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Activation of α-secretase by curcumin-aminoacid conjugates

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ABSTRACT

The extracellular senile plaques observed in Alzheimer's disease (AD) patients are mainly composed of amyloid peptides produced from the β-amyloid precursor protein (βAPP) by β- and γ-secretases. A third non-amyloidogenic α-secretase activity performed by the disintegrins ADAM10 and ADAM17 occurs in the middle of the amyloid-β peptide Aβ and liberates the large sAPPα neuroprotective fragment. Since the activation of α-secretase recently emerged as a promising therapeutic approach to treat AD, the identification of natural compounds able to trigger this cleavage is highly required. Here we describe new curcumin-based modified compounds as α-secretase activators. We established that the aminoacid conjugates curcumin–isoleucine, curcumin–phenylalanine and curcumin–valine promote the constitutive α-secretase activity and increase ADAM10 immunoreactivity. Strickingly, experiments carried out under conditions mimicking the PKC/muscarinic receptor-regulated pathway display different patterns of activation by these compounds. Altogether, our data identified new lead natural compounds for the future development of powerful and stable α-secretase activators and established that some of these molecules are able to discriminate between the constitutive and regulated α-secretase pathways.

1. Introduction

The main component of the senile plaques observed in AD patients is a 40- to 43-amino-acid-long β-amyloid (Aβ) peptide that is produced from the β-amyloid precursor protein (βAPP) through sequential cleavages by β- and γ-secretase [1]. Thus, intense efforts have focused on the development of specific and powerful inhibitors of these activities. However, the fact that both enzymes cleave numerous other physiologically relevant substrates renders their inhibition problematic because of deleterious secondary effects on the nervous system, the immune system and the gastrointestinal tract [2–5]. Therefore, a promising but yet underestimated strategy would be to activate the α-secretase processing of βAPP [6]. This cleavage is performed by the disintegrins ADAM10 and ADAM17 that are responsible for the constitutive and PKC-regulated pathways, respectively [7,8]. Interestingly, this cleavage can be seen as twice beneficial since it both occurs in the middle of the Aβ sequence and gives rise to the production of the neurotrophic and neuroprotective secreted sAPPα fragment [6]. In addition to protein kinase C (PKC) for which deficits were observed in Alzheimer's disease patients [9], M1/M3 subclass of muscarinic receptors have been shown to be key up-regulators of α-secretase processing of βAPP *in vitro* and *in vivo* [10,11].

Chronic or acute pharmacological stimulations of α-secretase would engender many deleterious consequences, especially when one considers the tumor-promoting activity of the PKC-activating phorbol esters. The demonstration that the natural compound bryostatin is able, at sub-nanomolar concentrations, to trigger PKC activity, promote sAPPα secretion and reduce Aβ production without inducing tumor represents a step forward in the search of potent and harmless PKC activators [12]. The alternative solution consisting in the activation of α-secretases themselves, would face a major issue since ADAM10 and ADAM17 target more than 80 different substrates, some of which being implicated in the development of severe pathologies [13]. Thus, it may appear more judicious to privilegiate the use of long-term and mild treatments, mainly through the consumption of natural compounds present in food. In this context it recently emerged that several natural compounds such as the plant extracts *Ginkgo biloba* and green tea–epigallocatechin-3-gallate can specifically activate the α-secretase cleavage of βAPP [14–15].

Curcumin (diferuloylmethane), the main component extracted from the plant *Curcuma longa* (turmeric), can penetrate the

Abbreviations: PKC, protein kinase C; ADAM, A disintegrin and metalloprotease; PDBu, phorbol-12,13-dibutyrate; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; HEK, human embryonic kidney.

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blood–brain barrier and was suggested as a promising therapy for AD since it displays anti-inflammatory, antioxidant and copper and iron chelation properties [16]. A role for curcumin in the regulation of βAPP biology and processing emerged during the past 3 years when curcumin was shown to impair Aβ42 production, to reduce βAPP protein levels, to suppress Aβ-induced BACE1 upregulation and to attenuate βAPP maturation in the secretory pathway, thereby decreasing Aβ levels [17,18]. However, the influence of curcumin on the α-secretase cleavage of βAPP has not been investigated so far.

Because of its low absorption, rapid metabolism, inherent instability and hydrophobic nature, clinical trials carried out with curcumin did not show any improvement of cognitive functions in humans with dementia [19]. Therefore, efforts have been made to increase its bioavailability and the use of adjuvants such as piperine ameliorates curcumin stability although no significant improvements were evidenced in mild-to-moderate AD patients [20]. This suggests that further chemical modifications of curcumin are needed to trigger some beneficial effects. In the present study, we examined the effects of curcumin, its metabolite tetrahydrocurcumin (THC) as well as the three aminoacid conjugates curcumin–isoleucine, curcumin–phenylalanine and curcumin–valine on the non-amyloidogenic α-secretase activity. We report that the aminoacid conjugates induce the constitutive α-secretase cleavage of both the synthetic substrate JMV2770 and βAPP through an increase in ADAM10 immunoreactivity. Moreover, it appears that these compounds are able to discriminate between the constitutive and the regulated α-secretase pathways.

2. Materials and methods

2.1. Antibodies, reagents and cell lines

Polyclonal anti-ADAM10 and anti-ADAM17 were from Millipore. Polyclonal anti-βAPP (A8717) was from Sigma. Monoclonal anti-β-amyloid (DE2B4) which was used to specifically detect sAPPα and monoclonal anti-actin were from Cell Signaling. DMEM complete medium and fetal bovine serum were from Gibthai-Invitrogen Corporation. Penicillin–streptomycin mix was from PAA. Tris buffer and Glycine were from Vivants. Dimethyl sulfoxide and sodium bicarbonate were from Sigma. Skim milk powder was from Criterion. ECL and ammonium persulphate were from GE Health care. SDS was from Amaresco. Metalloprotease inhibitor *o*-phenanthroline was from CalBiochem. The α-secretase fluorimetric substrate JMV2770 is an 11-amino-acid-long sequence of the cellular prion protein encompassing the peptidyl bond targeted by α-secretase. JMV2770 (Abz-Thr-Asn-Met-Lys-His-Met-Ala-Gly-Ala-Ala-Gln-EDDnp) contains the ortho-aminobenzoyl (Abz)/dinitro-phenyl (EDDnp) groups as the donor/acceptor and was synthetized as previously described [21]. HEK 293 cells overexpressing ADAM10, ADAM17, M1R and βAPP were cultured as previously described [22–25].

2.2. Curcumin and curcumin derivatives

Curcumin (~95% purity) was purchased from Ms Spicex chemicals Pvt. ltd. (Mysore, India). The aminoacid conjugates curcumin–isoleucine (Cur–Ile), curcumin–phenylalanine (Cur–Phe) and curcumin–valine (Cur–Val) were prepared on gram scale using the previously described protocol [26] (see Fig. 1 for the detailed structures of the compounds).

2.3. Fluorimetric assay on intact cells

HEK293 cells overexpressing ADAM10, ADAM17 or M1R were cultured in 35-mm dishes until cells reach 80% confluency. Cells were treated in duplicate without (control) or with 50 μM curcumin, THC, Cur–Ile, Cur–Phe and Cur–Val for 14 h at 37 °C in DMEM containing 1% FBS. After this treatment period, the PKC activator phorbol-12,13-dibutyrate (PDBu) (1 μM, ADAM17-overexpressing cells) or the muscarinic agonist carbachol (100 μM, M1R-overexpressing cells) were added for 2 h. Duplicates were then incubated for 30 min at 37 °C either with or without *o*-phenanthroline (100 μM in 1.5 ml of PBS). Then, JMV2770 substrate (10 μM) was directly added into the media and cells were maintained at 37 °C. After 10, 30 min and subsequently at every 30 min, 100 μl of media were removed and fluorescence was recorded in black 96-well plates at 320 and 420 nm excitation and emission wavelengths, respectively. After the last incubation time, cells were resuspended in 10 mM Tris, pH 7.4 and samples were kept for Western blot analysis.

2.4. Western blot analyses

Ten micrograms of proteins were loaded onto 8% (ADAM10 and ADAM17) or 10% (β-actin) SDS–PAGE and run at 120 V for 90 min and then transferred onto nitrocellulose membrane for 90 min at 100 V. Patterns of transferred proteins were checked with Ponceau Red staining and nitrocelluloses were incubated in 5% nonfat milk blocking solution for 30 min. Membranes were then incubated with primary antibodies directed against ADAM10 (1/1000 dilution), ADAM17 (1/1000) or β-actin (1/5000) on a platform shaker overnight at 4 °C. After three washes with PBST (PBS containing 0.05% Tween 20), membranes were incubated with HRP-conjugated anti-rabbit (ADAM10 and ADAM17) or anti-mouse (β-actin) secondary antibodies (1/5000) for 2 h and rinsed three times with PBST. Immunoreactivities were processed using ECL and signals were detected using an automatic X-ray developing machine. Films were then analyzed by densitometry and ADAM10 and ADAM17 levels were normalized using β-actin as an internal standard.

2.5. Effect of curcumin and its conjugates on βAPP processing

HEK293 overexpressing βAPP were cultured in 35-mm dishes until cells reach 80% confluency. Cells were treated without (control) or with 50 μM curcumin and its conjugates THC, Cur–Ile, Cur–Phe and Cur–Val for 14 h at 37 °C in DMEM containing 1% FBS. Media were then removed and replaced with DMEM without FBS and cells were allowed to secrete for 1 h. Both media (20 μl, sAPPα) and cell lysates (20 μg of proteins for βAPP; 10 μg of proteins for β-actin) were submitted to Western blot analysis. Samples were loaded onto 8% (sAPPα and βAPP) or 10% (β-actin) SDS–PAGE, run at 120 V for 90 min and transferred onto nitrocellulose membrane for 120 min at 100 V. Nitrocelluloses were incubated in 5% nonfat milk blocking solution for 30 min and incubated with primary antibodies directed against sAPPα (1/500 dilution), βAPP (1/5000) or β-actin (1/500) overnight at 4 °C. After three washes with PBST, membranes were incubated with HRP-conjugated anti-rabbit (βAPP) or anti-mouse (sAPPα and β-actin) antibodies for 2 h and rinsed three times with PBST. Immunoreactivities were processed using ECL and signals were detected using an automatic X-ray developing machine. Films were then analyzed by densitometry and βAPP levels were normalized using β-actin as an internal standard.

2.6. Statistical analysis

Statistical analyses were performed with the Prism software (Graphpad, San Diego, USA) by using the Newman–Keuls multiple comparison test for one-way ANOVA and the Student's *t* test for pairwise comparisons. All the results are expressed as means ± SEM values.

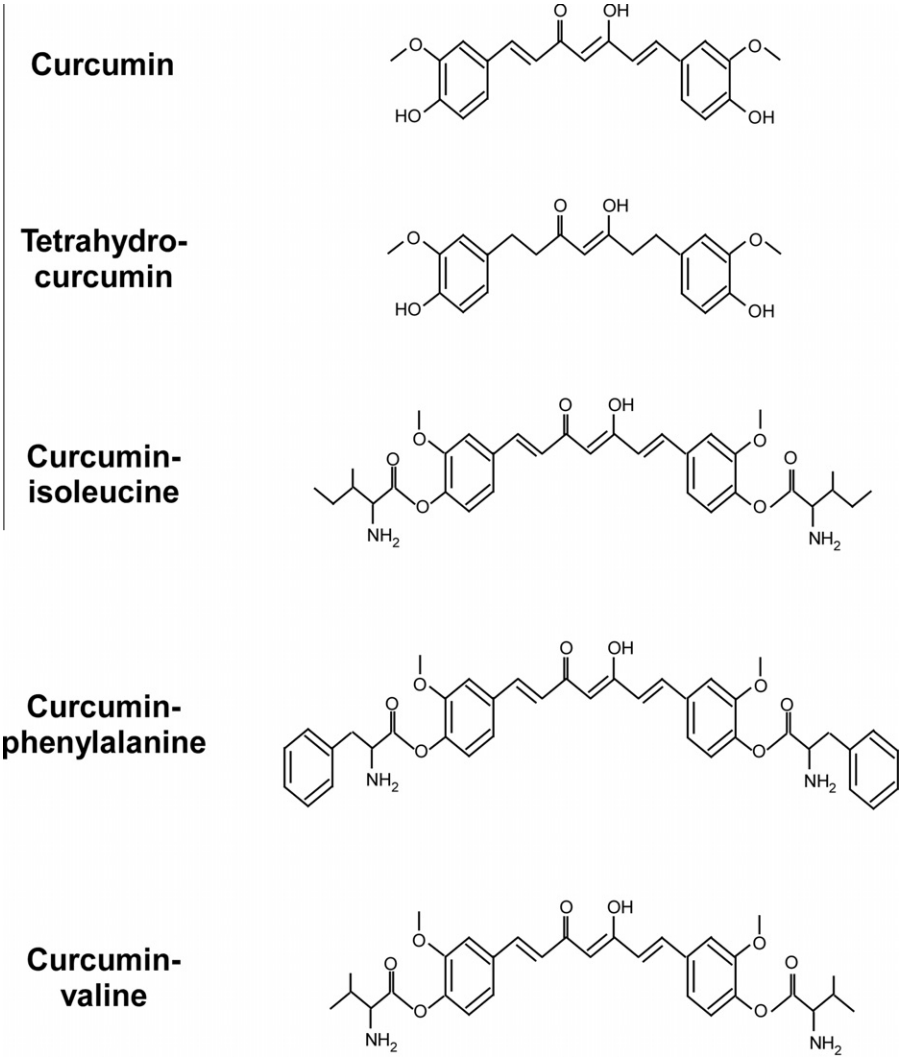

Fig. 1. Structure of curcumin, tetrahydrocurcumin and aminoacid conjugates.

3. Results

Because the constitutive α-secretase activity has been clearly assigned to ADAM10, we first investigated the ability of curcumin, THC, Cur–Ile, Cur–Phe and Cur–Val (Fig. 1) to promote α-secretase in cultured ADAM10-overexpressing HEK293 cells and thereby established that Cur–Ile, Cur–Phe and Cur–Val used at a 50-μM concentration for 14 h, but not curcumin and THC, were able to significantly enhance the *o*-phenanthroline-sensitive JMV2770 hydrolysis (Fig. 2A).

In order to determine whether the observed increase in JMV2770 hydrolysis was due to an augmentation of ADAM10 expression, we analyzed the effect of treatments with curcumin and its aminoacid conjugates on ADAM10 levels by Western blot. The results clearly showed that Cur–Phe, Cur–Ile and Cur–Val significantly increased ADAM10 immunoreactivity whereas curcumin and THC remained inactive when compared to the control (Fig. 2B).

We then wanted to establish a correlation between the results obtained on the catalytic activity of α-secretase (i.e. fluorescence related to JMV2770 hydrolysis) with the α-secretase cleavage of βAPP (i.e. sAPPα secretion). For that purpose, we treated HEK293 overexpressing human βAPP with curcumin and its derivatives at a concentration of 50 μM for 14 h. The results showed that the three aminoacid conjugates Cur–Val, Cur–Ile and Cur–Phe, but not curcumin itself or THC, significantly promote the α-secretase cleavage of βAPP as indicated by the increases in sAPPα when compared to control with no significant modification of βAPP immunoreactivities (Fig. 2C). Importantly, experiments performed under constitutive conditions (no muscarinic receptors or PKC activation) either in ADAM10-overexpressing cells (JMV2770 hydrolysis, Fig. 2A) or in βAPP-overexpressing cells (sAPPα secretion, Fig. 2C) showed similar effects of the compounds.

In order to evaluate the impact of curcumin and its modified analogs on the regulated α-secretase activity, we took advantage of the HEK293 cells that stably overexpress ADAM17, the protease that is mainly responsible for this pathway. Moreover, since PKC strongly up-regulate α-secretase activity, we incubated these cells with the PKC activator PDBu immediately after the treatment with curcumin, THC, Cur–Ile, Cur–Phe and Cur–Val and just before measuring JMV2770 hydrolysis. As observed in ADAM10-overexpressing cells (i.e. for constitutive activity), Cur–Val and Cur–Ile significantly enhanced JMV2770-hydrolyzing activity whereas curcumin and THC were still inactive (Fig. 3A). However, it appeared that Cur–Phe, that triggered a considerable increase in ADAM10-dependent constitutive α-secretase activity, remained inert under these particular conditions. Surprisingly, it appeared at first sight that curcumin and its aminoacid conjugates, used under the same conditions when compared to the fluorescence experiments, all increased ADAM17 immunoreactivity in ADAM17-overexpressing cells when compared to control (Fig. 3B). However, a careful

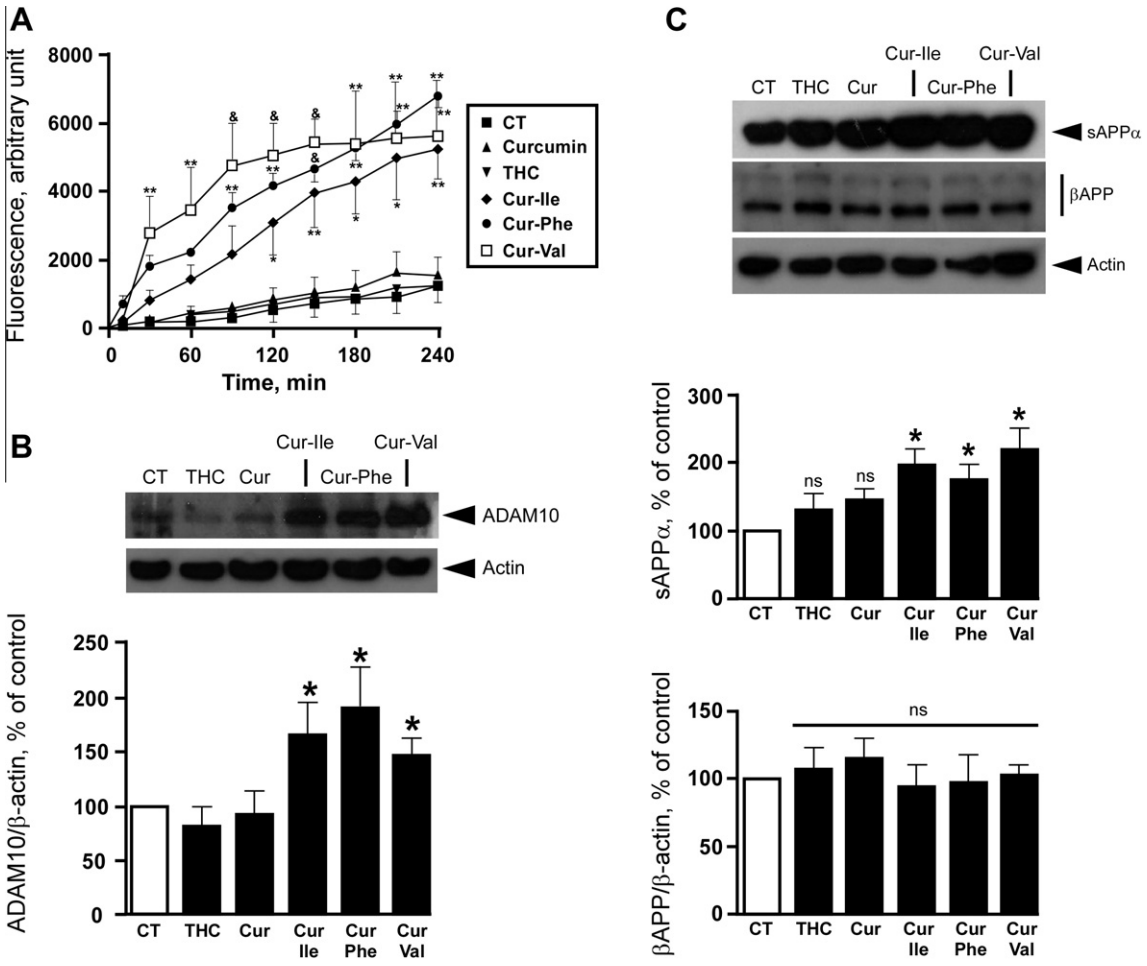

Fig. 2. Effect of curcumin and its derivatives on the constitutive hydrolysis of JMV2770 and βAPP by cultured HEK293 cells. Cultured HEK293 cells overexpressing ADAM10 (A, B) or wild-type βAPP (C) were treated without (CT) or with 50 μM of curcumin, THC, Cur-Ile, Cur-Phe or Cur-Val for 14 h at 37 °C. (A, B) Cells were then incubated with or without *o*-phenanthroline (100 μM) for 30 min. JMV2770 (10 μM) was then added to the media and its hydrolysis was measured as described in Section 2. Secreted sAPPα in the media as well as ADAM10, βAPP and β-actin in the cell lysates were analyzed by western blot as described in Section 2. (A) Effect of compounds on JMV2770 hydrolysis. (B and C) Effect of compounds on ADAM10 sAPPα and βAPP immunoreactivities with one representative gel and statistical analysis. Values in (A) represent the *o*-phenantholine-sensitive activities and are the means ± SEM of three independent experiments. Bars correspond to the densitometric analysis of ADAM10 (B), sAPPα and βAPP (C) (ADAM10 and βAPP being normalized with β-actin), are expressed as a percent of control (non-treated cells, CT) and are the means ± SEM of four independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{\&}p < 0.001$; ns, non-statistically significant.

statistical analysis of the data showed that THC does not significantly trigger ADAM17 level while the other compounds display variable effect according to significance (Fig. 3B).

For a complete characterization of the effect of these compounds on the regulated α-secretase activity, we decided to perform the same experiments under more physiological conditions (i.e. ADAM17 endogenous levels). For that purpose, we used HEK293 cells overexpressing type-1 muscarinic receptors. These cells were stimulated with the muscarinic agonist carbachol thereby allowing us to act upstream to PKC activation and to avoid the use of phorbol esters that are known to trigger toxic effects. Surprisingly, the results obtained showed a different spectrum of action of the compounds when compared to the two previous systems. Indeed, all the five compounds significantly increased the M1R-regulated α-secretase activity but with variable ability (Cur–Val > Cur–Ile > Cur–Phe > THC > curcumin) (Fig. 4).

4. Discussion

Alzheimer's disease is a complex pathology characterized by the loss of memory and cognition that is invariably accompanied by an Aβ over-production. However, the modulation of Aβ levels through the pharmacological regulation of secretases could prove difficult to apply as a therapy in humans due to strong deleterious side-effects. Therefore, natural compounds were considered as alternate therapeutic molecules for AD, such potent molecules being expected to reduce the amyloid load without triggering side-effects [27 for review]. For instance, studies carried out with *Centella asiatica* extract using mouse model of Alzheimer's disease showed that this extract decreased the levels of Aβ40 and Aβ42 in hippocampus [28]. Furthermore, *G. biloba* extract has been found to enhance the working memory and information processing [29] while the herb *Paeonia suffruticosa* and its active constituents not only inhibited the formation of Aβ fibrils but also destabilized the preformed fibrils [30].

One of the most promising natural molecules to be considered as anti-AD therapeutic compound is curcumin since it proved to convey beneficial effects according to oxidation, inflammation, apoptosis, Aβ production, βAPP expression and maturation as well as Aβ-dependent BACE1 expression both *in vitro* and *in vivo* [17,18,31,32]. However, despite its clinical safety at doses up to 12 g/day, curcumin displays poor bioavailability [20] and the main challenge is indeed to develop stable curcumin-based derivatives. The rationality behind the synthesis of aminoacid conjugates of curcumin was to increase its hydrophilicity and stability.

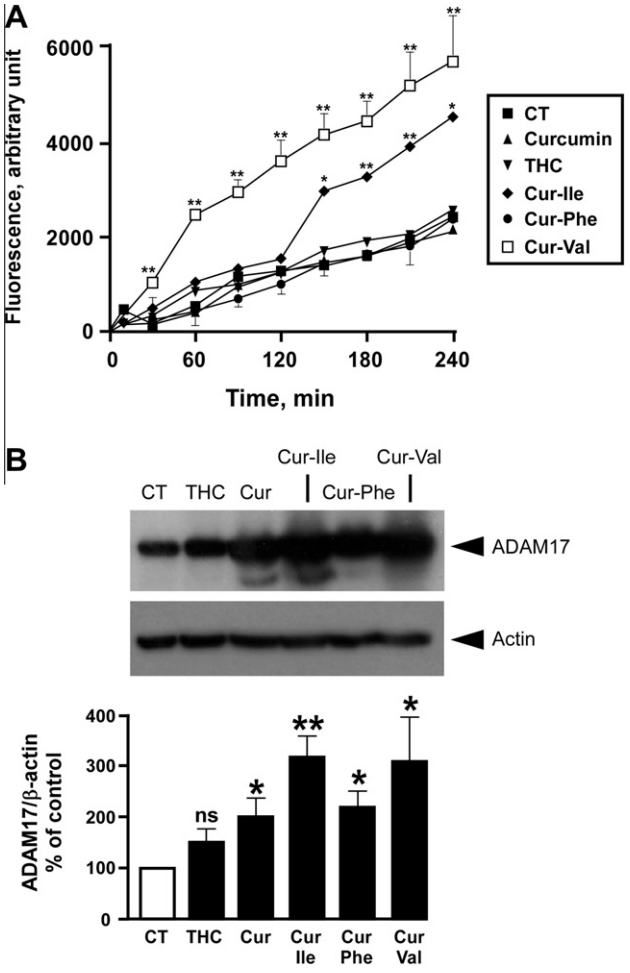

Fig. 3. Effect of curcumin and its derivatives on the PDBu-stimulated hydrolysis of JMV2770 by cultured HEK293 cells overexpressing ADAM17. Cultured HEK293 cells overexpressing ADAM17 were treated without (CT) or with 50 μM of curcumin, THC, Cur–Ile, Cur–Phe or Cur–Val for 14 h at 37 °C. Cells were then treated for 2 h with PDBu (1 μM) and subsequently incubated with or without *o*-phenanthroline (100 μM) for 30 min. JMV2770 was then added to the media and its hydrolysis was measured as described in Section 2. (A) Effect of compounds on JMV2770 hydrolysis. (B and C) Effect of compounds on ADAM17 immunoreactivity with one representative gel (B) and statistical analysis (C). Values in (A) represent the *o*-phenantholine-sensitive activities and are the means ± SEM of four independent experiments. Bars in (C) correspond to the densitometric analysis of ADAM17 (normalized with β-actin), are expressed as a percent of control (non-treated cells, CT) and are the means ± SEM of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$, ns, non-statistically significant.

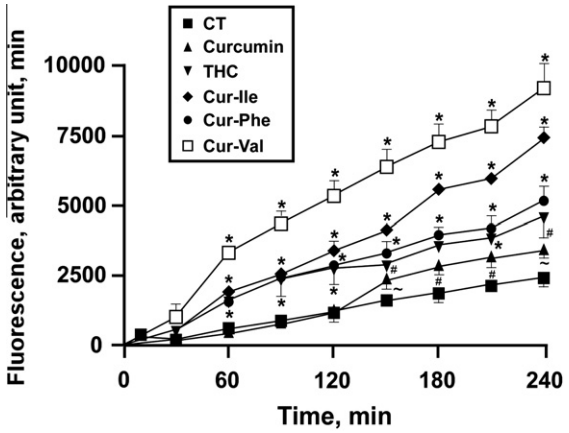

Fig. 4. Effect of curcumin and its derivatives on the carbachol-stimulated hydrolysis of JMV2770 by cultured HEK293 cells overexpressing M1 muscarinic receptors. Cultured HEK293 cells overexpressing M1 muscarinic receptors were treated without (CT) or with 50 μM of curcumin, THC, Cur–Ile, Cur–Phe or Cur–Val for 14 h at 37 °C. Cells were then treated for 2 h with carbachol (100 μM) and subsequently incubated with or without *o*-phenanthroline (100 μM) for 30 min. JMV2770 was then added to the media and its hydrolysis was measured as described in Section 2. Values represent the *o*-phenantholine-sensitive activities and are the means ± SEM of four independent experiments. $^{\sim}p < 0.05$; $^{\#}p < 0.01$; $^{*}p < 0.001$.

Our results first showed that Cur–Ile, Cur–Phe and Cur–Val are equally potent activators of the constitutive α-secretase activity whatever the substrates considered (JMV2770 and βAPP). The observation that curcumin and THC remained inefficient under the same conditions likely means that the three aminoacid conjugates display higher stability and/or better cell permeability over the 14-h treatment period. Although the exact mechanisms underlying the observed effects remain to be established, the fact that α-secretase activity augmentation is accompanied by a concomitant elevation of ADAM10 immunoreactivity likely suggests that the compounds need to penetrate into the cells to promote ADAM10 expression/stabilization.

When we next evaluated the ability of these compounds to modulate the regulated α-secretase activity in PDBu-stimulated ADAM17-overexpressing cells, we found that, although Cur-Ile and Cur-Val still behaved as powerful α-secretase activators, Cur-Phe, as curcumin and THC, was unable to promote JMV2770 hydrolysis. More surprising was the observation that ADAM17 immunoreactivity was significantly increased by curcumin and all the three conjugates. However, the highest augmentations observed with Cur–Ile and Cur–Val could explain that a threshold in ADAM17 expression is required to increase JMV2770 hydrolysis. Nevertheless, Cur–Phe seems to specifically activate the constitutive but not the PKC-dependent α-secretase pathway. Whether Cur–Phe specifically acts on ADAM10 activity remains to be established.

Finally, experiments carried out with carbachol-treated M1R-overexpressing cells unveiled a positive although variable effect (Cur–Val > Cur–Ile > Cur–Phe > THC > curcumin) of all the compounds regarding JMV2770 hydrolysis. Noteworthy, the fluorescence signal for JMV2770 hydrolysis is higher under carbachol-stimulated conditions (compare Fig2A and 3A with Fig4) eventhough M1R cells express endogenous (versus overexpressed) levels of ADAM17. This could explain why we evidenced a stimulatory effect of curcumin and THC that were not detectable in the previous experiments. Another possibility might be that curcumin and THC do not operate through ADAM10, ADAM17 or PKC but rather specifically interacts with M1 muscarinic receptors.

Additional experiments are now required to identify the molecular mechanisms that are indeed responsible for the hereabove described beneficial effect of curcumin derivatives. It will be of utmost importance to first establish whether the aminoacid conjugates modulate ADAM10 and/or ADAM17 at a transcriptional or at a post-transcriptional level. The impact of these compounds on the activities of ADAM10 and ADAM17 promoters as well as on mRNA levels are currently under investigation. Moreover, it will be crucial to validate these results in neuronal cell lines such as SY-5Y, N2a or primary neurons and in transgenic mouse model of Alzheimer's disease.

Altogether, beyond the fact that these new aminoacid conjugates of curcumin may serve as promising lead compounds for future drug development, our results show that some of these molecules are able to discriminate between the constitutive and the regulated α-secretase activities.

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