Potential CRE suppression by familial Alzheimer's mutants of APP independent of adenylyl cyclase regulation

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Abstract In familial Alzheimer's disease (FAD), mutations to I, F, and G have been discovered at V642 in the neuron-specific version of the amyloid precursor protein APP_{695}. It has been found that expression of each FAD mutant suppresses the transcriptional activity of the cAMP response element CRE in a $G\alpha_o$-dependent manner in a COS cell clone NK1 [Ikezu et al. (1996) EMBO J. 15, 2468–2475]. Here we show that adenylyl cyclase (AC) inhibition is probably not the prerequisite for this pathway. First, expression of each FAD mutant in NK1 cells had no effect on AC activity stimulated by cholera toxin and by mutationally activated $G\alpha_s$, although the same expression completely repressed the stimulated CRE. Second, a transfected activating mutant of $G\alpha_o$ inhibited CRE without detectable suppression of AC, whereas similarly transfected activating $G\alpha_{i2}$ inhibited both AC and CRE. Third, FAD mutant-induced inhibition occurred for CRE activity stimulated by dibutyryl cAMP. These data suggest that CRE suppression by FAD mutants of APP could occur independently of AC.

1. Introduction

AD (Alzheimer's disease), the most prevalent neurodegenerative disease, is characterized by the presence of extracellular senile plaques, whose major constituent is Aβ amyloid derived from APP (amyloid precursor protein). Among various splicing variants transcribed from a single gene, APP_{695}, consisting of 695 residues, is preferentially expressed in neuronal tissues. In patients with FAD (familial Alzheimer's disease), the three missense mutations V642I, V642F, and V642G have been identified in the transmembrane domain of APP_{695} [1]. These mutations co-segregate with the AD phenotype [2]. Games et al. [3] have reported that overexpression of V642F APP partially mimics the neuropathology of AD in transgenic mice. Therefore, structural alteration of APP is at least one established cause of AD. However, little has been known about how these mutations cause AD and what abnormality they induce in APP.

In its architecture, orientation, and localization, APP is similar to cell-surface receptors. APP has been implicated in cell-surface functions such as adhesion, neurite outgrowth, synaptic contact, and locomotion (reviewed in [4]). The cytoplasmic domain of APP binds Fe65 protein, which has a phosphotyrosine binding domain related to an oncogenic signal transducer Shc [5]. It also binds APP-BP1, a gene product similar to AXR1 in *Arabidopsis*; AXR1 is required for normal response to the plant growth hormone auxin [6]. These observations suggest that APP has not only the structure but also the function of a cell-surface receptor. Our own earlier study [7] found APP_{695} has an intrinsic G_o-stimulating domain at H657-K676 and forms a complex with G_o via H657-K676, suggesting that APP_{695} is a transmembrane G_o activator and a potential G_o-coupled receptor. It has been confirmed that the synthetic H657-K676 peptide activates G_o in vivo [8]. Co-localization of APP with G_o in growth cones and presynapses of neurons [9,10] is in good agreement with the notion that APP co-operates with G_o. In further support, APP and G_o have been implicated in similar or same functions of neurons (reviewed in [4]). Ferreira et al. [11] and Culvenor et al. [12] independently showed the cellular presence of APP in surface and clathrin-coated vesicles, lending additional credence to the notion that APP may be a functional signaling receptor. Recently, we have demonstrated that cross-linking of APP_{695} by anti-APP antibody activates G_o in reconstituted vesicles [13] and activates G-protein downstream Ser/Thr kinases in intact cells [14]. Therefore, APP_{695} can behave as a normal G_o-coupled receptor, although the physiological significance of this function remains unknown.

What happens to this signal of APP in AD? To address this question, we focused on FAD, which is homogenous in cause. We have shown that mutant APPs with each of the three FAD mutations V642I, V642F, and V642G, collectively referred to as FAD-APP here, are constitutively active receptors that can activate G_o ligand-independently [4,15]. In reconstituted vesicles, three recombinant FAD-APPs activated purified G_o without ligands in a pertussis toxin (PTX)-sensitive manner; for this activation, the cytoplasmic domain H657-K676, which has been shown to be a selective G_o activator [7,8], was required. We also reported that expression of the three FAD-APPs each suppresses transcriptional activity of the cAMP response element CRE [15]. This suppression was $G\alpha_o$-dependent [15]: in the usual COS cells that express no $G\alpha_o$, CRE was not suppressed by transfected FAD-APPs; in the NK1 clone expressing endogenous $G\alpha_o$, transfection of FAD-APPs suppressed CRE in a PTX-sensitive manner; in the NK1 cells overexpressing human $G\alpha_o$, similar expression of FAD-APPs resulted in greater, almost total suppression of CRE.

This negative transactivation of CRE by FAD-APP would be important because (i) deficient CRE function results in a loss of long-term memory in mice [16] and *Drosophila* [17]; and (ii) positive CRE function activates the expression of genes required for synaptic plasticity and long-term potentiation of synapses (reviewed in ref. [18]). Therefore, it is conceivable that FAD-APP continuously suppresses the tran-

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scriptional activity of CRE, potentially leading to long-term memory loss in patients with FAD. This study was conducted to further understand the mechanism whereby FAD-APPs suppress CRE through $G\alpha_o$. There are two possible mechanisms whereby $G\alpha_o$ negatively regulates CRE: one is through the inhibition of adenylyl cyclase (AC) and the other is AC-independent. In this study, we investigate the role of AC. The results suggest that FAD-APP could suppress CRE transcriptional activity through an AC-independent target of $G\alpha_o$.

2. Experimental procedures

All constructs used in this study were as described previously [15,19]. NK1 cells were grown in DMEM plus 10% fetal calf serum and antibiotics. This cell line is a naturally occurring, neuron-like transformant of COS-7 cells, which express endogenous $G\alpha_o$ [20]. The measurement of intracellular cAMP accumulation was performed as described [21]. A day before transfection, 5×10^4 cells were seeded on a 12-well plate. Unless otherwise specified, cDNAs (0.5 μg) were transfected with 1 μl LipofectAMINE and incubated for 24 h in a serum-free culture. After being washed, cells were labeled with 3 μCi of [^{3}H]adenine for another 23 h 30 min. After washing, cells were then treated with 2.5 μg/ml CTX (cholera toxin, Calbiochem) and 1 mM isobutylmethylxanthine in DMEM at 37°C for 30 min. Reactions were terminated by aspiration and the immediate addition of 5% ice-cold trichloroacetic acid. Acid-soluble nucleotides were separated on two-step ion-exchange columns, and specific accumulation of cAMP was expressed as (cAMP/ADP+ATP)$\times10^3$. Under similar conditions, AC activity was also measured by assaying cAMP contents by radioimmunoassay, in which co-transfection of pactβgal and standardization by the transfection efficiency were performed. The content of cAMP was expressed as *pmol/β-galactosidase IU*. These two assays for AC activity indicated similar results. For CAT assay, 10^5 cells were seeded onto a 6-well plate 24 h before transfection. One μg cDNA of interest, 0.2 μg pactβgal, and 0.3 μg CRE-CAT reporter were co-transfected with LipofectAMINE (3 μl); this condition allowed expression of FAD-APP comparable to that seen in other experiments without reporter co-transfection. At 24 h after transfection, media were changed. CTX (1 μg/ml) was added to these media and cells were incubated for another 24 h. CAT assay was performed as described [19]. CAT activity was normalized by β-galactosidase activity. As pactβgal expresses the β-galactosidase activity under the control of the β-actin promoter, we can detect specific alterations in CRE transcriptional activity by this normalization. The CRE is typified by the consensus palindromic sequence TGACGTCA and present in the promoters of many genes. Our CRE-CAT reporter, described previously [22], has CRE located in the promoter of the somatostatin gene, which is highly responsive to cAMP stimulation [23]. For the dibutyryl cAMP (dbcAMP) experiment, cells were treated with 1 mM dbcAMP (SIGMA) or 1 μg/ml CTX for the last 12 h, and CRE transcriptional activity was measured. Statistical analysis was performed with Student's t-test, with $p<0.05$ accepted as statistically significant.

3. Results

As the major mechanism for CRE suppression is inhibition of AC, we initially examined whether expression of FAD-APPs leads to the suppression of AC activity in NK1 cells. The left panel of Fig. 1 shows the effects of the three V642 mutants on cAMP formation. While CTX stimulated cAMP accumulation, transfection of wild-type or each FAD mutant of APP cDNAs resulted in no significant inhibition of this elevated cAMP production. Under similar conditions, transfection of FAD-APP totally suppressed CRE activity (Fig. 1, right), as reported previously [15]. Transfection efficiency of NK1 cells under the conditions we employed was 40–50%, as assessed with LacZ expression by transfected pactβgal. As the inhibition of CTX-stimulated CRE-CAT by transfected FAD-APP was nearly complete, the AC activity should have been half inhibited, if CRE suppression was only mediated by AC inhibition. Total lack of the effect of FAD-APPs on AC activity hence suggests that transfection efficiency may not be the major factor in this failure.

$G\alpha_s$-Q227L, termed gsp [24], is the GTPase-deficient mutant of $G\alpha_s$ that constitutively maintains an active conformation. To confirm that FAD-APPs were without effect on stimulated AC within the same transfected cells, we examined whether AC activity promoted by the transfected gsp could be antagonized by co-transfected FAD-APP. Transfection of the cDNA encoding $G\alpha_s$-Q227L resulted in significant stimulation of AC in NK1 cells (Fig. 2). Co-transfection of wild-

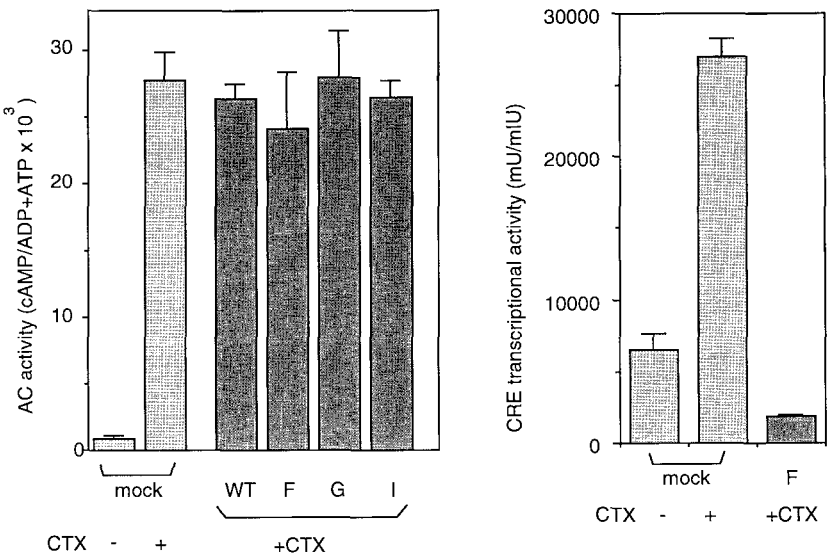

Fig. 1. Effect of FAD-APP on AC and CRE activities. Left: Effect of FAD-APP cDNA transfection on CTX-stimulated AC activity in NK1 cells. After either APP_{695}, FAD-APP (V642X APP_{695}, X = F, G, I; WT, wild-type APP_{695}) or empty plasmid was transfected into these cells, the cells were treated with or without CTX, and AC activity was measured. Right: Cells were transfected with or without V642F APP cDNA under similar conditions as in the left panel, and CAT activity was measured. All values in the figures of this study represent the means ± S.E. of three independent experiments with different transfections.

type APP had no effect on this promoted cAMP. When either FAD mutant of APP cDNA was co-transfected instead of an empty plasmid or APP, no gsp-promoted levels of cAMP were lowered, indicating that transfection-dependent stimulation of AC was not affected by co-transfected FAD-APPs. In these experiments, the total amounts of the plasmids were set to be constant, and expression of either FAD-APP was not impaired by the co-transfection of $G\alpha_s$-Q227L (data not shown). These data suggested no direct action of FAD-APPs on AC in situ. We attempted to check whether CRE activity stimulated by gsp was inhibited by co-transfected FAD-APP. However, we were not able to perform this experiment, as cells did not express well FAD-APP when the four plasmids encoding FAD-APP, $G\alpha_s$-Q227L, CRE-CAT, and pactβgal cDNAs were transfected.

We pursued different lines of evidence that FAD-APP suppresses CRE other than through AC in NK1 cells. In these cells, $G\alpha_o$ is highly likely to be a signal mediator of FAD-APPs affecting CRE [15]. Thus, we next examined whether $G\alpha_o$ activation can suppress CTX-stimulated activity of AC in NK1 cells. For this purpose, we used $G\alpha_o$-Q205L cDNA which encodes mutationally activated $G\alpha_o$ [19]. As reported previously [15], transfection of this mutationally activated $G\alpha_o$ cDNA resulted in robust suppression of CRE stimulated by CTX in NK1 cells (Fig. 3A). We then examined whether transfection of $G\alpha_o$-Q205L suppresses AC activity. Transfection of $G\alpha_o$-Q205L cDNA did not significantly suppress AC activity stimulated by CTX (Fig. 3B). In contrast, similarly transfected $G\alpha_{i2}$-Q205L, a mutationally activated $G\alpha_{i2}$, significantly suppressed AC and CRE activities both stimulated by CTX. As antibodies that equally recognize $G\alpha_o$ and $G\alpha_{i2}$ were not available, we could not compare the expression level

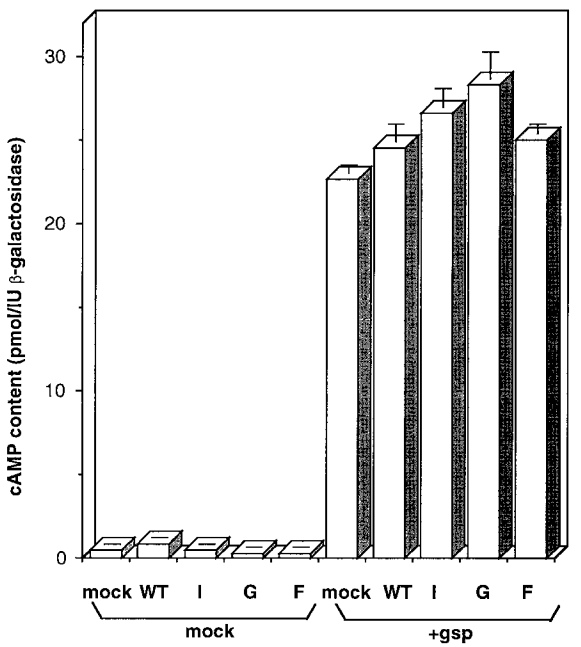

Fig. 2. FAD-APP cannot inhibit AC activity stimulated by gsp. Cells were transfected with gsp cDNA (0.5 μg) and either FAD-APP cDNA (0.5 μg; V642X APP_{695}, X = F, G, I; WT, wild-type APP_{695}). The total amount of DNA in control transfections was adjusted to 1 μg using an empty plasmid (mock). cAMP content was measured by cAMP radioimmunoassay without CTX by otherwise the same protocol. These experiments were repeated at least three times with similar results; a set of the representative data is shown.

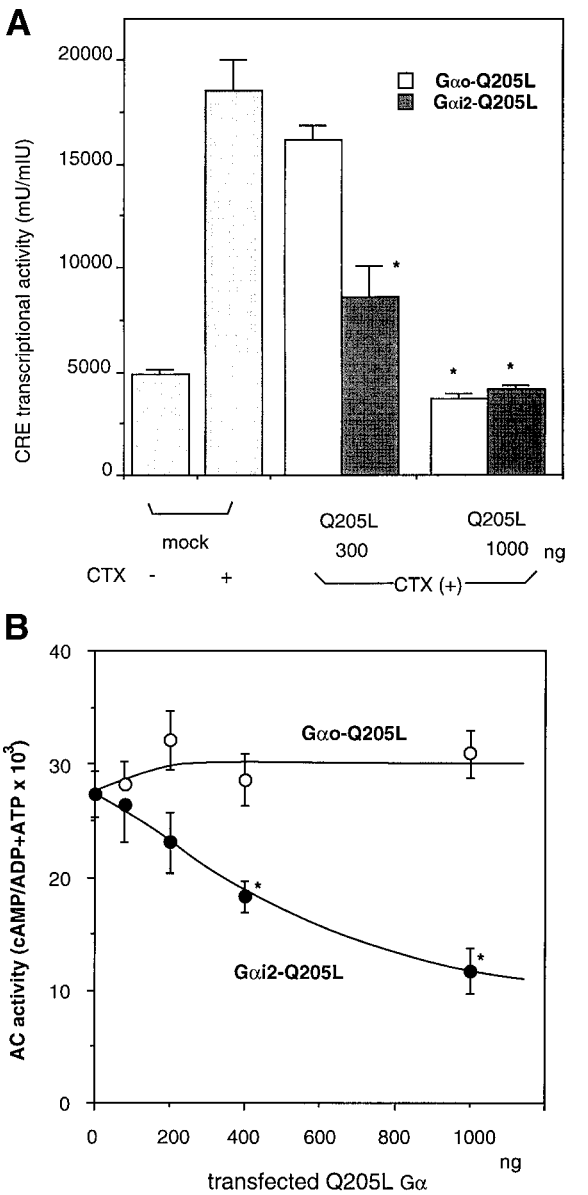

Fig. 3. Effect of GTPase-deficient $G\alpha_o$ on AC and CRE activities. Effect of GTPase-deficient Gα-Q205L cDNAs on CTX-stimulated AC activity in NK1 cells. After either $G\alpha_o$-Q205L, $G\alpha_{i2}$-Q205L (dose as indicated), or empty plasmid (total amount was adjusted at 1 μg) was transfected into these cells, the cells were treated with or without CTX, and CRE activity (A) and AC activity (B) were measured, as described in the legend of Fig. 1. The basal formation of cAMP in A was 0.628 ± 0.13. $^*p < 0.05$ vs CTX^+ in mock transfection.

of $G\alpha_o$-Q205L with that of $G\alpha_{i2}$-Q205L and with their effects in these experiments; thus, we could not totally exclude the possibility that the transfection efficiency of $G\alpha_o$-Q205L was too low to observe the inhibition of AC stimulated in total cells by CTX. However, this possibility was unlikely, because (i) similarly transfected $G\alpha_{i2}$-Q205L resulted in significant suppression of AC activity; (ii) transfected $G\alpha_o$-Q205L had no effect on AC activity, when it inhibited CRE to a maximal degree similar to that by $G\alpha_{i2}$-Q205L; and (iii) NK1 cells do not express type 1 AC (AC1) [21] whose activity $G\alpha_o$ can inhibit specifically [25]. Therefore, the present data suggest that $G\alpha_o$ suppresses CRE other than through AC inhibition, whereas $G\alpha_i$ suppresses CRE mainly through AC inhibition.

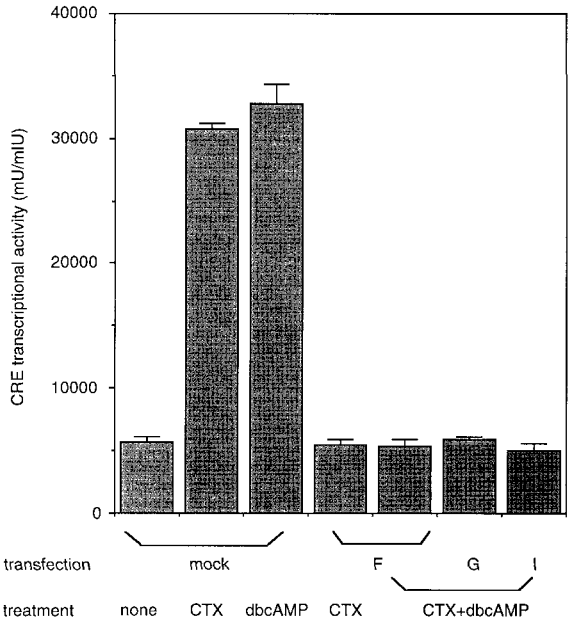

Fig. 4. FAD-APP can inhibit CRE activity stimulated by dbcAMP. After transfection with FAD-APP (V642X APP_{695}, X = F, G, I) or an empty plasmid (mock) under the same condition as in Fig. 1, cells were stimulated with or without CTX or dbcAMP for the last 12 h, and CRE transcriptional activity was measured.

These results provide evidence that components in the receptor-G protein transduction machinery can transmit inhibitory signals to nuclear CRE without modulating cytoplasmic AC.

If FAD-APPs suppress CRE through an AC-independent mechanism, they could also suppress CRE activity stimulated by exogenously added cAMP. We thus transfected NK1 cells with FAD-APP cDNA, and treated the transfected cells with 1 mM of dbcAMP, a cell-permeable cAMP analog, which attains maximal stimulation of CRE in these cells (data not shown). With mock transfection, cells increased the transcriptional activity of CRE by several fold in response to this cell-permeable cAMP. In contrast, cells transfected with each of FAD-APPs showed no increase in CRE activity in response to this cAMP stimulation (Fig. 4). These data provide clear evidence that the FAD-APP target is the system independent of AC in NK1 cells.

4. Discussion

Our previous study indicated that expression of FAD-APPs suppresses transcriptional activity of CRE through $G\alpha_o$ in the present NK1 system [15]. As cAMP is the major stimulator of CRE [23] and $G\alpha_o$ can potentially inhibit AC activity [26], inhibition of AC was the most likely mechanism for this suppression. However, neither expression of FAD-APP nor that of mutationally activated $G\alpha_o$, $G\alpha_o$-Q205L, inhibited AC activity, when either of them suppressed CRE activity in the same cells. These observations suggest that suppression of CRE by transfected FAD-APPs and $G\alpha_o$-Q205L may occur independently of AC regulation in NK1 cells.

We however emphasize that these transient transfection experiments may be limited. CTX stimulated AC in total cells, but we could detect the antagonism of FAD-APP or $G\alpha_o$-Q205L for AC, if any, only in transfected cells. Therefore, there was a discrepancy in the magnitude of action between CTX-induced stimulation and transfectant-induced inhibition. This is not the case with CRE. As the transcriptional activity of CRE was detectable only in the cells expressing transfected CRE-CAT reporter, the observed CRE change represented the change within cells expressing co-transfected FAD-APP or $G\alpha_o$-Q205L (because cells were transfected with the mixture of both DNA). If transfection efficiency was very low, we could detect little inhibition of CTX-stimulated AC by transfected FAD-APP, even when FAD-APP did inhibit AC activity in a single cell; besides, in this case, we could potentially detect significant inhibition of CTX-stimulated CRE by transfected FAD-APP. Therefore, one might argue that suppression of stimulated AC by transfected FAD-APP or $G\alpha_o$-Q205L could not be detected due to low transfection efficiency, despite their significant effects on AC in a single cell.

However, this argument seems unlikely in our case. Given the fact that transfection of $G\alpha_{i2}$-Q205L cDNA resulted in significant inhibition of CTX-stimulated AC, it was reasonable to assume that we were detecting transfection-dependent inhibition of AC stimulated by CTX in our system whose transfection efficiency was as high as ~40%. In addition, the inhibition of CTX-stimulated CRE-CAT by transfected FAD-APP or $G\alpha_o$-Q205L was nearly complete. Therefore, if the CRE suppression was only mediated by AC inhibition, the AC activity should have been unstimulated by CTX in the transfected cells (~40%) and completely stimulated by CTX in other cells (~60%), resulting in detectable suppression of CTX-stimulated AC by transfected FAD-APP or $G\alpha_o$-Q205L in total cells. In support, under conditions similar to those we employed in the present study, either transfected somatostatin type 3 receptor or insulin-like growth factor II receptor maximally inhibited AC activity stimulated by CTX to ~70% or ~50% levels, respectively [21,27]. The total lack of significant effects of FAD-APPs on AC suggests that transfection efficiency may not be the major factor for the negative observation, allowing for the notion that FAD-APPs repress CRE independently of AC regulation in our system.

One possible method of confirming this notion was to use NK1 cell lines stably overexpressing FAD-APPs. However, despite our repeated trials, we failed to establish such cell lines. This is however consistent with the fact that transfected FAD-APP causes cell death in NK1 cells [20]. We therefore tried to obtain alternative confirmation by making use of a different AC stimulator which only affects AC in FAD-APP-expressing cells: mutationally activated $G\alpha_s$ cDNA. As is the case with the CRE reporter gene, the activated $G\alpha_s$ gene would be taken up by cells together with FAD-APP genes complexed with a lipofection compound in the media. Therefore, it is highly likely that the $G\alpha_s$ mutant can only affect AC within the cells expressing APP or FAD-APPs. The results revealed that transfected FAD-APPs had no effect on AC stimulation by co-transfected $G\alpha_s$-Q227L. We again emphasize that in this system, AC was probably stimulated only in the cells expressing transfected genes. Therefore, the observed failure of AC inhibition by FAD-APP provides strong evidence that FAD-APP has no effect on stimulated AC inside the transfected cells.

Another line of confirmation was in the observation that transfected FAD-APPs could also suppress CRE activity stimulated by dbcAMP. Since this cell-permeable cAMP analog can act directly on protein kinase A, which stimulates CRE, the observed antagonism of CRE by FAD-APP against

cAMP indicates that FAD-APPs are able to suppress CRE by regulating the downstream targets of AC. Therefore, suppression of CRE by FAD-APP should not be the consequence of AC inhibition in our system.

What target and mechanism could underlie the actions of FAD-APP and $G\alpha_o$? At present, inhibition of AC and calcium channels are all known targets of $G\alpha_o$ [28]. The calcium channel inhibitable by $G\alpha_o$ is a voltage-sensitive type, whose distribution is limited to excitable cells, which NK1 cells are not. Given the AC-independent CRE regulation by FAD-APPs and $G\alpha_o$ shown here, the CRE-suppressing target of FAD-APP is therefore most likely a novel effector of $G\alpha_o$. It has been established that cAMP-dependent protein kinase A and calcium/calmodulin-dependent kinase (CaM kinase) IV each stimulates the CRE-binding protein CREB, the major regulator of CRE function, by phosphorylating its Ser^{133} residue [23,29]. In contrast, CaM kinase II activates the function of CREB by phosphorylating its *Ser133* residue but suppresses it by additionally phosphorylating it on *Ser142* [29, 30]. Therefore, CaM kinase II may be a likely candidate for the action mediator of FAD-APPs. Consistent with this possibility, CaM kinase II has been implicated in essential memory processes such as long-term hippocampal potentiation of synapses [31,32] and spatial learning [33]. It must be examined whether CaM kinase II is activated by the transfection of FAD-APP and $G\alpha_o$-Q205L, and whether inhibition of this enzyme precludes CRE from the suppression by FAD-APP.

Finally, it should be underscored that the results of this work do not exclude an intermediary role for AC in FAD-APP-induced CRE suppression in neurons. Inhibition of AC1 by $G\alpha_o$ does result in impaired function of cAMP, leading to CRE suppression [23]. However, using AC1-lacking NK1 cells, the present study shows, for the first time, that FAD-APPs and activated $G\alpha_o$ can suppress CRE without inhibiting AC activity. Hence, in neurons, which express AC1 in addition to other AC subtypes, FAD-APPs potentially suppress CRE by both AC-dependent and -independent mechanisms. The extent of FAD-APP-induced inhibition of CRE in neurons is therefore expected to be larger than in other types of cells. Given the fact that CRE regulates expression of so many genes, some of which are common across neurons and non-neurons, this dual mechanism could potentially underlie the higher vulnerability of nervous systems in AD and also allow for the extraneuronal occurrence [34] of AD disorders which mirror the abnormality in neurons.

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