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Brief Report

Capacitative Calcium Entry Deficits and Elevated Luminal Calcium Content in Mutant Presenilin-1 Knockin Mice

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Abstract. Dysregulation of calcium signaling has been causally implicated in brain aging and Alzheimer’s disease. Mutations in the presenilin genes (PS1, PS2), the leading cause of autosomal dominant familial Alzheimer’s disease (FAD), cause highly specific alterations in intracellular calcium signaling pathways that may contribute to the neurodegenerative and pathological lesions of the disease. To elucidate the cellular mechanisms underlying these disturbances, we studied calcium signaling in fibroblasts isolated from mutant PS1 knockin mice. Mutant PS1 knockin cells exhibited a marked potentiation in the amplitude of calcium transients evoked by agonist stimulation. These cells also showed significant impairments in capacitative calcium entry (CCE, also known as store-operated calcium entry), an important cellular signaling pathway wherein depletion of intracellular calcium stores triggers influx of extracellular calcium into the cytosol. Notably, deficits in CCE were evident after agonist stimulation, but not if intracellular calcium stores were completely depleted with thapsigargin. Treatment with ionomycin and thapsigargin revealed that calcium levels within the ER were significantly increased in mutant PS1 knockin cells. Collectively, our findings suggest that the overfilling of calcium stores represents the fundamental cellular defect underlying the alterations in calcium signaling conferred by presenilin mutations.

Key words: Alzheimer’s disease • endoplasmic reticulum • phosphoinositide signaling • store-operated calcium channel • store-operated calcium entry

Introduction

Alzheimer’s disease (AD) is the leading cause of age-related dementia (Selkoe, 1999). Certain familial forms of AD (FAD) are characterized by an autosomal-dominant inheritance pattern and a tragically early age of onset, and most of these have been linked to mutations in the two presenilin genes (PS1, PS2). The PS1 and PS2 genes encode highly conserved, polytopic integral membrane proteins that are widely expressed not only within the central nervous system (Cribbs et al., 1996), but also in many peripheral tissues (Sherrington et al., 1995). Intracellularly, in both neural and nonneuronal cells, the presenilins are localized predominantly to the ER (Cook et al., 1996).

The precise mechanisms by which presenilin mutations lead to AD neurodegeneration are currently unresolved. It is well established that presenilin mutations lead to increased production of the longer species of β-amyloid (Aβ) from the Aβ precursor protein (AβPP; Scheuner et al., 1996). A PP mismetabolism, however, need not represent the exclusive or most primary consequence of presenilin mutations. It remains possible that other pathological effects of presenilin mutations upstream of and/or independent of Aβ generation may also contribute to the mo-
lular and cellular changes characterizing A D neurode-
geneneration.

Mounting evidence has established that presenilin muta-
tions confer highly specific alterations in intracellular cal-
cium signaling pathways. For instance, a potentiation of
inositol 1,4,5-trisphosphate (InsP₃)-mediated calcium sig-
nals has been documented in an array of experimental sys-
tems expressing PS1 mutations, ranging from X enopus oo-
cytes to transgenic animals (G uo et al., 1996, 1998, 1999;
B egley et al., 1999; L eissing et al., 1999a,b). Similar alter-
ations have also been observed in studies of human fibro-
basts harboring FAD mutations (I to et al., 1994; G ibson et
al., 1996; E tchberrigaray et al., 1998). The calcium sig-
naling changes in FAD fibroblasts are highly selective and
specific for the disease, being present in affected individu-
als, but not in unaffected family members (E tchberrigaray
et al., 1998). M orover, mutations in PS2 produce al-
terations in calcium signaling indistinguishable from PS1
mutations (L eissing et al., 1999a,b), providing further sup-
port that these changes represent a common patho-

genic feature of all FAD-linked presenilin mutations.

Several lines of evidence suggest that the dysregu-
lation of calcium signaling conferred by presenilin mutations
plays a causal role in the pathogenesis of FAD, underlying
both the neuronal degeneration and the hallmark patho-
logical features of the disease. For instance, elevated levels
of cytosolic calcium ([Ca²⁺]) in cultured cells can modu-
late the processing of A βP and thereby increase A β pro-
duction (Q uerfurth and S elkoe, 1994). This underscores
the likelihood that calcium dysregulation is at least one
cause of increased A β production, and thus possibly con-
tributes to plaque formation. Furthermore, increased
[Ca²⁺] has also been shown to increase hyperphosphoryla-
tion of tau (M attson et al., 1991). Finally, altered calcium
homeostasis is centrally involved in the increased suscep-
tibility to cell death conferred by PS1 mutations (G uo et
al., 1996, 1998, 1999; M attson et al., 2000).

Despite the likely involvement of calcium disturbances
in the pathogenesis of FAD, very little is known about the
precise cellular mechanisms by which presenilin mutations
alter calcium signaling pathways. To address this issue, we
studied calcium signaling in fibroblasts from homozygous
mutant PS1 KI mice. In these genetically al-
tered mice, the endogenous mouse PS1 gene has been re-
placed by the human counterpart containing the FAD-
linked mutation, PS1M146V (G uo et al., 1999). This model
possesses many advantages over other experimental para-
digms, since the mutant human PS1 protein is expressed to
physiological levels, and the endogenous tissue and cellu-
lar expression pattern is maintained. H ence, concerns
about protein overexpression artifacts, ectopic expression,
or confounding influences of the wild-type protein are ob-
viated. H ere, we demonstrate that agonist-evoked calcium
signals are markedly potentiated in the PS1M146V-KI f1
fibroblasts. In addition, we report the novel finding that
PS1M146V-KI cells show deficits in capacitative calcium en-
try (CCE), i.e., the influx of extracellular calcium triggered
by depletion of intracellular calcium stores. Finally, we
provide evidence that both of these alterations are attribu-
table to an abnormal elevation of ER calcium stores in
the mutant cells. Thus, these findings provide a novel cel-

Materials and Methods

Cell Culture

The validation and characterization of the PS1M146V-KI mouse has been de-
described elsewhere (G uo et al., 1999). To isolate fibroblasts, snips of tail
from neonatal homozygous PS1M146V-KI animals and controls were
washed with 70% ethanol, minced in CM F (Ca²⁺⁻ and M g²⁺⁻free H BSS),
and incubated for 30 min at 37°C in 2.5 mL of T CH solution (0.125%
trypsin, 0.5 mM E D T A and 1 mg/mL collagenase Type V (S igma Ch emi-
cal C o.) in H B SS). A fter quenching the reaction by addition of D ME supple-
mented with 20% F BS, the supernatant was removed and spun at 225 g
for 5 min, and the pelleted cells were resuspended in D ME/20% F BS.
C ells were maintained at 37°C in a 5% C O₂ atmosphere. For calci-
imaging experiments, P1-P4 cultures were plated onto glass-bottomed 6-cm
perti dishes (M ateK Corp.) and grown to near confluency. 1-2 h before
imaging, c ells were loaded for 45 min at room temperature with 5 μM
Fura 2-A M supplemented with pluronic acid F-127 (M olecular P robes) in
Hepes buffered control solution (HCSS) containing 120 mM NaCl, 5.4
mM KCl, 0.8 mM MgCl₂, 2 mM CaCl₂, 15 mM glucose, 20 mM H epes, pH
7.3, and 0.5% phenol red (calci um-deficient H CSS contained 1 mM E
G TA and no CaCl₂). C ells were washed three times with H CSS and al-
lowed to incubate for at least 30 min before imaging. A l l reagents were
purchased from L ife T echnologies, Inc. unless otherwise noted.

Calcium Imaging

M easurements of [Ca²⁺], were obtained using the InCyt Im2™ C a lci-
imaging System (Intracellular Imaging Inc.) using excitation at 340 and 380 nm.
T he system was calibrated using stock solutions containing either no cal-
im (0 calcium plus 1 mM E G TA) or a saturating level of calcium (1 mM)
using the formula [Ca²⁺] = K [F o/F s] (225) [(R – R min)/R max – R i] (F o/F s).
D rugs were added by superfusion via a peristaltic pump and removed by
vacuum. A ll experiments were performed using fibroblasts harvested from
at least three different animals (n = 5-28 cells per experiment). Quantitative
data were analyzed by one-way A NOVA and are expressed as mean ± S E M.

Results

Potentiation of Agonist-evoked Calcium Signals in
Mutant PS1 Knockin Cells

W e studied calcium signals in fibroblasts isolated from
neonatal mutant PS1 KI mice and controls using the cyto-

doic acid calcium indicator Fura-2 A M. T o activate the phos-
phoinositide/calcium signaling cascade, cells were stimu-
lated with the c ell surface receptor agonists, bradykinin (B K;
F ig. 1) or bombesin (data not shown). In control fibro-
blasts, BK stimulation evoked calcium signals with two
characteristic phases: a transient rise in [Ca²⁺], lasting se-
conds, followed by a sustained phase of elevated [Ca²⁺],
lasting several minutes (F ig. 1 a).

C alcium signals in PS1M146V-KI fibroblasts differed from
controls in several salient respects. F irst, the rate of rise
of the calcium signal was significantly increased (F ig. 1, a and
b, inset). S econd, the peak of the transient phase was sig-
nificantly potentiated (F ig. 1, a and b). F inally, the sustained
phase of elevated [Ca²⁺], present in control cells was vir-
tually absent in the mutant fibroblasts (F ig. 1, a and b).

Impaired Capacitative Calcium Entry in Mutant PS1

Knockin Cells

The sustained elevation of [Ca²⁺], in control cells resem-

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bles CCE, in which depletion of ER calcium stores triggers the influx of extracellular calcium through store-operated calcium channels (SOCCs; Lewis, 1999). To determine whether the sustained phase resulted from calcium influx, we stimulated cells with BK in calcium-deficient medium. Removal of extracellular calcium abolished the sustained phase in control cells, but had little effect on the calcium signal in PS1<sub>M146V</sub>-KI cells, indicating that the sustained phase of elevated [Ca<sup>2+</sup>], in control cells is CCE, and that CCE is disrupted in cells harboring a mutation in PS1.

To quantify the magnitude of CCE after agonist stimulation, cells were initially stimulated with BK in calcium-deficient medium. Subsequently, after the initial calcium transients had returned to baseline, the calcium-deficient medium was replaced with medium containing 2 mM calcium (Fig. 2 a). Relative to controls, the magnitude of CCE upon calcium readition was significantly reduced in PS1<sub>M146V</sub>-KI cells after stimulation with 50 nM BK (Fig. 2, a and b). Intriguingly, in similar experiments using higher concentrations of BK (5 μM), the deficits in CCE in PS1<sub>M146V</sub>-KI cells, though still present, were significantly attenuated (see Fig. 3). Furthermore, no differences in CCE were observed after complete depletion of calcium stores with thapsigargin (TG; Figs. 2, c and d, and 3). Thus, as illustrated in Fig. 3, the magnitude of the deficits in CCE in PS1<sub>M146V</sub>-KI cells varied according to the degree and type of stimulation, with weak agonist stimulation eliciting the greatest deficits and complete store depletion eliciting no deficits whatsoever.

**Elevated Calcium-Store Content in Mutant PS1 Knockin Mice**

The preceding results suggested that the PS1<sub>M146V</sub>-KI cells possess functional SOCCs, but are impaired in their ability to trigger CCE when weakly stimulated. Since the trigger for CCE is the depletion of intracellular calcium stores, we...
hypothesized that CCE is impaired in PS\textsubscript{1\textsubscript{M146V}}-KI cells because their ER calcium levels are abnormally elevated. On this view, weak agonist stimulation, which elicits only a transient release of ER calcium through rapidly inactivating InsP\textsubscript{3} receptors (Parekh et al., 1997), fails to deplete stores in the PS\textsubscript{1\textsubscript{M146V}}-KI cells to the threshold required for CCE activation (see Fig. 5). To test this idea, the intracellular calcium stores of resting cells were released by treatment with TG or ionomycin in the nominal absence of extracellular calcium (Fig. 4). The TG-releasable calcium pool was significantly larger in PS\textsubscript{1\textsubscript{M146V}}-KI relative to controls, as indicated by increases in both the peak value of TG-evoked calcium transients (Fig. 4, a and b) and in their rate of rise (1.67 ± 0.12 nM s\textsuperscript{-1} versus 1.11 ± 0.11 nM s\textsuperscript{-1}, respectively, P < 0.01). Similar results were obtained when cells were treated with the calcium ionophore ionomycin in calcium-deficient medium (Fig. 4, c and d). These data indicate that intracellular calcium stores, including the ER, are increased in cells from PS\textsubscript{1\textsubscript{M146V}}-KI mice. Collectively, our results suggest that the potentiation of calcium transients and absence of CCE observed in mutant PS\textsubscript{1\textsubscript{M146V}}-KI cells are attributable to increased ER calcium levels.

**Figure 3.** Comparison of capacitative calcium entry evoked by weak or strong agonist stimulation or TG treatment. Data are shown for experiments using the protocol illustrated in Fig. 2, in which cells were stimulated with either 0.05 \( \mu \)M BK, 5 \( \mu \)M BK, or 1 \( \mu \)M TG. Note that the data are expressed as the increase in \([\text{Ca}^{2+}]_i\), relative to \([\text{Ca}^{2+}]_i\), 30 s before readdition of extracellular calcium. *P < 0.01.

**Figure 4.** Intracellular calcium-store content is elevated in PS\textsubscript{1\textsubscript{M146V}}-KI fibroblasts. a, Typical calcium signals evoked by 1 \( \mu \)M TG in calcium-deficient medium. b, Quantitative data for \( n = 7 \) experiments showing basal \([\text{Ca}^{2+}]_i\) and peak \([\text{Ca}^{2+}]_i\) induced by TG treatment. c, Typical calcium signals evoked by 1 \( \mu \)M ionomycin (IONO) in calcium-deficient medium. d, Quantitative data for \( n = 9 \) experiments showing basal \([\text{Ca}^{2+}]_i\) and peak \([\text{Ca}^{2+}]_i\) evoked by ionomycin treatment. *P < 0.01.

**Discussion**

In this study we investigated calcium signaling in fibroblasts from KI mice harboring a PS\textsubscript{1} mutation linked to FAD. Relative to controls, cytosolic calcium signals from PS\textsubscript{1\textsubscript{M146V}}-KI fibroblasts exhibited a significant potentiation in calcium released by agonist activation of the phosphoinositide signaling pathway. In addition, KI cells exhibited deficits in CCE evoked by agonist stimulation, but not by complete depletion of ER calcium stores. We conclude that both of these alterations are attributable to the elevation of ER calcium content in PS\textsubscript{1\textsubscript{M146V}}-KI fibroblasts.

The agonist-evoked calcium signals in PS\textsubscript{1\textsubscript{M146V}}-KI fibroblasts are virtually indistinguishable from comparable experiments with human fibroblasts from FAD patients harboring PS\textsubscript{1} mutations (compare our Fig. 1 a to Figure 1 a in Ito et al., 1994). Thus, the PS\textsubscript{1\textsubscript{M146V}}-KI animals faithfully mimic the calcium signaling changes seen in presenilin-associated FAD. Importantly, since the mutant PS\textsubscript{1} protein is expressed to physiological levels in these animals (Guo et al., 1999), none of the observed changes is attributable to protein overexpression.

It is notable that, unlike human FAD fibroblasts, the cells in this study were isolated from neonatal animals. This suggests that the changes in calcium signaling in FAD fibroblasts do not merely reflect secondary consequences of A D pathology, such as the accumulation of mitochondrial mutations during the lifetime of the individual that have been shown to affect calcium signaling in cybrids transformed with mitochondria from nonfamilial A D patients (Sheehan et al., 1997). Rather, our findings support the hypothesis that altered calcium signaling is an early and chronic consequence of PS\textsubscript{1} mutations, one that may play a causal role in the pathogenesis of FAD.

Although the present study was focused on fibroblasts, the potentiation of calcium transients has been described in neuronal cells from these same animals (Guo et al., 1999; Leissring, M.A., Y. A. kbari, and F.M. LaFerla, manuscript in preparation). This strongly suggests that the alterations in calcium signaling observed in peripheral cells from FAD patients may be directly involved in FAD neurodegeneration and memory loss (Disterhoft et al., 1994; Mattson et al., 2000). This finding also supports the use of fibroblasts as a model to study the pathological alterations in calcium signaling associated with presenilin mutations.

Our results suggest that elevated ER calcium levels are a fundamental cellular defect underlying the alterations in calcium signaling conferred by presenilin mutations. As illustrated in Fig. 5, we postulate that the higher levels of ER calcium in PS\textsubscript{1\textsubscript{M146V}}-KI cells would impair CCE by preventing agonist stimulation from depleting intracellular calcium stores beyond the threshold level required to activate CCE. Moreover, this model provides a satisfactory cellular mechanism to account for several observations made in other experimental systems. First, elevated ER calcium levels, by increasing the driving force on calcium across the ER, would be expected to increase the ampli-
tude of calcium release transients, as has been documented in many systems (Ito et al., 1994; Gibson et al., 1996; Guo et al., 1996, 1998; Etcheberrigaray et al., 1998; Leissring et al., 1999a,b). Second, an increased driving force on calcium would also increase the rate of ER calcium efflux and the average quantal content of elementary calcium release events, the fundamental building blocks making up global calcium signals (Matsson et al., 2000; Leissring, M.A., F.M. LaFerla, N. Callamaras, and I. Parker, manuscript submitted for publication). Third, because ER calcium levels can modulate the activity of IP$_3$ receptors (Missiaen et al., 1992), elevated calcium stores may also account for the increased sensitivity of cells expressing presenilin mutations to IP$_3$ stimulation. Finally, an overfilling of calcium stores may also explain the interesting observation that long-term potentiation is altered in mutant PS1 transgenic animals (Parent et al., 1999; Matsson et al., 2000), raising the specter that calcium dysregulation may underlie the memory impairments characterizing AD (Disterhoft et al., 1994).

The mechanism by which PS1 mutations elevate intracellular calcium stores is not yet known. Intriguingly, however, FAD fibroblasts harboring PS1 mutations have elevated levels of acylphosphatase, an enzyme that modulates the activity of the ER calcium-ATPases responsible for loading the ER (Liguri et al., 1996). Thus, overactivation of ER calcium pumps may underlie the overfilling of calcium stores. Future research into the molecular mechanisms by which presenilin mutations increase ER calcium levels could uncover novel targets for therapeutic intervention.

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Figure 5. Model of the perturbations in calcium signaling in PS1<sup>M146V</sup>-KI mice. a, Schematic diagram illustrating changes in cytosolic calcium signals after weak agonist stimulation, b. Similar diagram showing calcium levels within the lumen of the ER. A weaker agonist stimulation, luminal calcium levels in control cells fall well below the threshold level required for activation of CCE. In contrast, weak agonist stimulation barely triggers CCE in PS1<sup>M146V</sup>-KI fibroblasts because luminal calcium levels are abnormally elevated.

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