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Valproic acid and its congener propylisopropylacetic acid reduced the amount of soluble amyloid-β oligomers released from 7PA2 cells

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Abstract

The amyloid hypothesis of Alzheimer's disease suggests that synaptic degeneration and pathology is caused by the accumulation of amyloid-β (Aβ) peptides derived from the amyloid precursor protein (APP). Subsequently, soluble Aβ oligomers cause the loss of synaptic proteins from neurons, a histopathological feature of Alzheimer's disease that correlates with the degree of dementia. In this study, the production of toxic forms of Aβ was examined *in vitro* using 7PA2 cells stably transfected with human APP. We show that conditioned media from 7PA2 cells containing Aβ oligomers caused synapse degeneration as measured by the loss of synaptic proteins, including synaptophysin and cysteine-string protein, from cultured neurons. Critically, conditioned media from 7PA2 cells treated with valproic acid (2-propylpentanoic acid (VPA)) or propylisopropylacetic acid (PIA) did not cause synapse damage. Treatment with VPA or PIA did not significantly affect total $A\beta_{42}$ concentrations; rather these drugs selectively reduced the concentrations of $A\beta_{42}$ oligomers in conditioned media. In contrast, treatment significantly increased the concentrations of $A\beta_{42}$ monomers in conditioned media. VPA or PIA treatment reduced the concentrations of APP within lipid rafts, membrane compartments associated with Aβ production. These effects of VPA and PIA were reversed by the addition of platelet-activating factor, a bioactive phospholipid produced following activation of phospholipase A_2, an enzyme sensitive to VPA and PIA. Collectively these data suggest that VPA and PIA reduce Aβ oligomers through inhibition of phospholipase A_2 and suggest a novel therapeutic approach to Alzheimer's treatment.

Key words: Alzheimer's disease; amyloid-β; phospholipase A_2; platelet-activating factor; synapses; valproic acid

1. Introduction

Alzheimer's disease (AD) is a complex neurological disorder characterized by a progressive dementia resulting from synaptic failure (1). The amyloid hypothesis maintains that the accumulation of neurotoxic amyloid-β (Aβ) peptides following the proteolytic cleavage of the amyloid precursor protein (APP) (2) causes neurodegeneration (3). The production of Aβ is a key therapeutic target that can be investigated in cells transfected with the human APP gene; which metabolise APP to toxic forms of Aβ (4). 7PA2 cells are Chinese hamster ovary (CHO) cells transfected with human APP that produce Aβ that are of similar size and potency to soluble Aβ derived from AD patients (5). Aβ peptides in brains of AD patients are found in a mixture of complex physical forms. Not all forms of Aβ have equal biological significance; toxicity is dependent upon the state of Aβ, whether that is the length of peptide, state of aggregation, homogeneity of aggregates or specific Aβ conformations. Thus, there exist disease-relevant conformations of Aβ, while other conformations are less toxic or biologically inert (6). The identity of "toxic Aβ" remains highly controversial; Aβ oligomers of similar size demonstrate dissimilar toxicity (7) and consequently it is difficult to ascribe biological function to specific Aβ conformations as identified in biophysical methods. Therefore drug treatments could reduce non-toxic forms of Aβ without affecting the biologically active forms of Aβ. To overcome this problem the effects of Aβ released from treated 7PA2 cells was examined. Extensive synapse degeneration is observed in Alzheimer's patients (8) and the reductions in synaptic proteins correlate closely with the degree of dementia in AD (9,10). Small, soluble Aβ oligomers are thought to be the main form of Aβ that causes synapse degeneration (6). For these reasons the effects of conditioned media (CM) from 7PA2 cells upon synapses in cultured neurons was tested. Synaptic density was determined by quantifying the amounts of synaptophysin and cysteine string protein (CSP) using enzyme-linked immunoassays (ELISA). The amounts of synaptophysin has been used to access synaptic density in the brain (11,12) and in cultured neurons (13). Although the processing of APP is affected by phospholipase A_2 (PLA_2) inhibitors (14), conventional $cPLA_2$ inhibitors do not readily cross the blood-brain barrier and their use in AD is limited. Several reports demonstrated that valproic acid (2-propylpentanoic acid or VPA), a short branched-chain fatty acid acts like a PLA_2 inhibitor (13,15). Furthermore, although VPA has also been reported to reduce Aβ production (16,17) it was not clear which forms of Aβ were reduced. Here we report that the treatment of 7PA2 cells with VPA and its congener

Abbreviations - Alzheimer's disease (AD), amyloid-β (Aβ), amyloid precursor protein (APP), Chinese hamster ovary (CHO), conditioned media (CM), cysteine string protein (CSP), decanoic acid (DA), detergent-resistant membrane (DRM), detergent-soluble membrane (DSM) enzyme-linked immunoassays (ELISA), phospholipase A_2 (PLA_2), platelet-activating factor (PAF), propylisopropylacetic acid (PIA), prostaglandin (PG), standard deviations (SD), valproic acid (VPA).

propylisopropylacetic acid (PIA), but not decanoic acid (DA), reduced the production of toxic forms of Aβ. These drugs had only a small effect upon concentrations of Aβ; rather they caused a switching from the production of toxic Aβ oligomers to non-toxic Aβ monomers.

2. Materials and Methods

2.1. 7PA2 cells - CHO cells stably transfected with cDNA encoding human APP_{751} (referred to as 7PA2 cells) (5) were provided by Professor E Koo (University of California). These were cultured in neurobasal medium supplemented with B27 components (Invitrogen) in the presence of compounds for 72 hours. CM was collected, filtered, desalted and passed through a 50 kDa filter (Vivaspin-Sartorious). CM from CHO cells (CHO-CM) were used as controls. To determine cell viability thiazolyl blue tetrazolium bromide (Sigma) was added to cells at a final concentration of 50 µM for 3 hours at 37°C. The medium was removed, the formazan product solubilized in 200 µl of dimethyl sulfoxide, transferred to an immunoassay plate and absorbance read at 595 nm. Cell survival was calculated with reference to untreated cells (100% survival). Cell extracts were collected from treated cells washed 3 times with ice cold PBS and homogenised in extraction buffer (containing 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.2% SDS and mixed protease inhibitors (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride, Aprotinin, Leupeptin, Bestatin, Pepstatin A and E-46) (Sigma)) at 10^6 cells/ml. Cellular debris was removed by centrifugation (20 minutes as 16000 x g) and the supernatant collected. 7PA2-CM containing Aβ monomers were prepared by filtration of 2 mls 7PA2-CM through a 10 kDa filter (Sartorius). Oligomer preparations were the 7PA2-CM retained by the 10 kDa filter diluted back to the original volume (2 mls). For immunoblot analysis, 7PA2-CM/monomer or oligomer preparations were concentrated from 2000 to 100 µls using a 3 kDa filter (Sartorius). 10 µl of sample were mixed with an equal volume of in 0.5% NP-40, 5 mM CHAPS, 50 mM Tris, pH 7.4 and separated by electrophoresis on 15% polyacrylamide gels. Proteins were transferred onto a Hybond-P polyvinylidene fluoride membrane by semi-dry blotting and blocked using 10% milk powder. Aβ was detected by incubation with mAb 6E10 (Covance), biotinylated anti-mouse IgG, extravidin-peroxidase and enhanced chemiluminescence. Soluble brain extracts (containing peptides between 3 and 50 kDa) were prepared from brain tissue derived from Alzheimer's patients as described (13).

2.2 Brain extracts – Samples of temporal lobes from 3 patients with a pathologically-confirmed, clinical diagnosis of Alzheimer's diseasewas supplied by Asterand. Soluble extracts were prepared using methodology as described (18). Briefly, pieces of brain tissue of approximately 100 mg were added to tubes containing lysing matrix D beads (Q-Bio). Ice cold 20 mM Tris, pH 7.4 containing 150 mM NaCl was added to an equivalent of 100 mg brain tissue/ml, tubes were shaken for 10 minutes (Disruptor genie,

Scientific Instruments). This process was performed 3 times before tubes were centrifuged at 16,000 x *g* for 10 minutes to remove particulate matter. Soluble material was prepared by passage through a 50 kDa filter (Sartorius) (16,000 x *g* for 30 minutes). The remaining material was desalted (3 kDa filter (Sartorius)) 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 to 50 kDa). Preparations were stored at -80^{o}C. For cell experiments preparations were diluted in neurobasal medium containing B27 components. For immunoblots, preparations were separated by electrophoresis and visualised as outlined above.

2.3. Immunodepletions - To deplete preparations of Aβ they were incubated with 1 μg/ml mAb 4G8 (reactive with amino acids 17-24 of Aβ, Covance) or 1 μg/ml mAb LN27 (reactive with amino acids 45 to 53 of APP, (mock-depletion)) and incubated on rollers for 2 hours. Protein G microbeads were added (10 μl/ml) (Sigma) for 30 minutes and protein G bound-antibody complexes removed by centrifugation (1000 x *g* for 5 minutes) and passed through a 0.2 μm filter.

2.4. Isolation of detergent-resistant membranes (DRMs (lipid rafts)) - These membrane domains were isolated by their insolubility in non-ionic detergents as described (19). Briefly, cells 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. Nuclei and large fragments were removed by centrifugation (300 x g for 5 minutes at 4^{o}C). The post nuclear supernatant was incubated on ice (4^{o}C) for 1 hour (shaken at 10 minute intervals) and centrifuged (16,000 x g for 20 minutes at 4^{o}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 10^6 cells/ml, centrifuged (10 minutes at 16,000 x g) and the soluble material was reserved as the DRM fraction.

2.5. Western Blotting - Samples were mixed with Laemmli buffer containing β-mercaptoethanol, heated to 95^{o}C for 5 minutes and proteins were separated by electrophoresis on 15% polyacrylamide gels. Proteins were transferred onto a Hybond-P polyvinylidene fluoride membrane by semi-dry blotting. Membranes were blocked using 10% milk powder; APP was detected with rabbit polyclonal anti-APP (Sigma), caveolin with rabbit polyclonal anti-caveolin (Upstate) and platelet-activating factor (PAF) receptor with rabbit polyclonal anti-PAF receptor (Cayman chemicals). These were visualised using a combination of

biotinylated anti-mouse/goat/rat/rabbit IgG (Sigma), extravidin-peroxidase and enhanced chemiluminescence.

2.6. Primary neuronal cultures - Primary cortical neurons were prepared from the brains of mouse embryos (day 15.5) after mechanical dissociation. Neurons were plated at 5 x 10^5 cells/well in 48 well plates in Hams F12 containing 5% foetal calf serum for 2 hrs. Cultures were shaken (600 r.p.m for 5 mins) and non-adherent cells removed by 2 washes in phosphate buffered saline (PBS). Neurons were subsequently grown in neurobasal medium supplemented with B27 components (Invitrogen) and 5 nM nerve growth factor for 10 days. Immunohistochemistry showed that greater than 90% of cells were neurofilament positive. Neurons were incubated with 7PA2-CM/CHO-CM for 24 hrs as described (20). Neurons were washed twice in ice-cold PBS and homogenised in a buffer containing 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.2% SDS containing mixed protease and phosphatase inhibitors (as above) at 10^6 cells/ml. Nuclei and cell debris was removed by low speed centrifugation (300 x *g* for 5 mins). 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.

2.7. Synaptophysin ELISA - Maxisorb immunoplates (Nunc) were coated with anti-synaptophysin mouse monoclonal antibody MAB368 (Millipore) and bound synaptophysin was detected using rabbit polyclonal anti-synaptophysin (Abcam) followed by a biotinylated anti-rabbit IgG, extravidin-alkaline phosphatase and 1 mg/ml 4-nitrophenol phosphate solution (Sigma). Absorbance was measured on a microplate reader at 405 nm. Samples were expressed as "units synaptophysin" where 100 units were defined as the amount of synaptophysin in 10^6 control neurons.

2.8. CSP ELISA – Maxisorb immunoplates were coated with a monoclonal antibody (mAb) to CSP (Santa Cruz). Samples were added and bound CSP was detected using rabbit polyclonal anti-CSP (Santa Cruz) 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 were defined as the amount of CSP in 10^6 control neurons.

2.9. Sample preparation – To detach $A\beta_{42}$ from membrane components that blocked specific epitopes samples (300 µl) were mixed with 700 µl of propan-2-ol and sonicated. Proteins were precipitated by adding 250 µls 100%w/v trichloroacetic acid, incubating on ice for 30 mins and centrifugation (16,000 x *g* for 10 mins at 4ºC). The pellet was washed twice with ice cold acetone, dried, suspended in a buffer containing 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 10 mM EDTA and 0.2% SDS and sonicated.

2.10. $A\beta_{42}$ ELISA – Nunc Maxisorb immunoplates were coated with mAb 4G8 (epitope 17-24) (Covance) in carbonate buffer overnight. Plates were blocked with 5% milk powder in PBS-tween and samples were applied. The detection antibody was an $A\beta_{42}$ selective rabbit mAb BA3-9 (Covance) followed by biotinylated anti-rabbit IgG and extravidin alkaline phosphatase (Sigma). Total Aβ was visualised by addition of 1 ng/ml 4-nitrophenol phosphate solution and optical density was read in a spectrophotometer at 405 nm.

2.11. $A\beta_{40}$ ELISA - Maxisorb immunoplates were coated with mAb 4G8 (epitope 17-24) and blocked with 5% milk powder in PBS-tween. Samples were applied and $A\beta_{40}$ was detected with rabbit polyclonal PC-149 (Merck) followed by biotinylated anti-rabbit IgG and extravidin alkaline phosphatase. Total Aβ was visualised by addition of 1 mg/ml 4-nitrophenol phosphate and optical density was read in a spectrophotometer at 405 nm.

2.12. APP ELISA – Maxisorb immunoplates were coated with 1 µg/ml mouse mAb anti-APP (Clone 1G6 - epitope 573-596 (Biolegend)) and blocked with 5% milk powder in PBS-tween. Samples were applied and bound APP was detected using rabbit polyclonal raised against the N-terminal of APP (epitope 40 to 60 (Sigma)) followed by anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) and 1 mg/ml 4-nitrophenol phosphate. Absorbance was measured on a microplate reader at 405 nm and results were calculated by comparison to serial dilutions of cell extracts from control cells.

2.13. Cholesterol measurement - The concentrations of cholesterol in samples were measured using the Amplex Red cholesterol assay kit (Life Technologies) (21). Briefly, control and treated cells were washed (400 × *g*, 10 min) and lipids extracted by homogenization in hexane:isopropanol (3:2, v/v) 10 minutes in a cell disruptor (Disruptor genie, Scientific Instruments). Samples were centrifuged (10,000 × *g*, 1 min), supernatants collected and dried under nitrogen. Lipids were dissolved in 500 µl isopropanol:NP40 (9:1 v/v) and sonicated in a waterbath (30 minutes). Samples were pre-treated with catalase before the enzyme cocktail of the Amplex-red kit was added (0.1 M potassium phosphate buffer, pH 7.4; 0.25 M NaCl, 5 mM cholic acid, 0.1% Triton X-100, cholesterol oxidase, horseradish peroxidase, (± cholesterol esterase) and 0.4 mM 10-acetyl-3,7-dihydroxyphenoxazine) and incubated at 37°C for 30 minutes. 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.

2.14. Compounds – VPA and DA were obtained from Sigma. PIA was obtained from Ukrogsyntez Ltd. Stock solutions were dissolved in ethanol or di-methyl sulphoxide and diluted in medium to obtain final working concentrations. Vehicle controls consisted of equal dilutions of ethanol or di-methyl sulphoxide.

2.15. Statistical Analysis - Comparison of treatment effects was carried out using Student's paired t-tests. Error values are reported as standard deviations (SD) and significance was determined where $P<0.01$. Bivariate analysis using Pearson's coefficient (IBM SPSS statistics 20) were used to examine correlations between data sets.

3. Results

3.1. 7PA2 cells released toxic Aβ – Neurons incubated with CM from 7PA2 cells contained significantly less synaptophysin than did neurons incubated with CM from CHO cells (Figure 1A). Previous studies showed that the loss of synaptophysin was accompanied by the loss of other synaptic proteins including, CSP, vesicle-associated membrane protein-1 and synapsin-1 (20) indicative of synapse damage. To ascertain whether Aβ was the active component in 7PA2-CM it was removed immunoprecipitation with mAb 4G8 (reactive with Aβ) (Figure 1B). Immunodepletion with 4G8 reduced the concentrations of $A\beta_{42}$ (0.04 nM ± 0.04 compared with 2.21 nM ± 0.3, n=9, $P<0.01$) and $A\beta_{40}$ (0.23 nM ± 0.21 compared with 7.42 ± 0.6 nM, n=9, $P<0.01$) in 7PA2-CM. Mock-depletions did not significantly affect the concentration of $A\beta_{40}$ (6.95 nM ± 0.81 compared with 7.42 nM ± 0.6, n=9, P=0.2) or $A\beta_{42}$ (2.03 nM ± 0.21 compared with 2.21 ± 0.3 nM, n=9, P=0.21). We then assessed the role of CM on neurons. These immunoprecipitation studies demonstrate that synaptophysin loss was mediated by Aβ since the change caused by 7PA2-CM was lost following immunoprecipitation with mAb 4G8 (reactive with Aβ) but was unaffected by mock-depletion (Figure 1A). Synaptophysin loss from neurons was also monitored following exposure to soluble brain extracts derived from an Alzheimer's patient which was also Aβ-dependent (13). An immunoblot showed that soluble brain extract also contained Aβ monomers, dimers and trimers (Figure 1C). 7PA2-CM and brain extracts containing similar concentrations of $A\beta_{42}$ caused dose-dependent reductions in synaptophysin and CSP from neurons indicative of synapse damage (Figures 1D & 1E).

3.2. VPA reduced toxic Aβ in CM – Since VPA has been reported to reduce Aβ production in a transgenic murine model of AD (22) the effects of VPA and 2 related molecules (PIA and DA (23)) on Aβ production in 7PA2 cells was studied. 7PA2 cells were treated with 10 μM VPA, PIA or DA for 3 days. The survival

or growth of 7PA2 cells, as measured by thiazolyl blue tetrazolium, was not affected by treatment with 10 μM VPA (99% cell survival ± 4 compared with 100% ± 6, n=9, P=0.85), 10 μM PIA (99% cell survival ± 4 compared with 100% ± 6, n=9, P=0.75) or 10 μM DA (101% cell survival ± 5 compared with 100% ± 6, n=9, P=0.64). Critically, CM from 7PA2 cells treated with VPA or PIA did not cause the loss of synaptophysin (Figure 2A) or CSP (Figure 2B) from neurons, whereas the CM from cells treated with 10 μM DA caused as much synapse damage as did CM from control 7PA2 cells. This effect of VPA and PIA was dose-dependent (Figure 2C).

3.3. VPA reduced concentrations of $A\beta_{42}$ oligomers - 7PA2 cells release both $A\beta_{40}$ and $A\beta_{42}$ as demonstrated by sequence analysis and end-specific monoclonal antibodies (4,24). Treatment with 10 μM VPA did not significantly reduce the concentrations of $A\beta_{42}$ (2.43 nM ± 0.39 compared with 2.62 nM ±0.32, n=6, P=0.4) or $A\beta_{40}$ (8 nM ± 1.5 compared with 8.4 ± 1.2, n=6, P=0.72) found in CM when compared to control cells. Similarly, there were no significant differences between the concentrations of $A\beta_{42}$ (2.35 nM ± 0.3 compared with 2.62 nM ±0.32, n=6, P=0.28) or $A\beta_{40}$ (8.4 nM ± 1.2 compared with 8.4 ± 1.2, n=6, P=0.94) found in CM from cells treated with 10 μM PIA and controls. The surprising observation that CM from VPA and PIA-treated 7PA2 cells contained appreciable concentrations of $A\beta_{42}$ but did not cause synapse damage could be explained by observations that $A\beta_{42}$ exists in both disease-relevant forms and non-relevant pools (25). For example, synapse damage is thought to be caused by the soluble $A\beta_{42}$ oligomers rather than Aβ monomers (24,26).

The hypothesis that the toxicity of 7PA2-CM was dependent upon the concentration of Aβ oligomers was tested by separating 7PA2-CM into monomers and oligomers by passage through a 10 kDa filter. Immunoblots showed that the retained material contained Aβ oligomers with no Aβ monomers (Figure 3A). Treatment with VPA or PIA, but not DA, reduced the concentrations of $A\beta_{42}$ oligomers in a dose-dependent manner (Figure 3B). Concentrations of $A\beta_{40}$ oligomers were low and there were no significant differences between CM from control cells and cells treated with 10 μM VPA (0.26 nM ± 0.12 compared with 0.19 ± 0.12, n=6, P=0.49) or 10 μM PIA (0.26 nM ± 0.12 compared with 0.19 ± 0.12, n=6, P=0.46). There were significant inverse correlations between the concentrations of $A\beta_{42}$ oligomers in CM from VPA or PIA-treated cells (1.25 to 10 μM) and the amounts of synaptophysin (Figure 3C) or CSP (Figure 3D) in neurons incubated with these CM.

3.4. VPA increased concentrations of $A\beta_{42}$ monomers – Immunoblots showed that 7PA2-CM that passed through the 10 kDa filter contained a single band that migrated as a monomer (Figure 4A). Treatment of these cells with VPA or PIA caused a dose-dependent increase in $A\beta_{42}$ monomers (Figure 4B). These compounds did not alter the concentrations of $A\beta_{40}$ monomers; there were no significant differences

between CM from control cells and cells treated with 10 μM VPA (7.9 nM ± 0.95 compared with 7.56 ± 0.82, n=6, P=0.49) or 10 μM PIA (0.79 nM ± 0.95 compared with 7.42 ± 0.58, n=6, P=0.46). There were significant inverse correlations between the concentrations of $A\beta_{42}$ oligomers and monomers in CM from cells treated with VPA (1.25 to 10 μM), Pearson's coefficient= -0.92, P<0.01, or with PIA (1.25 to 10 μM), Pearson's coefficient= -0.94 P<0.01 (Figure 4C).

3.5. VPA did not disrupt $A\beta_{42}$ oligomers - The loss of Aβ oligomers and corresponding increase in Aβ monomers following treatment with VPA or PIA suggested that these compounds may interact directly with Aβ oligomers causing their dissociation into monomers. To test this hypothesis Aβ oligomer preparations (containing 10 nM $A\beta_{42}$) were incubated directly with 10 nM VPA or 10 nM PIA at 37ºC. After 3 days preparations were centrifuged through a 10 kDa filter to isolate $A\beta_{42}$ monomers. Monomeric forms of $A\beta_{42}$ were not detected in preparations incubated with control medium, VPA or PIA. In addition, there was no significant reductions in the concentrations of $A\beta_{42}$ oligomers between control preparations and preparations containing VPA (9.77 nM ± 0.25 compared with 9.69 nM ±0.21, n=6, P=0.62) or PIA (9.77 nM ± 0.25 compared with 9.81 nM ± 0.23, n=6, P=0.78).

The possibility that VPA/PIA affected the release of Aβ from cells was examined by measuring $A\beta_{42}$ in cell extracts. The concentrations of $A\beta_{42}$ oligomers in 7PA2 cells were significantly reduced by treatment with 10 μM VPA or with 10 μM PIA, but not by treatment with 10 μM DA (Table 1). Treatment with VPA and PIA also increased the amounts of cell-associated $A\beta_{42}$ monomers.

3.6. CM from VPA-treated 7PA2 cells reduced Aβ-induced synapse damage – Reports that Aβ monomers are neuroprotective (27) suggested that the $A\beta_{42}$ monomers released from treated cells may protect neurons. This hypothesis was tested by mixing CM from 7PA2 cells treated with VPA, PIA or DA with Aβ derived from a soluble brain extract containing 2 nM $A\beta_{42}$ and adding the mixture to neurons. CM from 7PA2 cells that had been treated with either 10 μM VPA or 10 μM PIA, but not 10 μM DA, significantly reduced the Aβ-induced synapse damage (Figure 5A). This effect of these CM was stimulus specific as they did not affect synapse damage caused by prostaglandin (PG)E_2 (Figure 5B). To confirm that these effects of CM were mediated by Aβ the CM from VPA and PIA-treated 7PA2 cells were depleted of Aβ and mixed with brain extracts containing 2 nM $A\beta_{42}$. The protective effect of CM from VPA and PIA-treated cells was removed by Aβ depletion, but not by mock-depletion (Figure 5C). There were significant correlations between the amounts of synaptophysin and concentrations of $A\beta_{42}$ monomers in CM from cells treated with VPA or PIA (1.25 to 10 μM), Pearson's coefficient= 0.94, P<0.01 (Figure 5D).

3.7. VPA and PIA and cholesterol synthesis – As VPA has been reported to affect cholesterol metabolism (28) the possibility that VPA and PIA reduced cholesterol synthesis was examined. Firstly, the cholesterol concentrations of 7PA2 cells were not significantly altered by treatment with 10 μM VPA or 10 μ PIA (Table 2). In addition, whereas the effects of the cholesterol synthesis inhibitor squalestatin on 7PA2 cells were reversed by the addition of 5 μM squalene (a non-toxic precursor that is rapidly converted to cholesterol), the inclusion of 5 μM squalene did not alter cholesterol concentrations in VPA or PIA-treated cells. Next we compared the effects of VPA and PIA to those of squalestatin upon the forms of Aβ released by treated cells. Although VPA and PIA had a similar effect upon concentrations of $A\beta_{42}$ oligomers and $A\beta_{42}$ monomers as squalestatin, the effects of squalestatin, but not those of VPA or PIA, were reversed by the inclusion of squalene (Table 2).

3.8. PAF reversed the effects of VPA and PIA upon the release of toxic Aβ – Reports that VPA and PIA reduced the activation of $cPLA_2$ (29) and that PLA_2 inhibitors affect the processing of APP (14) suggest that products of activated $cPLA_2$ may affect the release of toxic Aβ. The activation of $cPLA_2$ gives rise to the generation of multiple bioactive lipids, including prostaglandins and PAF. Here we tested whether PAF could reverse the effects of VPA or PIA on Aβ oligomer formation. CM from 7PA2 cells treated with a combination of 500 nM PAF and 10 μM VPA caused the loss of synaptophysin from neurons (Figure 6A). So did CM from 7PA2 cells mixed with a combination of 500 nM PAF and 10 μM PIA. The addition of PAF also reversed the inhibitory effects of VPA and PIA on the concentrations of $A\beta_{42}$ oligomers found in CM (Figure 6B). The effect of PAF on VPA and PIA-treated cells was dose-dependent; the addition of PAF reversed the VPA and PIA-induced suppression of $A\beta_{42}$ oligomers (Figure 6C) and the VPA and PIA-induced increase of $A\beta_{42}$ monomers (Figure 6D).

3.9. VPA and PIA altered the distribution of APP in cell membranes – Further studies examined the effects of VPA and PIA upon APP. Treatment with 10 μM VPA (106 units ± 3.2 compared with 100 units ± 2.6, n=9, $P<0.01$) or 10 μM PIA (106 units ± 3.4 compared with 100 units ± 2.6, n=9, $P<0.01$) caused a small but significant increase in the amounts of APP within cells. Since the processing of APP to toxic Aβ peptides is thought to occur within cholesterol-dependent micro-domains called lipid rafts we examined whether APP was found in lipid rafts. In control cells approximately 40% of total cellular APP was found within DRMs (lipid rafts) (Figure 7A). Significantly less APP was found within DRMs/rafts, and more within the DSMs, in cells treated with 10 μM VPA or 10 μM PIA (Figure 7A). Treatment of 7PA2 cells with 10 μM VPA or 10 μM PIA did not affect the amounts of other raft-associated proteins in DRMs including caveolin and the PAF receptor (Figure 7B). In cells treated with VPA or PIA (1.25 to 10 μM) there were significant correlations between the amounts of APP found in lipid rafts and the concentrations

of $A\beta_{42}$ oligomers found in CM (Figure 7C). There were also significant inverse correlations between the amounts of APP found in lipid rafts and the concentrations of $A\beta_{42}$ monomers found in CM (Figure 7D). The addition of 500 nM PAF reversed the VPA or PIA-induced reduction of APP within lipid rafts (Figure 7E).

4. Discussion

VPA, originally used to treat epilepsy, has been proposed to affect the production of Aβ and consequently the progression of AD. Here we show that treatment with either VPA or PIA reduced the concentrations of the Aβ oligomers in 7PA2 cells and CM. Critically VPA and PIA increased concentrations of Aβ monomers.

Firstly, 7PA2 cells were shown to release soluble forms of Aβ that have similar toxic properties to the Aβ found in soluble brain extracts derived from AD patients. These results are in accordance with prior reports that cell-derived soluble Aβ is similar in in size, stability and biological activity to soluble Aβ extracted from the brains of AD patients (24,30). The major finding of this study was that CM from 7PA2 cells treated with either VPA or PIA did not cause synapse damage. Remarkably, the loss of toxicity of CM from treated cells was accompanied by a relatively small reduction in total Aβ concentrations; demonstrating that there was not a simple relationship between the concentrations of Aβ in CM and their toxicity. Treatment with VPA or PIA significantly reduced the concentrations of $A\beta_{42}$ oligomers, the forms of soluble Aβ that are closely associated with synapse degeneration and clinical symptoms (31). Furthermore there were significant inverse correlations between the concentrations of $A\beta_{42}$ oligomers in CM from treated cells and the amounts of synaptic proteins (synaptophysin and CSP) in neurons incubated with such CM.

Treatment of 7PA2 cells with VPA or PIA significantly increased the concentrations of $A\beta_{42}$ monomers and there were significant inverse correlations between the concentrations of $A\beta_{42}$ monomers and $A\beta_{42}$ oligomers in CM. Although Aβ monomers and oligomers derived from 7PA2 cells or brain extracts are reported as stable (18,24,32,33) the possibility that VPA and PIA caused the dissociation of Aβ oligomers was investigated. Direct incubation of VPA or PIA with Aβ preparations did not change the amounts of Aβ oligomers or Aβ monomers indicating that these compounds did not have a direct effect upon Aβ oligomers or monomers. This finding suggests that VPA or PIA did not affect oligomerization of Aβ monomers in the CM. Either the oligomerization of Aβ occurs intracellularly (33) and are released as Aβ monomers, dimers and trimers, or Aβ monomers are taken up by cells and oligomerized before being re-released.

CM from VPA or PIA-treated cells reduced the Aβ oligomer-induced loss of synaptophysin and CSP from neurons. Immunodepletion and filtration studies demonstrated that these effects were mediated by Aβ monomers and there were also significant correlations between the concentrations of Aβ monomers in CM from treated cells and the amounts of synaptic proteins (synaptophysin and CSP). These observations are consistent with a report that Aβ monomers had a neuroprotective role (27). Notably, CM from treated cells did not affect synapse damage induced by PGE_2 indicating that their effects were selective.

VPA has been reported to have multiple effects including inhibition of histone deacetylase which in turn affects neprilysin, an enzyme that affects Aβ degradation (34). However, in this study VPA and PIA had optimal effects at concentrations of 10 μM whereas the inhibition of histone deacetylase required concentrations of 3 mM (35). Although VPA has also been reported to inhibit cholesterol synthesis (28) neither VPA nor PIA significantly altered total cellular cholesterol concentrations. Furthermore, their effects were not reversed by the addition of squalene, a precursor of cholesterol that reversed the effects of cholesterol synthesis inhibitors, indicating that the effects of VPA and PIA upon Aβ production were not via inhibition of cholesterol synthesis. We recently showed that VPA and PIA reduced the activation of $cPLA_2$ (29), an enzyme involved for cell signalling and the production of bioactive phospholipids including prostaglandins and PAF (13,15). In this study, PAF, a bioactive phospholipid produced following activation of $cPLA_2$, reversed the effects of VPA and PIA upon Aβ production, indicating that it was the key mediator involved in this pathway. These results are consistent with the hypothesis that VPA/PIA inhibits $cPLA_2$ reducing PAF and that PAF affected Aβ production.

Observations that the normal intracellular trafficking and endosomal sorting is dysregulated in AD (36) support the theory that endosome/lysosome dysfunction leads to the amyloidgenic processing of APP in endosomal and lysosomal compartments (37). Consequently Aβ production can be affected by factors that alter the trafficking of APP, β-site APP cleaving enzyme-1 and/or other components of the γ-secretase complex. Lipid rafts are a major source of $Aβ_{42}$ production (38) and approximately 40% of APP is found within lipid rafts (DRMs) in these cells. Treatment of cells with either VPA or PIA reduced the amounts of APP within lipid rafts leading to significant correlations between the amounts of APP in lipid rafts and the concentrations of $Aβ_{42}$ in CM. The positive correlation between amounts of APP in lipid rafts and the concentrations of $Aβ_{42}$ oligomers suggested that APP in lipid rafts is more likely to be processed into Aβ oligomers. Conversely, the negative correlation between the amounts of APP in lipid rafts and the concentrations of $Aβ_{42}$ monomers suggested that APP resident in the normal cell membrane is more likely

to be metabolised to Aβ monomers. It should be noted that VPA/PIA did not affect the residency of other lipid raft-associated proteins such as caveolin and the PAF receptor.

The trafficking of APP, and the consequent processing to Aβ peptides, is dependent upon multiple factors including membrane composition (39). Proteins found within lipid rafts often traffic via different pathways (40) to proteins found in the normal cell membrane, raising the possibility that APP in lipid rafts traffics to different cell compartments and interacts with different enzymes than APP in the normal cell membrane. Currently we can only speculate that treatment with VPA or PIA alters the trafficking of APP or other components that constitute a mature γ-secretase complex. It should be noted that these studies were performed on a non-neuronal cell line that overexpressed APP751. Neuronal cell lines mostly express APP695 (41) and their expression of other enzymes involved in Aβ production may also vary from the 7PA2 cells used here. While it would be unwise to extrapolate results from 7PA2 cells to the clinic these results raise interesting questions concerning APP metabolism that could be investigated further in neuronal cell lines and animal models.

4. Conclusions.

In summary we report that VPA and PIA significantly reduced the amounts of toxic forms of Aβ in the CM of 7PA2 cells. The effects of VPA/PIA were twofold; not only did they reduce the concentrations of Aβ oligomers, these drugs also increased concentrations of Aβ monomers in CM. Critically, CM from treated cells reversed the Aβ oligomer-induced synapse damage. These studies suggest that should sufficient concentrations of VPA or PIA penetrate the brain then they may be able to reduce synapse damage in AD.

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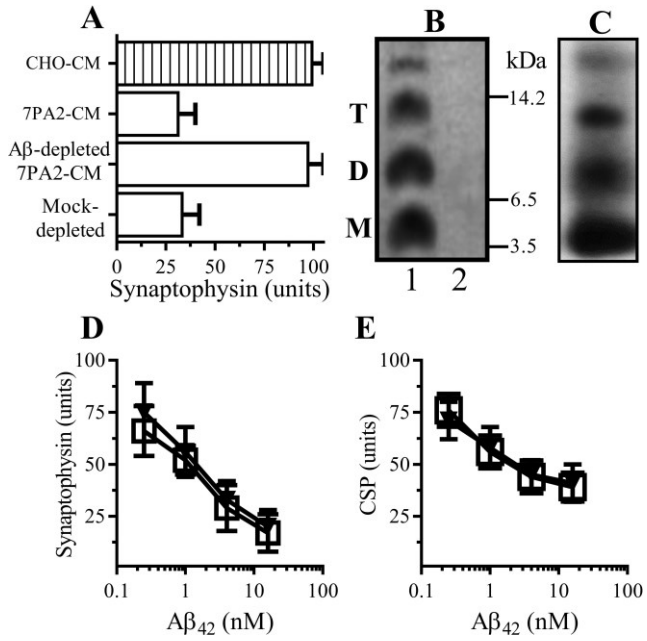

Figure 1 – 7PA2-CM contains Aβ that caused synapse damage – (A) The amounts of synaptophysin in neurons incubated with CHO-CM (vertical striped bar) or 7PA2-CM, Aβ-depleted 7PA2-CM or mock-depleted 7PA2-CM as shown (□). Values are means ± SD from triplicate experiments performed 3 times, n=9. (B) Immunoblot showing forms of Aβ including monomers (M), dimers (D) and trimers (T) in 7PA2-CM (1) and in Aβ-depleted 7PA2-CM (2). (C) An immunoblot showing forms of Aβ in the extract of soluble proteins from brain tissue of an AD patient. The amounts of synaptophysin (D) and CSP (E) in neurons incubated with 7PA2-CM (▼) or soluble brain extract (□) containing $A\beta_{42}$ as shown. Values are means ± SD from triplicate experiments performed 3 times, n=9.

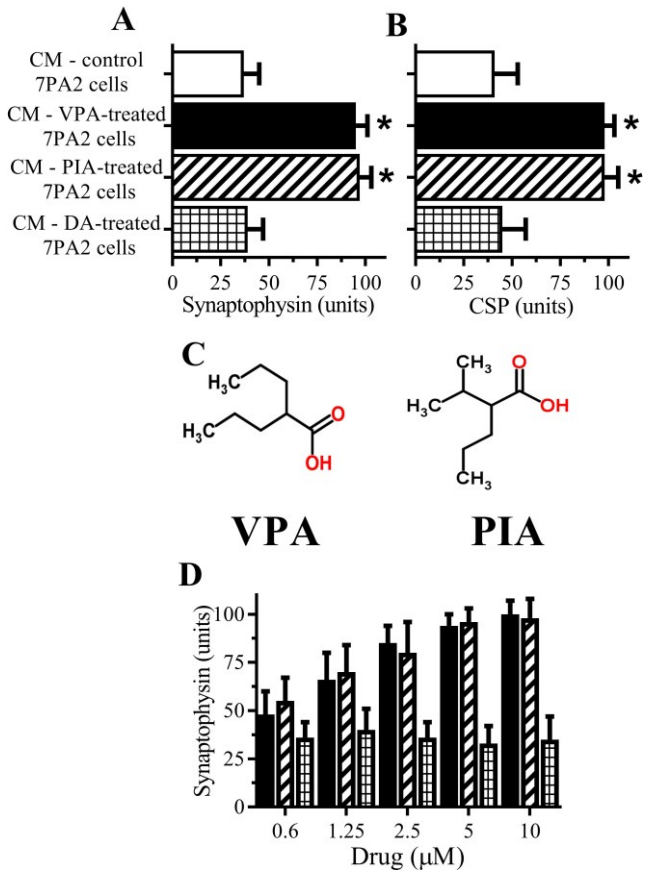

Figure 2. VPA and PIA reduced the amounts of toxic Aβ in CM – The amounts of synaptophysin (A) and CSP (B) in neurons incubated with CM from 7PA2 cells treated with control medium (□), 10 μM VPA (■), 10 μM PIA (diagonal striped bars) or 10 μM DA (hatched bars). Values are means ± SD from triplicate experiments performed 4 times, n=12. *= concentrations of synaptic proteins significantly higher than in neurons incubated with CM from control 7PA2 cells. (C) Schematic showing the structure of VPA and PIA. (D) The amounts of synaptophysin in neurons incubated with CM from 7PA2 cells treated with VPA (■), PIA (diagonal striped bars) or DA (hatched bars) as shown. Values are means ± SD from triplicate experiments performed 4 times, n=12.

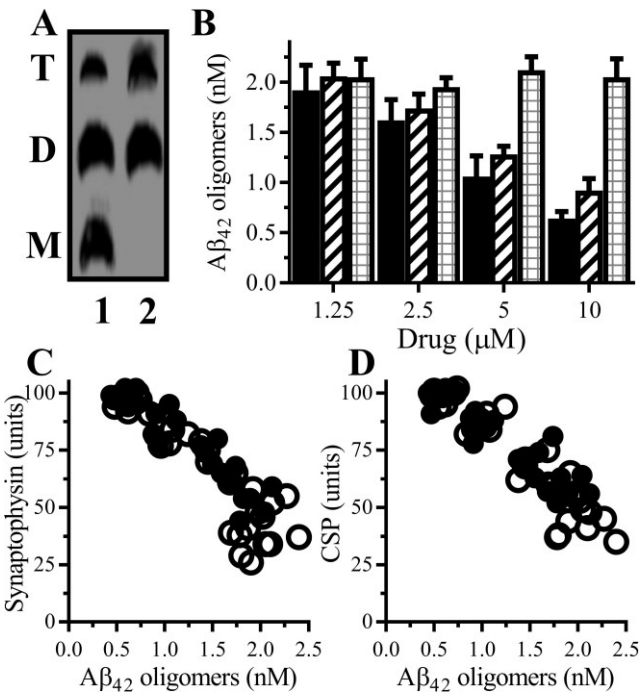

Figure 3 - VPA and PIA reduced the concentrations of Aβ oligomers in CM – (A) Immunoblot showing forms of Aβ including monomers (M), dimers (D) and trimers (T) in (1) 7PA2-CM and (2) 7PA2-CM >10 kDa (oligomer preparations). (B) The concentrations of $A\beta_{42}$ oligomers in CM from 7PA2 cells treated with VPA (■), PIA (diagonal striped bars) or DA (hatched bars) as shown. Values are means ± SD from triplicate experiments performed 4 times, n=12. There were significant inverse correlations between the concentrations of $A\beta_{42}$ oligomers in CM from 7PA2 cells treated with VPA (●) or PIA (○) (0.6 to 10 μM) and the amounts of synaptophysin, Pearson's coefficient= -0.89, $P<0.01$ (C) or CSP, Pearson's coefficient= -0.91, $P<0.01$ (D) in neurons incubated with these CM for 24 hours.

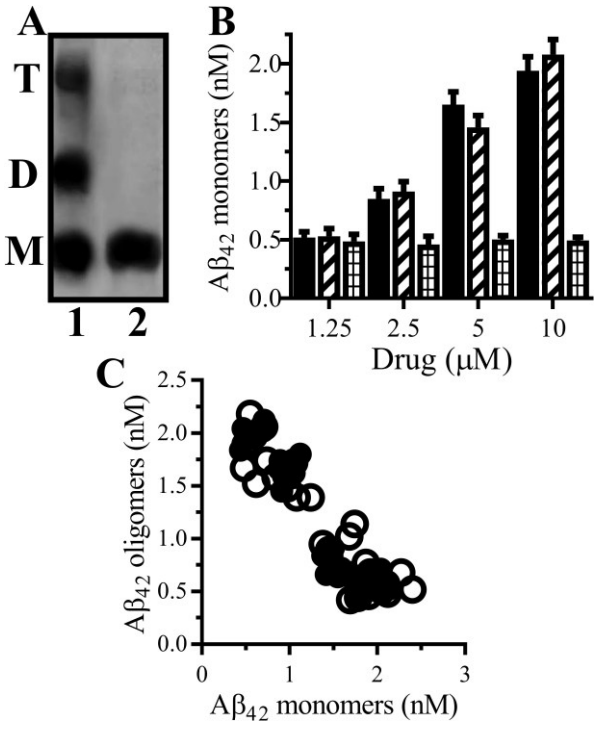

Figure 4 - VPA increased concentrations of Aβ monomers in CM - (A) Immunoblot showing forms of Aβ including monomers (M), dimers (D) and trimers (T) in (1) 7PA2-CM and in (2) 7PA2-CM <10 kDa (monomer preparations). (B) The concentrations of $A\beta_{42}$ monomers in CM from 7PA2 cells treated with VPA (■), PIA (diagonal striped bars) or DA (hatched bars) as shown. Values are means ± SD from triplicate experiments performed 4 times, n=12. (C) There was a significant inverse correlation between the concentrations of $A\beta_{42}$ monomers and $A\beta_{42}$ oligomers in CM from 7PA2 cells treated with VPA (○), Pearson's coefficient= -0.92, $P<0.01$, or PIA (●), Pearson's coefficient= -0.94, $P<0.01$ (1.25 to 10 μM).

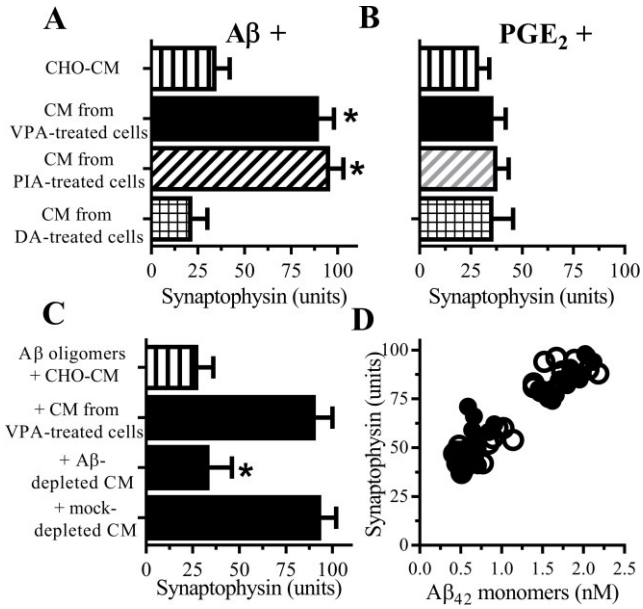

Figure 5 - CM from VPA/PIA-treated 7PA2 cells reduced Aβ oligomer-induced synapse damage – The amounts of synaptophysin in neurons incubated with (A) brain extract containing 2 nM $A\beta_{42}$ or (B) 10 nM PGE_2 mixed with CM from CHO cells (vertical striped bars) or CM from 7PA2 cells treated with either 10 μM VPA (■), 10 μM PIA (diagonal striped bars) or 10 μM DA (hatched bars). Values are means ± SD from triplicate experiments performed 3 times, n=9.*=synaptophysin higher than in neurons incubated with Aβ + CHO-CM. (C) The amounts of synaptophysin in neurons incubated with brain extract containing 2 nM $A\beta_{42}$ mixed with CM from CHO cells (vertical striped bar), CM from 7PA2 cells treated with 10 μM VPA or the same CM depleted of Aβ or mock-depleted (■). Values are means ± SD from triplicate experiments performed 3 times, n=9. *=synaptophysin significantly lower than in neurons incubated with Aβ oligomers + CM from VPA-treated cells. (D) There was a significant correlation between the amounts of synaptophysin in neurons treated with brain extract containing 2 nM $A\beta_{42}$ oligomers mixed with CM from 7PA2 cells treated with VPA (●) or PIA (○) (1.25 to 10 μM) and the concentrations of $A\beta_{42}$ monomers in these CM, Pearson's coefficient=0.94, $P<0.01$.

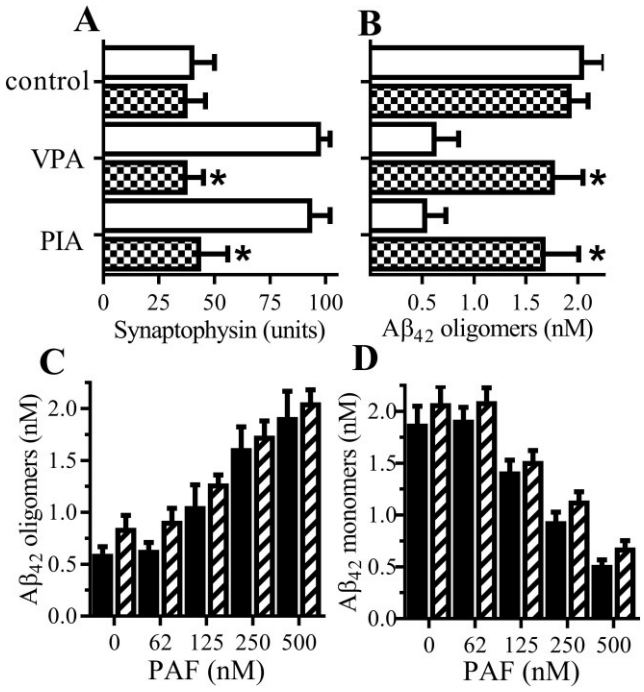

Figure 6 - PAF reversed the effects of VPA and PIA on Aβ$_{42}$ concentrations in CM – (A) The amounts of synaptophysin in neurons incubated with CM from 7PA2 cells treated with control medium, 10 μM VPA or 10 μM PIA mixed with control medium (□) or 500 nM PAF (checkerboard bars). Values are means ± SD from triplicate experiments performed 3 times, n=9. *=synaptophysin significantly lower than in neurons incubated with CM from VPA or PIA-treated cells. (B) The amounts of Aβ$_{42}$ oligomers in CM from 7PA2 cells treated with control medium, 10 μM VPA or 10 μM PIA mixed with control medium (□) or 500 nM PAF (checkerboard bars). Values are means ± SD from triplicate experiments performed 3 times, n=9. *=concentrations of Aβ$_{42}$ oligomers significantly higher than in CM from VPA or PIA-treated cells. The concentrations of Aβ$_{42}$ oligomers (C) and Aβ$_{42}$ monomers (D) in CM from 7PA2 cells treated with 10 μM VPA (■) or 10 μM PIA (diagonal striped bars) mixed with PAF as shown. Values are means ± SD from triplicate experiments performed 3 times, n=9.

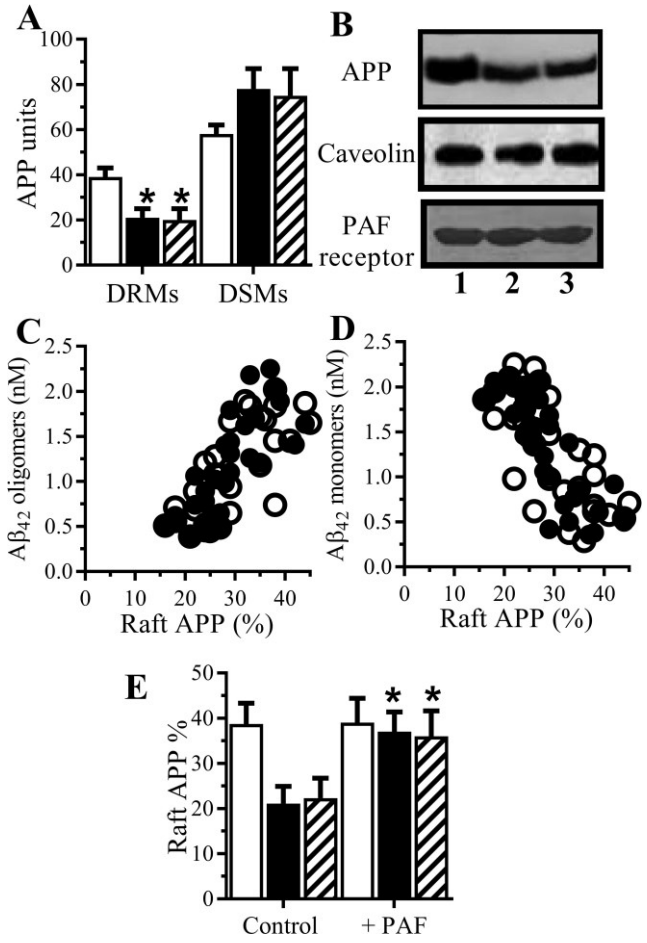

Figure 7 – VPA/PIA reduced amounts of APP within lipid rafts – (A) The amounts of APP in lipid rafts (DRM)s and detergent-soluble membrane (DSM)s from 7PA2 cells treated with control medium (□), 10 μM VPA (■) or 10 μM PIA (diagonal striped bar). Values are means ± SD from triplicate experiments performed 3 times, n=9. *=APP significantly less than in untreated cells. (B) The amounts of APP, caveolin and PAF receptor in DRMs (lipid rafts) isolated from 7PA2 cells treated with (1) control medium, (2) 10 μM VPA or (3) 10 μM PIA. (C) There was a significant correlation between the amounts of APP in lipid rafts (DRMs) and the concentrations of $A\beta_{42}$ oligomers in CM from 7PA2 cells treated with VPA (●) or PIA (○)(1.25 to 10 μM), Pearson's coefficient =0.91, $P<0.01$. (D). There was a significant inverse correlation between the amounts of APP in lipid rafts (DRMs) and the concentrations of $A\beta_{42}$ monomers in CM from 7PA2 cells treated with VPA (●) or PIA (○)(1.25 to 10 μM), Pearson's coefficient= -0.85, $P<0.01$. (E) The amounts of APP in DRMs from 7PA2 cells treated with control medium (□), 10 μM VPA (■) or 10 μM PIA (diagonal striped bars) mixed with control medium or 500 nM PAF as shown. Values are means ± SD from triplicate experiments performed 3 times, n=9. *= % of raft-associated APP significantly higher than in VPA or PIA-treated cells.

	7PA2-cells	
Treatment	**$A\beta_{42}$ oligomers (nM)**	**$A\beta_{42}$ monomers (nM)**
Control	12.47 ± 0.1.2	0.93 ± 0.33
VPA	8.75 ± 1.4*	1.27 ± 0.24*
PIA	7.97 ± 1.62*	1.19 ± 0.3*
DA	12.6 ± 1.42	0.91 ± 0.2

Table 1. VPA/PIA reduced concentrations of cell-associated Aβ – The concentrations of $A\beta_{42}$ oligomers and $A\beta_{42}$ monomers in cell extracts from 7PA2 cells treated with control medium, 10 μM VPA or 10 μM PIA for 3 days. Values are means ± SD from triplicate experiments performed 3 times, n=9. *=concentrations significantly different from controls.

		7PA2-CM	
Treatment	**Cholesterol (μM)**	**$A\beta_{42}$ oligomers (nM)**	**$A\beta_{42}$ monomers (nM)**
Control	0.82 ± 0.05	1.92 ± 0.18	0.5 ± 0.09
Squalestatin	0.55 ± 0.05*	0.53 ± 0.14*	1.88 ± 0.1*
squalene	0.85 ± 0.07	2.22 ± .23	0.46 ± 0.1
Squalestatin + squalene	0.84 ± 0.07	2.01 ± 0.16	0.61 ± 0.18
VPA	0.81 ± 0.04	0.58 ± 0.14*	1.73 ± 0.19*
VPA + squalene	0.81 ± 0.04	0.56 ± 0.07*	1.82 ± 0.24*
PIA	0.83 ± 0.05	0.57 ± 0.1*	1.65 ± 0.22*
PIA + squalene	0.84 ± .04	0.61 ± 0.09*	1.72 ± 0.25*

Table 2. Squalene did not reverse the effects of VPA/PIA upon Aβ concentrations in CM – 7PA2 cells were treated with 200 nM squalestatin, 5 μM squalene, 10 μM VPA, 10 μM PIA or combinations as shown for 3 days. The concentrations of cholesterol in treated cells was measured along with, the concentrations of $A\beta_{42}$ oligomers and monomers in CM. Values are means ± SD from triplicate experiments performed twice, n=6. *=concentrations significantly different from controls.