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-
Molecular and cellular changes characterizing A D neurodegeneration.

Mounting evidence has established that presenilin mutations confer highly specific alterations in intracellular calcium signaling pathways. For instance, a potentiation of inositol 1,4,5-trisphosphate (InsP$_3$)-mediated calcium signals has been documented in an array of experimental systems expressing PS1 mutations, ranging from X enopous oo-
cytes to transgenic animals (Guo et al., 1996, 1998, 1999; Begley et al., 1999; Leissring et al., 1999a,b). Similar alterations have also been observed in studies of human fibro-
blasts harboring F A D mutations (Ito et al., 1994; Gibson et al., 1996; Echberrigaray et al., 1998). The calcium sign-
caling changes in F A D fibroblasts are highly selective and specific for the disease, being present in affected individu-
ants, but not in unaffected family members (Echberrigaray et al., 1998). Moreover, mutations in P S 2 produce alter-
tations in calcium signaling indistinguishable from PS1 mutations (Leissring et al., 1999a,b), providing further support that these changes represent a common patho-
genic feature of all F A D -linked presenilin mutations.

Several lines of evidence suggest that the dysregulation of calcium signaling conferred by presenilin mutations plays a causal role in the pathogenesis of F A D , underlying both the neuronal degeneration and the hallmark patho-
logical features of the disease. For instance, elevated levels of cytosolic calcium ([Ca$^{2+}$i]) in cultured cells can modu-
late the processing of A P P and thereby increase A B production (Querfurth and Selkoe, 1994). This underscores the likelihood that calcium dysregulation is at least one cause of increased A B production, and thus possibly con-
tributes to plaque formation. Furthermore, increased [Ca$^{2+}$i] has also been shown to increase hyperphosphoryla-
tion of tau (M atson et al., 1991). Finally, altered calcium homeostasis is centrally involved in the increased suscepti-
Bility to cell death conferred by PS1 mutations (Guo et al., 1996, 1998, 1999; Matson et al., 2000).

Despite the likely involvement of calcium disturbances in the pathogenesis of F A D , 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 knockin (K I) mice. In these genetically al-
tered mice, the endogenous mouse PS1 gene has been re-
placed by the human counterpart containing the F A D-
linked mutation, P S 1M$_{146V}$ (Guo 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 cellular 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 P S 1M$_{146V}$-K I fibro-
blasts. In addition, we report the novel finding that P S 1M$_{146V}$-K I 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 attrib-
utable to an abnormal elevation of E R calcium stores in the mutant cells. Thus, these findings provide a novel cel-
lar mechanism to account for the calcium signaling alter-
ations conferred by presenilin mutations, which could lead to the development of novel therapeutics for presenilin-
associated F A D .

Materials and Methods

Cell Culture

The derivation and characterization of the P S 1M$_{146V}$-K I mice has been de-
scribed elsewhere (Guo et al., 1996). To isolate fibroblasts, snips of tail from neonatal homozygous P S 1M$_{146V}$-K I animals and controls were washed with 70% ethanol, minced in CMF (Ca$^{2+}$- and M g$^{2+}$-free H B S S), and incubated for 30 min at 37°C in 2.5 ml of T C H solution (0.125% trypsin, 0.5 mM E D T A and 1 mg/ml collagenase Type V (Sigma Chemical Co.) in H B S S. A fter quenching the reaction by addition of D M E 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 M E/20% F BS . Cells were maintained at 37°C in a 5% C O$_2$ atmosphere. For calcium im-
ageing experiments, P 1-P 4 cultures were plated onto glass-bottomed 6-cm petri dishes (M aTeK Corp.) and grown to near confluency. 1-2 h before imaging, cells were loaded for 45 min at room temperature with 5 pM F ura-2 A M supplemented with pluronic acid F-127 (M olecular Probes) in H epe buffered control solution (H CSS) containing 120 mM N aC l, 5.4 mM K C l, 0.8 mM M gC l$_2$, 2 mM CaC l$_2$, 15 mM glucose, 20 mM H epes, pH 7.3, and 0.5% phenol red (calcium-deficient H CSS contained 1 mM E G T A and no CaC l$_2$). Cells were washed three times with H CSS and al-
lowed to incubate for at least 30 min before imaging. A ll reagents were purchased from L ife T echnologies, Inc. unless otherwise noted.

Calcium Imaging

Measurements of [Ca$^{2+}$i] were obtained using the InCy t Im$^2$™ R atio I maging System (Intracellular Imaging Inc.) using excitation at 340 and 380 nm. The system was calibrated using stock solutions containing either no calcium (0 calcium plus 1 mM E G T A ) or a saturating level of calcium (1 mM) using the formula [Ca$^{2+}$i] = K$_{d}$(I + [Ca$^{2+}$]) ([R$^{-}$ - R$_{\text{min}}$]/[R$_{\text{max}}$ - R$_{-}$]) (F o / F s). Drugs 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

We studied calcium signals in fibroblasts isolated from neonatal mutant P S 1 K I mice and controls using the cyto-
solic calcium indicator F ura-2 A M . T o activate the phos-
phoinositide/calcium signaling cascade, cells were stimu-
lated with the cell 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 [Ca$^{2+}$i], lasting sec-
onds, followed by a sustained phase of elevated [Ca$^{2+}$i], lasting several minutes (F ig. 1 a).

Calcium signals in P S 1M$_{146V}$-K I fibroblasts differed from controls in several salient respects. First, the rate of rise of the calcium signal was significantly increased (F ig. 1, a and b, inset). Second, the peak of the transient phase was sig-
nificantly potentiated (F ig. 1, a and b). Finally, the sustained phase of elevated [Ca$^{2+}$i] present in control cells was virtu-
ally absent in the mutant fibroblasts (F ig. 1, a and b).

Impaired Capacitative Calcium Entry in Mutant PS1 Knockin Cells

The sustained elevation of [Ca$^{2+}$i] in control cells resem-
Elevated Calcium-Store Content in Mutant PS1 Knockin Mice

The preceding results suggested that the PS1\(^{M146V}\)-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 eliminated 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\(^{M146V}\)-KI cells, indicating that the sustained phase of elevated [Ca\(^{2+}\)]\(_i\), in control cells reflects calcium influx (compare Fig. 1, a and c). Notably, the initial calcium transients in both control and PS1\(^{M146V}\)-KI cells, which reflect calcium release from intracellular stores, were relatively unaffected by removal of extracellular calcium (compare Fig. 1, a and c). Therefore, the potentiation of the initial calcium transient seen in PS1\(^{M146V}\)-KI cells is attributable to increased release of calcium from intracellular stores rather than calcium influx.

To determine if the sustained calcium phase reflected CCE, we treated control cells with SKF-96365, an agent known to block CCE through SOCCs (Merritt et al., 1990). As illustrated in Fig. 1 d, SKF-96365 treatment eliminated the sustained phase of [Ca\(^{2+}\)]\(_i\), in control cells stimulated with BK in calcium-containing medium. Collectively, these data indicate that the sustained phase of elevated [Ca\(^{2+}\)]\(_i\) 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, 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 readdition was significantly reduced in PS1\(^{M146V}\)-KI cells after stimulation with 50 nM BK (Fig. 2, a and b). Intriguingly, in similar experiments using higher concentrations of BK (5 \(\mu\)M), the deficits in CCE in PS1\(^{M146V}\)-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\(^{M146V}\)-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.

Figure 1. A gonist-evoked calcium signals are altered in PS1\(^{M146V}\)-KI fibroblasts: evidence for impaired capacitative calcium entry. a, Typical traces showing calcium signals induced by 50 nM BK. b, Quantitative data from 11 experiments showing mean basal [Ca\(^{2+}\)]\(_i\), peak BK-evoked [Ca\(^{2+}\)]\(_i\), and [Ca\(^{2+}\)]\(_i\), 5 min after agonist stimulation. Cytosolic calcium signals in control cells contain two phases: a transient rise in [Ca\(^{2+}\)]\(_i\), followed by a sustained elevation of [Ca\(^{2+}\)]\(_i\), lasting many minutes. Note the absence of the sustained phase in the PS1\(^{M146V}\)-KI fibroblasts. The inset shows quantitative data for the rates of rise of the initial calcium transients. c, Representative calcium signals evoked by 50 nM BK in calcium-deficient medium. Note that control cells lack the sustained phase of elevated [Ca\(^{2+}\)]\(_i\), seen when extracellular calcium is present, whereas PS1\(^{M146V}\)-KI are relatively unchanged (see a). d, Typical BK-evoked calcium signals in control cells incubated in calcium-containing medium with or without SKF-96365 (10 \(\mu\)M). Note that SKF-96365 reduces the sustained elevation of [Ca\(^{2+}\)]\(_i\), normally seen in control cells. *P < 0.01.

Figure 2. Capacitative calcium entry is impaired in PS1\(^{M146V}\)-KI fibroblasts after weak agonist stimulation, but not complete depletion of calcium stores. a, Representative calcium signals evoked by addition of 50 nM BK in calcium deficient medium, followed by the readdition of 2 mM extracellular calcium. b, Quantitative data from 7 experiments showing basal [Ca\(^{2+}\)]\(_i\), peak BK-evoked [Ca\(^{2+}\)]\(_i\), and peak [Ca\(^{2+}\)]\(_i\), 30 s after readdition of extracellular calcium. The inset shows quantitative data for the rates of rise of the initial calcium transients. c, Representative calcium signals after the addition of 1 \(\mu\)M TG in calcium-deficient medium, followed by the readdition of 2 mM extracellular calcium. d, Quantitative data from 9 experiments showing basal [Ca\(^{2+}\)]\(_i\), peak TG-evoked [Ca\(^{2+}\)]\(_i\), and peak [Ca\(^{2+}\)]\(_i\), after readdition of extracellular calcium. *P < 0.01.
hypothesized that CCE is impaired in PS1M146V-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 InsP3 receptors (Parekh et al., 1997), fails to deplete transient release of ER calcium through rapidly inactivating InsP3 receptors (Parekh et al., 1997), fails to deplete intracellular calcium stores in the PS1M146V-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 PS1M146V-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⁻¹ versus 1.11 ± 0.11 nM s⁻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 PS1M146V-KI mice. Collectively, our results suggest that the potentiation of calcium transients and absence of CCE observed in mutant PS1M146V-KI cells are attributable to increased ER calcium levels.

Discussion

In this study we investigated calcium signaling in fibroblasts from KI mice harboring a PS1 mutation linked to FAD relative to controls, cytosolic calcium signals from PS1M146V-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 PS1M146V-KI fibroblasts.

The agonist-evoked calcium signals in PS1M146V-KI fibroblasts are virtually indistinguishable from comparable experiments with human fibroblasts from FAD patients harboring PS1 mutations (compare our Fig. 1 a to Figure 1 a in Ito et al., 1994). Thus, the PS1M146V-KI animals faithfully mimic the calcium signaling changes seen in presenilin-associated FAD. Importantly, since the mutant PS1 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 pathogenesis, 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 PS1 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. Akbari, 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 PS1M146V-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 release (see Fig. 1a) and would account for our observations in Xenopus oocytes that mutant PS1 increases both the rate of calcium efflux and the average quantal content of elementary calcium release events, the fundamental building blocks making up global calcium signals (Matson 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 barely triggers CCE in PS1(M146V)-KI fibroblasts because luminal calcium levels are abnormally elevated.

Figure 5. Model of the perturbations in calcium signaling in PS1(M146V)-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. Affer weak 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(M146V)-KI fibroblasts because luminal calcium levels are abnormally elevated.

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