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The presenilin 2 M239I mutation associated with familial Alzheimer's disease reduces Ca^{2+} release from intracellular stores

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Mutations in presenilin (*PS*) genes account for the majority of the cases of the familial form of Alzheimer's disease (FAD). PS mutations have been correlated with both over-production of the amyloid-β-42 (Aβ42) peptide and alterations of cellular Ca^{2+} homeostasis.

We here show, for the first time, the effect of the recently described PS2 FAD-associated M239I mutation on two major parameters of intracellular Ca^{2+} homeostasis: the Ca^{2+} storing capacity of the endoplasmic reticulum (ER) and the activation level of capacitative Ca^{2+} entry (CCE), the Ca^{2+} influx pathway activated by depletion of intracellular stores.

Ca^{2+} release from intracellular stores was significantly reduced in fibroblasts from FAD patients, compared to that found in cells from healthy individuals or patients affected by sporadic forms of Alzheimer's Disease (AD). No significant difference was however found in CCE between FAD and control fibroblasts. Similar results were obtained in two cell lines (HEK293 and HeLa) stably or transiently expressing the PS2 M239I mutation.

Keywords: Presenilin; Alzheimer's disease; Ca^{2+} homeostasis; Capacitative Ca^{2+} entry

Introduction

The most common forms of early onset FAD were linked to missense mutations in one of three genes encoding for amyloid precursor protein (APP), presenilin 1 (PS1) and presenilin 2 (PS2) which promote neuritic plaque accumulation and neuronal degeneration (Selkoe, 2000). *PS1* and *PS2* genes share a similar genomic organization and encode proteins that are 67% identical. Despite their similarities, there are genetic and physiological differences between the two molecules (Lai et al., 2003). While over 120 mutations have been identified in PS1, only 10 mutations have been found in PS2 that are definitively linked to FAD (Binetti et al., in press; Ezquerra et al., 2003; Finckh et al., 2000a; Levy-Lahad et al., 1995; Rogaev et al., 1995). In addition, PS1 mutations cause an aggressive form of FAD with a very early age of onset, whereas PS2 mutations are associated with a form of FAD that more closely resembles sporadic AD in terms of age of onset and disease duration (Finckh et al., 2000b; Sherrington et al., 1996). The majority of APP and PS mutations have been correlated with an increased cleavage of APP, resulting in overproduction of the highly amyloidogenic peptide Aβ42, the main constituent of neuritic plaques. Nonetheless, it has been reported that other FAD-linked APP mutations (Ancolio et al., 1999; Stenh et al., 2002), as well as a PS1 mutation associated with familial frontotemporal dementia (insR352; Amtul et al., 2002) did not affect or even diminished Aβ42 production. Perturbed Ca^{2+} homeostasis is also considered a common feature of FAD-linked PS mutations. In particular, mutant presenilins have been associated with both increased Ca^{2+} release from intracellular stores (Chan et al., 2000; Leissring et al., 1999, 2000; Schneider et al., 2001) and reduced CCE, the Ca^{2+} influx pathway that is activated upon store depletion (Smith et al., 2002; Yoo et al., 2000). More recently, a causal link has been proposed between alteration of cellular Ca^{2+} homeostasis and increased production of Aβ42 peptides (Yoo et al., 2000). Moreover, abnormalities in Ca^{2+} handling have been correlated with increased vulnerability of neurons to excitotoxicity and apoptosis induced by Aβ42 exposure and trophic factor deprivation (Guo et al., 1996, 1997, 1999). A number of alterations in metabolic and biochemical processes, including signal transduction systems, oxidative metabolism, APP processing, and Ca^{2+} homeostasis itself have been found in cultured skin fibroblasts derived from patients affected by sporadic and familial AD (Cecchi et al., 2002; Etcheberrigaray and Bhagavan, 1999; Gasparini et al., 1998; Gibson, 2002; Palotas et al., 2001; Zhao et al., 2002). We have here employed human primary fibroblasts obtained from patients bearing the recently described PS2 M239I mutation,

Abbreviations: PS, presenilin; FAD, Familial form of Alzheimer's Disease; ER, Endoplasmic Reticulum; CCE, Capacitative Ca^{2+} Entry; AD, sporadic form of Alzheimer's Disease; APP, Amyloid Precursor Protein; SERCA, Sarcoplasmic/Endoplasmic Reticulum Ca^{2+} ATPase; mKRB, modified Krebs–Ringer Buffer; AEQ, aequorin; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CPA, cyclopiazonic acid; Tg, thapsigargin; Cch, carbachol; $InsP_3$, inositol 1,4,5-trisphosphate; Hist, histamine.

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associated with autosomal dominant FAD (Finckh et al., 2000b), to study both CCE and intracellular Ca^{2+} stores. In these cells, with the Ca^{2+} indicator fura-2, we measured the amount of Ca^{2+} released from the ER and the properties of CCE upon treatment with sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitors. In addition, the effect of expression of such mutation on Ca^{2+} handling was evaluated in two different cell lines, to proof a specific role of the PS2 M239I mutation on Ca^{2+} homeostasis. Altogether, our findings suggest that this mutation plays a causal role in determining an altered Ca^{2+} handling in a way that differs significantly from that described for the large majority of FAD-linked PS mutations.

Materials and methods

Subjects

Patients included in this study were recruited and evaluated at the Memory Clinic of IRCCS "Centro San Giovanni di Dio-Fatebenefratelli", Brescia, Italy. Written informed consent was obtained from all subjects or, where appropriate, their caregivers, following the procedures approved by the hospital local ethical committee. Full description of the Italian pedigree with FAD-associated PS2 M239I mutation was reported elsewhere (Finckh et al., 2000b). We obtained fibroblast cell lines from two siblings carrying the PS2 M239I mutation: patients II-1 and II-6 (II-1, age 70 years; II-6, age 58 years). At the time of the first description (Finckh et al., 2000b), subject II-1 was unaffected. Additionally, we included in the present study subjects affected by sporadic AD ($n = 7$, mean age: 71.9 ± 11.2 years) and healthy controls (HC, $n = 8$, mean age: 67.5 ± 4.0 years). Global cognition was investigated by Mini-Mental State Examination (MMSE) as previously described (Folstein et al., 1975). Demographic and clinical features of subjects are reported in Table 1.

Table 1
Clinical and demographic characteristics

Subjects	Age (years)	Gender	Duration of disease (years)	MMSE
PS2 M239I (FAD)				
II-1	70	M	3	12/30
II-6	58	M	10	0/30
Healthy Controls (HC)				
Mean values ± SD	67.5 ± 4.0			28.25 ± 1.6
HC-1	65	F	–	30/30
HC-2	70	F	–	28/30
HC-3	70	M	–	30/30
HC-4	65	M	–	30/30
HC-5	69	F	–	27/30
HC-6	66	M	–	27/30
HC-7	74	M	–	28/30
HC-8	61	F	–	26/30
Alzheimer Disease (AD)				
Mean values ± SD	71.86 ± 11.2		6.43 ± 3.3	10.0 ± 9.7
AD-1	85	F	9	1/30
AD-2	66	F	8	15/30
AD-3	89	F	10	16/30
AD-4	66	M	6	2/30
AD-5	72	M	7	0/30
AD-6	67	M	5	26/30
AD-7	58	F	1	10/30

MMSE = Mini-Mental State Examination; F = female, M = male.

Human primary skin fibroblast cultures

Fibroblast primary cultures were derived from the dermal biopsy of FAD-PS2 M239I patients as previously described (Govoni et al., 1993). Fibroblasts from sporadic AD subjects and healthy controls were obtained from Fatebenefratelli Biological Repository (F-BR) of IRCCS "Centro San Giovanni di Dio-Fatebenefratelli", Brescia, Italy. Cells were cultured at 37°C in 5% CO_2/95% air in Eagle's minimal essential medium (MEM, Gibco, Invitrogen, San Diego, CA, USA), supplemented with 10% fetal calf serum (FCS, Gibco, Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin and with non-essential amino acids. The different fibroblast cell cultures were plated, at the same passage number, on cover slips (BDH, Milan, Italy) and used, after 2 days, for Ca^{2+} measurements.

Ca^{2+} measurements

Cells, plated on cover slips (24 mm diameter), were loaded with fura-2 by incubation with 2 μM fura-2/AM at room temperature for about 60 min in MEM containing 10% FCS and 0.04% pluronic. To prevent fura-2 leakage and sequestration, 250 μM sulfinpyrazone was present throughout the loading procedure and $[Ca^{2+}]_i$ measurement. The cover slips were washed with a modified Krebs–Ringer Buffer (mKRB, in mM: 140 NaCl, 2.8 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 11 glucose, pH 7.4, at 37°C), mounted on a thermostated chamber (Medical System Corp., New York, USA), placed on the stage of an inverted microscope (Zeiss, Axiovert 100 TV) equipped for single cell fluorescence measurements and imaging analysis (TILL Photonics, Martinsried, Germany). The sample was alternatively illuminated ($t = 10$ ms) by monochromatic light (at 340 and 380 nm) every second through a 40× oil immersion objective (NA = 1.30; Zeiss). The emitted fluorescence was passed through a dichroic beamsplitter (455DRPL), filtered at 505–530 nm (Omega Optical and Chroma Technologies, Brattleboro, VT, USA) and captured by a cooled CCD camera (Imago, TILL Photonics). Cells were challenged with different stimuli by either perfusion or quick mixing at 37°C. For presentation, the ratios (F340/F380) were off-line averaged (20–30 cells) and normalized to the resting value measured within the first minute of the experiment. All figures are representative of at least four independent experiments. Where indicated, in the mKRB, $CaCl_2$ was omitted and EGTA (150 μM) was added (Ca^{2+}-free medium), or NaCl was substituted by 140 mM KCl (K^+-based medium).

Cell lines and transfection

Human embryonic kidney (HEK) 293 cell lines stably expressing human wild-type (wt) or mutant (M239I) PS2, and control cells stably transfected with the void vector, were obtained by the Fugene methodology (Roche, Molecular Biochemicals, Indianapolis, IN, USA) following the manufacturer's instructions, employing 1 μg of pcDNA3 void vector (Invitrogen), or pcDNA3 construct containing the cDNA encoding either for human PS2

wt or PS2 M239I mutation. Cells were grown in F12/DMEM supplemented with 10% FCS containing penicillin (100 U/ml), streptomycin (100 μg/ml) and the selecting antibiotic gentamicin (800 μg/ml) (Gibco, Invitrogen). Resistant clones were isolated by using cloning cylinders and then analyzed by Western blot with antibody against PS2 (see below). Stable transfected HEK293 cells, expressing the different PS2 constructs, were maintained in gentamicin at a final concentration of 800 μg/ml. Additionally, to confirm the integration of cDNA, clones stably expressing mutant PS2 M239I were sequenced by using specific primers.

For Ca^{2+} measurements, cells from the three different HEK clones were plated into 13-mm glass cover slips and transiently transfected, using the Fugene methodology, with 1.5 μg aequorin (AEQ) cDNA, and used after 36 h as described below.

Transient transfections of HEK293 cells were performed by the Fugene methodology employing 0.5 μg of AEQ cDNA, together with 1.5 μg of pcDNA3 vector or pcDNA3 construct containing the cDNA encoding either for human PS2 wt or PS2 M239I mutation. After 36 h, cells were used for intracellular Ca^{2+}-AEQ measurements (see below).

HeLa cells were grown in DMEM supplemented with 10% FCS containing penicillin (100 U/ml), streptomycin (100 μg/ml) in 75-cm^2 Falcon flasks. Before transfection, cells were seeded onto 13-mm glass cover slips and allowed to grow to 50% confluence. At this stage, transfections were carried out using the Ca^{2+}-phosphate technique in the presence of 4 μg of DNA [3 μg PS2 (wt or M239I) cDNA or void vector (pcDNA3; Invitrogen, Milan, Italy) + 1 μg AEQ cDNA]. Intracellular Ca^{2+} measurements were carried out 36 h after transfection with the AEQ technique as previously described (Brini et al., 1995) and here briefly summarized.

Aequorin measurements

The cells, seeded on cover slips, were incubated with 5 μM coelenterazine for 1–2 h in mKRB and then transferred to the perfusion chamber. All the AEQ measurements were carried out in mKRB at 37°C. Agonists and other drugs were added to the same medium, as specified in the figure legends. The experiments were terminated by lysing the cells with 100 μM digitonin in a hypotonic Ca^{2+}-rich solution (10 mM $CaCl_2$ in H_2O) to discharging the remaining unused AEQ pool. The light signal was collected by a low-noise photomultiplier with a built-in amplifier-discriminator (Thorn-EMI photon counting board), stored on an IBM-compatible computer and off-line calibrated into $[Ca^{2+}]$ values, as previously described (Brini et al., 1995).

Protein extracts preparation and Western blot analysis

HEK293 and HeLa cells were washed twice with ice-cold phosphate-buffered saline (PBS) and harvested in ice-cold RIPA modified lysis buffer [25 mM Tris–HCl pH 7.2, 150 mM NaCl, 1 mM EDTA, 0.5% sodium salt deoxycholic (NaDOC), 1% nonidet-P40 (NP-40), 0.1% SDS, 200 μM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 20 μg/ml leupeptin, 1 μg/ml pepstatin A, 5 mM dithiothreitol (DTT)]. The homogenates were incubate 30 min on ice and centrifuged at 60,000 × *g* for 20 min at 4°C. Loading of the samples was normalized for the total content of cellular proteins determined by the BCA assay (Pierce, Rockford, IL, USA). Samples were run on a 10–20% Tris-tricine gels (Novex, Invitrogen) and then blotted onto PVDF membrane (Perkin-Elmer Life Sciences, Boston, MA, USA). Immunodetection of PS2 proteins was carried out with the polyclonal antibody Ab-2 PC235 (Oncogene, Merck, Darmstadt, Germany) that recognizes the PS2 C-terminal proteolytic fragments of 20 kDa. Detection was carried out by incubation with alkaline phosphatase-conjugated anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) for 1 h. The proteins were visualized by the chemiluminescence reagent CDP-star (Perkin Elmer Life Sciences). Densitometric analyses were performed by using NIH Image software. Means of densitometric measurements of three independent experiments, normalized by the void vector values, were compared by Student's *t* test.

Results

Primary fibroblasts from patients carrying the FAD-associated PS2 M239I mutation show altered Ca^{2+} release from intracellular stores and normal CCE

The aim of the present investigation was to define whether Ca^{2+} homeostasis is altered in cells carrying the FAD-linked PS2 M239I mutation. On this purpose, changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) were monitored with the Ca^{2+} indicator fura-2 (see Materials and methods) in primary skin fibroblasts from FAD-PS2 M239I patients, sporadic AD patients, and healthy controls (HC) (see Table 1). Fura-2 loaded cells were used to measure the effects of the PS2 M239I mutation on both Ca^{2+} content of intracellular stores and CCE, the Ca^{2+} influx across the plasma membrane which is elicited by store depletion (Penner et al., 1993). A typical protocol that allows to better distinguish between Ca^{2+} release and Ca^{2+} influx is described in Fig. 1A. By keeping the cells in a Ca^{2+}-free medium, store depletion was first induced by adding the SERCA inhibitor cyclopiazonic acid (CPA, 20 μM); CCE was subsequently monitored upon $CaCl_2$ addition (Ca^{2+}, 1 mM). The SERCA inhibitor, by inducing the passive release of Ca^{2+} from intracellular stores, caused a transient increase in $[Ca^{2+}]_i$. After 6–8 min, addition of $CaCl_2$, in the continuous presence of CPA, induced a second large $[Ca^{2+}]_i$ peak followed by a sustained $[Ca^{2+}]_i$ plateau due to Ca^{2+} influx across the plasma membrane. Indeed, removal of extracellular Ca^{2+} by the Ca^{2+} chelator EGTA (2 mM) caused a rapid return of the $[Ca^{2+}]_i$ to the resting level. Fig. 1A shows $[Ca^{2+}]_i$ changes in two representative experiments carried out with control (dashed trace) and FAD-PS2 M239I (continuous trace) fibroblasts. The transient increase in $[Ca^{2+}]_i$ elicited by CPA was significantly reduced in FAD cells relative to control ones. Similar results were obtained with thapsigargin (Tg), another SERCA inhibitor (data not shown). Thus, the rise in $[Ca^{2+}]_i$ induced by discharging intracellular Ca^{2+} stores is diminished in cells expressing the PS2 M239I mutation. In Fig. 1B, the entity of such reduction can be estimated from the average peak amplitudes above resting $[Ca^{2+}]_i$ values obtained upon CPA addition (38 ± 10%, mean ± SEM, $P < 0.05$; $n = 10$ FAD, $n = 62$ HC). Primary fibroblasts from sporadic AD patients, however, did not show any significant reduction in the Ca^{2+} content of intracellular stores when discharged by CPA (Fig. 1B), suggesting a direct involvement of the PS2 M239I mutation on this phenomenon.

The effect of the PS2 M239I mutation on Ca^{2+} influx activated by store depletion was measured with the same protocol described in Fig. 1A. No significant difference in Ca^{2+} entry was found between FAD and control fibroblasts, by measuring the peak value

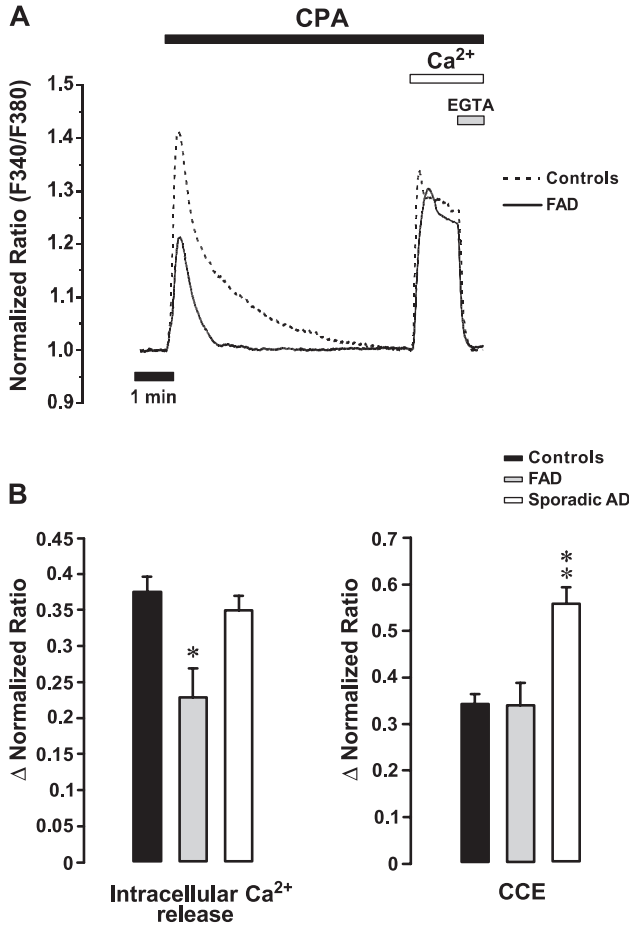

Fig. 1. Fibroblasts from FAD-PS2 M239I patients show a decreased Ca^{2+} release from intracellular stores and normal CCE. Panel A shows a typical experiment performed with fibroblasts from FAD-PS2 M239I patients (continuous trace) and healthy control subjects (dashed trace). Cells, loaded with fura-2 as described in Materials and methods, were perfused in mKRB in the absence of extracellular $CaCl_2$ and in the presence of EGTA 150 μM (Ca^{2+}-free medium). CPA (20 μM), $CaCl_2$ (1 mM), and EGTA (2 mM) were added when indicated by top bars. The ratio (F340/F380) was normalized to the averaged value obtained within the first minute of the experiment. Panel B shows the average peak amplitudes measured from resting values (Δ normalized ratio, mean ± SEM) for both intracellular Ca^{2+} release (left) and CCE (right); n = number of independent experiments (n = 62 controls, n = 10 FAD, n = 45 AD; * = $P < 0.05$, ** = $P < 0.001$, Student's t test).

reached upon $CaCl_2$ addition (Fig. 1B). In contrast, a marked increase in CCE was observed in fibroblasts from sporadic AD patients as compared to controls (63 ± 10%, mean ± SEM, $P < 0.001$, n = 45 AD, n = 62 HC; Fig. 1B).

It is well known that differences in plasma membrane potential strongly affect the rate and extent of CCE by altering the driving force for Ca^{2+} entry (Penner et al., 1993). To nullify any difference in membrane potential between the three cell groups, CCE was measured with the same protocol described in Fig. 1 but in a medium where NaCl was iso-osmotically substituted by KCl (K^+-based medium). Under these conditions, the membrane potential collapsed and differences among cells mostly abridged. Primary fibroblasts, bathed in a K^+-based medium, were first treated with CPA in the absence of Ca^{2+} and then challenged with a high concentration of $CaCl_2$ (10 mM) to obtain a more robust Ca^{2+} influx under a reduced electrical gradient. Even under depolarizing conditions, the effects of the PS2 M239I mutation on Ca^{2+} changes induced by CPA were similar to those found in the standard Na^+-containing medium (data not shown).

Decreased Ca^{2+} release from intracellular stores and normal CCE in HEK293 cells stably expressing the PS2 M239I mutation

The impact of the PS2 M239I mutation on Ca^{2+} homeostasis was also checked in HEK293 cells stably expressing the void vector (control cells), human wild-type (wt), or mutant (M239I) PS2. We decided to employ this experimental approach since in stable clones, the expression level of the exogenous protein is comparable with that of the endogenous one, thus better resembling the conditions present in primary fibroblasts. The cell clones were obtained as described in Materials and methods and analyzed by Western blot for the presence of the PS2 proteins. Immunodetection of PS2 protein showed a very similar expression level of the 20-kDa PS2 C-terminal proteolytic fragment in both PS2 wt and M239I clones, as well as in the control clone expressing endogenous PS2 (Fig. 2A).

Once integration of exogenous cDNA was confirmed by sequencing, the different clones were used for studying changes in the $[Ca^{2+}]_i$ with the technique based on the photoprotein AEQ. This methodology was preferred to the fura-2 technique, because HEK293 cells, differently from primary fibroblasts, are easily transfectable with high efficiency by the cDNA for AEQ, thus allowing cytosolic Ca^{2+} measurements at the cell population level (see Materials and methods).

To investigate the influence of the PS2 mutation on the Ca^{2+} content of intracellular stores, cells were challenged with CPA (20 μM) and carbachol (Cch, 100 μM), an agonist linked to inositol 1,4,5-trisphosphate ($InsP_3$) production. Compared to CPA alone, this type of stimulation produces a sharper and higher rise in the $[Ca^{2+}]_i$. This protocol appears better suited to monitor with AEQ the differences in Ca^{2+} dynamics between controls and PS2 expressing clones because the photoprotein is quite insensitive in the range 200–400 nM $[Ca^{2+}]_i$ (i.e., the peak values obtained with CPA alone), while it is very sensitive in the range 1–2 μM $[Ca^{2+}]_i$ (i.e., the peak reached with CPA + Cch). When CPA and Cch were applied together in a Ca^{2+}-free medium, a fast rise in $[Ca^{2+}]_i$, due to a prompt and complete discharge of intracellular stores, was followed by a rapid return to the pre-stimulatory level (Fig. 3A, first part of the traces). This fast Ca^{2+} response is typical of agonists coupled to phospholipase C activation and has been described in a large variety of cell types (Fasolato et al., 1988; Freichel et al., 2001; Pizzo et al., 1997; Pozzan et al., 1994; Putney, 1977). As shown in Fig. 3A, in the continuous presence of CPA, activation of CCE was detected as a second large $[Ca^{2+}]_i$ peak, upon $CaCl_2$ addition (Ca^{2+}, 1 mM). Similar to fibroblasts from FAD-PS2 M239I patients, in HEK293 cells stably expressing the PS2 M239I mutation (continuous trace), depletion of intracellular stores caused an increase in the $[Ca^{2+}]_i$ that was significantly smaller than that observed in control cells (dashed trace) (mean peak amplitudes ± SEM of 0.85 ± 0.06 μM vs. 1.02 ± 0.03 μM, $P < 0.05$, n = 15 PS2 M239I, n = 20 controls; Fig. 3B). Thus, also in this latter cell model, the PS2 M239I mutation appears to be linked to a reduced Ca^{2+} release from internal stores. In contrast, no difference was found in the PS2 wt clone compared to control cells transfected with the void

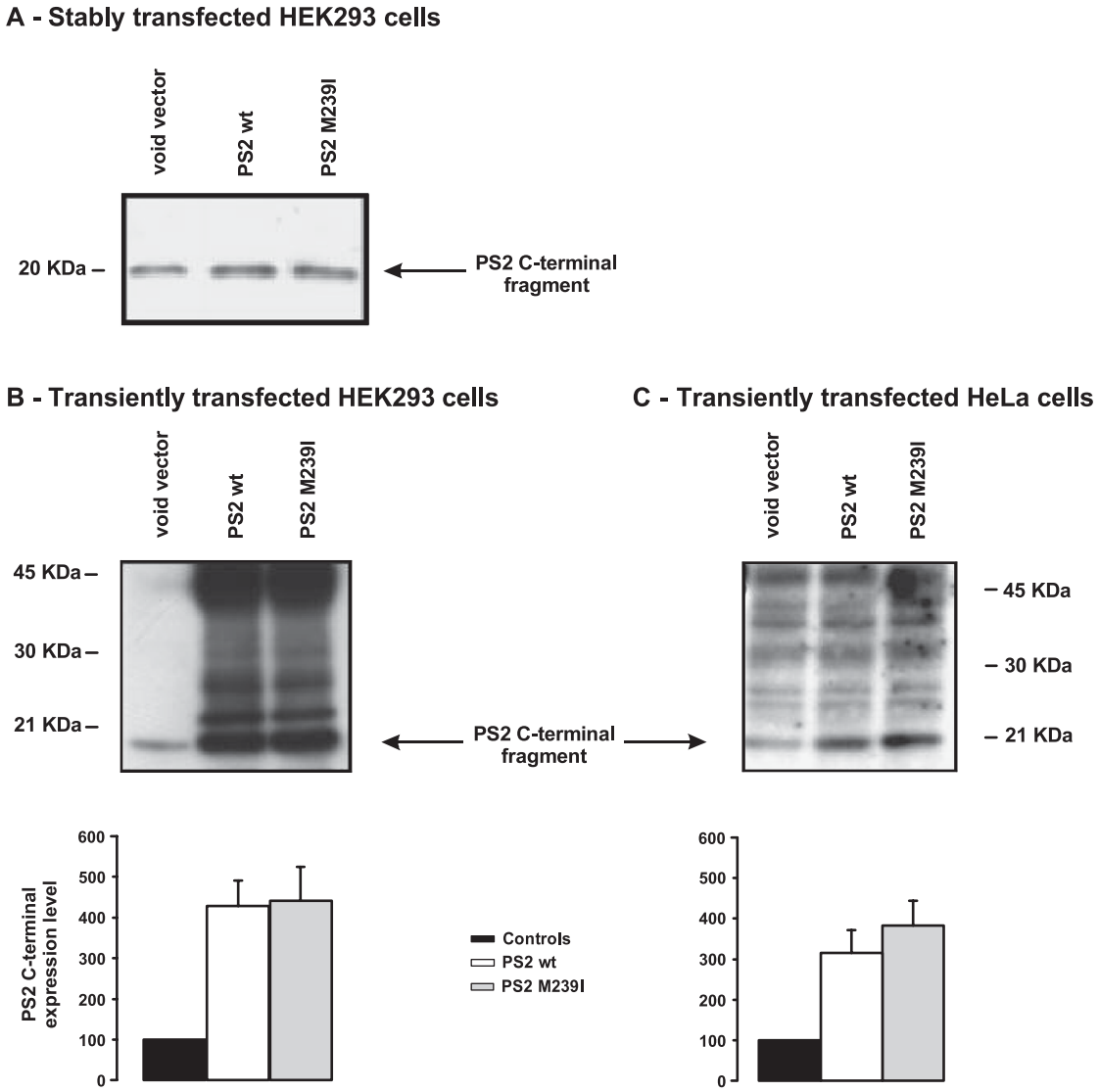

Fig. 2. HEK239 and HeLa cells stably or transiently transfected with the PS2 M239I mutation exhibit different expression levels of PS2. (A) Western blot analysis of HEK293 cell lines stably expressing human PS2 wt or mutant PS2 M239I and control cell line stably transfected with the void vector. (B and C) Western blot analysis of HEK293 and HeLa cells transiently transfected with human PS2 wt, mutant PS2 M239I, or the void vector. For each cell type, densitometric analysis of the bands corresponding to PS2 C-terminal fragment is shown in histograms (mean values ± SEM, $n = 3$). Cell lysates (15 μg per lane) were loaded on 10–20% denaturing Tris-Tricine gel and immunoblotted by using the polyclonal antibody Ab-2 PC 235 (Oncogene) that recognizes the 20 KDa PS2 C-terminal proteolitic fragment.

vector (mean peak amplitudes ± SEM of 0.98 ± 0.05 μM vs. 1.02 ± 0.03 μM, $P < 0.05$, $n = 16$ PS2 wt, $n = 20$ controls; Fig. 3B), thus excluding possible unspecific effects of exogenous PS2 expression on this Ca^{2+} pathway.

As far as CCE is concerned, again no significant difference was found among the three HEK293 clones (Fig. 3B), in agreement with the results obtained in FAD-PS2 M239I fibroblasts (Fig. 1B).

Transient over-expression of the PS2 M239I mutation reduces intracellular Ca^{2+} stores

To further confirm the effect of the PS2 M239I mutation on cellular Ca^{2+} handling, we over-expressed the mutated protein in HEK293 cells by transient co-transfection with the cDNA encoding for the photoprotein AEQ (see Materials and methods). By this approach, high levels of PS2 wt, as well as of the mutated protein, are expected without the disadvantages that are intrinsic to both stably expressing cell lines and primary cells from patients. In fact, the variability of individual clones or adaptive phenomena may erroneously be interpreted as specific properties of the protein(s) under investigation. The AEQ approach was again selected for its advantage of Ca^{2+} measurements at the population level. In fact, HEK293 cells are easily transfectable and it was previously demonstrated that, upon transient co-transfection, the two recombinant proteins are effectively expressed by the same subset of cells (Brini et al., 1995). In addition, since the majority of FAD-associated PS mutations are considered "gain of function" and thus linked to an increase in the γ-secretase activity (Haass and De Strooper, 1999), it could be predicted that over-expression of PS2 wt will, at least qualitatively, mimic the effect of the pathological

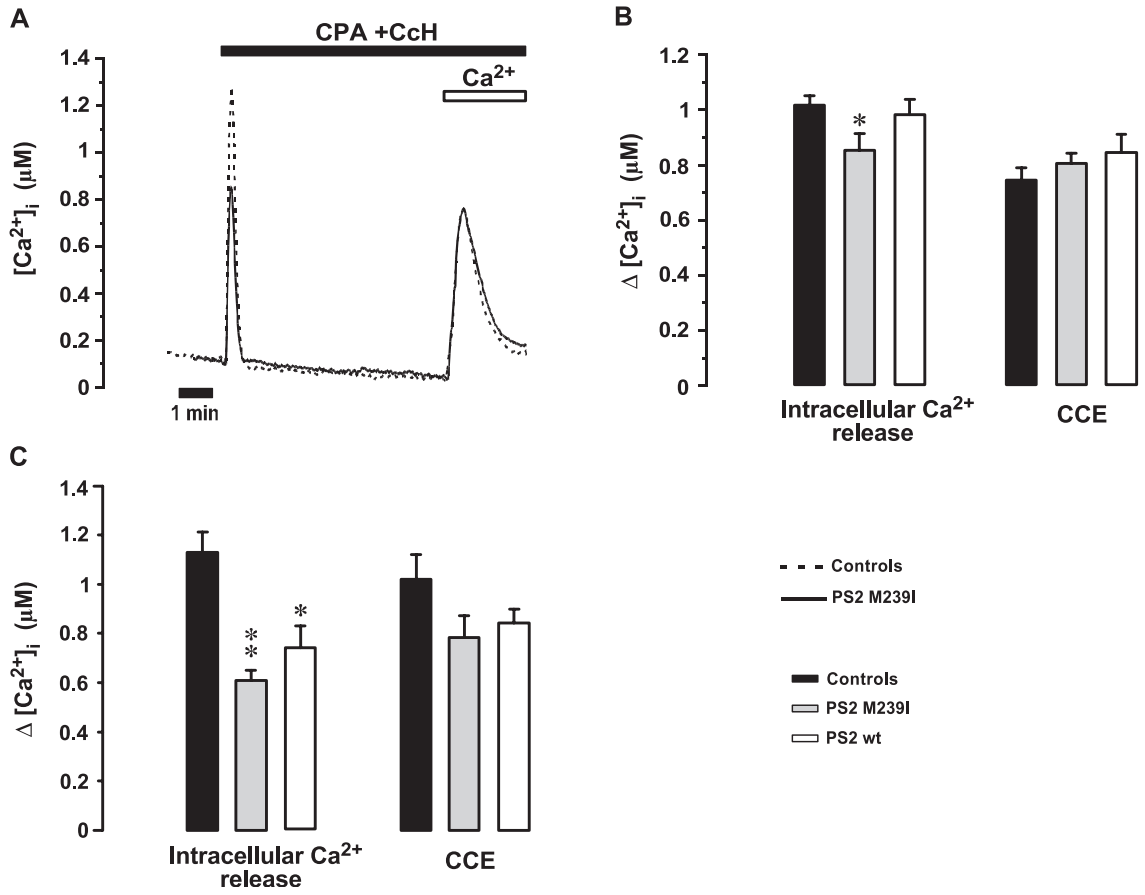

Fig. 3. HEK293 cells stably or transiently expressing the PS2 M239I mutation exhibit similar alterations of Ca^{2+} homeostasis. HEK293 clones, stably expressing the void vector (controls), the PS2 wt or the PS2 M239I mutation were transiently transfected with the cDNA encoding for AEQ, as described in Materials and methods, and then analyzed with a photon counting board; collected data were off-line calibrated into $[Ca^{2+}]_i$ values. Alternatively, in transient transfection experiments, HEK293 cells were exposed to both the cDNAs, for PS2 (wt, M239I or void vector) and AEQ, as described in Materials and methods. Panel A shows a typical protocol employed with these cells: upon perfusion in a Ca^{2+}-free medium, intracellular stores were depleted by adding CPA (20 μM) and Cch (100 μM); CCE started upon $CaCl_2$ addition (1 mM) (dashed trace, control clone; continuous trace, PS2 M239I clone). Panel B shows the average peak amplitudes measured from resting values ($\Delta[Ca^{2+}]_i$, μM, mean ± SEM) for both intracellular Ca^{2+} release (left) and CCE (right), in stable HEK clones; n = number of independent experiments (n = 20 control clone, n = 15 PS2 M239I clone, n = 16 PS2 wt clone; * = $P < 0.05$, Student's t test). Panel C shows the same values obtained with transient transfected HEK293 cells (n = 8 for controls, n = 11 for PS2 M239I and PS2 wt over-expressing cells; ** = $P < 0.001$, * = $P < 0.05$, Student's t test).

PS forms. In other words, we expect that over-expression of the PS wt should cause alterations of Ca^{2+} homeostasis similar to those caused by FAD-linked PS mutations. To obtain direct experimental evidence for this notion, HEK293 cells were transiently transfected; over-expression of PS2 wt and mutated PS2 M239I was then verified by Western blot. As expected, the expression level of the exogenous proteins (both PS2 wt and PS2 M239I) was much higher if compared to the level of the endogenous PS2 protein detected in control cells (Fig. 2B).

Fig. 3C shows the results obtained with transiently transfected HEK293 cells, following the protocol described above and represented in Fig. 3A: a reduction of Ca^{2+} release from intracellular stores was found in cells over-expressing the PS2 M239I mutation when compared to controls (mean peak amplitudes ± SEM of 0.61 ± 0.04 μM vs. 1.13 ± 0.08 μM, $P < 0.001$, n = 11 for PS2 M239I, n = 8 for controls; Fig. 3C). Interestingly, a similar effect was observed, as predicted, in cells over-expressing the PS2 wt.

Under this experimental condition, expression of PS2 M239I did not significantly modify the CCE, although a tendency to a reduction was observed (Fig. 3C), thus reinforcing the idea that the Ca^{2+} influx pathway activated by store depletion is not mainly affected by this PS2 mutation.

To validate these results in a different cell model, similar transient transfections were carried out in HeLa cells. This cell line is also suitable for Ca^{2+} measurements at the cell population level because of its high efficiency of transfection.

Fig. 4A shows two representative traces of $[Ca^{2+}]_i$ changes recorded in HeLa cells transiently co-transfected with cDNAs encoding for AEQ and the void vector (control, dashed trace) or the mutated PS2 (M239I, continuous trace). Maximal depletion of the ER Ca^{2+} content was obtained by stimulating the cells with CPA (20 μM) and the $InsP_3$-generating agonist histamine (Hist, 100 μM), here employed for the same technical reasons discussed above. Subsequent addition of $CaCl_2$ (Ca^{2+}, 1 mM) in the continuous presence of the stimulus allowed detection of CCE. Once again, PS2 M239I over-expressing cells showed a reduced Ca^{2+} release from intracellular stores compared to control cells (mean peak amplitudes ± SEM of 1.79 ± 0.08 μM vs. 2.83 ±

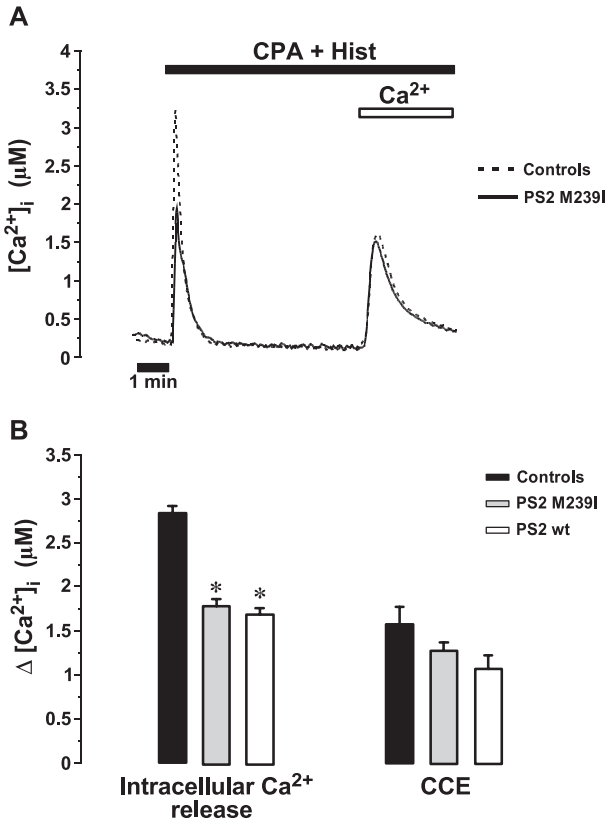

Fig. 4. Intracellular Ca^{2+} release is decreased in HeLa cells transiently transfected with the PS2 M239I mutation. HeLa cells were transiently co-transfected with the cDNA encoding for the PS2 wt, the PS2 M239I mutation, or the void vector (control), together with cDNA for AEQ. (Panel A) The experimental protocol is similar to that employed in Fig. 3: upon perfusion in a Ca^{2+}-free medium, cells were challenged with CPA (20 μM) and Hist (100 μM) to emptying the stores; $CaCl_2$ (1 mM) was then added to recording CCE (dashed trace, control cells; continuous trace, PS2 M239I transfected cells). Panel B shows the average peak amplitudes, measured from resting values ($\Delta[Ca^{2+}]_i$, μM, mean ± SEM) for both intracellular Ca^{2+} release (left) and CCE (right); n = number of independent experiments (n = 19 controls, n = 15 PS2 M239I, n = 15 PS2 wt; * = $P < 0.001$, Student's t test).

0.08 μM, $P < 0.001$, n = 15 for PS2 M239I, n = 19 for controls; Fig. 4B).

Also in this cell model, expression of PS2 M239I did not significantly alter the CCE, showing, however, the same tendency to a reduction observed in transiently transfected HEK293 cells (see Figs. 3C and 4B).

Discussion

The aim of the present study was to determine the influence on cellular Ca^{2+} homeostasis of the new FAD-PS2 M239I mutation (Finckh et al., 2000b). In fact, recent evidence suggests that abnormalities in cell Ca^{2+} signaling are common features in the pathogenesis of a range of neurodegenerative disorders, including AD (Mattson et al., 2000). It is well known that Ca^{2+} is one of the most relevant intracellular messengers, being essential in neuronal development, synaptic transmission, and plasticity, as well as in the regulation of various metabolic pathways at the brain level. However, in Alzheimer's pathology, the nature of alterations in Ca^{2+} homeostasis is highly controversial. In fact, increased (Hirashima et al., 1996; Ito et al., 1994), decreased (Grossmann et al., 1993; McCoy et al., 1993; Peterson et al., 1986, 1988; Tatebayashi et al., 1995), as well as unchanged (Borden et al., 1992; Gibson et al., 1997) Ca^{2+} responses have variously been reported in peripheral cells from AD patients, as compared to control cells, upon different stimulation protocols. Furthermore, opposite effects on both Ca^{2+} release from intracellular stores and CCE have also been reported in various cell models expressing different FAD-linked PS mutations (as well as FAD-associated APP mutations (Gibson et al., 1997)). In particular, mutations in PS1 (A246E and M146V) cause, upon agonist stimulation, higher Ca^{2+} release from intracellular stores and increased excitotoxicity in neurons from transgenic mice (Chan et al., 2000; Guo et al., 1999; Schneider et al., 2001). Furthermore, FAD-associated PS1 mutations have been shown to increase the level of ryanodine receptors and Ca^{2+} release in cortical neurons and PC12 cells (Chan et al., 2000), and endogenous PS2 has been reported to interact molecularly with sorcin, a modulator of the ryanodine receptor (Pack-Chung et al., 2000). Regarding CCE, it has recently been reported that the FAD-linked PS2 N141I mutation, as well as the PS1 M146L and ΔE9 mutations, all down-regulate CCE induced by Tg in cultured cortical neurons from transgenic mice and in stably transfected neuroblastoma SH-SY5Y cells (Smith et al., 2002; Yoo et al., 2000). In contrast, the PS1 A246E FAD-linked mutation increases Ca^{2+} influx induced by Tg or agonists in acutely dissociated hippocampal neurons from transgenic mice (Schneider et al., 2001), whereas no effect on CCE, induced by Tg, was found in PS1 M146V-knockin fibroblasts (Leissring et al., 2000). The emerging theme is that all the different mutations perturb ER Ca^{2+} homeostasis in a way resulting in sensitization of neurons to apoptosis and excitotoxicity (for a review, see Mattson et al., 2000).

We first analyzed primary fibroblasts from FAD-PS2 M239I and sporadic AD patients, as well as cells from healthy control subjects. We found that cells from patients carrying the PS2 M239I mutation showed a reduction in the Ca^{2+} content of intracellular stores compared to control cells. In contrast, sporadic AD fibroblasts did not differ in that parameter from control fibroblast cell lines, suggesting a determinant role of the PS2 M239I mutation in this phenotype.

As far as CCE is concerned, FAD-PS2 M239I cells showed a normal Ca^{2+} influx pathway activated by store depletion with respect to controls. In contrast, a marked increase in CCE was present in fibroblasts from sporadic AD patients compared to controls. This interesting, rather unexpected, result requires to be further investigated to define, in the sporadic AD patients belonging to this study, a possible influence of environmental/metabolic factors or genetic background on this specific Ca^{2+} pathway.

The PS2 M239I mutation affects cellular Ca^{2+} handling also in two other different cell models: stably and transiently transfected HEK293 cells and transiently transfected HeLa cells. Ca^{2+} release from intracellular stores was significantly reduced also in PS2 M239I stably or transiently expressing cells, strengthening the idea that this PS2 mutation influences the ER Ca^{2+} content. Again, as far as CCE is concerned, it was not significantly affected by expression of the PS2 M239I mutation in all the three experimental conditions analyzed, in agreement with the results obtained from primary FAD fibroblasts.

It is worth noting that the over-expression in HEK293 and HeLa cells of PS2 wt showed effects similar to those caused by the mutated form (Figs. 3C and 4B). This result was predictable given that the majority of FAD associated PS mutations are presumably linked to a toxic "gain of function" (Haass and De Strooper, 1999). This conclusion is further strengthened by the observation that PS knockout mice do not develop the Alzheimer's phenotype (Yoo et al., 2000). Thus, the over-expressed PS2 wt can mimic the effects on Ca^{2+} homeostasis elicited by the mutated PS2 M239I protein. On the other hand, unspecific effects, due to protein overload, can be excluded given that a "loss of function" PS2 protein, carrying the D257A mutation (Yu et al., 2000), did not affect Ca^{2+} release and CCE in transiently transfected cells (data not shown). In addition, the lack of effect of the PS2 wt in HEK cells stably expressing the protein could simply be explained by the fact that the protein level, as shown from Western blot, was comparable to that found in vector-transfected cells. At variance with this latter clone, in HEK and HeLa cells transiently over-expressing wt or mutated PS2, the level of the protein was clearly increased compared to control cells. Noteworthy, in transient transfections, the percentage of cells expressing exogenous PS2 proteins is about 50%, and thus the level of transfected protein per cell was underestimated. It is therefore conceivable that, because of the higher amount found in over-expressing cells, the PS2 wt protein functions as the mutated form, inducing similar alterations in Ca^{2+} homeostasis.

Our findings, showing that the PS2 M239I mutation causes similar alterations of Ca^{2+} homeostasis in three different experimental models, strongly favor the idea that this mutation directly alters Ca^{2+} handling. It is worth noting that its effect on Ca^{2+} content of intracellular stores substantially differs from that described for a wide number of FAD-linked PS mutations (for a review, see LaFerla, 2002). From the clinical point of view, neuropathological and clinical phenotype of FAD-associated PS2 M239I mutation can be considered relatively mild and thus different (in terms of age of onset and disease duration) from the more aggressive form of the disease caused by PS1 mutations (Finckh et al., 2000b). Various reports from the literature show a decreased, rather than an increased, Ca^{2+} response in FAD peripheral cells upon different stimulation protocols (Grossmann et al., 1993; McCoy et al., 1993; Peterson et al., 1986, 1988; Tatebayashi et al., 1995), thus contrasting the hypothesis of cellular Ca^{2+} overload in AD pathogenesis (LaFerla, 2002). These findings can be explained by assuming that the different PS mutations exert multiple effects on cell physiology. As an example, it has been shown that, in fibroblasts, mutations in different domains of PS1 modulate the signal transduction pathway of adenylate cyclase in opposite ways, resulting in either a decrease (Huang and Gibson, 1993) or an increase (Vestling et al., 1997) in adenylate cyclase activity upon β-adrenergic-receptor stimulation. These reports and the data here presented suggest the possibility that, in FAD, alterations in multiple signal transduction systems can lead to divergent phenotypic patterns because of the carried mutation. Alternatively, given that $InsP_3$- and ryanodine-sensitive stores can differ significantly among various cell types, the discrepancy in the literature could be due to methodological aspects, including the cell models and the experimental protocols employed.

Our conclusion that the PS2 M239I mutation reduces the Ca^{2+} content of intracellular stores with minor, or non-significant, effects on CCE has been reached by analyzing, at the same time, both Ca^{2+} release and Ca^{2+} influx in three different cell models and by employing different experimental protocols. By this integrated approach, we can state that the PS2 M239I mutation exerts a primary effect on ER Ca^{2+} homeostasis. The precise linkage between PS and other proteins directly involved in Ca^{2+} handling remains, however, to be established yet and requires further investigation.

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