Polygalasaponin F induces long-term potentiation in adult rat hippocampus via NMDA receptor activation

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Aim: To investigate the effect and underlying mechanisms of polygalasaponin F (PGSF), a triterpenoid saponin isolated from Polygala japonica, on long-term potentiation (LTP) in hippocampus dentate gyrus (DG) of anesthetized rats.

Methods: Population spike (PS) of hippocampal DG was recorded in anesthetized male Wistar rats. PGSF, the NMDAR inhibitor MK801 and the CaMKII inhibitor KN93 were intracerebroventricularly administered. Western blotting analysis was used to examine the phosphorylation expressions of NMDA receptor subunit 2B (NR2B), Ca2+/calmodulin-dependent kinase II (CaMKII), extracellular signal-regulated kinase (ERK), and cAMP response element-binding protein (CREB).

Results: Intracerebroventricular administration of PGSF (1 and 10 μmol/L) produced long-lasting increase of PS amplitude in hippocampal DG in a dose-dependent manner. Pre-injection of MK801 (100 μmol/L) or KN93 (100 μmol/L) completely blocked PGSF-induced LTP. Furthermore, the phosphorylation of NR2B, CaMKII, ERK, and CREB in hippocampus was significantly increased 5–60 min after LTP induction. The up-regulation of p-CaMKII expression could be completely abolished by pre-injection of MK801. The up-regulation of p-ERK and p-CREB expressions could be partially blocked by pre-injection of KN93.

Conclusion: PGSF could induce LTP in hippocampal DG in anesthetized rats via NMDAR activation mediated by CaMKII, ERK and CREB signaling pathway.

Keywords: polygalasaponin F; hippocampus; long-term potentiation; NMDA receptor (NMDAR); Ca2+/calmodulin-dependent kinase II (CaMKII); extracellular signal-regulated kinase (ERK); cAMP response element binding protein (CREB)

Introduction

Polygala japonica HOUTT (P japonica), a folk medicine herb used as expectorant, anti-inflammatory, antibacterial, ataractic, and antidepressant agents in the south of China. These activities may be due to the presence of various saponins in P japonica, since studies have indicated that the saponins found in Polygala have antipsychotic and expectorant effects[1, 2]. Polygalasaponin F (PGSF), a triterpenoid saponin, was first isolated from P japonica by Zhang et al in 1995[3]. Previous studies indicate that PGSF possesses evident anxiolytic and sedative-hypnotic activities, and has cognition improving and cerebral protective effects[4]. A structural analogues of PGSF, polygalasaponin XXXII (PGS32) has been found to improve the hippocampus-dependent learning and memory and induce Long-term potentiation (LTP) in adult rats[5].

LTP of synaptic transmission is currently the best available cellular model for learning and memory in the mammalian brain[6]. LTP has also been proposed as a tool for screening nootropic drug candidates. Although LTP can be induced in a variety of brain regions, hippocampal LTP is the most investigated form of synaptic plasticity, because the hippocampus is involved essentially in learning and memory processes[7, 8]. In particular we chose dentate gyrus (DG) of the hippocampus, which is critically involved in hippocampus dependent memory formation[9].

Accumulating evidence indicates that the NMDA receptor (NMDAR) plays an essential role in the induction of LTP in the hippocampal DG area[10]. Evidence indicates that NMDARs are regulated by tyrosine phosphorylation and that LTP in DG is correlated specifically with tyrosine phosphorylation of the NMDAR subunit 2B (NR2B), which is a major functional component of the hippocampal NMDAR[11, 12].

One effect of NMDAR activation is the influx of calcium (Ca2+), which can bind to calmodulin (CaM)[13]. The Ca2+/CaM complex activates many downstream signaling molecules. Ca2+/CaM-dependent protein kinase II (CaMKII) is one of its target proteins to be implicated in synaptic plasticity[14]. CaMK-II is present in high concentrations in the postsynaptic density, a cytoskeletal structure beneath the postsynaptic membrane in hippocampus[15]. Activation of CaMKII by Ca2+/CaM

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initiates its autophosphorylation on threonine residue 286, which makes it independent of Ca²⁺ and renders it constitutively active[10, 17]. CaMKII is essential for the induction of LTP in the hippocampus. The hippocampal LTP is blocked by CaMKII inhibitors[18]. In addition, Lledo PM et al reported that postsynaptic application of CaMKII produces an increase in synaptic efficacy that mimics LTP[19].

Increasing evidence indicates that the long-lasting potentiation of synaptic efficacy requires an activation of MAPK/ERK in mammals. ERK phosphorylation has been shown to occur in a variety of memory models and following different LTP paradigms in the hippocampus[20, 21]. It is now well established that ERK activation is via multiple upstream kinases, among which CaMKII is the major one[22].

One transcription factor, cAMP response element binding protein (CREB), is a nuclear target of many kinases[23]. Once phosphorylated, CREB appears to mediate the transduction of neuronal stimulation into gene expression, which is also a necessary component for hippocampus-dependent memory formation in mammals[24, 25].

Based on the above ideas, the present study was aimed to examine whether PGSF plays its cognition-enhancing effect through improvements of basic synaptic transmission in the DG and explore the underlying mechanisms.

Materials and methods

Materials

Anti-phospho-CaMKII antibody, anti-CaMKII antibody, anti-phospho-ERK antibody, anti-ERK antibody, and anti-rabbit IgG secondary antibody, anti-mouse IgG secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-CREB antibody, anti-CREB antibody, anti-phospho-NR2B antibody, and anti-NR2B antibody were obtained from Cell Signaling Biotechnology (Hertfordshire, UK). MK801 (a high-affinity NMDAR antagonist) and KN93 (an inhibitor of CaMKII) were obtained from Sigma. Enhanced chemiluminescent (ECL) substrate was from Pierce (Rockford, IL, USA). PGSF with purity greater than 98% was obtained from phytochemistry department in Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China, and dissolved in dimethyl sulfoxide (DMSO) to make stock solution at 0.1 mol/L and diluted with physiological saline before use.

Animals

Male Wistar rats (230–260 g) in this study were provided by the Experimental Animal Center of the Chinese Academy of Medical Sciences, Beijing, China. Rats were housed in a temperature- and light-control room (23±1 °C, 12 h light cycle) 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 (Beijing, China).

Electrophysiological assays

Surgical preparation

The animals were prepared as previously described[26, 27]. Briefly, rats were anesthetized with urethane carbamate (1.5 g/kg, ip) before being fixed on an SR-6N stereotoxic apparatus (Narishige Science Instrument, Japan). Three holes were sequentially drilled at 0.8 mm, 3.8 mm, and 7.5 mm posterior to the bregma and 1.8 mm, 2.5 mm, and 4.2 mm lateral to the mid-line for an outer guide cannula, a monopolar recording electrode, and a bipolar stimulating electrode, respectively. The cannula was placed into the lateral cerebral ventricle at a depth of 2.5–3.0 mm, the recording electrode was placed in the granular cell layer of DG at a depth of 3.0–3.5 mm, and the stimulating electrode was lowered into the perforant path (PP) to a depth of 3.0–3.5 mm. The synaptic responses were monitored by a VC-11 memory oscilloscope (Nihon Kohden, Japan). Once the locations of the cannula and electrodes were verified, they were kept in place for the whole experimental duration.

Measurement of evoked potentials

The population spike (PS) amplitude was employed to assess the excitation level of the granular cell population in the DG. An evoked response was generated in the DG granular cell layer by stimulating the PP at low frequency (0.033 Hz) with single constant current pulses (150 μs in duration) triggered by an SEN-7203 electrical stimulator (Nihon Kohden, Japan) through a SS-202J isolator (Nihon Kohden, Japan). After input/output curve determination, the baseline responses were evoked by a stimulus with an intensity to produce 20% of the maximal PS amplitude.

Intracerebroventricular (icv) drug delivery

Drugs or vehicle injections were delivered via a cannula in the lateral cerebral ventricle after 30 min of electrophysiological baseline measurement from the DG of the contralateral hemisphere. Injections were performed using a 5 μL volume over a 5 min period via a Hamilton syringe. The dose of PGSF was calculated based on the theoretical concentration that the drug would have in the brain (assuming a brain volume of approximately 2 mL). Thus, for an estimated brain concentration of PGSF at 1 μmol/L (equivalent to the dose of 2×10⁻³ pmol per rat), 5 μL of 0.4 mmol/L PGSF was injected and for the brain concentration of PGSF at 10 μmol/L (equivalent to the dose of 2×10⁻² pmol per rat), 5 μL of 4 mmol/L PGSF was injected. Control rats were injected with 0.4% DMSO. For inhibitor experiments, MK801 and KN93 were dissolved in DMSO and diluted with physiological saline to give the desired final concentration (100 μmol/L). The final concentration of DMSO was 0.4%.

Western blotting analysis

After electrophysiological recording, hippocampus from the experimental rats was removed immediately and stored at -70 °C until use. Western blotting analysis was employed to detect p-NR2B, p-CaMKII, p-ERK, and p-CREB protein...
levels in the hippocampus after LTP induction by 1 μmol/L PGSF and to examine the effect of MK801 on the change of p-CaMKII, and the effects of KN93 on p-ERK and p-CREB protein levels. Tissues of hippocampus were homogenized in a buffer of 150 mmol/L NaCl, 25 mmol/L Tris-HCl, 1 mmol/L EGTA, 1 mmol/L EDTA, pH 7.4, 1% Triton X-100, 1 mmol/L PMSF. Protein concentrations were measured with a BCA protein assay kit (Pierce). Samples were separated by a SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto PVDF membranes (Millipore). The membranes were blocked with 3% BSA in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 2 h at room temperature, and then incubated at 4 °C overnight with primary antibodies (1:1000 dilutions) followed by species-appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000 dilutions) for 2 h at room temperature. The immunoreactive bands were visualized using enhanced chemiluminescence (ECL) system (FUJIFILM, Tokyo, Japan). The density of each band was quantified by Gelpro 32 software.

Statistical analysis
All data were shown as the mean±SEM. Group differences of PS amplitude in the electrophysiological assays were evaluated using two-way analysis of variance (ANOVA). Group differences in the Western blotting assays were performed by one-way ANOVA. P-values of <0.05 were regarded as statistically significant.

Results
Effects of PGSF on basic synaptic transmission in the hippocampal DG of anesthetized rats
We examined the effects of PGSF (1 μmol/L and 10 μmol/L) on synaptic transmission in the hippocampal DG area of anesthetized rats. We stimulated the PP coming from entorhinal area and recorded the evoked PS in DG region of rat hippocampus. As shown in Figure 1, the baseline amplitude of the PS stabilized within the first 30 min in both the control and the PGSF groups; two-way ANOVA showed no significant difference between the control and PGSF groups \[F(2, 90)=0.03, P=0.971\], as well as the six time points \[F(5, 90)=1.999, P=0.086\]. Moreover, the PS amplitude did not fluctuate significantly for 60 min after vehicle (0.4% DMSO) administration. In contrast, after the injection of 1 μmol/L PGSF, the PS amplitude increased to 170%±15% of baseline within 5 min. At 15, 30, and 60 min, the PS amplitude increased to 180%±17%, 210%±22%, and 227%±27% of baseline, respectively. Furthermore, PS amplitude increased to 276%±30% of baseline within 5 min after 10 μmol/L PGSF application and at 15, 30, and 60 min, the PS amplitude increased to 312%±31%, 332%±43%, and 401%±36% of baseline, respectively. There was a significant main effect of the control and PGSF groups \[F(2, 180)=269.956, P<0.001\] (1 μmol/L PGSF group vs control, \(P<0.001\); 10 μmol/L PGSF group vs control, \(P<0.001\)). The more than 200% increase of PS amplitude over baseline for longer than 30 min indicated the formation of PGSF-induced LTP in the hippocampal DG area in vivo.

Figure 1. Effects of PGSF on basic synaptic transmission in the hippocampal DG of anesthetized rats. (A) Anatomical diagram of a rat skull. The points R and S represent the approximate locations of the recording electrode and the stimulating electrode, respectively. (B) Measurement of PS amplitude. a, stimulus artifact; b, initial point of excitatory postsynaptic potential (EPSP); c, middle point of bd; d–f, end points of the peaks; length of g–e, amplitude of PS. (C) Time-course plots of perforant path-evoked PS of the control group and PGSF groups. The baseline was recorded from 30 min before administration. At 30 min, 5 μL 0.4 mmol/L PGSF or 5 μL 4 mmol/L PGSF (icv, final concentration was 1 μmol/L or 10 μmol/L) was injected in the test subjects, whereas 0.4% DMSO was injected in control animals. The arrow shows the time point of icv injection. All values are presented as the mean±SEM of 6 rats. \(^bP<0.05, ^cP<0.01\) vs control group, two-way ANOVA.

Effects of PGSF on the phosphorylation of NR2B, CaMKII, ERK, and CREB in hippocampus of rats after PGSF-induced LTP
In order to elucidate the molecular mechanisms underlying the apparent facilitation of long-term potentiation induced by PGSF in hippocampus of adult rats, we detected the phosphorylation changes of several proteins which are related
closely to LTP. First, the level of p-NR2B was evaluated with Western blotting at different time points after LTP induction by 1 μmol/L PGSF. Results showed that p-NR2B increased at 5 min (1.59-fold increase, \( n=6, P<0.05 \)) peaked at 15 min (2.65-fold increase, \( n=6, P<0.01 \)) and maintained for 60 min (1.62-fold increase, \( n=6, P<0.05 \)) after LTP induction (Figure 2A, 2B). Next, we analysed the phosphorylation state of CaMKII at distinct time points. We found that p-CaMKII strongly increased at 5 min (2.89-fold increase, \( n=6, P<0.01 \)). The increased phosphorylation peaked at 15 min (3.78-fold increase, \( n=6, P<0.01 \)) and was sustained for at least 60 min (2.56-fold increase, \( n=6, P<0.01 \)) after LTP induction (Figure 2C, 2D). Similar to the expression of p-CaMKII, the increased p-ERK was observed at 5 min after LTP induction (1.78-fold increase, \( n=6, P<0.01 \)), peaked at 30 min (2.43-fold increase, \( n=6, P<0.01 \)) and persisted for at least 60 min (1.59-fold increase, \( n=6, P<0.01 \)) after LTP induction (Figure 2E, 2F). Finally, CREB phosphorylation level was detected. Results identified a strong increment at 30 min (1.83-fold increase, \( n=6, P<0.01 \)) and remained to 60 min (1.59-fold increase, \( n=6, P<0.01 \)) after LTP induction (Figure 2G, 2H).

Effects of MK801 and KN93 on LTP induced by PGSF

Because the above studies have found that the NR2B and CaMKII were activated after induction of the hippocampal LTP by PGSF, we next evaluated the role of NMDAR and CaMKII in the induction of PGSF-induced LTP. After establishing a stable baseline for 30 min, MK801 was applied 30 min before PGSF injection, two-way ANOVA showed no significant difference between the control and MK801 groups \( [F(2, 90)=2.158, P=0.121] \), as well as the six time points \( [F(5, 90)=0.330, P=0.894] \) (Figure 3A). The PS amplitude of MK801-treated group decreased to 96±9% of baseline from 177±16% in the PGSF group at 5 min after PGSF delivering. The PS amplitude also remained near baseline levels at the other time points. There was a significant main effect of the three groups \( [F(2, 360)=209.617, P<0.001] \) (MK801-treated group vs PGSF group, \( P<0.001 \); vs control, \( P>0.05 \)), indicating that the induction of PGSF-induced LTP was completely blocked by pre-treatment with MK801.

In the same way, KN93 was applied 30 min before PGSF injection, two-way ANOVA showed no significant difference between the control and the KN93 groups \( [F(2, 90)=1.83, P=0.166] \), as well as the six time points \( [F(5, 90)=0.36, P=0.961] \) (Figure 3B). The PS amplitude decreased to 112±8% of baseline from 177±16% in the PGSF group at 5 min after PGSF delivering. The PS amplitude also remained near baseline levels at the other time points. There was a significant main effect of the three groups \( [F(2, 360)=174.773, P<0.001] \) (KN93-treated group vs PGSF group, \( P<0.001 \); vs control, \( P>0.05 \)), indicating that the induction of PGSF-induced LTP was completely blocked by pre-treatment with KN93.

Effect of MK801 on phosphorylation of CaMKII induced by PGSF

It is well-known that NMDAR is a key receptor for CaMKII activation, and based on the results we have found that activations of the NMDAR and CaMKII are required for PGSF-
induced potentiation. Therefore, we investigated whether the enhancement of phosphorylation of CaMKII induced by PGSF was dependent on the NMDAR.

The above results indicated that p-CaMKII was upregulated significantly 5 min after PGSF application, so we observed the effect of MK801 on p-CaMKII at this time point. The result showed that the phosphorylation of CaMKII was decreased from 2.86 fold to 0.96 fold in the presence of MK801, compared with the PGSF-treated group (Figure 4A, 4B), indicating that CaMKII activation induced by PGSF was totally dependent upon the NMDAR.

Effects of KN93 on phosphorylation of ERK and CREB induced by PGSF

Given that the ERK and CREB were also activated after PGSF-induced potentiation, we next evaluated the signaling pathway for ERK and CREB activation induced by PGSF. It is well-known that CaMKII is located upstream of ERK. Therefore, we explored whether the enhancement of p-ERK induced by PGSF was dependent on CaMKII. In the above results, p-ERK was upregulated significantly 5 min after PGSF application, so we observed the effect of KN93 on p-ERK at this time point. The result showed that the phosphorylation of ERK was decreased from 1.68 fold to 1.20 fold in the presence of KN93, compared with the PGSF-treated group (Figure 5A, 5B). The phosphorylation level of ERK was partly, but significantly reduced by pretreatment of KN93, indicating that the activation of ERK is partially dependent on CaMKII.

In the meanwhile, we tested whether CREB was also CaMKII dependent. As p-CREB was upregulated significantly 30 min after PGSF application, we observed the effect of KN93 on p-CREB at this time point. The result showed that pretreatment of KN93 attenuated the phosphorylation of CREB from 1.77 fold to 1.28 fold, compared with the PGSF-treated group (Figure 5C, 5D), suggesting that the activation of CREB is also partially dependent on CaMKII.

Discussion

In the present study, we present the first evidence that PGSF could facilitate LTP in the hippocampal DG. We found that basic synaptic transmission was potentiated within 5 min after PGSF application, so we observed the effect of MK801 on p-CaMKII at this time point. The result showed that the phosphorylation of ERK was decreased from 1.68 fold to 1.20 fold in the presence of KN93, compared with the PGSF-treated group (Figure 5A, 5B). The phosphorylation level of ERK was partly, but significantly reduced by pretreatment of KN93, indicating that the activation of ERK is partially dependent on CaMKII.

As described in Results, MK801 strongly abolished the effect of PGSF on the induction of LTP[28]. This is in agreement with previous studies that activation of the NMDAR was essential for the expression of LTP in the DG[29]. Thus, particular attention has been paid to NMDAR and CaMKII. MAPK/ERK and CREB were the downstream pathway of CaMKII activation. PGSF-induced potentiation might be modulated via enhanced tyrosine phosphorylation of NR2B subunit of NMDAR.
well documented that tyrosine phosphorylation of NR2B is crucial for the induction of LTP in various types of synapses [32]. Tyrosine phosphorylation of NR2B can stabilize NMDAR on the cell surface and thereby increases the NMDAR responses. It can upregulate NMDAR current and prevent the removal of signaling molecules from the NMDAR complex [33–35]. Therefore, the action of NMDAR could be facilitated via the enhanced phosphorylation of NR2B induced by PGSF.

A great deal of evidence has led to the hypotheses that CaMKII is a mnemonic molecule. It has been obtained that the CaMKII plays an important role in LTP [36, 37]. In this study, we found that the phosphorylation of CaMKII strongly increased at 5 min after potentiation, peaked at 15 min, and was sustained for at least 60 min after LTP induced by PGSF. In addition, as described in Results, the facilitating effect of PGSF on the NMDAR-mediated LTP induction disappeared after pretreatment with CaMKII blocker KN93, thus indicating that the action of CaMKII was indispensable for PGSF-induced LTP. This strengthens our confidence that CaMKII plays a key role in PGSF-induced LTP. Furthermore, the NMDAR inhibitor MK801 perfectly prevented the upregulation of CaMKII phosphorylation induced by PGSF in vivo, suggesting that CaMKII phosphorylation was mediated through NMDAR activation. These results indicated that PGSF induces the long-lasting potentiation of synaptic efficacy, which may exert a cognitive effect in the adult rat brain, through the NMDAR followed by CaMKII activation.

It is well established that MAPK/ERK pathway is an essential component of NMDAR signal transduction controlling the neuronal synaptic plasticity [38]. In our experimental conditions, we found a rapid and strong increment in ERK phosphorylation after LTP induction, which lasted for at least 60 min. The time course was parallel with that of CaMKII activation. According to the time of reaching the maximum activation of CaMKII and ERK, we speculated that CaMKII was one of the upstream kinases of ERK activated by PGSF. Therefore, KN93 was pretreated before PGSF was applied. The results demonstrated that the activation of ERK was partially inhibited by KN93, suggesting that ERK was the downstream signaling molecule of CaMKII activation, which was consistent with other reports [39].

CREB is a key molecular involved in synaptic plasticity and memory [40]. CRE-mediated transcription is stimulated in the hippocampus during hippocampus dependent memory formation [41]. In the current study, CREB phosphorylation was increased 30 min after LTP induction. Moreover, we found that CREB phosphorylation was partially blocked with KN93 in vivo, suggesting that the CaMKII pathway at least in part mediated the activation of CREB induced by PGSF. The striking effect of CaMKII on the activation of ERK and CREB strongly supports that CaMKII is a critical molecule of the signal cascade responsible for synaptic potentiation induced by PGSF.

In addition, a close association between facilitated hippocampal LTP and enhanced learning and memory has been demonstrated [42, 43]. Therefore, PGSF probably enhanced learning and memory function through improvements of basic synaptic transmission in hippocampal DG. PGSF has tremendous potential for the development of therapeutic drugs that can be used to treat cognitive deficit diseases.

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Author contribution
Feng SUN designed the study, performed the research and wrote the paper; Jian-dong SUN provided assistance in experiment design and revised the paper; Ning HAN performed partial research; Yu-he YUAN assisted in experiment design; Dong-ming ZHANG and Chuang-jun LI extracted the PGSF; and Nai-hong CHEN designed the research and revised the paper.
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