

Research report

Ca²⁺ Stores and capacitative Ca²⁺ entry in human neuroblastoma (SH-SY5Y) cells expressing a familial Alzheimer's disease presenilin-1 mutation

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Abstract

Presenilins are involved in the proteolytic production of Alzheimer's amyloid peptides, but are also known to regulate Ca²⁺ homeostasis in various cells types. In the present study, we examined intracellular Ca²⁺ stores coupled to muscarinic receptors and capacitative Ca²⁺ entry (CCE) in the human neuroblastoma SH-SY5Y cell line, and how these were modulated by over-expression of either wild-type presenilin 1 (PS1wt) or a mutant form of presenilin 1 (PS1 ΔE9) which predisposes to early-onset Alzheimer's disease. Ca²⁺ stores discharged by application of 100 μM muscarine (in Ca²⁺-free perfusate) in PS1wt and PS1 ΔE9 cells were significantly larger than those in control cells, as determined using Fura-2 microfluorimetry. Subsequent CCE, observed in the absence of muscarine when Ca²⁺ was re-admitted to the perfusate, was unaffected in PS1wt cells, but significantly suppressed in PS1 ΔE9 cells. However, when Ca²⁺ stores were fully depleted with thapsigargin, CCE was similar in all three cell groups. Western blots confirmed increased levels of PS1 in the transfected cells, but also demonstrated that the proportion of intact PS1 in the PS1 ΔE9 cells was far greater than in the other two cell groups. This study represents the first report of modulation of both Ca²⁺ stores and CCE in a human, neurone-derived cell line, and indicates a distinct effect of the PS1 mutation ΔE9 over wild-type PS1.

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1. Introduction

Alzheimer's disease (AD) is characterised in post mortem brain tissue by the presence of plaque-like structures composed of amyloid peptides (Aβs), and the slow accumulation of Aβs is generally believed to account for the progressive, age-related development of this form of dementia [6,10,18]. Aβs are generated by the sequential cleavage of amyloid precursor protein by specific enzyme 'activities' termed β- and γ-secretase, with γ-secretase providing the final step in Aβ production [4,23]. The

central importance of γ-secretase in particular to AD has prompted intense investigations into its molecular identity over recent years, and accumulating evidence points to presenilins (PS1 and PS2) as accounting for this enzymatic activity [4,18]. These membrane-spanning proteins are believed to be active as γ-secretase following their own cleavage into N- and C-terminal fragments (NTF and CTF, respectively), which associate together and contribute to a γ-secretase 'complex' which is yet to be fully characterised, but also contains other proteins including the recently identified nicastrin [9,25]. Most importantly, mutations of PS1 and PS2 account for most cases of early-onset familial AD (FAD [6,10,18]).

Recent studies from numerous, independent groups have also shown that presenilins, particularly PS1, are intimately

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linked to cellular Ca^{2+} homeostasis, and that FAD-related mutations of PS1 can alter inositol trisphosphate (IP_3)-coupled intracellular Ca^{2+} stores as well as Ca^{2+} influx pathways [1,7,8,24]. Such effects of PS1 appear independent of $\text{A}\beta$ production [24], and indeed it is not known whether PS1 acts in this way as the intact protein or as NTF and CTF. However, this function of PS1 may contribute to apoptotic neuronal cell death in AD, since disruption of Ca^{2+} homeostasis is accepted as an important mechanism underlying such loss of neurones [3,11,12]. Despite the recent advances in our understanding of the influence of PS1 on Ca^{2+} homeostasis, there are notable areas of contention (see Section 4), and no single study has examined the role of PS1 in regulating both Ca^{2+} stores and Ca^{2+} influx pathways in the same excitable tissue. Here, we report the effects of PS1 on Ca^{2+} homeostasis in the human neuroblastoma, SH-SY5Y [21,22]. We demonstrate that stores liberated by activation of muscarinic receptors, and capacitative Ca^{2+} entry (CCE) activated by partial, but not complete, depletion of Ca^{2+} stores, are modulated in cells over-expressing either wild-type PS1 (PS1wt) or in cells expressing the FAD PS1 mutant, ΔE9 [19]. The effect of this ΔE9 mutation on PS1 processing is consistent with the deletion of the normal endoproteolytic cleavage site of PS1 found within exon 9 [20].

2. Materials and methods

Human neuroblastoma SH-SY5Y cells were cultured as previously described [14,15] in a mixture of Ham's F12 medium and Eagle's minimal essential medium (1:1 mixture), supplemented with 10% (v/v) fetal calf serum, 1% nonessential amino acids and 0.1% gentamicin. Stable transfections of cells over-expressing either PS1wt or PS1 ΔE9 were generated as previously described [19] and cultured under identical conditions as untransfected cells, except that 200 $\mu\text{g}/\text{ml}$ geneticin was present to maintain selection of transfected cells. All cells were incubated at 37 °C in a humidified incubator gassed with 95% air and 5% CO_2 , passaged every 7 days and used for up to 20 passages. When required for fluorimetric studies, cells were harvested in phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+} and sub-cultured on poly-D-lysine-coated glass coverslips at a seeding density of 3×10^4 cells/ml.

Changes in $[\text{Ca}^{2+}]_i$ were measured in SH-SY5Y cells attached to glass coverslips after 4 days in culture using the methods described previously [13,14,17]. In brief, cell layers were incubated with HEPES-buffered saline (HBS) containing 4 μM Fura2-AM at room temperature for 1 h. HBS was composed of (in mM): NaCl 135, KCl 5, MgSO_4 1.2, CaCl_2 2.5, HEPES 5, and glucose 10 (pH 7.4, osmolarity adjusted to 300 mosmol with sucrose, 21–24 °C). Fragments of coverslip were then placed in a perfusion chamber and intracellular Ca^{2+} concentration

($[\text{Ca}^{2+}]_i$) measured with a Joyce Loebble Phocal apparatus (Applied Imaging, Newcastle, UK). Cells were continually perfused with HBS and $[\text{Ca}^{2+}]_i$ was indicated by the ratio of fluorescence emitted at 510 nm due to alternating excitation at 340 nm and 380 nm using a rotating filter wheel. Ca^{2+} -Free HBS contained 1 mM EGTA and no added CaCl_2 . Since calibration of fluorescence into absolute $[\text{Ca}^{2+}]_i$ values can be subject to artefactual inaccuracies [5], data are presented as ratio signals.

Several parameters were determined from collected data. Changes in $[\text{Ca}^{2+}]_i$ were taken from measuring peak or plateau values and expressing them as the change in fluorescence ratio from basal levels, determined for each recording. Decay times are expressed as $t_{1/2}$ values, i.e. the time taken for a response to decline to 50% of its peak value. The size of muscarine-evoked stores was taken from the integral of transient responses recorded in Ca^{2+} -free perfusate (see Fig. 2). Mean time courses of CCE were measured every 10s for averaging as indicated in Figs. 2 and 3B. All results are expressed as means \pm S.E.M., together with example traces, and statistical comparisons were made using unpaired *t*-tests.

For Western blotting, SHSY5Y cells were grown to confluence as detailed above, in 75-cm² flasks, washed free of media, harvested into $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS and centrifuged at 4 °C, 2500 rpm for 10 min. The pellet was then re-suspended and the cells lysed in 1 ml M-Per mammalian protein extraction reagent (Perbio Science UK) containing Complete Mini protease inhibitors (Roche Bioscience). Protein levels in the cell lysates were measured using the method of Bradford [2].

Cell proteins (typically 10 μg protein per lane) were separated on 15%, 0.75-mm thick polyacrylamide SDS gels and transferred onto PVDF membranes (30v overnight). Membranes were blocked with 5% non-fat milk protein/2% BSA in PBS-Tween (0.05%) for 1 h and immunostained with antibodies raised against C-terminal (344–358) or N-terminal (2–15) fragments of human presenilin-1 for 3 h. The blots were washed and incubated with anti-rabbit Ig conjugated to HRP (Amersham, UK) and bands visualised using the ECL detection system and Hyperfilm ECL (Amersham, UK). Band intensities were measured using Scion Image analysis software.

3. Results

Muscarinic receptor activation leads to generation of IP_3 and subsequent liberation of Ca^{2+} from intracellular stores in SH-SY5Y cells, as well as many other cell types. The depletion of intracellular stores in turn leads to activation of CCE [12,15–17]. To examine separately these two processes by which cytosolic $[\text{Ca}^{2+}]_i$ is raised, we firstly discharged Ca^{2+} stores by applying muscarine (100 μM) whilst cells were perfused with Ca^{2+} -free solution. In all cells examined, this caused a transient rise of $[\text{Ca}^{2+}]_i$

which declined to basal levels in the continued presence of muscarine (Fig. 1A). Subsequent applications of muscarine failed to evoke any change in $[Ca^{2+}]_i$ (data not shown; see also Ref. [14]). Following washout of muscarine, re-admission of Ca^{2+} to the perfusate caused a rise of $[Ca^{2+}]_i$, which we and others have previously shown [13,14,16] is due to CCE (Fig. 1A). From these studies, several parameters were quantified in the three cell groups. Firstly, peak rises of $[Ca^{2+}]_i$ due to release from stores evoked by muscarine were found to be significantly enhanced in PS1 $\Delta E9$ expressing cells, but not in those over-expressing PS1wt, as compared with control (untransfected) cells (Fig. 1B). However, there was a significant slowing in the rate of decay of these Ca^{2+} transients in both groups of PS1 transfected cells, as compared with controls (Fig. 1C). Furthermore, the total amount of Ca^{2+} liberated by muscarine—as determined by integration of the Ca^{2+} transients—was also significantly enhanced in these two cell groups, as compared with controls (Fig. 1D).

As illustrated in Fig. 1A, readmission of Ca^{2+} to the perfusate following store depletion causes a rise of $[Ca^{2+}]_i$ attributable to CCE. To examine this in detail, we averaged these changes of $[Ca^{2+}]_i$ every 10 s for each group of cells, and results are plotted in Fig. 2. As compared with control cells, CCE observed in those cells over-expressing PS1wt occurred with a similar time-course and magnitude. By contrast, cells expressing the FAD PS1 mutant $\Delta E9$ displayed significantly smaller and slower rises of $[Ca^{2+}]_i$

following store depletion and readmission of Ca^{2+} to the perfusate. It has been previously suggested that altered CCE, recorded using the same protocols as shown in Fig. 1A, might be attributable to incomplete discharge of Ca^{2+} from internal stores following receptor activation. To investigate this, we depleted stores completely by exposing cells to the endoplasmic reticulum Ca^{2+} ATPase inhibitor, thapsigargin. Prevention of re-uptake of Ca^{2+} into intracellular stores causes a slow, passive and complete depletion in these cells [14,17]. Following incubation in Ca^{2+} -free solution containing 1 μM thapsigargin for 20 min, CCE was monitored on readmission of Ca^{2+} to the perfusate. Example recordings are shown in Fig. 3A for each of the cell groups, and averaged data plotted in Fig. 3B. Clearly, following complete store depletion, over-expression of PS1wt or PS1 $\Delta E9$ had no significant effect on the time course or magnitude of CCE.

In order to confirm over-expression of PS1, we employed both CTF- and NTF-directed antibodies in Western blot studies. Results are exemplified and summarised in Fig. 4. PS1 was detected with either antibody at two points using SDS PAGE, corresponding to fragmented and intact PS1. Using either antibody, it was apparent that PS1 levels were significantly increased in SH-SY5Y cells stably transfected with PS1wt or PS1 $\Delta E9$ ($P < 0.005$ and $P < 0.002$, respectively). It was also noteworthy that the fraction of PS1 observed in the fragmented form was less in PS1wt over-expressing cells, and smallest of all in PS1

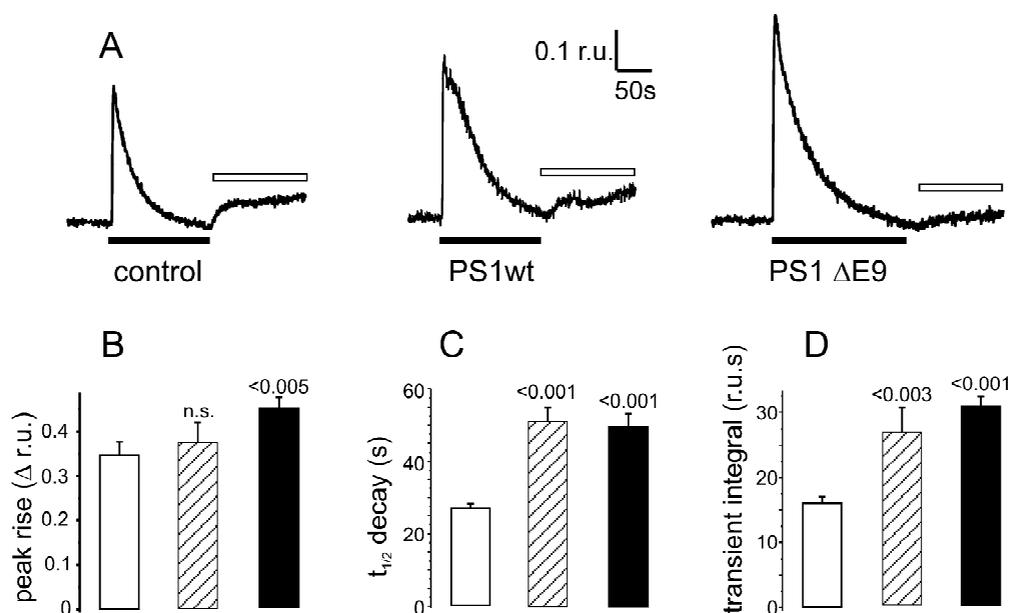


Fig. 1. (A) Example $[Ca^{2+}]_i$ changes evoked in response to 100 μM in control SH-SY5Y cells (left trace), cells over-expressing wild-type PS1 (PS1wt; middle trace) and cells over-expressing the FAD PS1 mutant $\Delta E9$ (PS1 $\Delta E9$; right trace). Muscarine was applied for the period indicated by the solid horizontal bar below each trace, in the absence of extracellular Ca^{2+} (replaced with 1 mM EGTA). For the periods indicated by the open bars, Ca^{2+} was re-admitted to the perfusate. Scale bars apply to all three traces (r.u.; ratio units). (B–D) Mean (with vertical S.E.M. bars) measured parameters determined from experiments exemplified in (A) in control cells (open bars, $n=17$), PS1wt cells (hatched bars, $n=12$) and PS1 $\Delta E9$ cells (solid bars, $n=24$). Parameters determined were peak Ca^{2+} rise (B), rate of decay of Ca^{2+} transient seen during muscarine application (C) and the integral of the transients (D). P values shown above bars indicate statistically significantly different values from control cells, determined using unpaired t -tests.

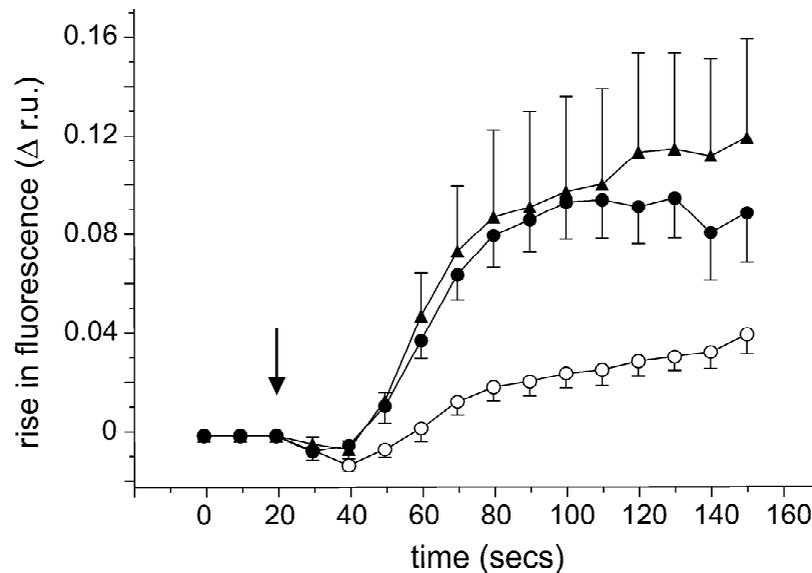


Fig. 2. Mean (\pm S.E.M.) time courses of rises of $[Ca^{2+}]_i$ due to capacitative Ca^{2+} entry in control cells (solid circles, $n=17$), PS1wt cells (solid triangles, $n=12$) and PS1 Δ E9 cells (open circles, $n=24$). These data were averaged from experiments exemplified in Fig. 1A, and show the rise of $[Ca^{2+}]_i$ observed when Ca^{2+} was re-admitted to the perfusate (at point indicated by arrow) after intracellular stores were depleted by exposure of cells to 100 μ M muscarine in the absence of extracellular Ca^{2+} . For comparative purposes, data were sampled every 10 s.

Δ E9 cells. Thus, although these cells could produce large amounts of PS1, they appeared less efficient in cleaving the elevated PS1 into fragmented forms.

4. Discussion

These studies demonstrate that over-expression of either PS1wt or the FAD mutant PS1 Δ E9 leads to increases in the Ca^{2+} content of intracellular stores coupled (via IP_3 formation) to muscarinic receptors in SH-SY5Y cells. This was manifested as an increase in the time-course and integral of the Ca^{2+} transients evoked by muscarine in Ca^{2+} -free perfusate (Fig. 1). This finding, obtained in a human neuronal cell line, is in accordance with previous studies conducted in peripheral murine fibroblasts [7] and *Xenopus* oocytes [8]. Thus, over-expression of either wild-type or mutant form of PS1 led to enhanced overloading of muscarinic receptor-coupled intracellular (endoplasmic reticulum) Ca^{2+} stores. It should be noted, however, that the magnitude and kinetics of such Ca^{2+} transients can be influenced not only by the size of Ca^{2+} stores, but also by Ca^{2+} buffering and extrusion mechanisms which have yet to be explored as targets for modulation by elevated or mutant forms of PS1. The mechanism by which PS1 regulates Ca^{2+} store size is presently unknown. Indeed, it is not known whether the intact or fragmented form of PS1 is responsible for this important intracellular function. Our Western blot studies (Fig. 4) indicate that both PS1wt and PS1 Δ E9 transfected cells expressed far greater levels of both intact and fragmented forms of PS1. However,

proportionally far more of the PS1 Δ E9 form remained in the intact form than the PS1wt form, yet both exerted similar effects on muscarine-sensitive Ca^{2+} store size (Fig. 1).

Our results also indicate that CCE following store depletion induced by muscarinic receptor activation was dramatically suppressed in PS1 Δ E9 transfected cells, yet not in those over-expressing PS1wt. This indicates a distinct effect of the FAD mutation Δ E9 over wild-type PS1. Whether this is due to the mutation per se, or to the fact that the mutant form underwent far less cleavage and so remained in the intact form, cannot be distinguished at present. Nevertheless, the finding is in general accordance with other, recent studies, yet detailed comparisons are not ideal, and interpretation of results appears to be a subject of contention. Thus, Yoo et al. [24] demonstrated in mouse cortical neurones and SH-SY5Y cells that PS1 deficiency and 'loss-of-function' PS1 mutants potentiated CCE following complete store depletion. In addition, they demonstrated that FAD PS1 mutant over-expression suppressed CCE, although the mutations used were distinct from the Δ E9 mutant used here. Finally, these workers showed that Ca^{2+} release activated Ca^{2+} channels (CRAC channels), believed to be the channels underlying CCE, were greatly diminished or even absent in non-neuronal (CHO) cells expressing another FAD PS1 mutant, but not wild-type PS1 [24]. Leisring et al. [7] also demonstrated suppressed CCE in peripheral fibroblasts of transgenic mice over-expressing a FAD mutant PS1. However, this suppressed CCE was due to incomplete discharge of Ca^{2+} from internal stores, rather than dysfunction of CCE itself, since

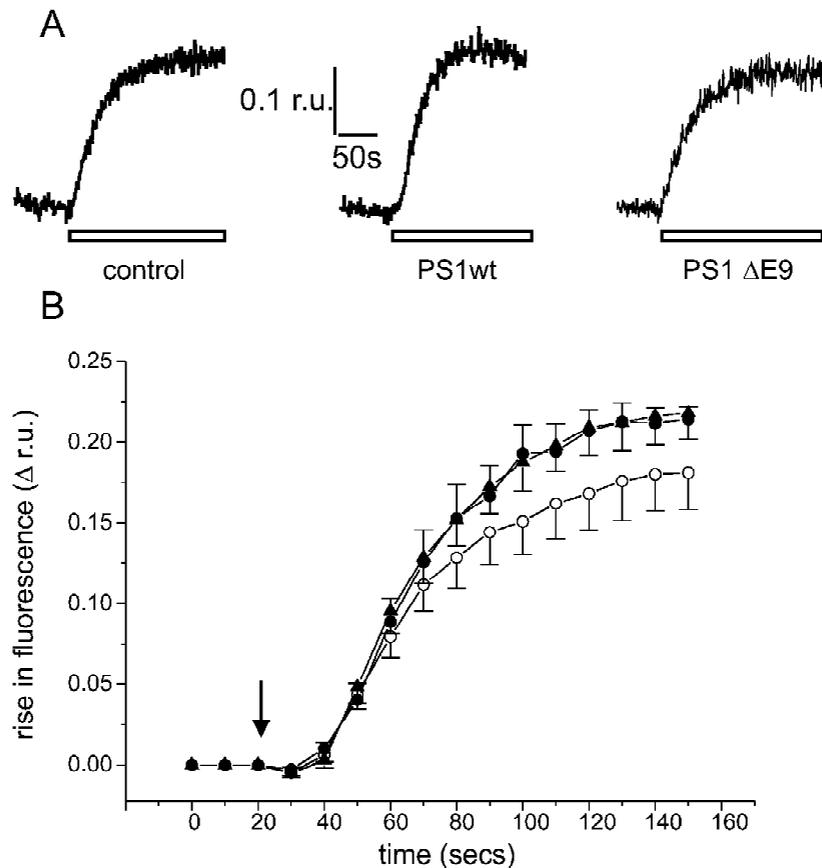


Fig. 3. (A) Example rises of $[Ca^{2+}]_i$ observed in response to re-addition of 2.5 mM Ca^{2+} to the perfusate in control SH-SY5Y cells (left trace), PS1wt cells (middle trace) and PS1 $\Delta E9$ cells (right trace). In each case, cells were previously exposed to 1 μ M thapsigargin in Ca^{2+} -free solution (containing 1 mM EGTA) for 20 min to deplete intracellular stores, before Ca^{2+} was re-applied for the periods indicated by the open bars. Scale bars apply to all three traces (r.u.; ratio units). (B) Mean (\pm S.E.M.) time course of rise of $[Ca^{2+}]_i$ due to capacitative Ca^{2+} entry in control cells (solid circles, $n=9$), PS1wt cells (solid triangles, $n=12$) and PS1 $\Delta E9$ cells (open circles, $n=13$). These data were averaged from experiments exemplified in A. For comparative purposes, data were sampled every 10 s and Ca^{2+} was re-admitted to the perfusate at the time point indicated by the arrow.

after complete emptying of stores with thapsigargin, CCE was similar in control and transgenic mice. The incomplete discharge of stores occurred because stores contained greater levels of Ca^{2+} . Our findings, obtained in a human neurone-derived cell line, are in good agreement with the work of Leissring and colleagues. Thus, we found elevated Ca^{2+} stores in both PS1wt and PS1 $\Delta E9$ over-expressing cells. CCE, observed following complete store depletion with thapsigargin, was not significantly affected (Fig. 3). However, CCE (observed following muscarine application) was severely suppressed in $\Delta E9$ expressing cells, whilst PS1wt-expressing cells showed similar CCE to controls. These differences cannot presently be accounted for, and various explanations could account for these observations. Thus, for example, the CRAC channels underlying CCE in PS1 $\Delta E9$ expressing cells may be altered in terms of their sensitivity to the emptying of Ca^{2+} stores following muscarine application, possibly by disruption of the channels from the signalling pathway (which at present is unidentified) that relays information concerning the status

of store Ca^{2+} levels to the CRAC channels. It is also noteworthy that Western blot results (Fig. 4) revealed that a much greater fraction of PS1 remained in the intact form in $\Delta E9$ mutant-expressing cells. Evidence to date suggests that fragmented PS1 is required for γ -secretase activity [9]; whether or not intact PS1 is required for modulation of Ca^{2+} homeostasis has yet to be determined, and is worthy of further investigation.

In summary, our findings indicate that Ca^{2+} stores are strongly influenced by PS1 levels, and that physiological store depletion (i.e. agonist-activated, rather than thapsigargin-induced) can be modulated by over-expression of wild-type PS1 and a FAD PS1 mutation. CCE, monitored following agonist-evoked release of Ca^{2+} from stores, was unaffected in PS1wt cells as compared with controls, but was markedly suppressed in PS1 $\Delta E9$ cells. Total amounts of over-expressed PS1 protein were similar in wild-type and mutant transfected cells, but the degrees of PS1 cleavage were markedly different. We are therefore unable at present to distinguish between effects being due to the

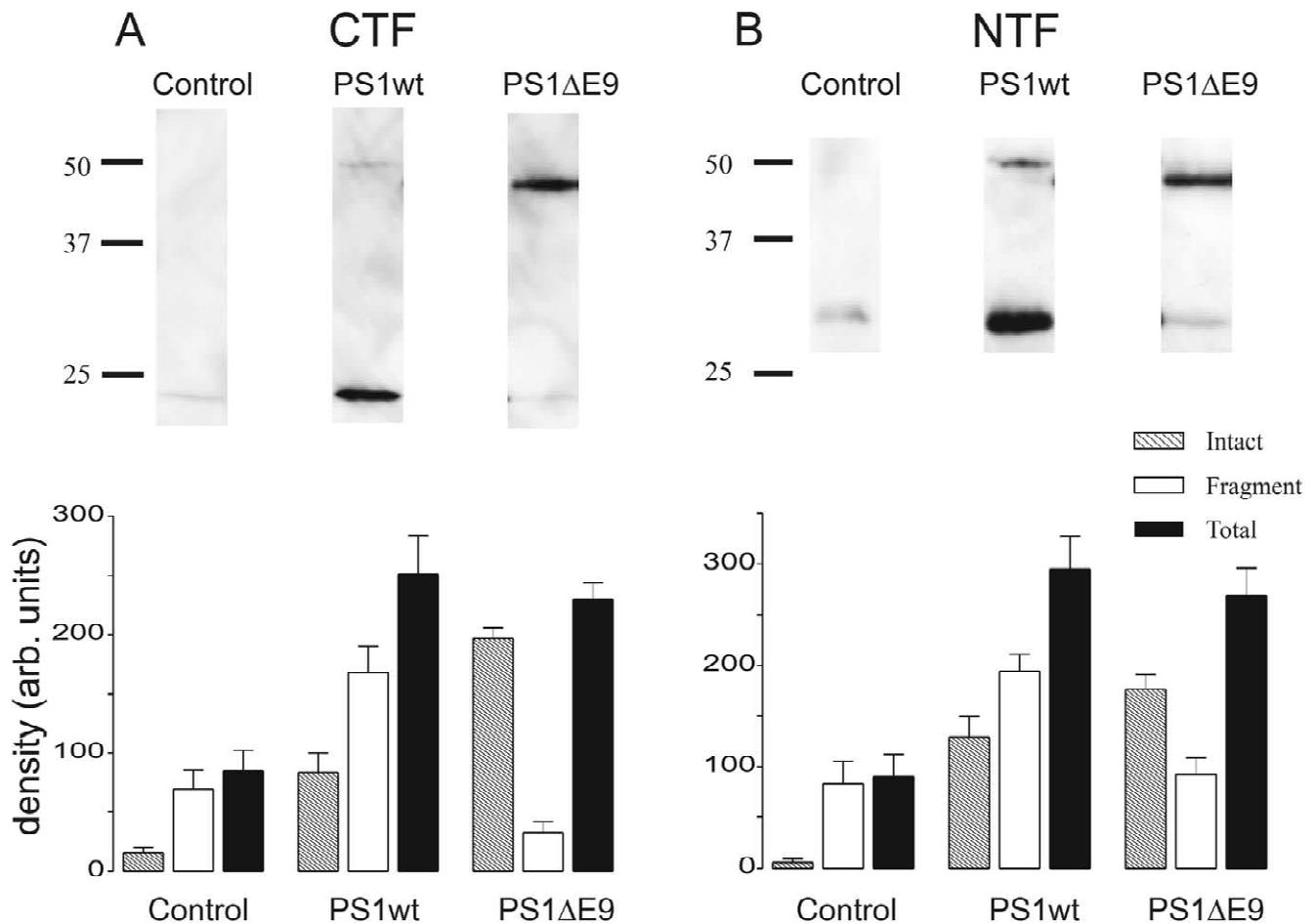


Fig. 4. (A) Example Western blots illustrating presenilin-1 intact (upper bands) and fragment (lower bands) levels detected with a C-terminal fragment (CTF) directed antibody in control cells, PS1wt mutants and PS1ΔE9 mutants, as indicated. Below are plotted averaged densitometric values (arbitrary units, based on a 0–256 grey-scale densitometric analysis, with vertical S.E.M. bars) determined from seven experiments as exemplified above, plotting intact (hatched bars), fragment (open bars) and total (solid bars) PS1 levels. (B) As A, except that blots were obtained using an N-terminal directed antibody.

mutation itself or due to differential cleavage. Our results, obtained exclusively in human neuronal tissue are consistent with the recently developed concept [8,24] that disruption of Ca^{2+} homeostasis is a likely mechanism underlying cell damage and death in AD.

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