Apolipoprotein E4 Stimulates cAMP Response Element-binding Protein Transcriptional Activity through the Extracellular Signal-regulated Kinase Pathway*

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Inheritance of the e4 allele of the apolipoprotein E gene (APOE4) is a major risk factor for the development of Alzheimer’s disease (AD). Although the association between APOE4 and AD is well documented, the mechanism by which apolipoprotein E exerts an isoform-specific effect on neurons in disease is unknown. In this report, we demonstrate that apoE4 stimulates the transcriptional activity of cAMP-response element-binding protein (CREB) by activating the extracellular signal-regulated kinase (ERK) cascade in rat primary hippocampal neurons. In contrast, apoE3 was unable to stimulate CREB transcriptional activity and unable to activate the ERK pathway. Elevation of intracellular Ca2+ levels are also involved because treatment with receptor-associated protein, nifedipine, MK801, removal of Ca2+ from the medium and dantrolene all served to inhibit calcium elevation and attenuate the activation of CREB. Treatment with an apoE peptide was also found to facilitate transcription of the CREB-dependent genes, c-fos and Bcl-2. In contrast to treatment with apoE3, our findings suggest apoE4 and apoE-peptide induce a novel signaling pathway.

Alzheimer’s disease is a devastating neurological disorder characterized by progressive memory loss and cognitive deficits. To date, four genes have been reported to be associated with Alzheimer’s disease. Downstream of CREB, the fos and myc are involved in this same pathway. Downstream of CREB, the fos and myc are involved in this same pathway. Downstream of CREB, the fos and myc are involved in this same pathway. Downstream of CREB, the fos and myc are involved in this same pathway. Downstream of CREB, the fos and myc are involved in this same pathway. Downstream of CREB, the fos and myc are involved in this same pathway.

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EXPERIMENTAL PROCEDURES

Reagents—Recombinant apolipoprotein E3 and apolipoprotein E4 were purchased from Calbiochem (San Diego, CA); U0126 and cAMP-dependent protein kinase–specific inhibitor from Promega (Madison, WI); nifedipine and dantrolene from Sigma; (+)-MK801 hydrogen malate and KN92 from Research Biochemicals International (Natick, MA); RAP and anti-apolipoprotein E antibody (IgG5-E1) from Pregen Biotechnik (Heidelberg, Germany); anti-CREB antibody, anti-phospho-CREB antibody, anti-ERK antibody, and anti-phospho-ERK antibody from New England Biolabs (Beverly, MA); anti-c-Fos antibody from Santa Cruz Biotechnology (Santa Cruz, CA); mouse anti-actin mono-
clonal antibody (C4) from Chemicon International (Temecula, CA) and anti-Bcl-2 antibody from MBL (Nagoya, Japan).

ApoE Peptide Synthesis—A 30-amino acid apoE peptide, which is a tandem repeat of apoE amino acid residues 141-155 (LRKLKRRKLRLRADDL) was synthesized by the solid-phase method as described previously (17) and greater than 95% purity was determined by high performance liquid chromatography and mass spectrometry.

Cell Culture—Primary cultures of rat hippocampal neurons were prepared from Wistar rats at embryonic day 18 as described previously (18). Cells were routinely propagated in Dulbecco's modified Eagle's medium (Sigma) with 10% fetal calf serum (Life Technologies, Inc.). All experiments were performed on cells cultured for 5 to 8 days.

Primary astrocyte cultures were prepared from neonatal apoE-deficient mice as described previously (19) with minor modifications. In brief, apoE-deficient mice were purchased from Taconic Farms (Germantown, NY) and backcrossed to C57BL/6 for at least six generations, brains were removed from neonatal apoE-deficient mice within 24 h of birth, meninges were removed, and brain tissues were digested at 37 °C with Dispase II (Rochem Molecular Biochemicals, Germany) in PBS. The mixture was centrifuged (3000 × g for 10 min), plated in 175-cm² culture flasks (two brains/flask), and cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. After 10 days, cells were incubated for 48 h with cytosine arabinofuranoside (10 µg/ml; Wako, Osaka, Japan) to prevent fibroblast overgrowth. Astrocytes were then separated from microglia and oligodendroglia by agitation on a shaking platform (Bioshaker BR-30L, Tautek, Tokyo, Japan) and identified by immunoreactivity with an anti-GFAP antibody. Cultures used for experiments were >98% astrocytes based on these techniques.

Stimulation of Primary Astrocytes and Quantification of TNFα—Inhibition of secretion of TNFα from primary astrocytes by apoE was quantified as described previously (20). Briefly, primary astrocytes from apoE-deficient mice were plated on 96-well tissue culture dishes at a density of 20,000 cells/well and incubated in serum-free media (OptiMEM I, Life Technologies, Inc.) containing 1% N2 supplement (Life Technologies, Inc.). The following day, recombinant apoE3, apoE4, or control protein (denatured apoE3 by boiling for 30 min) was added. 24 h later, 100 ng/ml lipopolysaccharide (Sigma) was added. 60 h later, 50 µl of medium was removed and TNFα secretion measured by Quantikine M Mouse TNFα ELISA kit (R & D systems) as described in the manufacturer's protocol.

Promoter-reporter Assay—To detect the pathway activated in the presence of apoE peptide, we utilized the Mercury Pathway Profiling Systems (CLONTECH). Briefly, rat hippocampal neurons were cultured in 96-well dishes, transiently transfected with promoter-reporter plasmids using LipofectAMINE 2000 (Life Technologies, Inc.) as described in the manufacturer's protocol. These plasmids contained the secreted alkaline phosphatase (SEAP) reporter gene downstream of several copies of specific transcription factors binding sequences such as AP1, CRE, HSE, Myc, NFkB, and serum response element. Transfected neurons were allowed to recover for 24 h before 2 µM apoE peptide was added to the media. Alkaline phosphatase activities in the media, at indicated times after addition of apoE peptide, were measured using Great EscAPE SEAP Chemiluminescence Detection Kit (CLONTECH) following the manufacturer's protocol.

Western Blot Analysis—Cells were cultured in 6-well dishes, washed three times with PBS, and lysed with 200 µl of lysis buffer (50 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1% SDS, and 100 mM NaCl). 20-µg samples were boiled for 5 min, electrophoresed on 12.5% SDS-polyacrylamide electrophoresis gels and transferred onto Immobilon membrane (Millipore Corp., Bedford, MA). The membrane was incubated in blocking buffer (1 x PBS, 5% nonfat dried milk) for 1 h at room temperature and then probed with a primary antibody in blocking buffer overnight at 4 °C. After four washes in PBS containing 0.3% Tween 20, blots were probed with the secondary antibody in blocking buffer for 1 h at room temperature, and washed again in PBS containing 0.05% Tween 20. Detection of signal was performed with an enhanced chemiluminescence detection kit (Amersham International, Little Chalfont, United Kingdom).

Protein Quantification—The amount of protein (CREB, ERK, phosphorylated CREB, phosphorylated ERK, e-fos, Bcl-2, and actin) was quantified by scanning the density of immunodetected bands on Immobilon membrane using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Measurement of Intracellular Calcium Levels—Fura-2 AM (Molecular probes, Eugene, OR) and an Argus 50CA system (Hamamatsu Photonics, Japan) were used to quantify the cytoplasmic free calcium as described in the manufacturer's protocol.

RESULTS

Recombinant ApoE and Recombinant ApoE3 Were Equally Active in Suppressing the Secretion of TNFα from Astrocytes—The recombinant apoE4 and apoE3 proteins used for this study were not degraded as determined by Western blot analysis of denatured and reduced apoE proteins (Fig. 1A). To show that these recombinant proteins also retained biological activity, we examined whether they equally inhibited the secretion of TNFα from astrocytes as described by Laskowitz et al. (20). Preincubation of primary astrocytes cultures prepared from neonatal apoE-deficient mice with recombinant apoE3 or with recombinant apoE4, prior to stimulation with 100 ng/ml lipopolysaccharide, each decreased the concentration of TNFα released into the conditioned medium in a dose-dependent fashion as compared with untreated cells (Fig. 1B). There was no significant difference in TNFα levels between cells treated with the same concentration of apoE3 or apoE4. Boiled and denatured apoE3 was used as control protein and it did not suppress the secretion of TNFα.

ApoE Peptide Facilitates CRE-driven Transcription—To detect the signaling pathway activated in the presence of apoE peptide, we utilized the Mercury Pathway Profiling Systems (CLONTECH). Several cis-acting enhancer elements, such as AP1 (activator protein 1), CRE (cAMP responsive element), HSE (heat shock element), Myc, NFkB (nuclear factor of kB), and serum response element included in this system were assessed in rat primary hippocampal neurons exposed to 1 µM apoE peptide. Among these elements, transcription from the CRE element was found to be activated (Fig. 2).

ApoE Peptide and ApoE4, but Not ApoE3, Facilitated Phosphorylation of CREB at Ser-133—Since apoE peptide stimulated transcription from the CRE element, then the CREB transcription factor could be activated by apoE treatments. Phosphorylation of CREB at Ser-133 leads to its activation as a transcription factor (21, 22). To monitor the activation of CREB directly, Western blot of cell lysates from rat hippocampal neurons stimulated with 1 µM apoE peptide for the indicated times were probed with anti-CREB and anti-phospho-CREB (p-CREB) antibodies. 15 min after addition of apoE peptide, phospho-CREB levels substantially increased followed by a gradual decrease over the next 12 h while the total amount of CREB did not appear to change (Fig. 3A). In addition to apoE peptide, treatment with 1 µM apoE4 protein also appeared to significantly increase phospho-CREB levels by 1 h after treatment, while 1 µM apoE3 protein did not appear to change phospho-CREB levels significantly at any time (Fig. 3, B-D).
Although the molar concentration of apoE4 and apoE peptide added to the media was equivalent, the kinetics (time course) of CREB phosphorylation following treatment with apoE4 protein appeared to be slower than that stimulated by apoE peptide treatment.

ApoE Peptide and ApoE4, but Not ApoE3, Facilitated ERK Activity in Rat Hippocampal Neurons—Since activated CREB is phosphorylated at Ser-133 and apoE treatment increases levels of phosphorylated CREB, then apoE may stimulate a pathway resulting in kinase-mediated phosphorylation of CREB. One candidate for CREB phosphorylation at Ser-133 is through the ERK pathway that is activated by a double phosphorylation of ERK at Thr-202 and Tyr-204. We examined ERK activation by measuring phosphorylated ERK levels following treatment with apoE peptide, apoE3, and apoE4 proteins. Treatment with apoE peptide or with apoE4 protein resulted in significantly increased levels of phosphorylated ERK while apoE3 treatment failed to increase levels (Fig. 4). ApoE peptide increased phospho-ERK levels by 15 min of treatment while apoE4 protein increased them by 1 h of treatment. Like the phosphorylation of CREB, phosphorylation of ERK following apoE4 protein treatment appeared to be slower than that following apoE peptide treatment.

ApoE Peptide Facilitated the Expression of c-fos and Bcl-2 in Rat Hippocampal Neurons—To confirm that the apoE peptide stimulated phosphorylation that resulted in increased functional activity of CREB, we examined whether apoE peptide treatment led to transactivation of the CRE-dependent genes, c-fos and Bcl-2. As shown in Fig. 5, increased levels of both c-fos and Bcl-2 result from treatment with apoE peptide, whereas the level of a control protein, actin, does not appear to increase.

Apolipoprotein E Modulates Activation of CREB

FIG. 2. ApoE-peptide treatment increases CREB-responsive reporter activity (transcriptional activity of CREB). A CRE-SEAP reporter plasmid containing three copies of CRE elements linked to a TATA-like promoter from the HSV-TK gene and fused to the SEAP gene (CLONTECH) was transfected into rat primary hippocampal neurons on day 7 of in vitro culture using the LipofectAMINE 2000 procedure (Life Technologies, Inc.). The activity of SEAP measured as chemiluminescence (Great EscApe, CLONTECH) in the medium increased in a time-dependent fashion after treatment with 2 μM apoE peptide indicating that apoE peptide facilitates activation of the transcriptional activity of CREB. Error bars represent the S.E. (n = 6). *, p < 0.05 compared with time 0 control using Student’s t test.

FIG. 3. Treatment with apoE4 or apoE-peptide increases phospho-CREB levels. Rat hippocampal neurons at day 7 in culture were treated with 2 μM apoE peptide (A), 2 μM apoE3 (B), or 2 μM apoE4 (C) for the indicated times. Cell lysates (20 μg/lane) were electrophoresed on SDS-PAGE, Western blotted, and probed with anti-phospho-CREB antibody or anti-CREB antibody. The amount of phosphorylated CREB and total CREB was quantified by scanning densitometry of immunoreactive bands. The ratios of phosphorylated CREB to total CREB are plotted as a mean ± S.E. of at least four separate experiments. *, p < 0.05 compared with unstimulated control using Student’s t test (A and D). **, p < 0.05 using Student’s t test. apoE4 > apoE3 (D).

Rat Hippocampal Neurons—To confirm that the apoE peptide stimulated phosphorylation that resulted in increased functional activity of CREB, we examined whether apoE peptide treatment led to transactivation of the CRE-dependent genes, c-fos and Bcl-2. As shown in Fig. 5, increased levels of both c-fos and Bcl-2 result from treatment with apoE peptide, whereas the level of a control protein, actin, does not appear to increase.

ApoE Receptor, NMDA Receptor, and L-type Voltage-dependent Ca2+ Channel Are Involved in the Pathway—To further elucidate the pathway where CREB is phosphorylated after
apoE treatment, we examined the effects of RAP, which is a competitive blocker of apoE for its receptor; MK801 which is a selective antagonist of the NMDA receptor; and nifedipine which is a selective antagonist of the L-type voltage-dependent calcium channel (LVDC). Pretreatment with RAP followed by apoE appeared to attenuate the increase in phospho-CREB levels seen with apoE treatment alone suggesting that the apoE receptor is involved in the pathway (Fig. 6A). Similarly, pretreatment with MK801 or with nifedipine appeared to attenuate the increase in phospho-CREB levels, suggesting that the NMDA receptor and/or the LVDC are also involved in the pathway (Fig. 6B).

Activation of MEK and Protein Kinase A Is Involved in the Pathway—To find out which of the possible pathways involving apoE receptors, LVDC and/or NMDA receptors, contribute to the phosphorylation of CREB, we examined the effect of inhibiting kinases associated with these receptors by treatment with the U0126 inhibitor of MAPK/ERK kinase (MEK) and with the c-AMP-dependent protein kinase inhibitor of protein kinase A (PKA). Pretreatment with one of these selective inhibitors, as well as pretreatment with both, mostly inhibited CREB phosphorylation suggesting that activation of MEK and PKA are involved in this pathway (Fig. 6C).

ERK Cascade Is a Major Pathway Leading to the ApoE Peptide-stimulated Phosphorylation of CREB—To further investigate the apoE stimulation pathway, we examined the effect of inhibiting kinases associated with the receptor signaling by treatment with the SB203580 inhibitor of p38 MAP kinase, KN62 inhibitor of calmodulin-dependent protein kinases (CaMK II/IV), and the U0126 inhibitor of MEK. Of these selective inhibitors, CREB phosphorylation was mostly inhibited by pretreatment with U0126 while pretreatment with KN62 or SB203580 did not appear to have any significant effect on the phosphorylation status of CREB. This result suggests that the ERK cascade is a major pathway leading to the apoE-stimulated phosphorylation of CREB (Fig. 6D).

Elevation of Intracellular Ca\(^{2+}\) following ApoE Peptide Treatment Involves the ApoE Receptor, NMDA Receptor, and L-type Voltage-dependent Ca\(^{2+}\) Channel—To demonstrate if the elevation of intracellular Ca\(^{2+}\) parallels the phosphorylation of CREB, we measured the concentration of intracellular Ca\(^{2+}\) under unstimulated or apoE peptide-stimulated conditions. Treatment with apoE peptide significantly raised the concentration of intracellular calcium in rat hippocampal neurons. Preincubation with RAP, MK801, or nifedipine significantly attenuated this elevation, suggesting that the apoE receptor, NMDA receptor, and LVDC are involved in the pathway to calcium elevation as was observed for the activation of CREB (Fig. 7A).

A Major Part of the Intracellular Ca\(^{2+}\) Elevation May Be from an Intracellular Source—To determine whether calcium influx from the extracellular space or from the endoplasmic reticulum (ER) contributes to our apoE-mediated Ca\(^{2+}\) increases, we measured the intracellular Ca\(^{2+}\) elevation in rat primary hippocampal neurons cultured in medium lacking Ca\(^{2+}\) and containing 1 mM EGTA (Fig. 7B). Under these conditions, apoE-stimulated calcium elevation was significantly attenuated suggesting that calcium influx from extracellular sources significantly contributes to the elevation of intracellular Ca\(^{2+}\). To confirm this finding, we also measured the contribution of calcium release from the endoplasmic reticulum to the increase in intracellular Ca\(^{2+}\). Rat primary hippocampal neurons were preincubated with 10 μM dantrolene, an inhibitor of ryanodine-sensitive Ca\(^{2+}\) channel found on the ER membrane, followed by apoE peptide treatment. Interestingly, preincubation with dantrolene also significantly attenuated the elevation of intracellular Ca\(^{2+}\). Combining extracellular Ca\(^{2+}\)-free conditions and preincubation with dantrolene also attenuated the calcium elevation, even though there was no significant difference between the calcium levels measured under extracellular Ca\(^{2+}\)-free conditions without dantrolene preincubation and the extracellular calcium-free condition with dantrolene preincubation. Taken together, we hypothesize that the calcium influx from extracellular sources triggers the release of Ca\(^{2+}\) from intracellular endoplasmic reticulum sources through its
ryamodine-sensitive Ca\(^{2+}\) channels. Calcium influx from extracellular space seems to be essential for signaling the apoE-stimulated elevation of intracellular Ca\(^{2+}\) levels, but contributes only a small amount to the rise in intracellular calcium levels compared with the calcium released from the ER.

**DISCUSSION**

Although evidence of the association between the e4 allele of the *APOE* gene and Alzheimer’s disease is overwhelming, the mechanism by which the apoE4 protein isoform influences onset and progression of the disease and its pathology is unknown. Of the many suggested mechanisms, we have focused on reports that apoE3 and apoE4 protein isoforms have differential effects on neuronal plasticity and survival (13, 23, 24). Compared with the apoE3 protein isoform, apoE4 protein, proteolytic fragments of apoE4 protein, and peptides corresponding to the receptor-binding domain of apoE proteins appear to actively injure, and certainly do not support maintenance of healthy neurites and neuronal cells (13–16). On the larger scale, the failure of apoE4 protein isoforms and their related fragments to support neuronal plasticity and maintenance may infer a mechanism that underlies the association between *APOE* gene alleles and disease.

Under many conditions that eventually result in neuronal death, the cell struggles to induce protective mechanisms even though destructive forces inevitably march forward. In this report, we have demonstrated one such scenario where apoE4, but not apoE3, activates an ERK cascade that results in activation of CREB and induction of many different genes including the cell-protective gene, *Bcl-2*. In prior reports, we showed that overexpression of only Bcl-2 protein could inhibit neuronal death following a toxic insult (25). In this case, treatment of neurons with recombinant apoE4 protein or with synthetic apoE peptide resulted in CREB phosphorylation and increased Bcl-2 expression, events which were not observed following recombinant apoE3 protein treatments. Time course experiments showed that the increase in phospho-CREB levels following apoE4 protein treatments appeared to be slower than that by apoE peptide, although their molar concentrations were the same. This time lag is consistent with previous results where protease inhibitors reduced the neurotoxicity of apoE4 through a presumed mechanism where the full-length apoE4 protein must be digested over time to produce a toxic fragment of apoE4. Despite the increases in phospho-CREB and Bcl-2, we also observed apoE4/apoE peptide-mediated increases in calcium levels that were also observed by others in the context of their association with neuronal death (16, 26).

The apoE4/apoE-peptide-mediated increase of intracellular Ca\(^{2+}\) can be generated by calcium influx from the extracellular space and release of calcium from intracellular stores. Significant attenuation of increased intracellular calcium levels was observed when we cultured rat primary hippocampal neurons in the medium lacking Ca\(^{2+}\) and containing EGTA as a Ca\(^{2+}\)
chelator, suggesting that calcium influx from extracellular sources follows apoE4/apoE peptide treatments. When we measured the intracellular Ca$^{2+}$ elevation following preincubation with 10 µM dantrolene, an inhibitor of ryanodine-sensitive Ca$^{2+}$ channel on ER membranes, Ca$^{2+}$ elevation was also significantly attenuated, suggesting the participation of the calcium influx from ER in the elevation of intracellular Ca$^{2+}$.

Interestingly, extracellular Ca$^{2+}$-free conditions did not seem to have any additional effect on attenuating the Ca$^{2+}$ elevation following preincubation with dantrolene, suggesting that calcium influx from the extracellular space may serve to trigger a larger amount of Ca$^{2+}$ release from the ER through ryanodine-sensitive Ca$^{2+}$ channel in a “Ca$^{2+}$-induced Ca$^{2+}$-release” fashion. Thus, calcium influx from extracellular space may be less
than calcium release from intracellular sources such as the endoplasmic reticulum.

The apoE peptide-mediated increase in phospho-CREB levels and of intracellular Ca\(^{2+}\) levels was inhibited by pretreatment with RAP, MK801, or nifedipine, suggesting that apoE receptors, NMDA receptor, and LVDCC are involved in rat hippocampal neuron responses to apoE. Tolar et al. (16) reported that RAP and MK801 attenuated the elevation of intracellular Ca\(^{2+}\) caused by truncated apoE. They also reported that nifedipine did not provide any protection against the apoE-mediated rise in intracellular Ca\(^{2+}\) in rat hippocampal neurons, but data was not shown. Wang et al. (26) reported that RAP, MK801, and diltiazem, another inhibitor of LVDCCs, failed to block the apoE peptide-induced calcium influx in rat primary cultured neurons. Assuming that neurotoxicity is caused by calcium influx following apoE treatment, our data are consistent with the data provided by Tolar et al. (16) in that RAP and MK801 attenuated calcium influx (16). This result suggests that LRP or some other apoE receptors found in neurons may be linked to NMDA receptor. Our finding that preincubation with nifedipine almost completely inhibited the apoE peptide-induced calcium influx is reasonable because calcium entry through NMDA receptors may depolarize the plasma membrane and open LVDCCs that results in larger amounts of calcium influx. Compared with the calcium entry from LVDCCs, calcium entry from NMDA receptors may be negligible (27). Nevertheless, this calcium elevation can activate kinases such as ERK and calmodulin kinase II that function to phosphorylate CREB (27).

Further inhibition experiments showed that pretreatment with a MEK inhibitor and/or a PKA inhibitor mostly prevented the apoE peptide stimulated phosphorylation of CREB, suggesting that activation of both MEK and PKA kinases are involved in the pathway. Ca\(^{2+}\) elevation stimulates CREB phosphorylation by activation of a PKA-dependent, Rap1-MEK-ERK pathway (28). Pretreatment with KN62 or SB203580 did not appear to have any effect on the phosphorylation of CREB.

FIG. 7. Intracellular calcium levels increase after apoE-peptide treatment. As a measure of intracellular calcium levels, Fura 2 fluorescence in 7-day-old rat hippocampal neuronal cultures increases rapidly after treatment with apoE-peptide (bar labeled a). This apoE-peptide stimulated fluorescence is inhibited by pretreatment with RAP, MK801 at 10 or 100 \(\mu\)M, or nifedipine at 10 or 100 \(\mu\)M. Peak values of intracellular calcium were observed about 40 s after exposure to apoE-peptide in at least 10 neurons per experiment in each of four separate experiments. Compared with the apoE-peptide alone, significant inhibition of calcium fluorescence at the \(p < 0.01\) are calculated with a Student’s \(t\) test and marked by a double asterisk (a). ApoE-peptide induces a rapid elevation of intracellular calcium fluorescence in rat hippocampal neurons (bar a) that is inhibited by culturing in Ca\(^{2+}\)-free growth medium or in the presence of growth medium containing calcium and 10 \(\mu\)M dantrolene. Peak concentrations of intracellular calcium in at least 10 neurons for each of four experiments are plotted. Significance of the difference between apoE-peptide alone and treatment conditions were calculated with Student’s \(t\) test where double asterisks represent \(p < 0.01\) (B).

FIG. 8. A model of apoE-mediated CREB activation in hippocampal neurons. When apoE4, but not apoE3, binds to apoE receptor on the surface of hippocampal neuron, NMDA receptors open. Its mechanism is unclear. Activation of NMDA receptor causes small amounts of Ca\(^{2+}\) entry into postsynaptic spines. The resulting depolarization triggers the opening of L-type voltage-dependent calcium channels which exist in cytoplasmic membranes of the neurons. The activation of L-type voltage-dependent calcium channels, which is open during strong depolarization, permits the entry of larger amount of Ca\(^{2+}\) along the dendrites and into the cell bodies, causing the opening of ryanodine-sensitive Ca\(^{2+}\) channel on ER membrane and the influx of larger amount of Ca\(^{2+}\) from ER in Ca\(^{2+}\)-induced Ca\(^{2+}\) release fashion. The Ca\(^{2+}\) entry from extracellular space may be negligible, comparing that from ER calcium elevation allows activation of PKA-dependent Rap1-ERK pathway and phosphorylation of CREB.
influx and intracellular Ca\(^{2+}\) levels to rise. Preincubation with apoE receptor treatment of apoE in rat hippocampal neurons upon exposure to these receptor involved in neuronal functions like long-term potentiation (32–34). Stimulation of these receptors has also been associated with increased intracellular Ca\(^{2+}\) levels, potentiation of kinase activities, and CREB activation.

To clarify if calcium influx caused by apoE treatment causes neurototoxicity and parallels phosphorylation of CREB, we measured the elevation of intracellular Ca\(^{2+}\) caused by apoE peptide in rat hippocampal neurons upon exposure to these receptor antagonists. As others has reported (16), treatment of apoE receptor with increased intracellular Ca\(^{2+}\) is modulated by a variety of effectors. \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/kainate receptors and NMDA receptors are associated with synapses and LVDCCs are involved in neuronal functions like long-term potentiation (32–34). Stimulation of these receptors has also been associated with increased intracellular Ca\(^{2+}\) levels, potentiation of kinase activities, and CREB activation.

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