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Platelet and erythrocyte membrane changes in Alzheimer's disease

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Previous reports have suggested that the physical properties of cell membranes and calcium homeostasis in both the central and peripheral nervous system are changed in Alzheimer's disease (AD). This study has examined the biophysical properties of erythrocyte and platelet membranes by measuring the fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) and possible related changes in lipid peroxidation. In addition, we have studied calcium homeostasis by measuring thrombin-stimulated changes in intraplatelet free calcium and Ca^{2+}-ATPase activity in AD and healthy age and sex-matched controls. Our results show that there was no significant difference in the fluorescence anisotropy of DPH in erythrocyte membranes isolated from the three groups. There was also no significant difference in lipid peroxidation levels in erythrocytes and plasma of AD patients compared to controls. However, there was a significant reduction in the fluorescence anisotropy of DPH in platelet membranes from AD patients, compared with healthy controls. Recent evident suggests that the increase in platelet membrane fluidity results from alterations in internal membranes. We measured the specific activities of enzyme markers associated with intracellular and plasma membranes in platelets from AD patients and healthy controls. There was a significant reduction in the specific activity of antimycin A-insensitive NADH–cytochrome-*c* reductase (a specific marker for smooth endoplasmic reticulum (SER)), in AD patients compared to controls, but no change in the specific activity of bis(*p*-nitrophenyl)phosphate phosphodiesterase (a specific marker for plasma membrane). We have also shown that SER mediated $[Ca^{2+}]$ homeostasis is possibly impaired in AD platelets, i.e., the percentage of thrombin-stimulated increase in intraplatelet $[Ca^{2+}]$ above basal levels was significantly higher in AD compared to matched controls and there were significant reductions in the specific activities of Ca^{2+}/Mg^{2+}-ATPase and Ca^{2+}-ATPase (but not Mg^{2+}-ATPase) in AD platelets. Finally electron microscopic analysis of platelets showed that there was a significant increase in the incidence of abnormal membranes in AD patients compared to controls. The ultrastructural abnormalities seem to consist of proliferation of a system of trabeculated cisternae bounded by SER. These results suggest that both SER structure and function might be defected in AD platelets, which could explain the fluidity changes observed here.

Introduction

Alzheimer's disease (AD) can only be diagnosed with certainty at autopsy, or in rare cases by brain biopsy. Although adherence to strict diagnostic criteria [1] is usually confirmed at postmortem [2], all that can be said about a possible case is that it is one of probable AD. The neuropathological hallmarks of AD are senile plaques and paired helical filaments. There are also numerous neurochemical changes (which include a reduction in choline acetyltransferase in cerebral cortex and hippocampus, a decrease in GABA and somatostatin levels and damage to the ascending adrenergic system) [3], but AD etiology remains obscure.

There have recently been reports of both central and peripheral membrane changes in AD [4–6]. It was first reported by Zubenko [5] that the fluorescence polarization of 1,5-diphenyl-1,3,5-hexatriene (DPH) (an indicator of membrane 'microviscosity') was decreased in platelets isolated from patients suffering from AD compared to healthy age and sex-matched controls. Hicks et al. [7] in this laboratory replicated Zubenko's findings and additionally found no change in multi-infarct de-

Abbreviations: DPH, diphenylhexatriene; AD, Alzheimer's disease; MID, multi-infarct dementia; S.D., standard deviation; *P*, probability; SER, smooth endoplasmic reticulum; $[Ca^{2+}]_i$, intraplatelet free calcium concentration; IP_3, inositol 1,4,5-trisphosphate.

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mentia (MID) patients or in platelet phospholipid composition in any of the groups. Following these initial studies, it seemed possible that there might be a diagnostically useful general peripheral membrane change in AD and the work of Chia et al. [4] suggested that this might result from changes in lipid peroxidation which have been used as an index of ageing in biological membranes.

The aims of this study were (i) to ascertain whether there was a widespread change in peripheral membrane fluidity by extending our studies to erythrocytes, (ii) to test whether lipid peroxidation was significantly changed in AD and (iii) to examine the nature of the structural and functional changes in platelet membranes. A preliminary report of some of the findings has been published previously as an abstract [8].

Materials

1,6-Diphenyl-1,3,5-hexatriene (DPH), cytochrome *c*, antimycin A, α-tocopherol acetate, β-NADH, bis(*p*-nitrophenyl) phosphate, fura-2/AM, ATP, Malachite green, thrombin, thiobarbituric acid and 1,1,3,3-tetraethoxypropane were obtained from Sigma Chemical Co (U.K.). All other reagents were of analytical grade and were obtained from BDH Chemical Co. (U.K.).

Methods

Subject selection

Alzheimer patients. These were patients suffering from a progressive dementing illness of at least 6 months duration without evidence of an alternative diagnosis. Patients were excluded if they had a history or signs suggestive of cerebrovascular disease, or if there was any evidence of an underlying cause of the dementia such as cerebral tumour, syphilis, endocrine disorder, etc. All patients had a CT brain scan. None of the subjects received any anti-inflammatory analgesic for at least 1 week before blood sampling. All patients were drug free for our studies. These patients were recruited from a group attending the Memory Clinic at the Maudsley Hospital [9] with a view to inclusion in a clinical trial. Four patients have unfortunately died during the course of this study and the postmortem diagnosis of AD was confirmed in all the cases.

Controls. These were also matched with the above for age and sex. Subjects were excluded if they had a past or present history of alcohol or drug abuse. Recruitment was from local elderly persons clubs and day-centres.

Multi-infarct dementia. These were also matched with the above for age and sex. Diagnosis was based on a history of a progressive dementing illness of at least 6 months duration associated with features suggestive of cerebrovascular disease, namely abrupt onset, stepwise deterioration, history of stroke, focal neurological symptoms. None of the patients were receiving medication at the time of the study.

Erythrocyte preparation

Freshly drawn heparinized human blood was centrifuged at $200 \times g$ for 10 min at room temperature. Plasma and buffy coats were removed and, the red cells washed three times in isotonic NaCl using the same centrifugation condition. The washed red cells were maintained at 0–4°C for the ghost preparation (carried out according to the method of Bjerrum [10]). The cells were lysed by injecting 2 ml of 50% haematocrit from a syringe into an ice-cooled centrifuge tube containing 20 ml haemolyzing medium A (4 mM $MgSO_4$, 3.8 mM CH_3COOH, (pH 3.6–3.8)). After 1 min, the cell suspension was centrifuged at $23000 \times g$ for 12 min (Sorvall RC-5B). The supernatant (mostly haemoglobin) was removed as completely as possible and the tonicity of the medium was increased by adding 200 μl of medium B (2000 mM KCl)/ml of ghost suspension. The ghost were washed after 2 min with 10 volumes of medium C (4 mM $MgSO_4$, 1.2 mM CH_3COONa, 2 mM NaH_2PO_4/Na_2HPO_4 (pH 7.0)) and centrifuged at $23000 \times g$ for 12 min. This washing procedure was repeated four times until the ghosts had a pinkish white colour. The membranes at this stage were used for our biochemical analyses.

Platelet preparation

Whole venous blood samples (normally 10 ml) were taken from each subject into a heparinized tube. Platelets were isolated by differential centrifugation in Anticoagulant Citrate Dextrose (ACD) buffer (36 mM citric acid, 5 mM KCl, 90 mM NaCl, 5 mM glucose, 10 mM EDTA (pH 6.8)) by the method of Menashi et al. [11]. This involved a preliminary centrifugation step ($200 \times g$ for 10 min) to obtain platelet-rich plasma (PRP) and a pellet consisting mainly of red cells. The red cells were washed three times in the ACD buffer and centrifuged exactly as above. The PRPs from all the washes were combined and centrifuged again at $200 \times g$ for 10 min to remove any residual red cells. The PRPs were finally centrifuged at $2000 \times g$ for 20 min to isolate the platelets. The platelet pellet was washed twice in phosphate-buffered saline.

Lipid peroxidation (thiobarbituric-acid test (TBA))

1.0 ml plasma or erythrocyte ghost membranes (0.1–2 mg of membrane protein) were incubated in physiological buffer alone or with H_2O_2 (200 mM) plus $FeSO_4$ (2 mM) for 1 h at 37°C. These suspensions were then mixed thoroughly with 2.0 ml of TBA-trichloroacetic acid (TCA)-hydrochloric acid (HCl) (0.375% (w/v) TBA, 15% (w/v) TCA and 0.25 M HCl). The mixture was heated for 15 min in a boiling water bath. After cooling the flocculent precipitate was removed by cen-

trifugation at $1000 \times g$ for 10 min. The absorbance of the sample was determined at 535 nm against a solvent blank [12]. Malondialdehyde standard was prepared by acid hydrolysis of 1,1,3,3-tetraethoxypropane.

Vitamin E (α-tocopherol) assay by high performance liquid chromatography (HPLC)

300 μl of plasma was vortex mixed for 30 s with 300 μl of ethanol. *n*-Hexane (600 μl) was added and the mixture was mixed for further 30 s. The whole suspension was then mechanically shaken for 30 min in the dark, after which it was centrifuged at $3000 \times g$ in a microcentrifuge (MSE Micro Centaur) for 10 min. The upper (hexane) layer was removed and dried in a glass tube under a stream of nitrogen gas. Immediately before HPLC samples were resuspended in 100 μl of ethanol and, 30 μl of each sample was injected on to the column [13]. The column used was a Spherisob 5-ODS2 (Technical) column with dimensions 12.5 cm × 4.6 mm. We used 7% (v/v) dichloromethane in methanol as the mobile phase with 1 ml/min flow rate. The absorbance was measured using a single path UV monitor with a 280 nm filter.

Fluorescence polarization

The lipid soluble fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) was used in this study. DPH was made up as a 1 mM stock solution in tetrahydrofuran and stored at −27°C until required. Immediately before use, it was diluted 152-fold with phosphate-buffered saline (PBS) and 1 ml of this diluted (6.6 μM) solution was then incubated with an equal volume of platelet or erythrocyte suspension (both cell suspensions were adjusted to 0.2 absorbance at 600 nm). After incubating for 60 min at 37°C, the suspension was transferred to an Aminco-Bowman spectrophotofluorometer modified with a thermostatically controlled cuvette holder and polarizing filters for excitating and emitted light. DPH fluorescence was excited at 360 nm and the emission was measured at 30 nm with all four possible excitation/emission polarizing filter combinations. Fluorescence polarization (P) and anisotropy (A) were then calculated as described by Lakowicz [14] as indicated in Eqns. 1 and 2, respectively. Each sample was measured in triplicate with DPH and duplicate with solvent blank for scatter control.

$$P = ([I_{vv}/I_{vh}]/[I_{hv}/I_{hh}] - 1)/([I_{vv}/I_{vh}]/[I_{hv}/I_{hh}] + 1) \quad (1)$$

$$A = (2 \cdot P)/(3 - P) \quad (2)$$

Measurement of intraplatelet free calcium

Platelets were prepared as described previously from freshly drawn human (AD and controls) blood. Intraplatelet calcium ($[Ca^{2+}]_i$) was measured by fluorescence spectroscopy using the Ca^{2+} indicator fura-2 [15,16]. Intact platelets (10^8) were loaded (in physiological buffer: 145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes and 10 mM glucose, pH 7.4 at 37°C) with membrane permeant fura-2 acetoxymethyl ester (fura-2/AM, 1 μM) at 37°C for 1 h (the fura-2 signal reached a plateau at this time). Excess unconverted fura-2/AM was removed by centrifugation (Microcenteur, $1000 \times g$, 10 min). Platelets were resuspended in physiological buffer and transferred to an Aminco Bowman spectrofluorometer fitted with a thermostatically controlled cuvette holder (set at 37°C). $[Ca^{2+}]_i$ was measured fluorimetrically and calculated from the formula

$$[Ca^{2+}]_i = [(F - F_{min})/(F_{max} - F)] \times K_d$$

The K_d for fura-2 free acid is 225 nM [17]. Maximal fluorescence (F_{max}) was measured after platelets lysis with 10 μl/ml of sodium dodecyl sulphate (20% (w/v)). Adjustment of the pH to 8.2 with 1 M NaOH plus addition of 10 μl/ml of 0.5 M EGTA (final concentration of 5 mM, which produces a free Ca^{2+} concentration of 1.3 nM) provided minimal fluorescence (F_{min}). Monochromator settings were 322 (excitation) and 500 nm (emission), parallel controls were carried with the addition of fura-2 solvent, dimethyl sulphoxide. Thrombin (1 unit/ml) was added directly to the cuvettes and incubated for 1 min (this condition produced maximal effect on $[Ca^{2+}]$; [15]) after which, further fluorescence measurements were made.

Enzyme assays

Bis(*p*-nitrophenylphosphate) phosphodiesterase was assayed according to Lanillo and Cabezas [18]. The 1.0 ml reaction mixture contained 0.1 ml of platelet suspension or buffer and 3 mM bis(*p*-nitrophenylphosphate) in 200 mM acetate buffer (pH 5.5). The reaction was terminated with 2.0 ml of 200 mM NaOH after 1 h at 37°C. The production of *p*-nitrophenol was determined spectrophotometrically at 410 nm.

Antimycin A-insensitive NADH–cytochrome-*c* reductase was assayed as described by Tolbert [19]. The 1.0 ml reaction mixture contained 0.1–0.3 ml platelet suspension, 0.08 mM cytochrome *c*, 0.8 mM NADH, 0.7 mM antimycin A and 0.2 mM KCN in 80 mM phosphate buffer (pH 7.0). The reaction was initiated by the addition of NADH and the absorbance at 550 nm followed at 25°C.

Ca^{2+}-ATPase(s) activity was measured according to the method of Kallner [20]. For determination of ATPase activity, the following reaction mixture was used: 25 mM Tris-HCl (pH 7.4), 100 mM KCl, 3 mM $MgCl_2$, 3 mM ATP, 0.05 mM $CaCl_2$ and different protein fractions in a final volume of 0.5 ml. After incubation at 25°C for 60 min, 0.05 ml of the medium

was added to 2 ml Malachite green reagent used for the colorimetric determination of inorganic phosphorus [20,21]. The Ca^{2+}-dependent ATPase activity was calculated by subtracting the ATPase activity in the presence of 5 mM EGTA and without adding $CaCl_2$ from that obtained with added $CaCl_2$. Results are expressed as nmol inorganic phosphorus liberated per min per mg protein.

Electron microscopy

Platelet pellets in plastic microcentrifuge tubes were fixed in Karnovsky's fluid (2.5% glutaraldehyde, 5% formaldehyde, 0.2 M cacodylate and distilled water (pH 7.3)) for 30 min at room temperature. Samples were then treated with secondary fixative (1% osmium tetroxide in 0.2 M cacodylate) for 30 min at room temperature, after which they were stained with saturated uranyl acetate in absolute alcohol for 30 min. Samples were dehydrated through 50, 70, 90 and 100% ethanol for 15 min each and embedded in Spurr resin [22]. Thin sections were cut with a diamond knife on an LKB Ultramicrotome and then examined in a Hitachi H600 electron microscope. Representative micrographs were taken of each platelet sample blind to the subject's clinical status. The structure of the endoplasmic reticulum in each sample was then subjected to morphometric analysis to reveal the amount of trabeculated cisternae.

Two types of morphometric analysis were carried out:

(1) Point counting. A grid of dots (points) arranged in a regular array was superimposed on the transmission micrographs. The number of dots falling on abnormal platelets are then counted.

(2) Random sampling. A grid of identical squares is superimposed randomly on an area of a transmission electron micrograph. All the cells are counted in the squares and then the abnormal cells are counted within that population. For both estimations, low power magnification of platelets was used, providing a large sample of platelets for analysis on each micrograph. Four micrographs from each sample were analysed for both morphometrical estimations.

Protein estimation

Protein concentration was determined according to Bradford [23] using bovine serum albumin as a standard.

Statistical analysis

The unpaired Student's *t*-test and unpaired randomization test [24] were used.

Results and Discussion

It has been reported that patients with AD have reduced platelet 'microviscosity' (measured by fluorescence anisotropy of DPH) compared with age- and sex-matched controls [5]. Furthermore studies of myelin in AD patients using X-ray diffraction have revealed increased membrane disorder [4] which would also be compatible with increased 'fluidity' (reciprocal of 'microviscosity').

The initial study by Hicks et al. [7] in this laboratory essentially replicated Zubenko's data on platelet fluidity changes in AD. That study also included multi-infarct dementia patients as secondary controls.

It is now accepted that the fluidity of biological membranes can have a marked effect on their properties, modulating the activity of membrane bound enzyme and other membrane molecules such as ion channels and receptors [25]. A number of factors can influence membrane fluidity, such as the chain length and degree of unsaturation of the acyl groups of phospholipids, peroxidation of fatty acids and the membrane cholesterol content.

The initial aim of this study was to investigate the fluidity of erythrocyte ghost membranes. One reason for this was to show whether a general peripheral membrane change existed in AD, which could be used as a possible antemortem diagnostic criterion. Our data (Table I) showed that there was no significant change in erythrocyte membrane fluidity in AD and MID patients compared with healthy age-matched controls [8]. At the same time we also examined lipid peroxidation to test whether this was altered in AD peripheraly as Chia et al. [4] have previously reported central changes. Increased peroxidation could be due either to excessive free-radical generation or reduced antioxidant protection (e.g., by vitamin E). We tested these hypotheses by measuring lipid peroxidation and vitamin E levels in plasma and erythrocyte ghost membranes. Our results again showed no significant differences; (i) in vitamin E levels (nmol/mg protein) in plasma and erythrocyte ghost membranes from AD patients (plasma: $n = 14$,

TABLE I

Fluorescence anisotropy of DPH in platelets and erythrocyte ghost membranes

Values are the means ± S.D. [a] vs. [b]: Significant, $P = 0.01$. [c] vs. [d]: Significant, $P = 7.1 \cdot 10^{-6}$.

	DPH anisotropy		
	Alzheimer	Control	MID
Erythrocyte	0.2026 ± 0.0236 ($n = 43$)	0.2033 ± 0.0101 ($n = 9$)	0.2178 ± 0.043 ($n = 4$)
Platelet			
Hicks et al. (1987)	0.1922 ± 0.0208 [a] ($n = 27$)	0.2083 ± 0.0218 [b] ($n = 19$)	0.1986 ± 0.0155 ($n = 15$)
Platelet	0.1953 ± 0.0092 [c] ($n = 40$)	0.2063 ± 0.0081 [d] ($n = 29$)	

0.408 ± 0.056 (mean ± S.E.); erythrocyte ghost membranes: $n = 8$, 0.105 ± 0.007 (mean ± S.E.)) and controls (plasma: $n = 6$, 0.648 ± 0.168 (mean ± S.E.); erythrocyte ghost membranes: $n = 6$, 0.097 ± 0.009 (mean ± S.E.), (ii) in basal peroxidation levels (malondialdehyde; nmol/mg protein) in plasma and erythrocyte ghost membranes from AD patients (plasma: $n = 9$, 0.194 ± 0.009 (mean ± S.E.); erythrocyte ghost membranes: $n = 11$, 0.041 ± 0.005 (mean ± S.E.)) and controls (plasma: $n = 7$, 0.217 ± 0.006 (mean ± S.E.); erythrocyte ghost membranes: $n = 10$, 0.035 ± 0.003 (mean ± S.E.)) and (iii) in stimulated ($FeSO_4 + H_2O_2$) peroxidation levels (malondialdehyde; nmol/mg protein) in erythrocyte ghost membranes from AD patients ($n = 11$, 0.101 ± 0.007 (mean ± S.E.)) and controls ($n = 10$, 0.108 ± 0.002 (mean ± S.E.)).

At this time Zubenko et al. [6] reported the results of electron microscopic investigation of platelet morphology in AD. They observed an increase in the percentage of abnormal platelets in AD patients compared to controls. The ultrastructural abnormalities seemed to involve proliferation of a system of trabeculated cisternae bounded by smooth endoplasmic reticulum (SER). We have confirmed these observations in our laboratory (Fig. 1, Table II). It seemed possible that this intracellular membrane defect might be associated with the increased fluidity observed in AD platelets and might also explain the erythrocyte fluidity data (erythrocytes lack intracellular membranes and hence there are no significant differences in erythrocyte membrane fluidity between AD patients and controls).

The morphological abnormalities observed in AD platelets led us to look for biochemical markers for intracellular membrane changes. We first repeated membrane the fluidity measurements (using DPH) on platelets from AD patients and controls, and obtained essentially the same results (Table I) as published previously by this laboratory and elsewhere [5–7].

TABLE II

Morphometrical analysis of electron transmission micrographs of platelets

Values are the means ± S.D. [a] vs. [b]: Significant, $P = 0.002$. [c] vs. [d]: Significant, $P = 0.008$.

	% Abnormal platelets	
	dot (point counting)	square (random sampling)
Control	11.5 ± 5.4 [a] ($n = 3$)	12.7 ± 4.1 [c] ($n = 3$)
Alzheimer	28.9 ± 18.6 [b] ($n = 3$)	22.5 ± 10.7 [d] ($n = 3$)

We then measured total platelet protein and the specific activities of antimycin A-insensitive NADH–cytochrome-*c* reductase (a specific marker for SER) and bis(*p*-nitrophenyl)phosphate phosphodiesterase (a specific marker for plasma membrane) in AD patients and age-matched controls. We found a significant increase in total platelet protein ($P < 0.0005$), no significant difference in phosphodiesterase specific activity, but a significant reduction in the specific activity of antimycin A-insensitive NADH–cytochrome-*c* reductase in AD patients compared to age-matched controls (Table III). Our findings show an increase in total protein with no apparent change in platelet number (data not shown) and a change in the relative levels of the plasma membrane and SER markers. These findings would reinforce our morphological data and further suggest abnormal membrane distribution in AD platelets.

Thus it is possible that AD involves a proliferation of an abnormal SER-related membrane system which does not show normal levels of enzyme markers such as that used in this study. In order to examine this possibility in more detail, we have obtained surface and intracellular platelet membrane fractions using Percoll gradi-

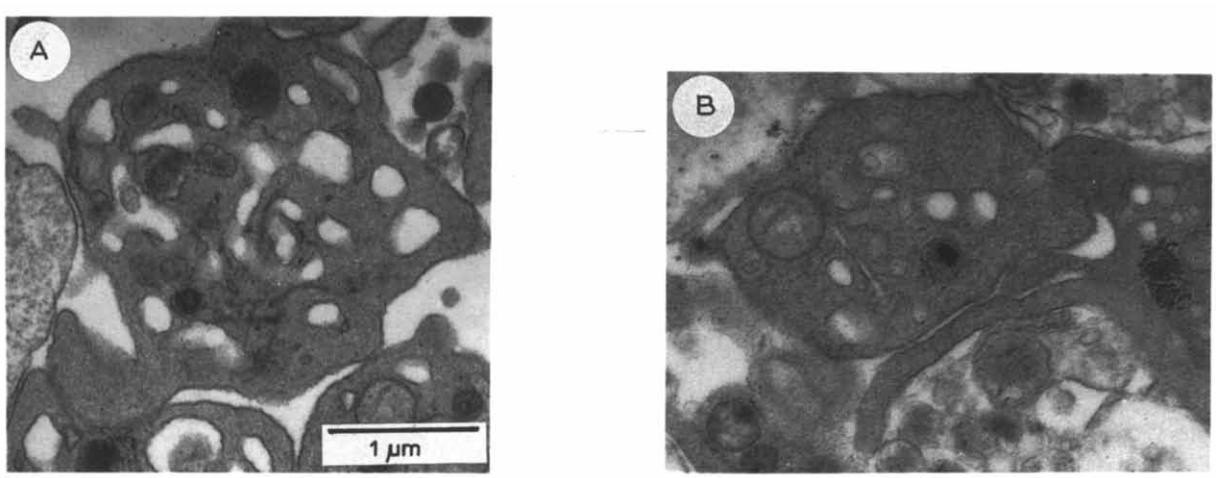

Fig. 1. Transmission electron micrographs of a typical platelet from an AD patient (A) and a healthy control (B). Magnification for A and B, ×25000. Scale bar is 1 µm.

TABLE III

Analysis of plasma and intracellular membrane marker enzymes

Values are the means ± S.D. n.s., not significant. [a] vs. [b]: Significant, $P = 0.014$.

	Specific enzyme activity (nmol/min per mg protein)	
	bis(*p*-nitrophenyl)-phosphate phosphodiesterase (plasma membrane)	antimycin A-insensitive NADH–cytochrome-*c* reductase (SER)
Alzheimer	0.091 ± 0.031 ($n = 8$)	74.88 ± 28.90 [a] ($n = 15$)
Control	0.105 ± 0.051 [n.s.] ($n = 9$)	106.82 ± 35.50 [b] ($n = 15$)

ents [26]. Although the fractionation was successful, the small amount of material obtained from the limited (10 ml) blood samples precluded accurate biochemical analysis. However, Menashi and colleagues [11] have described an electrophoretic technique with which they separated platelet membranes into a fraction consisting of internal cell membranes and two fractions of external membranes. In their studies, the internal membrane fraction exhibited higher membrane fluidity than external membranes, as judged by DPH fluorescence polarisation (we have similar observations with membranes fractionated on the Percoll gradient; data not shown). Therefore, the relative increase in internal platelet membranes as revealed by electron microscopy may account for the increase in the fluidity of AD platelet membranes.

The SER is involved in various important cellular processes. These include regulation of calcium homeostasis, N-glycosylation of polypeptides (e.g., β-amyloid precursor protein), maturation and localization of cellular proteins [27]. A defect or mutation that gives rise to an abnormality in SER could interfere with its cellular functions.

We examined SER functioning in platelets by measuring the relative increases in the intraplatelet basal free $[Ca^{2+}]$ (using the fluorescent EGTA analogue fura-2) with thrombin (1 unit/ml) stimulation in AD and matched controls. Our results show that thrombin stimulated increase in intraplatelet $[Ca^{2+}]$ is changed in AD platelets compared to matched controls; Fig. 2 shows this change. The thrombin stimulated percent increase in $[Ca^{2+}]$ was approximately twice as great in AD platelets as in matched controls.

Thrombin has been shown to be linked to phospholipase C hydrolysis of polyphosphoinositides in platelets [28], resulting in generation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) which, in turn release calcium from the non-mitochondrial stores [29] and activate protein kinase C [30], respectively. Calcium

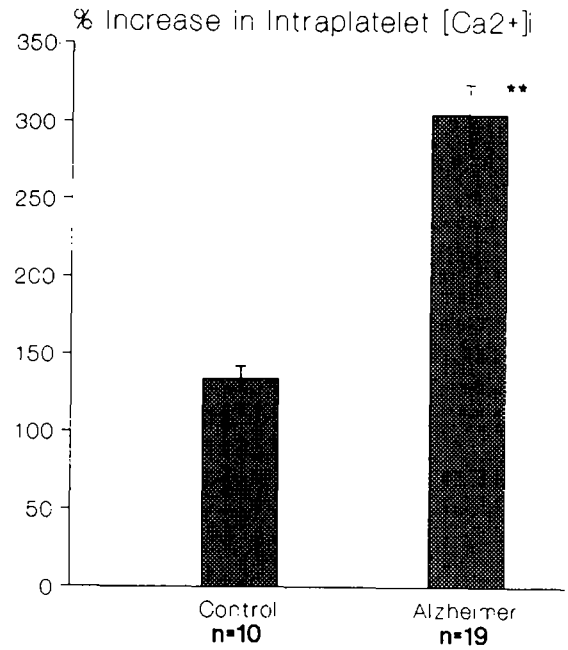

Fig. 2. The effect of thrombin (1 unit/ml) on intraplatelet free calcium concentration ($[Ca^{2+}]_i$) presented as percentage increase above basal levels. The bars represent mean values ± S.E. The means ± S.E. and number of subjects in each group are: control (134 ± 8%; $n = 10$); Alzheimer's disease (305 ± 19%; $n = 19$). * Significantly different from control group ($P < 1 \cdot 10^{-5}$, Student's *t*-test).

liberated from these stores (e.g., endoplasmic reticulum) is then closely regulated by Ca^{2+}-ATPase located on the plasma and endoplasmic reticulum membranes. We measured the specific activities of Ca^{2+}/Mg^{2+}-ATPase, Mg^{2+}-ATPase and Ca^{2+}-ATPase in AD and control platelets. Our results show (Fig. 3) that there was a significant decrease in the specific activities of Ca^{2+}/Mg^{2+}-ATPase and Ca^{2+}-ATPase but not in the Mg^{2+}-ATPase in the AD platelets compared to matched

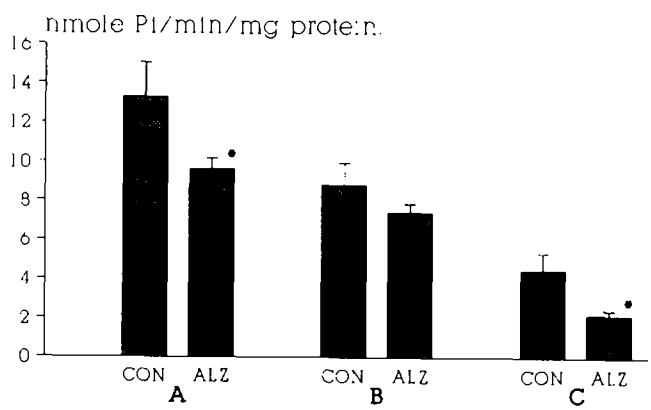

Fig. 3. Specific activities of Ca^{2+}/Mg^{2+}-ATPase (A), Mg^{2+}-ATPase (B) and Ca^{2+}-ATPase (C) in platelets from AD (Alz) and controls (Con). The means ± S.E. and number of subjects in each group are: (A) (Con, $n = 18$, 13.28 ± 1.76; Alz, $n = 24$, 9.58 ± 0.57), (B) (Con, $n = 18$, 8.80 ± 1.16; Alz, $n = 24$, 7.40 ± 0.44) and (C) (Con, $n = 18$, 4.48 ± 0.85; Alz, $n = 24$, 2.18 ± 0.26). * Significantly different from control group ($P < 0.05$, Student's *t*-test).

controls. These data suggest that calcium homeostasis is altered in AD platelets. They also complement our electron microscopic analysis, which shows SER proliferation in AD platelets. The increase in SER might be responsible for excess calcium storage in AD platelets which is released by thrombin stimulation. An increased level of stimulated release coupled to a decrease in the ATPase activity would combine to keep the intracellular free calcium at abnormal concentrations. These functional changes may be related to structural changes in the SER described above.

Peripheral calcium homeostasis is altered by ageing in AD. For example, ionized intracellular calcium in fibroblasts is decreased by ageing and further reduced in AD [31]. The reduction in cytosolic free calcium may alter other cellular functions (e.g., protein phosphorylation) [32]. A deficiency in calcium homeostasis may also be important in the pathophysiology of central nervous system dysfunctions. For example, there is increased bound calcium in neurones that possess neurofibrillary tangles [33] and abnormal cytoskeletal components which may be due to altered calcium/calmodulin-dependent protein kinase and calcium-activated proteinase (e.g., calpain) activity in AD [34].

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