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(−)Clausenamide facilitates synaptic transmission at hippocampal Schaffer collateral-CA1 synapses

Na Ning, Jin-Feng Hu, Jian-Dong Sun, Ning Han, Jun-Tian Zhang *, Nai-Hong Chen **

State Key Laboratory of Bioactive Substances and Functions of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, PR China

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ABSTRACT

Clausenamide is a chiral compound isolated from leaves of the traditional Chinese herb *Clausena lansium* (lour) Skeels. It has been shown that (−)clausenamide, but not (+)clausenamide, improved learning and memory in amnesia animal models. However, the precise mechanism of clausenamide's actions remains unknown. Here we used an electrophysiological approach to observe the effect of (−)clausenamide on facilitating field excitatory postsynaptic potential (f-EPSP) in the CA1 area of hippocampal slices from rats. The results showed that (−)clausenamide enhanced synaptic transmission at doses 0.1, 1 and 10 μM. The increase of f-EPSP induced by (−)clausenamide was completely inhibited by preincubation with nimodipine (L-voltage-dependent calcium channel blocker, 10 μM), but there was no change when nimodipine was added after (−)clausenamide application. However, ryanodine (ryanodine receptors blocker, 100 μM) attenuated the slope of f-EPSP before or after (−)clausenamide incubation. The data suggested that (−)clausenamide promoted calcium influx to trigger intracellular calcium release which was responsible for potentiating synaptic transmission. Intracellular calcium release induced by (−)clausenamide promoted the activation of CaMKIIα at concentrations of 0.1, 1 and 10 μM, and pretreatment with KN93 (CaMKIIα inhibitor, 10 μM) completely blocked the enhancement of synaptic transmission induced by (−)clausenamide. cAMP response element-binding protein (CREB) was activated by (−)clausenamide and inhibited by KN93 preincubation, but H89 (PKA inhibitor, 10 μM) had no effect, indicating that (−)clausenamide facilitated synaptic transmission by a PKA-independent pathway. Collectively, (−)clausenamide facilitated synaptic transmission by promoting calcium influx to trigger intracellular calcium release, subsequently activating CaMKIIα-CREB signal pathway.

1. Introduction

Synaptic plasticity in mammalian hippocampus has been considered to be the neurobiological foundation of learning and memory (Bliss and Collingridge, 1993). Long-term potentiation (LTP) of synaptic transmission is currently the best available cellular model for learning and memory in mammalian brain. It's now believed that calcium and the activated downstream signals, including calcium/calmodulin-dependent protein kinase II/IV (CaMKII/IV), extracellular signal-regulated kinase (ERK1/2) and cAMP response element-binding protein (CREB), played central roles in the induction and maintenance of LTP (Berberich et al., 2007; Kandel, 2001; Sweatt, 2004; Thomas and Huganir, 2004). Neuronal cytoplasmic calcium accumulates by extracellular calcium influx through calcium channel in the postsynaptic membrane, such as voltage-dependent calcium channel(VDCC) and N-methyl-D-aspartic acid glutamate (NMDA) receptors, or release from intracellular stores, such as mitochondria and endoplasmic reticulum (Berridge et al, 2003; Burnashev, 1998; Tang and Zucker, 1997).

CaMKIIα, a chief kinase in postsynaptic density (PSD), is an intracellular calcium responder. Studies confirmed that LTP was deficient in CaMKIIα-knockout mice, indicating that CaMKIIα might underlie long-term changes in synaptic activity (Hinds et al., 1998; Silva et al., 1992). Accumulated cytoplasmic calcium also led to an increase in cyclic adenosine 3′,5′-monophosphate (cAMP) level in area CA1 of the hippocampus (Chetkovich et al., 1991). As a principal target for cAMP, PKA regulates transcription by phosphorylating several different transcription factors, one of which is CREB (Bacskai et al., 1993). Several lines of evidence confirmed that CREB acted as a molecular switch on synaptic plasticity and long-term memory in drosophila, aplysia and mammals (Bourtchuladze et al., 1994; Dash et al., 1990; Martin et al., 1997), because transcription of the required

* Corresponding author. Fax: +86 10 63165177.
** Correspondence to: N.-H. Chen, Key Laboratory of Bioactive Substances and Resources Utilization, Ministry of Education, Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences and Beijing Union Medical College, 1 Xiannongtan Street, Xuanwu district, Beijing 100050, China. Tel./fax: +86 10 63165177.
E-mail addresses: zhangjt@imm.ac.cn (J.-T. Zhang), chennh@imm.ac.cn (N.-H. Chen).

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de novo gene was mediated by CREB family genes (Barzilai et al. 1989). Besides PKA, there were also many other kinases observed to affect the phosphorylation of CREB in long-term memory formation, like ERK1/2 and CaMKIIα (Sun et al 1994; Sweatt 2004).

Clausenamide was isolated from the aqueous extract of leaves of Rutaceae *Clausena lansium* (Lour.) Skeels. The previous studies showed that (−)clausenamide, but not (+)clausenamide, improved the learning and memory in amnesia animal models (Duan and Zhang, 1998., Tang and Zhang, 2002, Zhu et al., 2004), and also potentiated basal synaptic transmission and high frequency stimulation-induced LTP on either anesthetized or freely moving rats through VDCC (Xu et al., 2005). However, the downstream signal effectors of (−)clausenamide facilitating synaptic transmission were unclear. In this paper, the signal transduction pathway of (−)clausenamide on potentiating synaptic transmission was investigated to explain its effects on improvement of learning and memory.

2. Materials and methods

2.1. Animals

Male Wistar rats with ages of 4 weeks to 6 weeks (90–140 g) in this study were provided by the Experimental Animal Center of Chinese Academy of Medical Sciences SCXK (Jing) 2009-0007. They were housed in groups of 3 per cage under controlled laboratory conditions (temperature 22–26 °C, light cycle 12 h, air humidity 50–60%) and had free access to food and water. All animals were handled in accordance with the standards established in the Guide for the Care and Use of Laboratory Animals, published by the Institute of Laboratory Animal Resources of the National Research Council (United States) and approved by the Animal Care Committee of the Peking Union Medical College and the Chinese Academy of Medical Sciences.

2.2. Treatment of multi-electrode dish

The treatment of multi-electrode dish (Panasonic, MED64 probe, P530A) was based on the description by Zhao et al. (2009). The microelectrode on the bottom of dish was arranged in an 8×8 pattern,

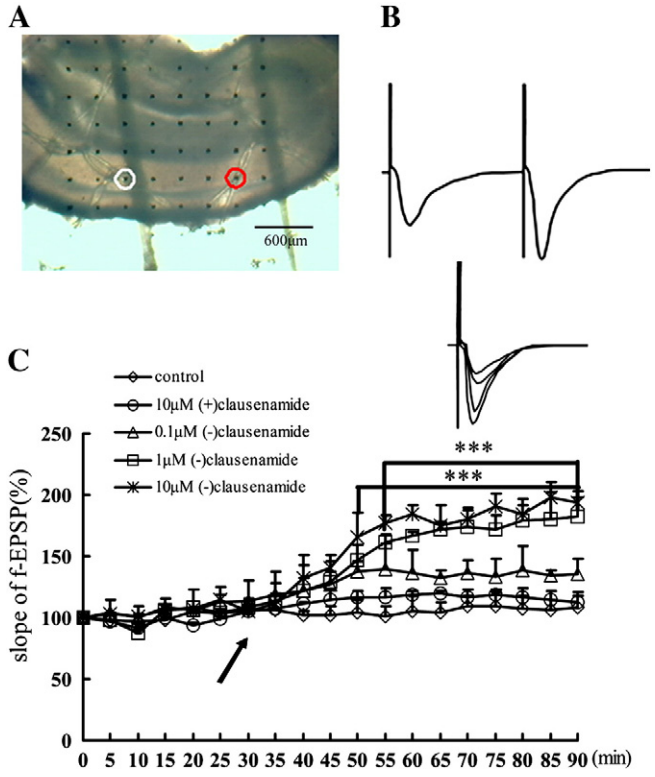

Fig. 1. Effect of clausenamide on the slope of f-EPSP in hippocampal slices. (A) A photograph showed a hippocampal slice which was positioned on a Med64 probe with 8×8 arrays (interelectrode distance: 300 μm). The red circle indicates an electrode selected for electrical stimulation of Schaffer collateral fibers and the white one indicates the recording electrode. The image was taken by an inverted microscope. (B) Example pair pulses stimulation on the selected electrode. (C) (−)clausenamide potentiated the slope of f-EPSP in a dose dependent manner while (+)clausenamide showed no effect. The arrows point to the application time. The points recorded every 5 min showed the averaged value of f-EPSP on at least three hippocampal slices. Data were expressed as mean ± S.E.M. *** P<0.001 vs control group.

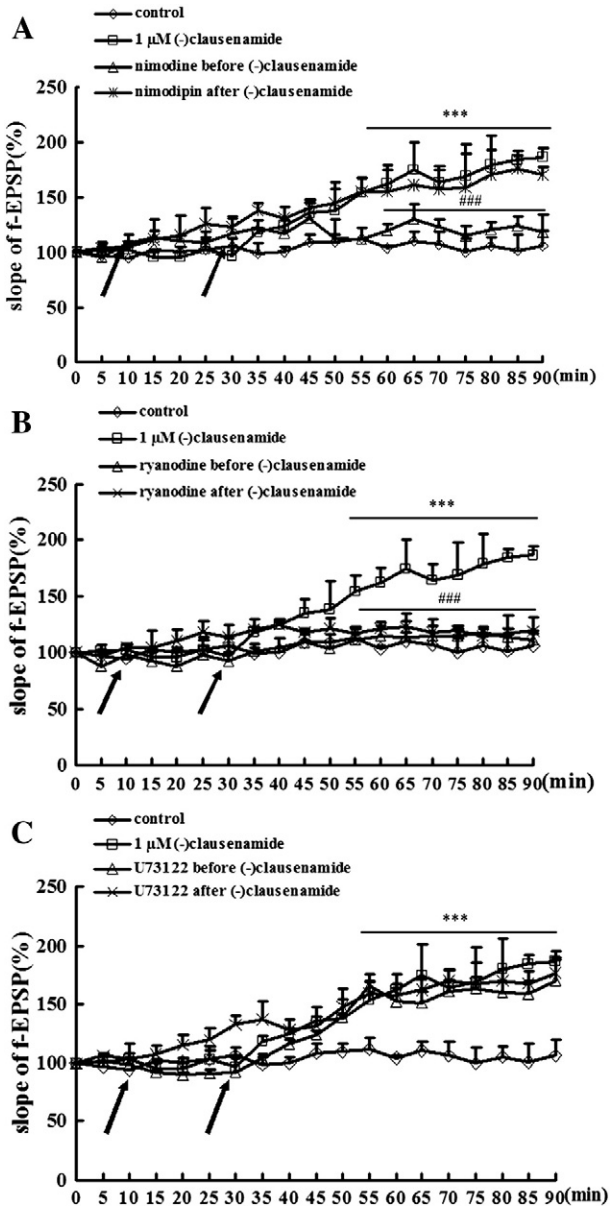

Fig. 2. Increase of f-EPSP slope induced by (−)clausenamide was attributed to the intracellular calcium release evoked by calcium influx from VDCC. (A) nimodipine treated group; (B) ryanodine treated group; (C) U73122 treated group. The arrows point to the compounds application time. In preincubation groups, nimodipine, ryanodine or U73122 were preincubated with the hippocampal slices for 20 min before (−)clausenamide application, and the recording was kept for 1 h. Data were expressed as mean ± S.E.M. *** P<0.001 vs control group, ### P<0.001 vs (−)clausenamide group.

and the distance was 300 μm between the adjacent electrodes. MED64 probe was treated with 0.1% polyethyleneimine (Sigma, St. Louis, MO; P-3143) in 25 mM borate buffer (pH 8.4) overnight at room temperature to establish sufficient adhesion of the probe surface to the slice. The probe surface should be rinsed three to five times with sterile distilled water before immediate using.

2.3. Preparation of acute hippocampal slices

The artificial cerebrospinal fluid (aCSF) containing 124 mM NaCl, 3.3 mM KCl, 1.2 mM KH_2PO_4, 2.4 mM $MgSO_4$, 10 mM glucose, 26 mM $NaHCO_3$, and 2.5 mM $CaCl_2$ had a pH of 7.3 adjusted by gassing with 5% CO_2/95% O_2 for 30 min. The general procedures for preparing acute hippocampal slices were similar to those described previously (Oka et al., 1999). Briefly, male Wistar rats were decapitated, the whole brain was rapidly removed and immediately soaked in ice-cold, oxygenated preparation buffer of aCSF for approximately 1–2 min. Appropriate portions of the brain were then trimmed and the remaining brain block was placed on the ice-cold stage of a vibrating tissue slice (VIBRA TOME, 1000 Plus Sectioning System) filled with oxygenated and frozen aCSF. The hippocampal slices were ranging from Bregma −2.52 mm to Bregma −4.08 mm according to the atlas of the Rat Brain in the study (Zhao et al., 2009), with a thickness of 300–350 μm. The slices were immediately soaked in an incubation chamber containing oxygenated aCSF for 2 h at 28 ± 0.5 °C.

2.4. Electrophysiological recording

After incubation, a hippocampal slice was selected and positioned on the array of 64 planar microelectrodes through a charge-coupled camera connected to an inverted microscope. The slice was perfused with oxygenated, fresh aCSF continuously at the rate of 2–3 ml/min with peristaltic pump (PERI-STAR™, WPI, USA). The intensity of the test stimulation was then adjusted to elicit 30–40% of the maximum based on the I/O curves by a series of stimulation intensities from 20 to 100 μA (duration 0.2 ms). Each microelectrode around the position of Schaffer collateral fibers was tested by pair pulse tests (interval 40 ms, with the same duration and stimulating intensity as recording), and the ones around CA1 which showed that the amplitude of the second waveform was higher than the first one were taken for recording (Fig. 1B). Those slices that did not meet this criterion were excluded from the final analysis. After that, monopolar,

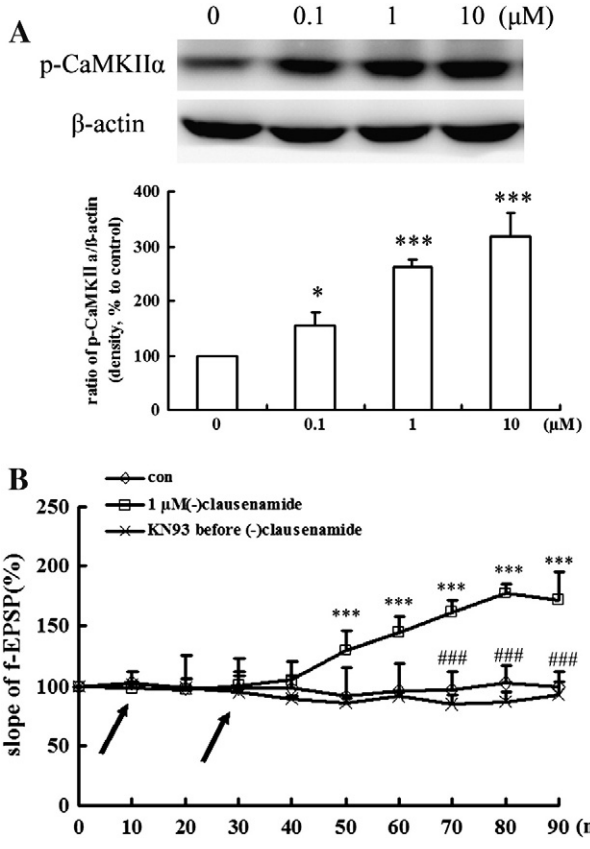

Fig. 3. CaMKIIα activation was involved in (−) clausenamide potentiating synaptic transmission in hippocampal slices. (A) CaMKIIα was phosphorylated by (−)clausenamide in a dose dependent manner. The quantitative analysis determined by densitometry was summarized. (B) Inhibitor of CaMKIIα completely suppressed the (−) clausenamide potentiating synaptic transmission in hippocampal slices. The arrows point to the application time. Data were expressed as mean ± S.E.M. * $P<0.05$, *** $P<0.001$ vs. control group; , ### $P<0.001$ vs. (−)clausenamide group.

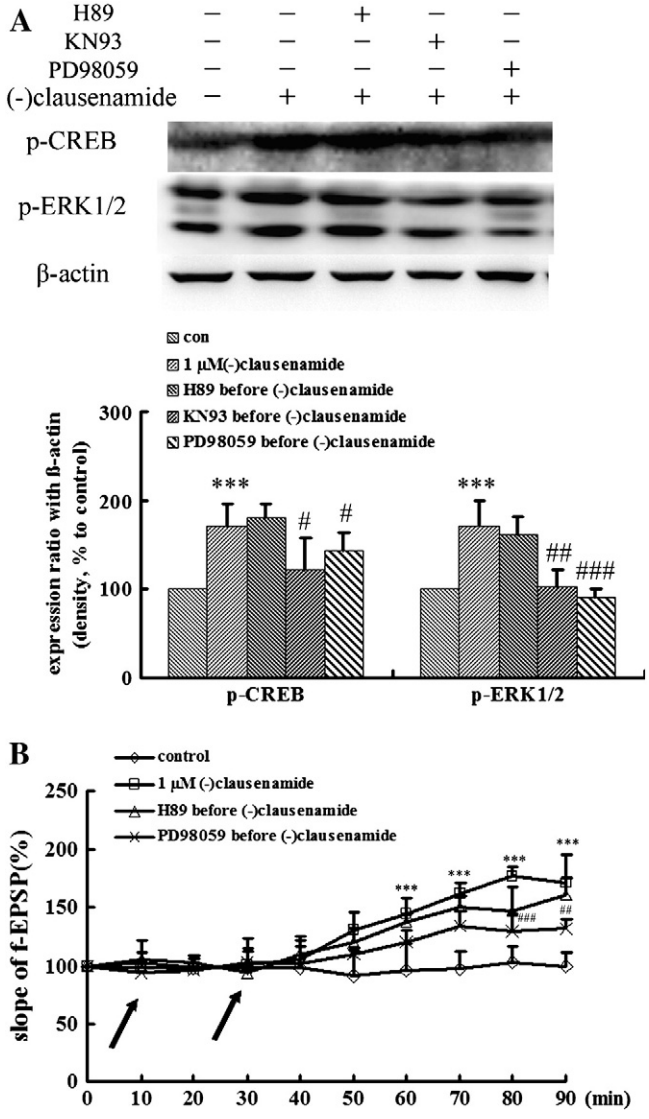

Fig. 4. CaMKIIα-ERK1/2-CREB was involved in (−)clausenamide enhancing synaptic transmission. (A) Representative images of immunoblots using antibodies against p-ERK1/2, p-CaMKIIα, p-CREB after treatment in hippocampal slices. (B) Effect of inhibitors on f-EPSP induced by (−)clausenamide. Hippocampal slices were pretreated for 20 min with H89 (10 μM), KN93 (10 μM) or PD98059 (30 μM) before perfusion with (−)clausenamide(1 μM). The arrows point to the application time of inhibitors and (−)clausenamide. The quantitative analysis determined by densitometry was summarized. Data expressed as mean ± S.E.M., *** $P<0.001$ vs. control group; # $P<0.05$, ## $P<0.01$, ### $P<0.001$ vs. (−)clausenamide group.

biphasic constant current pulses were applied to the Schaffer collateral fibers at 0.0167 Hz for at least 30 min until stable. Five successive responses evoked at the tested recording site were averaged automatically in real time. Each result was taken from at least three slices.

2.5. Experimental procedures

(−)Clausenamide was diluted to 0.1, 1 or 10 μM with pre-heated and -oxygenated aCSF, kept the temperature at 28 °C by water bath. Nimodipine (VDCC blocker, 10 μM), ryanodine [ryandine receptors (RyRs) blocker, 100 μM] or U73122(1-[6-[((17β)-3-Methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione, PLCγ inhibitor, 10 μM) was added either before or after (−)clausenamide application respectively, and the recording was kept 60 min. For the signal pathway analysis, the slice was perfused with H89(N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride, PKA inhibitor, 10 μM) , KN93 (N-[2-[N-(4-Chlorocinnamyl)-N-methylaminomethyl]phenyl]-N-(2-hydroxyethyl)-4-methoxybenzenesulfona - mide phosphate salt, CaMKIIα inhibitor, 10 μM) or PD98059 (2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one, ERK1/2 inhibitor, 30 μM) for 20 min, and then the perfusion was changed into (−)clausenamide diluents with the very inhibitor at the same concentration as preincubation. Then the slices were taken out gently by brush for western blotting analysis.

2.6. Western blotting

After electrophysiological experiment, at least three hippocampal slices from each group were homogenized thoroughly in RIPA lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate and 0.1% SDS]. Protein concentrations were measured by BCA kit (Vigorous). The lysates were solubilized in SDS sample and separated by 9% SDS-PAGE, then transferred to PVDF membrane (Millipore). The membrane was blocked by 3% BSA and incubated with anti-phospho-ERK1/2, anti-phospho-CaMKIIα, anti-phospho-CREB, or anti-β-actin antibody, followed by horseradish peroxides (HRP)-conjugated secondary antibody, and detected by the ECL plus detection system (Molecular Device, Lmax). The density of each band was quantified using image analysis software (Science Lab 2005 Image Gauge; Fuji Film Co. Ltd, Tokyo, Japan).

2.7. Data analysis

The slope of field excitatory postsynaptic potentials (f-EPSP) was analyzed off line by the MED64 Conductor. The recording of beginning was defined as 100%. Data are expressed as mean ± S.E.M. Statistical differences of the slope of f-EPSP were analyzed using two-way analysis of variance (ANOVA) followed by Newman–Keuls *post hoc* test. Group differences in biochemical assays were evaluated using one-way ANOVA followed by Duncan's multiple-range test. P-values of <0.05 were regarded as statistically significant.

3. Results

3.1. (−)Clausenamide facilitated f-EPSP in CA1 region of hippocampal slices

With appropriate test stimulations on Schaffer collaterals, a negative-going waveform was consistently observed in CA1 region (Fig. 1A). On average, the latency for the peak of negative-going field potentials was 5–8 ms. After stable, the baseline slope of f-EPSP was kept recording for 30 min in both the control and the clausenamide groups. Two-way ANOVA (Fig. 1C) showed no significant difference between the groups in the first 30 min [$F(3, 70) = 1.718$, $P = 0.156$], as well as the time points [$F(6, 70) = 1.301$, $P = 0.268$]. Moreover, the f-EPSP did not fluctuate significantly for 60 min after vehicle (0.1% DMSO-aCSF) or (+)clausenamide administration. However, the significant difference showed between groups after the (−)clausenamide application [$F(3, 120) = 486.399$, $P<0.001$]. The slope of f-EPSP in 1 μM (−)clausenamide treated group increased significantly at 10 min after application and reached the plateau at 25 min, and the facilitation lasted for at least 60 min. 10 μM (−)clausenamide also increased the slope of f-EPSP significantly which was almost two times of the control group's at 60 min(194.1 ± 8.9%), but showed no statistically significant difference with 1 μM (−)clausenamide. Thus, 1 μM (−)clausenamide was selected in the following study (Fig. 1).

3.2. (−)Clausenamide facilitated synaptic transmission through calcium release

To make clear whether (−)clausenamide promoted synaptic transmission by elevating the calcium concentration, we tested the role of different calcium channels by pharmacological blockade. There was no significant difference between groups in the first 30 min [$F(3, 56) = 1.094$, $P = 0.086$]. Nimodipine was added in aCSF before (−)clausenamide application, and the result showed that synaptic transmission was completely inhibited (119.2 ± 15.2%, Fig. 2A). Curiously, treatment with (−)clausenamide before nimodipine addition failed to affect the slope of f-EPSP compared to (−)clausenamide treated group, indicating that initiating external calcium influx through L-VDCC was necessary for facilitation of synaptic transmission by (−)clausenamide. However, ryanodine markedly reduced the f-EPSP slope in any treated group, with the inhibition rate of 111.7 ± 5.8% and 119.2 ± 12.1% in preincubated group and postincubated group, respectively (Fig. 2B). In contrast, U73122 failed to affect the slope of f-EPSP in the situation of both preincubation and postincubation (170.3 ± 17.4%, 176.3 ± 12.9%, Fig. 2C), suggesting that the PLCγ-IP_3 pathway was not involved in the mechanism of (−)clausenamide on facilitation of synaptic transmission.

3.3. The phosphorylation of CaMKIIα was involved in potentiating synaptic transmission induced by (−)clausenamide

CaMKIIα is an intracellular calcium responder which is activated by the Ca^{2+}/calmodulin. The phosphorylation of CaMKIIα in hippocampal slices with bath application of (−)clausenamide was therefore observed in the study. Based on the results of protein analysis, the expression of phosphorylated CaMKIIα was 155.2 ± 24.3%, 261.9 ± 15.9%, or 319.0 ± 42.6% respectively in 0.1, 1, 10 μM (−)clausenamide treated

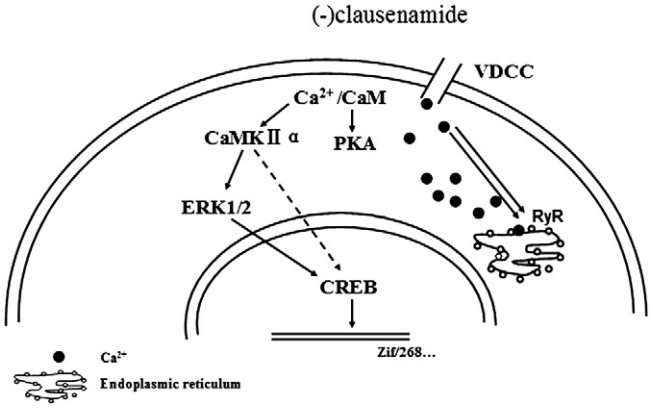

Fig. 5. (−)Clausenamide potentiated f-EPSP in hippocampal slices from rats by activating CaMKIIα-ERK1/2-CREB signal pathway. Intracellular calcium release from endoplasmic reticulum might be invoked by a small amount of calcium influx through a voltage-dependent calcium channel activated by (−)clausenamide.

group when the control group was defined as 100%. Thus, (−)clausenamide promoted the phosphorylation of CaMKIIα in hippocampal slices in a dose dependent manner. Pretreatment with KN93 for 20 min before (−)clausenamide addition completely blocked the increase of f-EPSP induced by (−)clausenamide (92.5 ± 12.1%, Fig. 3B), uncovering the central role of CaMKIIα in the enhancement of synaptic transmission by (−)clausenamide.

3.4. (−)Clausenamide activated ERK1/2−CREB cascade by CaMKIIα

To determine the downstream responder of (−)clausenamide, some potential signal molecules established to be related to learning and memory were detected after (−)clausenamide exposure, and CREB was observed to be significantly activated by (−)clausenamide. As showed in Fig. 4, when the hippocampal slices were bath applied with KN93 for 20 min before (−)clausenamide addition, activation of ERK1/2 and CREB was attenuated almost to the baseline, and pretreatment with PD98059 partially blocked the effect of (−)clausenamide on f-EPSP. H89 failed to affect the activation of CREB (171.4 ± 23.9%, Fig. 3B) and the enhancement of f-EPSP, indicating that PKA was not involved in the process of potentiating synaptic transmission by (−)clausenamide (Fig. 4).

4. Discussion

Changes in synaptic strength after repeated communication between neurons are a key mechanism of learning and memory in the central nervous system. It has been recognized that regulating the efficacy of synaptic transmission is essential for continual remodeling of neural networks. LTP, lasting for several hours to days after tetanic stimulation in hippocampus, is commonly accepted to be the most prominent model for elucidating molecular and cellular mechanisms of learning and memory (Bliss and Collingridge, 1993). Recently, studies showed that improvement of basal synaptic transmission in acute slices required lower-frequency electrical test stimulation (or presynaptic activity) or activation of NMDA receptors. Chemically-induced facilitation of synaptic transmission offered a lower threshold to electrical stimulation to induce LTP, thus ameliorating the cognitive dysfunction in Alzheimer disease patients (Otmakhov et al., 2004). Thus, the effect of (−)clausenamide on synaptic transmission in acute hippocampal slices was tested with the 8 × 8 multi-electrode probe in the present study. The results demonstrated that (−)clausenamide potentiated the basic synaptic transmission without the tetanic stimulation in CA1 area of hippocampal slices, which might provide the explanation for the improvement of cognitive deficiency by (−)clausenamide (Zhu et al., 2004).

Calcium plays crucial roles in many forms of activity-dependent synaptic plasticity. In a previous study, (−)clausenamide was demonstrated to promote the intracellular calcium release from calcium stores (Tang and Zhang, 2004), such as endoplasmic reticulum. Calcium release from endoplasmic reticulum is mediated by two main types of receptors: RyRs and inositol-triphosphate receptors (IP_3Rs) (Gafni et al, 1997; Meissner, 1986). We therefore tested the effects of specific inhibitors for RyRs or IP_3Rs on synaptic facilitation. Blockade of RyRs with ryanodine suppressed the synaptic facilitation induced by (−)clausenamide, whereas blockade of IP_3Rs with U73122 showed no discernable effect. The results suggested that RyRs, rather than the PLCγ/IP_3Rs, were involved in the facilitation of synaptic efficacy induced by (−)clausenamide. Shakiryanova et al. (2007) also reported that a small amount of calcium influx through VDCC triggered a much larger calcium spark by opening multiple RyRs. The fact that the nimodipine treatment before bath application with (−)clausenamide completely blocked the facilitation of synaptic transmission and the activation of CaMKIIα confirmed that external calcium influx induced by (−)clausenamide was necessary for the enhancement of synaptic efficacy.

Increased calcium binds several proteins to form active complexes. The activated calcium/calmodulin complex itself can combine with CaMKIIα, resulting in its auto-phosphorylation at Thr286. (−)Clausenamide activated CaMKIIα in hippocampal slices. Inhibition of CaMKIIα with KN93 completely suppressed the enhancement of synaptic efficacy and the activation of CREB induced by (−)clausenamide, indicating that the activation of CaMKIIα was involved in the (−)clausenamide facilitating synaptic transmission. CREB promotes the transcription of genes which was related to synaptic plasticity, neuronal survival and so on. Studies established that the activation of CREB was modulated by ERK1/2. Once activated, ERK1/2 phosphorylated p90 ribosomal S6 kinases 1–3 (RSKs 1–3), which translocated to the nucleus and phosphorylated CREB at Ser133 to activate CRE-regulated gene expression (Impey et al., 1999; Sweatt, 2004). KN93 preincubation inhibited the activation of ERK1/2 and CREB, while PD98059 application only partially suppressed the enhancement of synaptic efficacy and the activation of CREB, suggesting that CREB was phosphorylated by activated CaMKIIα and ERK1/2 while the latter was involved in the CaMKIIα/CREB pathway. Another outcome of calcium/calmodulin activation is the modulation of signal transduction molecules, such as PKA. However, H89, an inhibitor of cAMP-dependent PKA, failed to affect the enhancement of synaptic transmission, which suggested that PKA was not involved in the mechanism of (−)clausenamide on facilitating synaptic plasticity.

It was found that the potentiated synaptic transmission induced by (−)clausenamide occurred at a large fraction of synapses in the slice, and we just took the glutamatergic fibers in CA1 region to do the research. In this region, (−)clausenamide promoted calcium influx through VDCC to trigger intracellular calcium release, subsequently activated CaMKIIα-CREB signal pathway (as shown in Fig. 5). The molecular mechanism of (−)clausenamide on potentiating basal synaptic transmission in hippocampal slices might be useful for explaining the biochemical and morphological changes that has been proven to correlate to hippocampal long-term memory.

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