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Research report

Enhanced BK-induced calcium responsiveness in PC12 cells expressing the C100 fragment of the amyloid precursor protein

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Abstract

Several lines of evidence have implicated the amyloid precursor protein (APP) and its metabolic products as key players in Alzheimer's disease (AD) pathophysiology. The approximately 100 amino acid C-terminal fragment (C100) of APP has been shown to accumulate intracellularly in neurons expressing familial AD (FAD) mutants of APP and to cause neurodegeneration when expressed in transfected neuronal cells. Transgenic animals expressing this fragment in the brain also exhibit some neuropathological and behavioral AD-like deficits. Here, we present evidence that PC12 cells expressing the C100 fragment either via stable transfections or herpes simplex virus-mediated infections show alterations in calcium handling that are similar to those previously shown in fibroblasts from AD patients. This alteration in calcium homeostasis may contribute to the deleterious effects of C100 in PC12 cells. Our data also lend support for a pathophysiological role for C100 since it induces an alteration thought to play an important role in AD pathology.

Keywords: PC12; C100; Bradykinin; Calcium

1. Introduction

Numerous studies have implicated the amyloid precursor protein (APP) and its cleavage products as major players in Alzheimer's disease (AD) pathophysiology [16,19,45,48,54]. However, the precise mechanisms by which they exert their actions remain unknown. The 40–42 amino acid product of APP termed β-amyloid (Aβ), the main protein component of senile plaques, has been shown to have a number of deleterious effects in cultured cells. These include direct cellular toxicity [8,41,42,56], disruption of intracellular calcium regulation [32], formation and/or modulation of ion channels [1,10,12,18], and other miscellaneous effects ([28,43]; for reviews, see Refs. [33,45,54]). It has also been demonstrated that familial AD (FAD) APP mutations flanking the Aβ segment lead to increased production of amyloidogenic fragments in cellular models and in circulating fluids of (symptomatic and asymptomatic) individuals carrying the mutations [5,6,23,44,49]. Mutations in the presenilins (PS1 and PS2) have also been shown to cause an increased Aβ 1–42/Aβ 1–40 ratio [4,44,46,51]. Both APP (chromosome 21) and presenilin (chromosomes 1 and 14) mutations are thought to be causative and are transmitted by an autosomal dominant mode of inheritance [19,46,51]. Some researchers have shown that a smaller fragment (aa 25–35) of the Aβ retained some of the properties of the full fragment [35,56].

An amyloidogenic fragment comprising 100 amino acids in the C terminus of APP termed C100, which contains the entire Aβ 42 amino acid sequence and which is a product of endosomal/lysosomal processing presumably involving the β-secretase [7], has also been linked to the pathophysiological process. It was initially reported that PC12 (pheochromocytoma) cells transfected with this fragment gradually degenerated when induced to differentiate [55]. C100 has been reported to be toxic to neuronal cells in

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culture [13,55] and is itself amyloidogenic [29,53]. Moreover, this fragment has been shown to induce AD-like pathology in vivo. Rat brains transplanted with cells expressing this fragment showed cortical atrophy; some animals had Alz-50 immunoreactivity and axonal disorganization in parts of the hippocampus ipsilateral to the transplant [38]. Transgenic mice expressing the C100 fragment exhibited age-dependent neuronal degeneration, abnormal intracellular Aβ immunoreactivity, and deficits in spatial learning [3,37,39,40].

It has been shown that the toxicity of C100 is pH-dependent and inversely proportional to the peptide's aggregation state [27]. These characteristics of the mode of action of C100 seem distinct from those of the Aβ-mediated toxicity [38]. Indeed, a direct comparison of the capacity of C100 and Aβ to induce LDH secretion in PC12 cells proved the former more potent (or more toxic) than the latter [24]. The C100 fragment has also been reported to induce non-specific ionic currents in *Xenopus* oocytes [11] and to form cationic channels in lipid bilayers [25]. These properties appear similar to some of the effects of Aβ, shown to induce channel formation [1] and to induce inward currents [15]. However, C100 has not been reported to mimic the specific blocking effects of Aβ (in general at nanomolar concentrations) on potassium channels [10,18].

A recent study showed that expression of FAD mutants of APP in neurons caused increased intracellular accumulation of the C100 fragment and increased secretion of the Aβ 1–40 and Aβ 1–42 fragments [34], suggesting a direct link between C100 and FAD. Perturbations of intracellular calcium handling have long been implicated as relevant in AD [26], with supporting evidence from both non-neuronal and neuronal cell models [9,31]. To determine whether C100 alters calcium handling in neuronal cells, we studied IP_3-mediated calcium release in PC12 cells expressing the C100 fragment. We report here that the transfected cells have, as shown previously in AD fibroblasts [20], an enhanced calcium response to bradykinin (BK), a peptide that causes IP_3 generation.

2. Materials and methods

2.1. Cells and cell culture

Two independently-isolated pheochromocytoma PC12 cells expressing C100 from the retroviral Doj vector were used (see Ref. [55] for the transfection protocol). They will be referred to as PC12-C100#1 and PC12-C100#2. The parental cell line served as the control. All cell lines were maintained in polylysine coated T75 plastic flasks or in positively charged flasks in DMEM supplemented with 10% horse serum, 5% calf serum and 1% of a mixture of penicillin (5000 units/ml in G sodium) and streptomycin (5000 μg/ml). For calcium imaging experiments, cells were seeded (150–200,000 cells/ml) onto polylysine-coated 25 mm cover slips. C100-transfected and control cell lines were treated with nerve growth factor (NGF, Boehringer) in some experiments. NGF was directly added to the dishes at a final concentration of 50 ng/ml. C100-transfected PC12 cells treated with NGF deviate from the normal course of differentiation after day 3, while they largely die by day 7 in culture [55]. To detect early changes induced by NGF, we studied cells after 3 days post-plating (DPP 3), before gross signs of cell death were evident. We also studied the cells 7 days after NGF addition (DPP 7) to investigate long-term effects. Thapsigargin (Tg; Calbiochem), an inhibitor of calcium ATPase [50], was tested at 1 or 10 μM on control and C100-transfected cells to determine whether the effect of C100 on calcium elevations involved calcium responses (or release) by mechanisms different than IP_3. The calcium ionophore, ionomycin (Calbiochem) was tested at 10 μM to discern differences, if any, in calcium buffering systems between control and C100-transfected cells. Each of the drugs used in the study was dissolved in dimethyl sulfoxide (DMSO, Sigma) to make stock concentrations from which the final concentrations were obtained for testing.

2.2. Generation of recombinant HSV vectors and infection of PC12 cells

To study the effect of acute expression of C100 (5 h and overnight), nontransfected PC12 cells were infected with HSV-1 vectors expressing C100 and flagC100 and *Escherichia coli* (*E. coli*) β-galactosidase (as a negative control). We prepared replication defective HSV vectors expressing C100 (HSV/C100) and a flag-tagged version of C100 (HSV/flagC100; [40]) and β-galactosidase (HSV/Lac; negative control) in the expression vector pHSVPrpUC as described [34]. The titer of the helper virus component of each stock was $1–1.2 \times 10^6$ plaque forming units (pfu)/ml on 2–2 cells. The titer of the recombinant virus component of each stock, as assayed by expression of the exogenous gene in PC12 cells, was consistently 3×10^7 infectious units (iu)/ml.

PC12 cells were infected with HSV recombinants 3 days post-plating. Immunoblots to demonstrate levels of expression of the HSV recombinant genes were performed on PC12 cells maintained under the same conditions as those used for the calcium imaging experiments. Cells were lysed in the following buffer — 100 mM Tris–HCl pH7.4, 20 mM NaCl, 10 mM EDTA, 10 mM EGTA with the following protease inhibitors: 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM benzamidine, and 10 mM betaglycerolphosphate. The immunoblots were performed as described [34] using antibody C8 (gift of D. Selkoe).

2.3. Calcium imaging

Intracellular calcium changes were monitored using standard fluorescent techniques [52]. Culture medium was

removed and cells were washed at least 3 × with basal salt solution (BSS in mM: NaCl 140, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1.5, HEPES 10, Glucose 5, pH = 7.4). The fluorescent probe was loaded by incubating cells in 1 μM fura 2-AM (Molecular Probes) in 1 ml BSS at room temperature (RT). One hour later, cells were washed thoroughly with BSS or BSS-0 Ca^{2+} (in mM: NaCl 140, KCl 5, $CaCl_2$ 0.1, $MgCl_2$ 1.5, EGTA 1, HEPES 10, Glucose 5, pH = 7.4; free Ca^{2+} 5 nM) and 1 ml of fresh BSS (or BSS-0 Ca^{2+}) was added for $[Ca^{2+}]_i$ baseline measurements. One milliliter of BK (Calbiochem) diluted in BSS was added to the experimental dish to achieve the desired final BK concentration of 10, 100, 500 or 1000 nM. Fluorescent images at 334 (numerator) and 380 nm (denominator) were acquired so the rate of acquisition was approximately one ratio per second for about 300 s with a Zeiss-Attofluor Ratio Arc Imaging System (Zeiss). A 40 × Zeiss Fluor (oil immersion) objective lens was used. The number of dishes tested per experimental condition ranged from 7 to 18. All experiments were repeated on at least three separate occasions. The total number of cells analyzed per treatment condition ranged from 208 to 755 cells.

2.4. Ca^{2+} loading and release measurement

Calcium fluxes across microsomes were detected by measuring changes in intra-microsomal ^{45}Ca content as described [2,14]. All manipulations were performed on triplicate samples from separate flasks of control or PC12-C100#1 transfected cells. Cells from each flask were homogenized by sonication in 0.5 ml 10 mM Tris–HCl (pH 7.4) + 1 mM PMSF. The homogenates were centrifuged at 10,000 × *g* and the supernatants were centrifuged at 100,000 × *g*. Microsomes were loaded with $^{45}CaCl_2$ by incubating 250 μl microsomes in buffer A (final conc.: 20 mM MOPS pH 7.2, 100 mM KCl, 20 mM NaCl, 3.5 mM $MgCl_2$, 3 mM ATP, 20 mM phosphocreatine, 1 mM glucose-6-phosphate, 5 mM NaN_3). $^{45}CaCl_2$ (New England Nuclear) (20 μM) and creatine kinase (10 U/ml) were then added and the mixture was incubated at RT for 20 min, followed by centrifuging (100,000 × *g*) at 4°C for 10 min. The pellet was resuspended in buffer A + creatine kinase. IP_3 (5 μM) was added to half the sample and after further incubation for 5 min at RT, the microsomes were collected by centrifugation (100,000 × *g*, 10 min, 4°C). The pellet was resuspended by sonication and ^{45}Ca was measured in both supernatant and pellet using a scintillation counter.

2.5. BK receptor binding assay

Confluent cultures of PC12 cells were collected in medium and washed 3 × with phosphate-buffered saline (PBS). A crude membrane fraction was prepared by sonicating in PBS + 1 mM PMSF, centrifuging at 100,000 × *g* for 20 min, and resuspending the pellet in PBS. Membranes (20 μl) were incubated for 4 h at four EC PBS containing 0.5% BSA, 1 mg/ml soybean trypsin inhibitor, 1 mM PMSF, and 26 nM [2,4-prolyl-3,4(*n*)^{3}H] BK, (Amersham Pharmacia Biotech). Control samples containing 10 μM unlabeled BK were incubated identically to correct for non-specific binding. After incubation, 100 μl of 33% PEG-8000 + 3.3 M NaCl were added. The samples were incubated for 30 min at 4°C and then collected by vacuum filtration on GF/C glass fiber filters, washed 2 ×

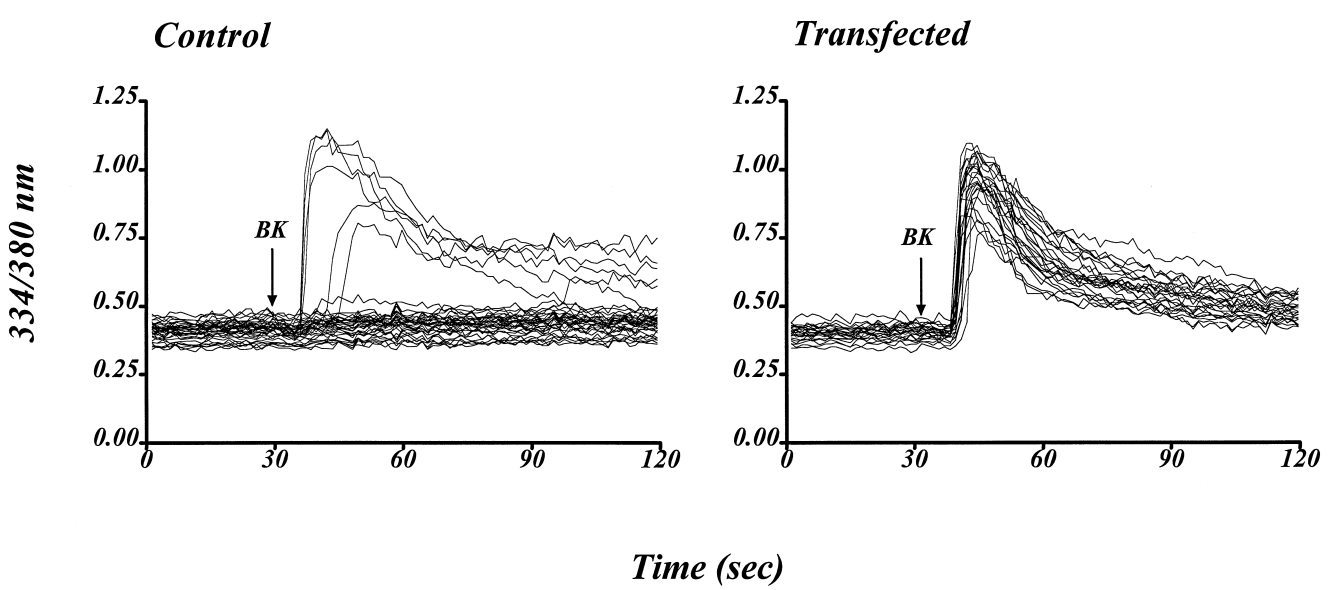

Fig. 1. Representative responses to 1 μM BK in a dish containing control (left panel) and C100-transfected (right panel) PC12 cells. While a small proportion of control cells responded to BK stimulation, there was a higher proportion of transfected cells with BK-induced calcium elevations. Arrow indicates time of drug application.

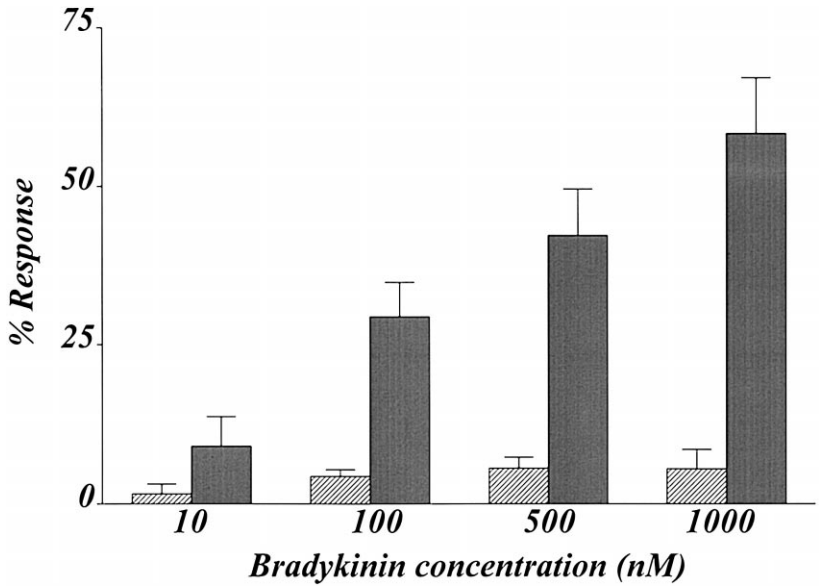

Fig. 2. Dose-dependent effect of BK concentration in control and C100#1-transfected PC12 cells on day 3 post-plating. Transfected cells (solid bars) have a significantly higher proportion of ''LARGE'' responders (mean ± S.E.) than control cells (hatched bars) at almost all concentrations of BK. One-way ANOVA test analyses were performed on the data.

with 15% PEG-8000/1.6 M NaCl, and counted in a scintillation counter. To measure binding affinity, BK was added at concentrations ranging from 10 nM to 100 μM. Affinity and B_{max} were calculated using the Marquardt algorithm in Tnplot (version 2.34).

2.6. IP_3 receptors and serca assays

Anti SERCA-2 antibody and anti IP_3R-3 antibody were purchased from Affinity Bioreagents and Transduction Labs, respectively. All manipulations were performed on triplicate samples from separate flasks of control or PC12-C100#1 transfected cells. Microsomes were prepared as described above. The resulting microsomal pellet was resuspended by gentle homogenization in 0.5 ml buffer. For immunoblotting, aliquot of PC12 cells homogenates containing 50 μg protein were separated by SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose, and stained with the respective antibodies. Densitometric analysis of triplicate samples was done using Tnimage (version 2.8.1b). Results are expressed as mean ± S.E. of the estimate.

2.7. Statistics

Conventional parametric statistical methods were used [36] since data followed a Gaussian distribution. One-way

Table 1
Calcium responses to BK in control and transfected PC12 cells. The experiments were performed in untreated and treated (50 ng/ml NGF) PC12 cells after 3 or 7 days post-plating (DPP). Results are represented as mean percentage ± S.E. of ''LARGE'' (i.e., double the baseline calcium level) and ''ALL'' (i.e., all discernible responses) responders following 1 μM BK stimulation. One-tailed unpaired *t*-test was performed in each group of data

Treatment	Cell-line	Large responders	All responders
Untreated DPP3	Control	5.50 ± 3.02	12.90 ± 4.46
	C100#1	58.36 ± 8.78***	89.68 ± 2.84***
NGF DPP3	Control	5.06 ± 2.04	14.97 ± 3.76
	C100#1	40.78 ± 12.10**	91.04 ± 3.05***
Untreated DPP7	Control	4.30 ± 0.94	10.40 ± 2.02
	C100#1	29.05 ± 14.02 n.s.	95.24 ± 1.91***
NGF DPP7	Control	6.25 ± 3.27	21.85 ± 4.71
	C100#1	63.75 ± 11.07***	98.94 ± 0.53***
Untreated DPP3	C100#2	67.07 ± 5.55***	99.51 ± 0.34***

** $p < 0.01$.
*** $p < 0.001$.

Table 2
Calcium responses to BK in control and HSV-infected PC12 cells. The experiments were performed in control PC12 cells after 3 days post-plating. Results are represented as mean percentage ± S.E. of ''LARGE'' and ''ALL'' responders following 1 μM BK stimulation. One-tailed unpaired *t*-test was performed to compare responses between HSV/Lac treated cells and the HSV/C100 or HSV/flagC100-treated cells in each group of data

Incubation time	HSV infection	Large responders	All responders
5 h	Lac	5.28 ± 1.39	11.97 ± 2.04
	C100	11.75 ± 2.21**	20.96 ± 2.64**
	flagC100	12.71 ± 2.84*	27.99 ± 3.53**
Overnight	Lac	4.36 ± 0.95	6.44 ± 1.92
	C100	11.56 ± 2.62*	13.63 ± 2.55*
	flagC100	14.24 ± 2.53**	26.25 ± 2.88***

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

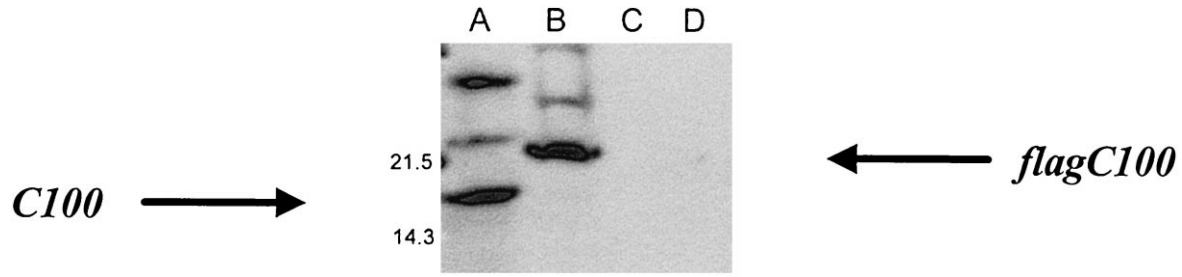

Fig. 3. Immunoblots of HSV/C100 and HSV/flagC100 expression in PC12 cells. A total of 10 μg of protein was run in each line. The blot was probed with C8, a polyclonal antibody directed to the C-terminal 20 amino acids of APP. Lane A: HSV/C100, lane B: HSV/flagC100, lane C: HSV/Lac and lane D: MOCK (no infection).

ANOVA [36] was performed on two sets of data — dose-dependent effects of BK concentration and the additional experiment with the two independent transfectants (PC12-C100#1 and PC12-C100#2). If there was a significant effect of transfection, differences within each concentration were determined using Tukey's multiple comparison post-test [36]. For all other experiments, unpaired *t*-test [36] was performed with Welch's correction in cases where variances were significantly different for each set of data. For the *t*-test analyses, one-tailed significance are considered for all except the Tg and ionomycin experiments since transfected cells were predicted to have higher calcium responses compared to controls due to the toxic nature of the C100 fragment. For data from Tg and ionomycin experiments, two-tailed analyses were performed since there was no a priori prediction for the direction of the calcium response. All analyses were performed with Graph Pad Prism™ (version 2).

3. Results

3.1. Calcium responses to BK challenge in control and stably transfected PC12 cells

BK challenge caused measurable calcium responses in control and C100-transfected cells (Fig. 1). However, a significantly higher percentage of PC12-C100#1 cells than controls responded to BK with calcium elevations of at least double the base line level (''LARGE'' responders; Fig. 2). This effect of C100 expression on the frequency of ''LARGE'' responses was significant at almost all concentrations of BK on DPP 3, although more marked at higher concentrations (Fig. 2; $F = 14.84$, $p < 0.001$; 10 nM — NS; 100 nM — $p < 0.05$; 500 and 1000 nM — $p < 0.001$). Therefore, all subsequent experiments were carried out with 1 μM BK as the test concentration.

The effect of C100 expression following 1 μM BK pharmacological challenge was largely independent of DPP, in that the proportion of ''ALL'' (all discernible calcium elevations) responses to BK was significantly higher in PC12-C100#1 transfected cells at both DPP 3 and 7 compared to controls (Table 1; DPP 3: $t = 14.51$, $p < 0.001$; DPP 7: $t = 30.26$, $p < 0.001$). However, the proportion of ''LARGE'' responses to BK (Table 1) was significantly higher in PC12-C100#1 transfected cells vs. control at DPP 3 ($t = 5.70$, $p < 0.001$) but not at DPP 7 ($t = 1.76$, $p = 0.06$).

The same protocol was repeated in cells treated with NGF, since it has been shown that some of the deleterious effects of the C100 fragment in PC12 cells were linked to the cellular differentiation process [55]. The effect of C100 expression was also observed with NGF treatment. The proportion of cells that were ''LARGE'' responders (Table 1) was significantly higher in PC12-C100#1 cells compared to controls at both DPP 3 ($t = 2.91$, $p < 0.01$) and DPP 7 ($t = 4.98$, $p < 0.001$). The PC12-C100#1 cells had a significantly higher proportion of ''ALL'' responders (Table 1) compared to the controls (DPP 3: $t = 14.81$, $p < 0.001$; DPP 7: $t = 16.27$, $p < 0.001$). Since days post plating did not influence the effect of C100 expression on BK response with or without NGF treatment (except at DPP7 for ''LARGE'' responders), additional experiments were carried out on DPP 3 only.

The calcium responses were preserved in calcium-free (0-Ca^{2+} BSS) medium (1 μM BK at DPP 3), with a significantly higher proportion of responders (''ALL'') in transfected cells (PC12-C100#1 69.17 ± 6.69; Control 10.24 ± 2.77, $t = 8.14$; $p < 0.001$).

Table 3
Biochemical changes in transfected PC12 cells. ^{45}Ca release was measured in isolated microsomes in an ATP-regenerating system. The ^{45}Ca release represents approx. 50–60% of the total ^{45}Ca label. Receptor levels were determined by densitometry of western blots. Values are mean of three measurements from cultures of PC12 cells from separate flasks ± S.E. of estimate

Incubation time	Control	C100#1
IP_3-induced ^{45}Ca release (CPM)	99,600 ± 11,400	169,500 ± 30,000*
IP_3R (arbitrary units)	8.34 ± 0.44	5.03 ± 0.19**
SERCA-2 (arbitrary units)	32.7 ± 5.2	37.7 ± 2.4

* $p < 0.05$.
** $p < 0.005$.

To confirm the effect of C100 expression on 1 μM BK-induced calcium elevations, we repeated the experiment with an additional independent PC12 transfected cell line (PC12-C100#2) on DPP 3 and compared it to control and PC12-C100#1 cells (Table 1). Both PC12-C100#1 and -C100#2 cells had a significantly higher proportion of ''LARGE'' responders compared to the control (Table 1; $F = 39.80$, $p < 0.001$; all post-test comparisons: $p < 0.001$). Among the ''ALL'' responders, there was a significant effect of C100 expression vs. control for each of the two C100 transfectants (Table 1; $F = 622.9$, $p < 0.001$; control vs. PC12-C100#1 or PC12-C100#2: $p < 0.001$; PC12-C100#1 and PC12-C100#2: NS).

3.2. Effect of BK application after HSV / C100 and HSV / flagC100 infection

The experiments described to this point were carried out on stably transfected PC12 cells expressing C100 from retroviral vectors. It was important to show that a completely different system of overexpression of C100 would yield a similar phenotype. We, therefore, generated HSV-1 vectors expressing C100 [34], a flag-tagged version of C100 [40], and *E. coli* β-galactosidase as a control. PC12 cells were plated (ca. 150–200,000 cells/ml) and 3 days later were infected with the appropriate recombinant virus at a multiplicity of infection (moi) of [1.5]. Five or 14 h

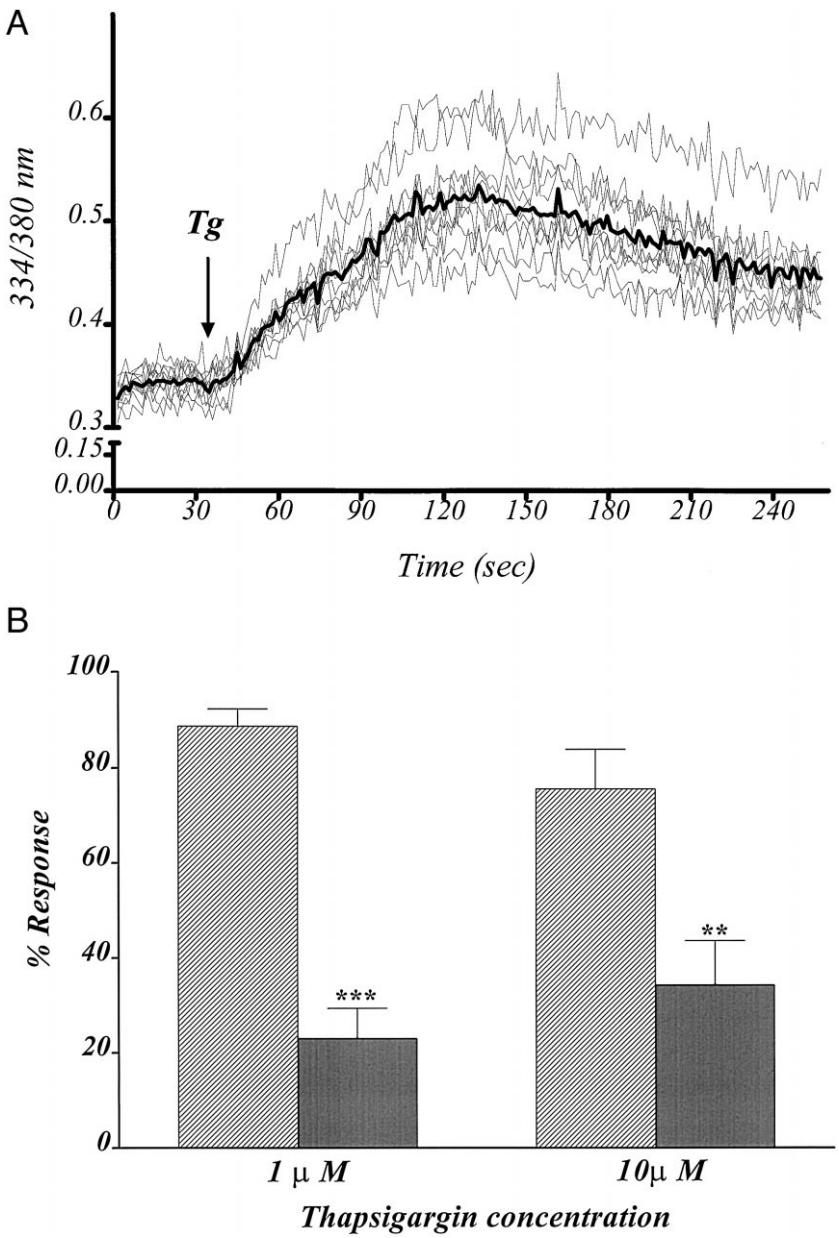

Fig. 4. Tg-induced calcium responses. (A) Representative responses to Tg challenge. Average response shown in bold trace. (B) In contrast to BK-induced responses, the proportion of discernible responses is higher in control cells (hatched bars) compared to transfected cells (solid bars). Two-tailed unpaired *t*-test was performed on the data. ** $p < 0.01$; *** $p < 0.001$.

post-infection, the cells were challenged with BK and calcium responses were measured. The proportion of ''LARGE'' responders (Table 2) after BK challenge was significantly higher in cells incubated for 5 h with either HSV/C100 ($t = 2.48$, $p < 0.01$) or HSV/flagC100 ($t = 2.35$, $p < 0.05$) compared to cells treated with HSV/Lac. The significant difference in calcium responses between HSV/C100 or HSV/flagC100-infected cells vs. HSV/Lac-infected controls was conserved among the ''ALL'' responders (Table 2; HSV/C100: $t = 2.64$, $p < 0.01$; HSV/flagC100: $t = 3.93$, $p < 0.01$). Similarly, after overnight incubation with the viruses, control HSV/Lac-treated cells had significantly lower BK-induced calcium elevations (Table 2) compared to cells treated with HSV/C100 (''LARGE'': $t = 2.59$, $p < 0.05$; ''ALL'': $t = 2.25$, $p < 0.05$) or HSV/flagC100 (''LARGE'': $t = 3.66$, $p < 0.01$; ''ALL'': $t = 5.90$, $p < 0.001$). Immunoblots confirming the levels of HSV/C100 and HSV/flagC100 expression are shown in Fig. 3.

3.3. Biochemical changes in C100 stably transfected PC12 cells

Consistent with the imaging experiments, IP_3-induced release of $^{45}Ca^{2+}$ from microsomes was increased by 70% in transfected cells (Table 3). This effect could not have been caused by differences in plasma membrane influx because the Ca^{2+} release was measured in isolated microsomes. In addition, calcium imaging experiments showed robust calcium signals in the absence of external calcium (see above). Mitochondrial contribution to the calcium signal can also be ruled out since they were inactivated by NaN_3. Binding studies showed a slight, although not statistically significant, decrease in number of BK receptors in transfected cells. B_{max} for control and transfected was 292 ± 2 and 242 ± 3, respectively (values from three or more replicates, data are expressed as fmol/mg protein $\pm$ 95% confidence limits). Similarly, there was a small, not significant decrease in the affinity of the receptors in transfected cells [Km: control 284 ± 14; transfected 349 ± 21, values (nM) from four replicates]. Surprisingly, however, IP_3R levels as determined by immunoblot analysis, were decreased by 40% in transfected cells (Table 3). No differences in immunoreactivity for the SERCA pump were observed (Table 3).

3.4. Responses to Tg and ionomycin in control and C100 stably transfected cells

Tg, an inhibitor of the calcium ATPase [50], and the calcium ionophore (ionomycin) were used to challenge control and C100-transfected cells to determine whether *non* IP_3-mediated calcium release and buffering systems, respectively, were affected by C100 expression. In control cells, Tg induced calcium responses of different magnitude, shape and duration from those elicited by BK (Fig. 4A). The pattern of calcium response of control vs. PC12-C100#1-transfected cells to Tg was opposite of that observed with the BK challenge. The overall proportion of control cells (Fig. 4B) responding to both concentrations of Tg tested was significantly higher compared to PC12-C100#1 cells (1 μM: $t = 9.03$, $p < 0.001$; 10 μM: $t = 3.28$, $p < 0.01$). C100 expression did not have an effect on ionomycin-induced responses (data not shown). Almost every one of the cells tested from both groups had discernible calcium elevations upon ionomycin stimulation (control: 99.76 ± 0.24; PC12-C100#1: 100.0 ± 0.00).

4. Discussion

We have presented evidence for an additional, potentially pathological, effect of the APP C100 fragment in PC12 cells. Two independent methods, calcium imaging in intact cells and $^{45}Ca^{2+}$ fluxes in microsomes, were used to demonstrate an exaggerated calcium response to IP_3 generation. An exaggerated response to BK and other substances that induce IP_3-mediated calcium release has been documented in fibroblasts from AD patients [17,20,22]. Enhanced IP_3 production in AD fibroblasts correlated with increased numbers and affinity of BK receptors [21]. Elevated IP_3-mediated calcium release was recently reported in human neuroblastoma cells transformed with mitochondrial DNA from AD patients [47]. Thus, alterations at some level of the IP_3-mediated calcium release cascade seem to be a prevalent feature in AD. Although one study showed changes in the BK receptor itself, the present and previous studies [22] do not show significant changes in receptors associated with the plasma membrane. In PC12 cells, the binding studies rule out a direct effect of C100 on the BK receptors as one of its mechanism of action.

Tg, which blocks the calcium ATPase pump and thus induces calcium release via mechanisms different from those of IP_3 [50], induced larger responses in control cells than in C100-transfected cells, so that its effects were opposite to those of BK. These results indicate that the total calcium pools (which overlap with the IP_3-sensitive stores) may be smaller in transfected cells than in control PC12 cells. The lack of differences in the SERCA pump expression, further supports this interpretation. Thus, the enhancement of the IP_3 pathway is capable of rendering larger calcium responses to BK in C100-transfected cells than in controls despite smaller calcium pools in the former. Interestingly, a reduction in total IP_3-R immunoreactivity was observed in the cells with enhanced responses (transfected), strongly suggesting that changes in number of receptors in C100 expressing cells cannot explain the phenomenon. Since the calcium elevations in response to the ionophore in both cell lines recovered at a similar rate, we can conclude that there are no major

differences in calcium buffering systems (i.e., the combined actions of binding proteins, pumps and mitochondrial storage) between the transfected and control lines. In addition to excluding the previously discussed steps as the primary sites of action of C100, the combined data suggest that the enhanced response might be due to an elevated production of IP_3 and/or increased sensitivity of the IP_3 receptor itself. The lack of change in the BK receptors makes differences in IP_3 production less likely. Changes in IP_3 receptor number and/or calcium content can also be ruled out since they were both *reduced*. Therefore, enhanced IP_3 receptor sensitivity seems the most likely explanation for the observed phenomenon in transfected cells. Further support for this interpretation comes from the increased IP_3-induced calcium release in isolated microsomes from transfected cells compared to controls.

NGF treatment did not significantly modulate the effects of C100 on the calcium response. Although cellular degeneration in C100 transfected PC12 cells is linked to the differentiation process, the ion channel-related effects of C100 in other cells do not require the presence of NGF. Similarly, C100 causes alterations in the calcium homeostasis of PC12 cells by a mechanism that is independent of NGF-induced differentiation.

In summary, our combined evidence indicates that increased intracellular levels of the C100 fragment may be responsible for conferring on AD cells an altered IP_3 sensitivity leading to an altered calcium handling. This enhanced calcium response could itself be a direct mechanism of toxicity, or it could contribute to processes that ultimately cause cell death. Perturbed calcium homeostasis is thought to play a pivotal role in the process of neurodegeneration in AD (reviewed in Ref. [30]). The demonstration that C100 induces significant alterations of calcium homeostasis in neuronal cells suggests that this peptide is relevant to the pathophysiology of AD.

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