrane by semi-dry blotting and blocked using 10% milk powder. A β was detected by incubation with 1 μ g/ml mAb 6E10, reactive with amino acids 1-16 of A β (Covance, SIG-39340), biotinylated anti-mouse IgG, extravidin-peroxidase and enhanced chemiluminescence.

Immunodepletions

Brain extracts were incubated with 1 μ g/ml mAb 4G8 (reactive with amino acids 17–24 of A β , Covance, SIG-39220) or 1 μ g/ml mAb 3F4 (reactive with human prion proteins, Millipore, MAB1562; mock-depletion) and incubated at 4°C on rollers for 24 h. Protein G microbeads were added (10 μ l/ml) (Sigma-Aldrich) for 2 h, protein G bound-antibody complexes removed by centrifugation and the depleted media filtered before use.

Sample preparation for end-specific ELISAs

To detach A β from cellular components that occlude specific epitopes, samples (50 μ l) were mixed with 250 μ l 70% formic acid and sonicated. A 50 μ l aliquot was added to 50 μ l 10 M Tris-HCl with protease inhibitors (as above) and sonicated before addition to ELISA.

A β ₄₀/A β ₄₂ ELISA

Nunc Maxisorb immunoplates were coated with 1 μ g/ml mAb 4G8 and blocked with 5% milk powder. Samples were added for 1 h. In separate plates, A β ₄₀ was detected with 2 μ g/ml rabbit polyclonal PC-149 (Merck) and A β ₄₂ with a 1 μ g/ml rabbit mAb BA3-9 (Covance, SIG-39168), followed by biotinylated anti-rabbit IgG, extravidin alkaline phosphatase (Sigma-Aldrich) and 1 mg/ml 4-nitrophenol phosphate solution. Absorbance was measured at 405 nm and compared to a dose response of synthetic A β ₄₀/A β ₄₂ (Bachem).

PrP^C:A β complex ELISA

Maxisorb immunoplates were coated with 1 μ g/ml mAb 4F2 reactive with PrP^C (a gift from Professor Jaques Grassi). Plates were blocked with 5% milk powder and samples were added for 1 h. A β bound to PrP^C was detected by 1 μ g/ml biotinylated mAb 6E10 (reactive with epitopes 1–16 of A β ,

Covance), followed by extravidin-alkaline phosphatase and 1 mg/ml 4-nitrophenol phosphate solution. Absorbance was measured at 405 nm. Samples were expressed as a % of the maximum OD in control synaptosomes.

Drugs

Stock solutions were dissolved in ethanol or DMSO, and diluted in culture medium to obtain final working concentrations. Vehicle controls consisted of equivalent dilutions of ethanol or DMSO.

Statistical analysis

Comparison of treatment effects was carried out using Student's paired *t*-tests, and one-way and two-way ANOVA with Bonferroni's post hoc tests (IBM SPSS Statistics 20). Data are presented as mean \pm s.d. and *P*<0.01 was considered significant. Correlations between data sets were analysed using Pearson's bivariate coefficient (IBM SPSS Statistics 20).

Competing interests

The authors declare no competing or financial interests.

Author contributions

Methodology: E.W., C.O., C.B.; Formal analysis: E.W., C.O., C.B.; Investigation: E.W., C.O., C.B.; Writing - original draft: C.B.; Writing - review & editing: C.B.; Supervision: C.B.

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