

Amyloid precursor protein-mediated modulation of capacitive calcium entry

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Alzheimer's disease (AD) is a progressive neurodegenerative disorder. In the present study, the function of amyloid precursor protein (APP) in modulating capacitive calcium entry (CCE), a refilling mechanism for depleted intracellular calcium stores, was investigated. CCE in neural 2a (N2a) cells stably expressing wild-type human APP was lower than in wild-type N2a cells, while CCE in APP knockout mouse embryonic fibroblast (MEF) cells was higher than in their wild-type counterparts. We demonstrate that wild-type APP depresses CCE. Furthermore, using N2a cells transfected with C-terminal APP fragments, we show that these fragments anchored in the cell membrane play an important role in CCE depression.

Alzheimer's disease, amyloid precursor protein, capacitive calcium entry

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Amyloid precursor protein (APP) plays an important role in the pathophysiology of Alzheimer's disease (AD), one of the most common neurodegenerative diseases affecting an increasing population worldwide [1,2]. APP can be processed to generate proteolytic fragments through two distinct intracellular metabolic pathways. One is the non-amyloidogenic pathway, through which APP is cleaved by α -secretase, generating a large extracellular soluble APP α (sAPP α) and an intracellular C-terminal fragment (C83). The other is the amyloidogenic pathway, where APP is sequentially cleaved by β - and γ -secretase. β -Secretase cleavage produces extracellular soluble APP β (sAPP β) and C99 fragments. The C99 fragment is further cleaved by γ -secretase to liberate the amyloid intracellular domain (AICD) and β -amyloid peptide (A β), which is deposited to form senile plaques in AD brains [1,3].

APP gene mutations have long been known to be major causes of the familial form of AD, and have attracted exten-

sive study [4,5]. Although the relationship between APP mutations and AD is already well established, the normal physiological functions of APP are still unclear. An increasing body of evidence suggests that APP might be involved in various cellular activities, including intracellular signal transduction, gene expression, cellular organelle function, axonal transport and synaptic plasticity [6–11]. In addition, recent studies have shown that virtually every proteolytic fragment of APP, including the secreted ectodomain, A β , C99 and AICD, can differentially modulate calcium signaling [12], and even lead to intracellular calcium dyshomeostasis [13,14].

Capacitive calcium entry (CCE) is a store depletion-activated calcium influx pathway [15], through which calcium enters the cells via store-operated calcium channels (SOCs) on the plasma membrane, replenishing ER calcium stores [13]. CCE was first identified as a major route of calcium influx in non-excitabile cells, but is now known to be present in smooth muscle cells, neurons and other excitable cells as well [16–19]. CCE prolongs the duration of intracellular Ca²⁺ elevation and assists in refilling depleted ER

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Ca²⁺ stores [20]. As a major mechanism of cellular Ca²⁺ signaling, CCE has been implicated in a number of neuro-pathological conditions, including AD [19]. It has been reported that knockout of presenilins (PS1 and PS2) alters ER calcium stores [21]. In addition, their corresponding familial AD mutations act as negative regulators of CCE, which might contribute to the onset of dementia [22–24].

In contrast to extensive studies on the relationship between presenilins and CCE, the roles of full-length APP and its proteolytic derivatives on CCE regulation remain to be elucidated. We therefore investigated the regulatory effects of APP on CCE. Time-lapse recordings of intracellular Ca²⁺ signals were performed in both neural 2a (N2a) cells and mouse embryonic fibroblast (MEF) cells. N2a cells stably expressing wild-type human APP695 (APPwt) and APP gene knockout MEF cells (APP^{-/-}) were used here to assess the effects of APP on CCE modulation. Our results demonstrate that CCE in N2a APPwt cells is lower than in N2a wild-type (WT) cells, which indicates that wild-type APP overexpression can depress CCE. Moreover, CCE in MEF APP^{-/-} cells was elevated compared with control cells, further supporting a negative modulatory role of APP in the CCE pathway. To examine whether the inhibitory effect of APP on CCE was mediated by the full-length protein and/or its fragments, we performed genetic and pharmacological manipulations on N2a cells. In accordance with results previously published by our group [25], abrogation of A β resulted in a further suppression of CCE in N2a APPwt cells, implying its potentiating effect on CCE. Cell culture medium treatment experiments revealed that the extracellular soluble APP fragments were unable to depress CCE. Compared with these fragments, CCE was significantly lower in N2a cells transfected with C-terminal APP fragments, suggesting that fragments anchored to the cell membrane might be responsible for the CCE suppressing effect.

1 Materials and methods

1.1 Antibodies and chemicals

Mouse monoclonal antibody 6E10, recognizing APP, was obtained from Signet Laboratory (USA). Antibody 4G8 against A β and rabbit polyclonal antibody recognizing the C-terminal of APP were from Sigma (USA). Goat anti-mouse IgG and goat anti-rabbit IgG, conjugated with alkaline phosphatase, as well as *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT) were from Sigma. G418 was from Amresco (USA).

1.2 Cell cultures and transfection

The cells and vectors, including mouse neural 2a (N2a) cells stably expressing wild-type human APP695 (APPwt), Swedish mutant of APP (APP^{swe}), and their wild-type (WT) controls, i.e. blank vector-transfected N2a cells, were gen-

erous gifts from Dr. Huaxi Xu in the Burnham Institute, USA [25]. The cell culture medium for these cells contained equal parts of DMEM (Gibco, USA) and Opti-MEM (Gibco), as well as 5% fetal bovine serum (FBS) and 0.1% penicillin & streptomycin. Embryonic fibroblasts isolated from APP knockout mice (APP^{-/-}) and their wild-type counterparts (APP^{+/+}) were cultured in DMEM containing 10% FBS and 0.1% penicillin & streptomycin [8]. Transfection of C99 was performed using lipofectamine 2000 reagent (Invitrogen, USA). The transfected cells were screened with 300 μ g/mL G418 for more than 10 d.

1.3 Calcium imaging

Intracellular [Ca²⁺] measurements were performed using the indicator Fluo3/AM (Calbiochem, USA). Fluo3/AM was solubilized in DMSO, aliquoted and stored at -20°C. Cells were loaded with 5 μ mol/L Fluo3/AM in HBSS (145 mmol/L NaCl, 2.5 mmol/L KCl, 1 mmol/L MgCl₂, 20 mmol/L HEPES, 10 mmol/L Glucose, 50 μ mol/L EGTA) containing bovine serum albumin (1%) at 37°C for 30 min. After 30 min of washing, they were viewed using an upright microscope (BX61, Olympus, Japan). Fluorescence intensities were acquired at 3-s intervals. Digital images were collected using a CCD camera (CoolSNAP HQ, Photometrics, USA) with a 20 \times water immersion objective, and subsequently analyzed using MetaMorph (Molecular Devices, USA). Each experiment was performed on at least four cultures. Experiments were performed at room temperature.

1.4 Western blotting analysis

Cultured cells were collected and treated with cell lysis buffer. A commercially available BCA kit (Pierce, USA) was used to determine protein concentrations. Samples containing similar amounts of protein were diluted with 4 \times SDS-PAGE loading buffer and boiled at 100°C for 5 min. After separation by 10% SDS-PAGE, the protein samples were electrophoretically transferred onto a piece of nitrocellulose membrane. Primary antibodies, 6E10 (1:1000 dilution) or C-terminal APP antibody (1:1000 dilution), were then incubated with the membrane at 4°C overnight. Immunoreactive bands on the membrane were then detected by incubating with alkaline phosphatase conjugated goat-anti-mouse IgG (1:10000 dilution) or goat-anti-rabbit IgG (1:5000 dilution). For C99 detection, Tris-Tricine SDS-PAGE was performed. Most of the procedures were the same as SDS-PAGE, except that for Tris-Tricine SDS-PAGE, the samples were diluted with 4 \times Tris-Tricine SDS-PAGE sample buffer and proteins were separated using 20% Tris-Tricine gels.

1.5 Reverse transcription PCR

Total RNA was extracted from cells using the Trizol RNA

extraction reagent (Invitrogen). First-strand cDNA was synthesized from 2 μg of total RNA in a final volume of 20 μL using a first-strand cDNA synthesis kit (Toyoba, Japan). PCR reaction was conducted using 3 μL aliquots of the RT reaction and specific primers for *TRPC1–7*, *TRPV6* and *STIM1* mouse sequences [26,27]. Forward and reverse primers were as follows: *TRPC1*, 5'-CAAGATTTTGGGAAA-TTTCTGG-3' and 5'-TTTATCCCTCATGATTTGCTAT-3'; *TRPC2*, 5'-GACGACATGATCCGGTTCATGTTTC-3' and 5'-CTCGATCTTCTGGAAGGAGTTGGTG-3'; *TRPC3*, 5'-GTGGCAATAATACTCCTAA-3' and 5'-CATGACCGC-CTAGCTTAA-3'; *TRPC4*, 5'-TCTGCAGATATCTCTGG-GAAGGATG-3' and 5'-AAGCTTTGTTTCGAGCAAATTT-CCATTC3'; *TRPC5*, 5'-ATCTACTGCCTAGTACTACTG-GCT-3' and 5'-CAGCATGATCGGCAATGAGCTG-3'; *TRPC6*, 5'-AAAGATATCTTCAAATTCATGGTC-3' and 5'-CACGTCCGCATCATCCTCAATTTTC-3'; *TRPC7*, 5'-GACTACTTCTGCAAGTGAATGAGTGC-3' and 5'-TT-CCACAAGTGTAGCACGTACTCCC-3'; *TRPV6*, 5'-ATC-CGCCGCTATGCACA-3' and 5'-AGTTTTTCTCCTGAA-TCTTTTTCCAA-3'; *STIM1*, 5'-AGACATTTATGGCGT-TGAA-3' and 5'-GAAAGGAAGGGAGGTGAA-3'. PCR was performed using Expand High Fidelity Taq polymerase (Promega, USA). Thermocycling consisted of 35 cycles of denaturation at 94°C for 45 s, annealing at 53 to 62°C (optimized for each fragment amplified) for 45 s and extension at 72°C for 15 s, followed by a final extension at 72°C for 10 min.

1.6 sAPP β ELISA

sAPP β levels in the media of N2a APPwt and N2a APPswe cells were assessed using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (Covance, USA). Briefly, 50 μL of concentrated cell culture medium or standard controls from the kit were added to microtiter plates covered with primary antibody at 4°C overnight. Secondary antibody conjugated with HRP was then applied and incubated for another 1 h at room temperature. After washing, the wells were loaded with chemiluminescence substrate solution. The plate was then read immediately with a luminometer.

1.7 Statistical analysis

Data were presented as mean \pm SD. Student's *t*-test was used to perform statistical analysis of the results. $P < 0.05$ was considered significant.

2 Results

2.1 Wild-type APP overexpression attenuates CCE

CCE was measured in N2a WT cells in parallel with N2a cells stably expressing wild-type human APP (APPwt) (Figure 1(a)). To induce CCE, cells were first incubated in

Ca²⁺-free medium containing cyclopiazonic acid (CPA) to deplete ER Ca²⁺ stores, and subsequently replenished with Ca²⁺-containing medium (1.8 mmol/L) to facilitate calcium entry [22–24]. Figure 1(b) shows intracellular calcium changes in two representative experiments carried out with N2a WT (continuous trace) and APPwt (dashed trace) cells. The average peak amplitude of CCE, expressed as normalized ratio above baseline, was significantly decreased in N2a APPwt cells compared with N2a WT cells (Figure 1(c)), implying that wild-type APP, and/or some of its fragments, might depress CCE.

CCE in the APP gene knockout MEF cells (APP^{-/-}) was also compared with that in wild-type MEF cells (APP^{+/+}). Significantly greater CCE was found in MEF APP^{-/-} cells compared with MEF APP^{+/+} control cells (Figure 1(d),(e)). This suggests that CCE is apparently enhanced by the absence of APP, indicating that APP might be necessary to maintain CCE at normal levels. Since APP can be processed by secretases to produce several fragments in addition to full-length APP, levels of APP fragments may also be elevated in N2a APPwt cells, and down-regulated in MEF APP^{-/-} cells. These results suggest that full-length APP and/or its fragments have the ability to suppress CCE.

2.2 Abrogation of A β further depresses CCE in N2a APPwt cells

The suppression of CCE in N2a APPwt cells could be mediated by full-length APP and/or its fragments. To discriminate between these possibilities, N2a cells were manipulated with gene transfection or pharmacological treatments before CCE assessments. As mentioned above, our previous results demonstrated that A β can potentiate CCE in N2a cells stably expressing Swedish mutant APP (APPswe) [25]. This finding has been bolstered by the results obtained in N2a APPwt cells here. A β antibody 4G8, which can completely block the effect of extracellular A β , was added to the culture medium of N2a APPwt cells for 24 h (at 0.05%, v/v). Following this treatment, CCE in N2a APPwt cells was further depressed compared with N2a APPwt controls not treated with 4G8 (Figure 2(a),(b)). Furthermore, a 24 h incubation of N2a APPwt cells with 2 $\mu\text{mol/L}$ DAPT, which can inhibit γ -secretase activity and therefore stop the secretion of A β , had a similar effect on CCE (Figure 2(c)–(e)). Abrogation of A β in N2a APPwt cells resulted in a further attenuation of CCE compared with either N2a WT or APPwt control cells (Figure 2(b),(e)). Given the CCE-enhancing effect of A β , we propose that full-length APP and/or its proteolytic derivatives other than A β might mediate the depression of CCE, with a more powerful impact.

2.3 Membrane-anchored APP fragments depress CCE

APP can be processed by β -secretase or α -secretase, releasing large soluble fragments, sAPP β and sAPP α , into the

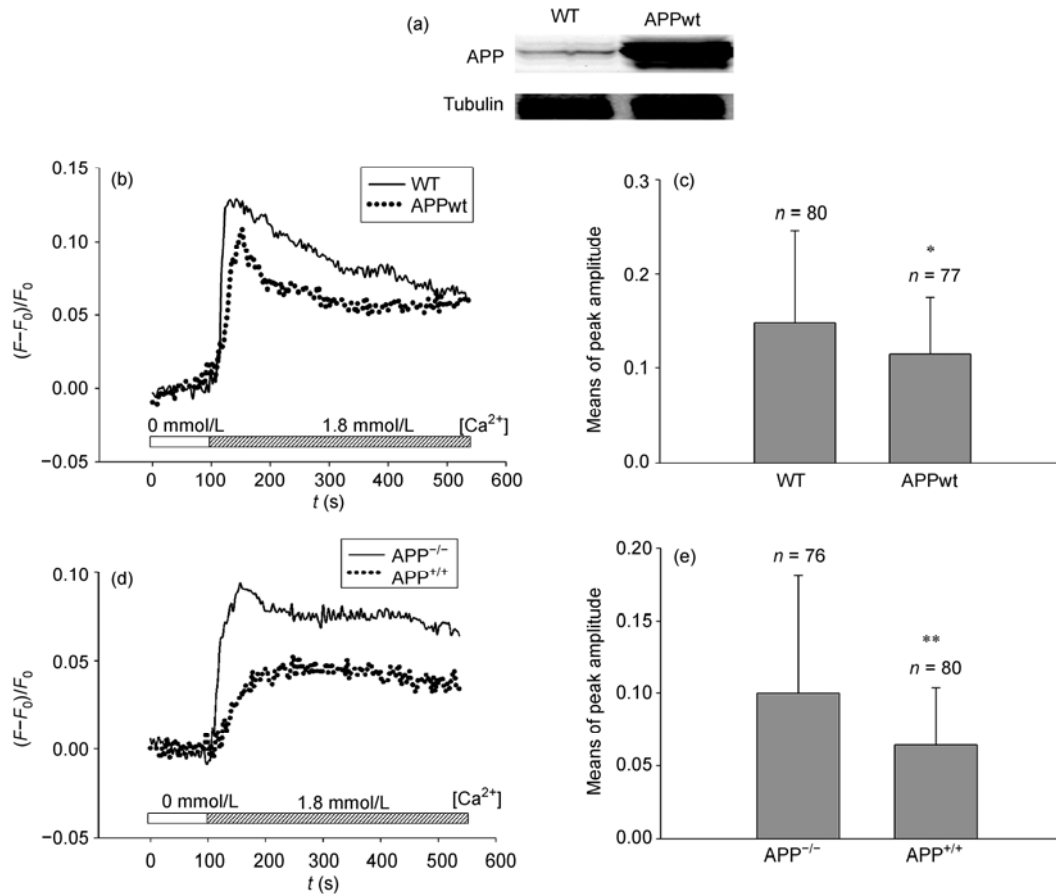


Figure 1 CCE in N2a APPwt cells was lower than in N2a WT controls. (a) APP expression levels were higher in N2a APPwt cells than in WT cells. Cell lysates from different cells were analyzed by Western blotting with anti-APP monoclonal antibody (6E10) (upper panel); (b) representative time courses of CCE in N2a WT and APPwt cells; (c) the average peak amplitudes of CCE in N2a WT and APPwt cells; (d) representative time courses of CCE in APP knockout MEF cells (APP^{-/-}) and wild-type MEF cells (APP^{+/+}); (e) the average peak amplitudes of CCE in MEF APP^{-/-} and APP^{+/+} cells. *n*, number of cells; **P* < 0.05, ***P* < 0.01.

extracellular space. To determine if these secreted fragments are involved in the depression of CCE in N2a APPwt cells, we performed a number of experiments. Culture media were collected from N2a APPsw cells, which mainly contain sAPP β , after a 24-h incubation period, as well as from N2a APPwt cells, which mainly produce sAPP α (Figure 3(a)). N2a WT cells were then incubated with culture medium from N2a APPsw or APPwt cells, separately, in the presence of 0.1% mouse anti-A β antibody 4G8 to abrogate the effect of A β . After 24 h, CCE was measured in both of these N2a WT cultures. No obvious changes in CCE were observed in N2a WT cells when they were incubated with these media containing different quantities of sAPP α and sAPP β (Figure 3(b),(c)), suggesting that the secreted fragments of APP may not participate in CCE depression in N2a APPwt cells.

The effect of the membrane-anchored APP fragments on CCE was then studied in N2a cells. To examine the role of C-terminal APP fragments in the regulation of CCE, N2a cells were transfected with a C99 construct (APP_{CTF}) or an empty vector control before CCE measurements. The 300 μ g/mL G418 was used to screen the cell lines for a period

greater than 10 d. Western blotting analysis showed that both C99 and C83 were overexpressed in the cell line (Figure 3(d)). Before CCE estimation, N2a APP_{CTF} cells were treated with 2 μ mol/L DAPT for 24 h to exclude the influence of A β or AICD fragments generated endogenously. The average peak amplitude of CCE was found to be dramatically attenuated in N2a APP_{CTF} cells (Figure 3(e),(f)). These results demonstrate an important role of C-terminal APP fragments, C99 and/or C83, in CCE depression.

2.4 CCE depression in N2a APPwt cells is independent of the transcription of store-operated calcium channels (SOCs)

To clarify the mechanism underlying the depressed CCE in N2a APPwt cells, we examined the expression of SOC in N2a APPwt and WT cells. RT-PCR was used to measure transcription of the SOC recently identified in animal cells [26,27], including *TRPC 1-7*, *TRPV 6* and *STIM1*. Four of these were found to be transcribed in both N2a WT and APPwt cells (Figure 4(a)). However, none showed remarkable differences at the mRNA level between N2a APPwt

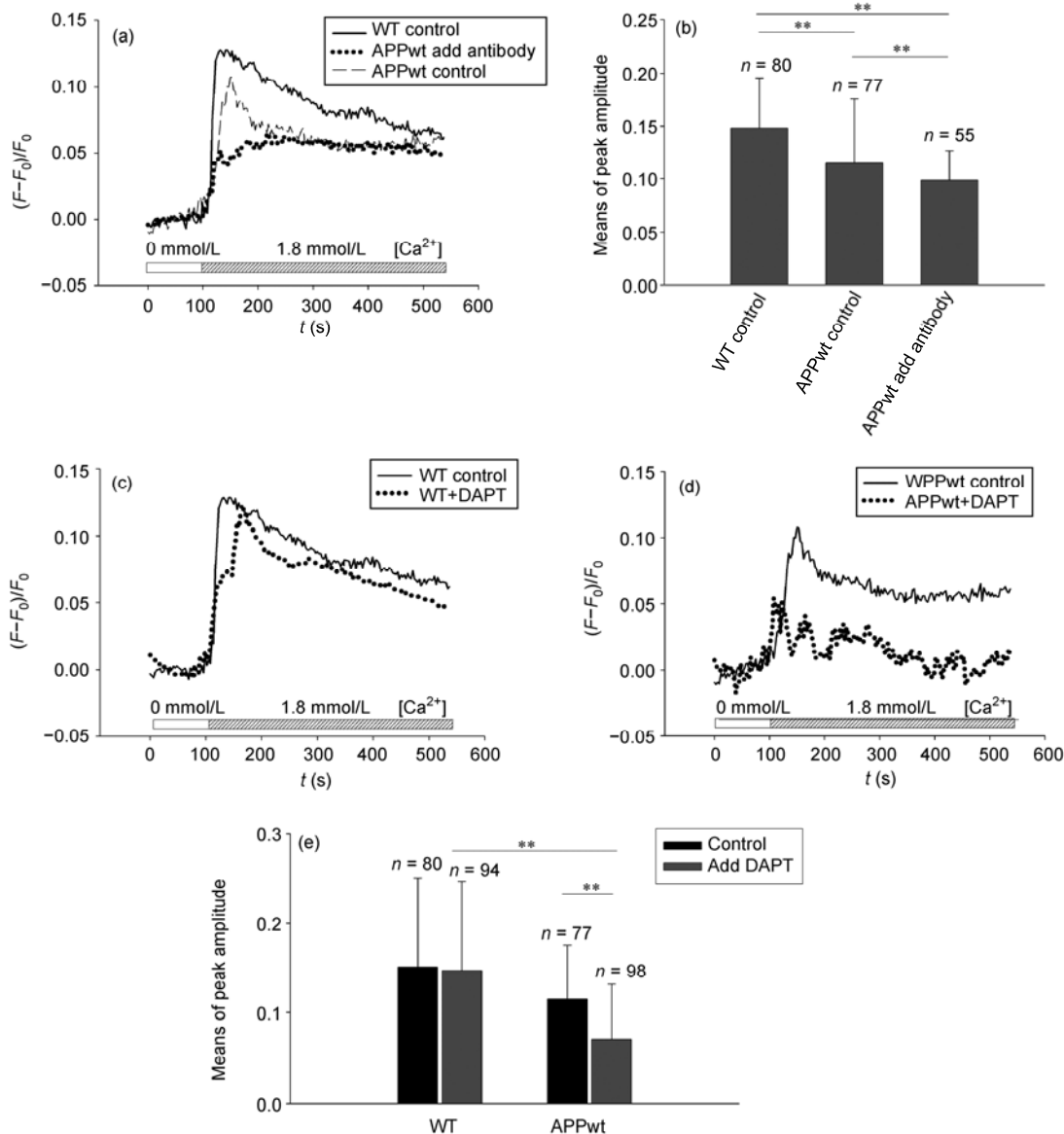


Figure 2 Removal of A β further depresses CCE. (a) Representative time courses of CCE in N2a WT cells and APPwt cells with or without A β antibody treatment; (b) the average peak amplitudes of CCE in N2a WT and APPwt cells with or without A β antibody treatment; (c) representative time courses of CCE in N2a WT cells with or without DAPT treatment; (d) representative time courses of CCE in N2a APPwt cells with or without DAPT treatment; (e) the average peak amplitudes of CCE in N2a WT and APPwt cells with or without DAPT treatment. n , number of cells; ** $P < 0.01$.

and WT cells (Figure 4(b)). This suggests that transcriptional regulation of store-operated channels is not involved in the mechanism by which the APP C-terminal fragments depress CCE.

3 Discussion

APP, a type I intrinsic membrane protein, is widely expressed and distributed in the central nervous system and peripheral tissues [28]. While the roles of APP mutations in the pathogenesis of AD have been thoroughly studied, the normal physiological functions of the protein remain unclear and debatable [29,30]. Recently published reports

highlight the importance of APP in various cellular processes, ranging from intracellular signal transduction to synaptic plasticity [6,11]. APP can be processed by α -, β - and γ -secretases to generate various proteolytic products, including secreted APPs, AICD and A β . The effects of some of these APP proteolytic derivatives on cellular calcium homeostasis have been studied [13,14]. However, whether APP proteins and/or its fragments regulate CCE remains to be clarified. CCE is one of the primary mechanisms regulating calcium influx into the cytoplasm. It regulates a number of vital cellular processes, such as gene transcription, cell cycle regulation, apoptosis and calcium oscillation [15,18,31]. Disturbed CCE signaling has been found to play a role in the neurodegenerative process

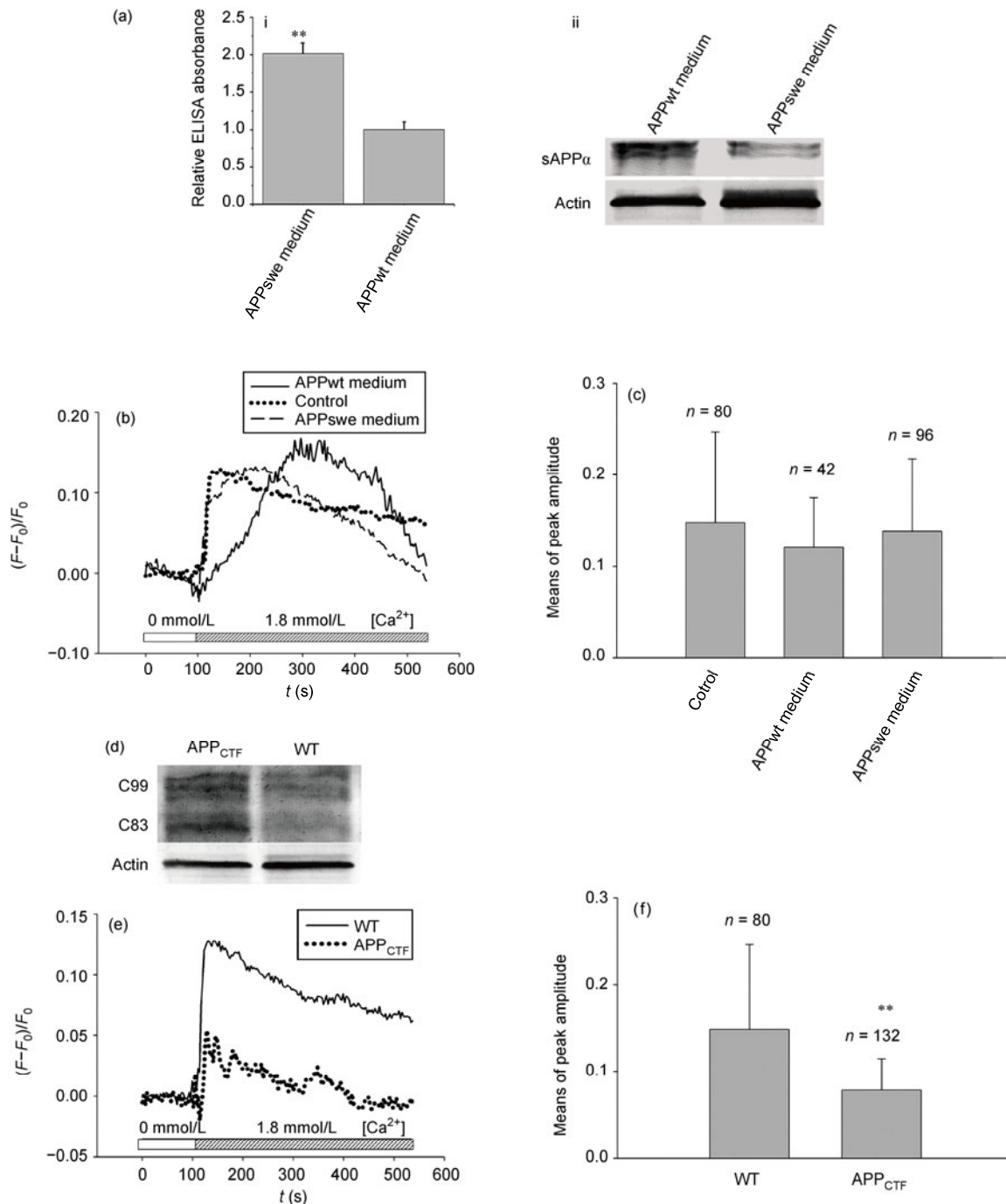


Figure 3 The effects of secreted and C-terminal fragments of APP on CCE. (a) Relative contents of sAPP β and sAPP α in the media of N2a APPwt and APPswe cells. Media from different cell cultures were collected and analyzed by ELISA to detect sAPP β (i, $n = 3$) and by western blotting with antibody 6E10 to detect sAPP α (ii, which is representative of three independent experiments); (b) representative time courses of CCE in N2a WT cells treated with N2a APPwt or APPswe medium; (c) the average peak amplitudes of CCE in N2a WT cells treated with N2a APPwt or APPswe cell culture medium; (d) APP-CTF fragment expression levels were higher in N2a cells transfected with the C-terminal APP construct (APP_{CTF}). Cell lysates from different cells were analyzed by western blotting with a C-terminal APP-specific antibody; (e) representative time courses of CCE in N2a WT and APP_{CTF} cells; (f) the average peak amplitudes of CCE in N2a WT and APP_{CTF} cells. n , number of cells; $**P < 0.01$.

underlying AD [22–24].

In this study, we investigated the effect of APP on CCE. We found that wild-type human APP overexpression can depress CCE. This finding may provide substantial insight into the physiological functions of APP. However, a study

by Herms et al., which focused on the modulatory role of mutant presenilin-1 on CCE, implied that APP knockout does not significantly change CCE [23], which is not in agreement with our findings. This discrepancy could be because of differences in experimental protocols and/or the

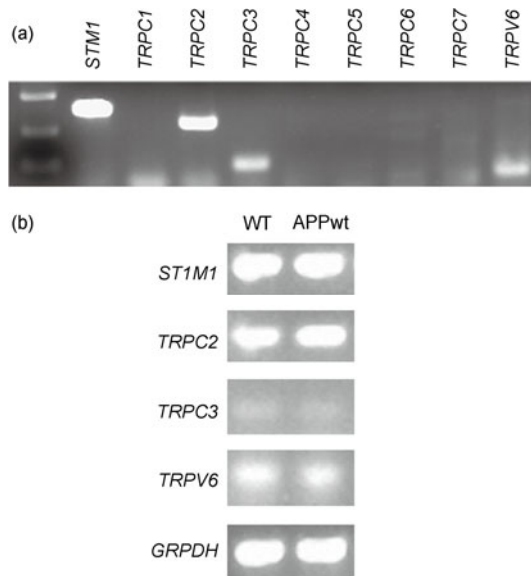


Figure 4 APP does not affect mRNA levels of store-operated calcium channels. (a) mRNA expression levels of representative store-operated calcium channels in N2a WT cells determined using RT-PCR; (b) mRNA expression levels of four store-operated calcium channels in N2a WT and APPwt cells.

model cells used.

As APP is processed by secretases to produce several fragments, the CCE depressing effect of wild-type APP observed here could be the combined effect of full-length APP and/or its fragments. The effects of APP fragments on CCE were therefore investigated here. We have already demonstrated the CCE-potentiating effect of A β , the most toxic fragment of APP [25,32]. CCE in N2a APPwt cells could be further depressed by treatment with A β antibody or DAPT, suggesting that full-length APP and/or APP fragments other than A β can depress CCE in N2a APPwt cells, and this depressing effect is even stronger than the CCE-potentiating effect of A β .

The extracellular secreted fragments of APP have been demonstrated to exhibit neuroprotective properties, such as preventing tau hyperphosphorylation, protecting against oxidative impairment and enhancing memory [12,33,34]. They might attenuate the calcium response to protect hippocampal neurons from excitotoxicity caused by cellular insults, such as A β or excessive glutamate [35,36]. Secreted APPs may also protect cells with PS1 mutations by stabilizing intracellular calcium levels [37]. However, we failed to find any significant effect of these secreted APPs on CCE response (it is worth noting that the initial rate of CCE in N2a WT cells treated with APPwt medium was somewhat lower than in N2a WT controls despite the unchanged average peak amplitude; this observation requires further study for clarification). These results prompted us to investigate the action of C-terminal APP fragments on CCE in N2a cells by transfecting them with C99 constructs. C99 transfection induced a lower CCE compared with control

cells. As C99 fragments are the obligatory precursors of A β peptides, the cleavage of C99 fragments by γ -secretase was inhibited using DAPT before CCE measurement. Therefore, it appeared that C99 fragments mediated the observed alterations in CCE signaling. Since C99 can be cleaved by α -secretase to generate C83 fragments, both C99 and C83 are overexpressed in N2a APP_{CTF} cells. We were therefore unable to determine whether C99 or C83, or both, contribute to the depression of CCE in N2a APP_{CTF} cells.

The mechanisms of CCE regulation are complex [38]. Our recently published data indicate that there is no significant difference in ER calcium store content between N2a APPwt and WT control cells [39]. Therefore, other mechanisms must be responsible for the modulation of CCE by C-terminal APP fragments. One of these might be altered expression of store-operated channels. To examine this possibility, we examined the transcriptional levels of several SOCs in N2a WT and APPwt cells. Among the SOCs expressed in both N2a WT and APPwt cells, we failed to find any obvious differences in mRNA expression between these two cell lines. Thus, the inhibitory action of C-terminal APP fragments on CCE is likely to be caused by an alternative mechanism. Further studies are required to clarify the molecular mechanisms underlying this modulation in detail.

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