Neuroprotective Effect of Piperine on Primarily Cultured Hippocampal Neurons

Min Fu, Zhao-hui Sun, and Huan-cong Zuo*

Medical College of Tsinghua University; Beijing 100084, China.
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It was previously reported that piperine (PIP) significantly blocks convulsions induced by intracerebroventricular injection of threshold doses of kainate, but had no or only slight effects on convulsions induced by L-glutamate, N-methyl-D-aspartate and guanidinosuccinate. In traditional Chinese medicine, black pepper has been used for epileptic treatment; however, the exact mechanism is still unclear. We reported here in that appropriate concentration of PIP effectively inhibites the synchronized oscillation of intracellular calcium in rat hippocampal neuronal networks and represses spontaneous synaptic activities in terms of spontaneous synaptic currents (SSC) and spontaneous excitatory postsynaptic currents (sEPSC). Moreover, pretreatment with PIP expects protective effect on glutamate-induced decrease of cell viability and apoptosis of hippocampal neurons. These data suggest that the neuroprotective effects of PIP might be associated with suppression of synchronization of neuronal networks, presynaptic glutamic acid release, and Ca²⁺ overloading.

Key words  piperine; calcium oscillation; spontaneous synaptic current; spontaneous excitatory postsynaptic current; glutamate-induced excitotoxicity; hippocampal neuron

Black pepper (Piper nigrum) is the most common spice in the world. In traditional medicine, black pepper has been used as analgesic and anti-inflammatory drug and for the treatment for epilepsy and snake venom poisoning. Piperine (PIP) is a major alkaloid of black pepper. Recent studies on the pharmacological actions of PIP have demonstrated its cytoprotective effect and antioxidant activity, immune-modulatory and antitumor activity, and inhibitory action on drug-metabolizing enzymes in rodents. The results from animal experiments showed that PIP possesses antidepressive hyphen like activity and cognitive-enhancing effect after chronic oral administration. It was demonstrated that the antidepressant properties of PIP are mediated via the regulation of serotonergic system; chronic treatment of PIP enhances the serotonin levels in the hypothalamus and hippocampus. It is also reported that up regulation of progenitor cell proliferation of hippocampus and cytoprotective activity might be mechanisms involved in the antidepressive-like effect of PIP, which may be closely related to elevation of hippocampal brain-derived neurotrophic factor (BDNF) level. In traditional Chinese herbal medicine, black pepper has a long tradition as a treatment for epilepsy. Data from animal kindling experiments also demonstrated anticonvulsant activity of PIP on seizures induced by excitatory amino acid receptor agonists. However, the underlying mechanisms are still unclear.

Synchronization activity was found in many types of neuronal tissues in vivo and in vitro, including the olfactory bulb, thalamus, hippocampus, and neocortex. The synchronous activities were believed to play an important role during neuronal development, migration, and information processing. Oscillatory synchronization has been implicated in several cognitive functions including feature binding and scene segmentation, memory formation and recall, and attention. Moreover, it has been reported that synchronization processes are associated with neuropsychiatric disorders such as epilepsy, schizophrenia, dementia, and Parkinson’s disease. It is well known that epileptic seizures involve excessive discharge and abnormal electrical behavior of neurons. However, excessive discharge alone dose not necessarily cause seizure; synchronization of a network of neurons seems involved. Massive synchronized firings were observed among neurons of sensorimotor cortex, hippocampus, amygdalae and subthalamic nucleus during seizure episodes. It is well accepted that the hippocampus is critical in the formation of declarative memories. Interestingly, electrophysiological studies demonstrated that this region also has a particularly low seizure threshold, where globally synchronous synaptic activity occurred. In primary cultures of embryonic hippocampal neuronal networks, spontaneous and synchronous depolarizations were detectable in the form of intracellular calcium oscillations. This experimental model, which has been widely characterized in terms of synaptic development and functionality, provided a convenient and accessible system to investigate pharmacological effects of various factors on neuronal networks activities.

In this paper, we investigated the effects of PIP on cultured hippocampal neuronal networks to enrich evidence for the neuropharmacological activities of PIP and for its clinical usefulness against epilepsy.

MATERIALS AND METHODS

Materials Dulbecco’s modified eagle’s medium (DMEM), fetal bovine serum, equine serum, neurobasal medium, B27 supplements, and 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) were from Invitrogen (Carlsbad, CA, U.S.A.). Poly-D-lysine, L-glutamine, Hoechst 33342 powder, and MK-801 were from Sigma (St. Louis, MO, U.S.A.). PIP (powder, reference substance for content determination, HPLC≥98%; molecular weight: 285.33) was from the China National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and dissolved in dimethylsulfoxide (DMSO). CytoTox-ONE™ Homogeneous Membrane Integrity Assay kits were purchased from Promega (Madison, WI, U.S.A.). Other reagents were purchased from domestic reagent companies.

Cell Culture and Treatments Hippocampal neuron cul-

* To whom correspondence should be addressed. e-mail: zuohc@mail.tsinghua.edu.cn © 2010 Pharmaceutical Society of Japan
The primary cultured hippocampal neurons were pre-treated with 15 μg/ml PIP for 24 h, followed by exposure to 125 μM glutamate with 10 μM of glycine in supplemented neuronal culture medium for 15 min in a humidified incubator for 15 min. After excitotoxicity was induced, the cells were incubated with the neuronal culture medium in a humidified incubator for 18 h. Two groups of neurons served as control: one group underwent pretreatment of MK-801, the glutamate receptor antagonist; the other received neither PIP pretreatment nor glutamate stimulation.

**Calcium Imaging** The cells grown on the glass bottom in 35-mm dishes were directly imaged by Nikon (Tokyo, Japan) inverted microscope (TE300) using a 40× numerical aperture 1.30 oil immersion Plan Fluor objective (Figs. 2Aa, b). The hippocampal cells were loaded with 6 μM Fluo-4-AM (Molecular Probe, Invitrogen, U.S.A.) in Krebs-Ringer’s saline (in mM: 150 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 glucose, and 10 N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES), pH 7.4). A Lambda DG-4 high-speed wavelength switcher (Sutter Instrument, Novato, CA, U.S.A.) was used for Fluo-4 excitation at 480 nm and cooled CCD camera (CoolSnap FX; Roper Scientific, Princeton, NJ, U.S.A.) was used for image acquisition. MetaFluor imaging software (Universal Imaging Corporation, Downingtown, PA, U.S.A.) was used for hardware control, image acquisition, and image analysis. Using a heater positioned near the microscope stage, the hippocampal cells were maintained at 30±2 °C during imaging. The sampling rate was one frame every 2 s with exposure time of 80 ms and CCD binning of 4×4.

**Electrophysiology** Before the experiments, the culture dishes were rinsed twice and perfused with extracellular solution (in mM: 140 NaCl, 3 CaCl2, 1 MgCl2, 5 KCl, 10 HEPES, 10 glucose, pH 7.4) for recording spontaneous synaptic currents. The patch-pipette solution contained (in mM): 140 KCl, 10 HEPES, 10 ethylene glycol bis(2-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA), 2 Na2ATP, pH 7.3. The spontaneous excitatory postsynaptic currents (sEPSC) were isolated by application of 0.05 mmol/l bicusculine, the competitive antagonist to the γ-aminobutyric acid (GABA_A) receptors in the extracellular solution, and the intracellular pipettes solution contained (in mM): 140 KCl, 10 HEPES, 10 EGTA, 2 MgCl2, 2 Na2ATP, 1 CaCl2, pH 7.3. The whole-cell patch clamp experiments were performed at room temperature (22±2 °C) using Axopatch-200B amplifier (Axon Instruments, Foster City, CA, U.S.A.). The patch electrodes (Fisherbrand, Pittsburgh, U.K.) were pulled on a PP-83 micropipette puller (Narishige, Tokyo, Japan). The typical resistance of glass electrodes was 4—8 MΩ when filled with intracellular pipette solution. The signals were recorded by pClamp9 software (Axon Instruments, Foster City, CA, U.S.A.) and analyzed by clampfit9 software (Axon Instruments, Foster City, CA, U.S.A.).

**Quantitative Analysis of Frequency of Synchronized Calcium Spikes and Spontaneous Synaptic Currents** Quantitative measurements of [Ca^{2+}], were obtained by measuring the average Fluo-4 fluorescence intensity of a 3×3 pixel^2 analysis box placed at the center of the cell body; the intensity values were then subtracted to the average background intensity measured in cell-free region. The changes of [Ca^{2+}], in each cell were represented by changes of relative Fluo-4 fluorescence (∆F/F_0), where F_0 was the baseline intensity. Ca^{2+} spikes were defined as rapid elevation of ∆F/F_0≥20%. Under our imaging settings, fields of 4—8 neurons were typically recorded and subsequently analyzed. Data from at least three dishes from different batches of cultures were pooled together and analyzed for significant statistical differences. We calculated the frequency of both the Ca^{2+} spikes and the spontaneous currents by counting the
number of spikes and firings over a 2-min period of the recordings at a defined time-point. The frequency values after drug application were normalized to the control frequency value and expressed as percentages; a value of 100% indicated no change.

**Hoechst 33342 Staining**  The hippocampal neurons were fixed in 4% paraformaldehyde at room temperature for 20 min. After staining with 10 μg/ml Hoechst 33342 for 20 min, the cells were observed under a fluorescence microscope (TE300, Nikon, Japan) on ultraviolet illumination.

**LDH Release Assay**  Cytotoxicity was quantified by measurement of LDH released in the medium by using CytoTox-ONE™ Homogeneous Membrane Integrity Assay kit according to the manufacturer’s instructions. Briefly, hippocampal neurons were cultured in 96-well plates. Cell lysis solution 2 μl was added to untreated cells, which were selected as the control of maximum LDH activity. After 10 min, 100 μl of the supernatant of each well was transferred to the corresponding well of a fresh 96-well plate and was mixed with 100 μl of the LDH substrate. After incubation with protection from light at room temperature for 15 min, the reaction was stopped by adding 50 μl of stop buffer and the absorbance was measured at 590 nm by Safire2™ multimode readers (TECAN Inc., U.S.A.). The LDH release percentage was calculated by the formula: 100×(culture medium background)/(maximum LDH release−culture medium background). Data from 6 wells per one experiment from three separate experiments were used for statistic analysis.

**Statistic Analysis**  The compiled data were expressed and graphed as mean±S.E.M., with n denoting the number of neurons studied. For calcium imaging and electrophysiological data, the data were analyzed for significant statistical differences by Student’s t-test. SigmaPlot software (SPSS Inc., IL, U.S.A.) was used to fit concentration-response data to the equation: $Y = 1/(1 + (IC_{50}/C)^n)$, where $C$ is the concentration of drug, $Y$ is the inhibition percentage, and $n$ is the Hill coefficient. For the Hoechst 33342 staining and LDH release assay, Significant differences among the treatments were analyzed ANOVA test followed by Tukey test ($\alpha=0.5$) (Sigma-Stat, Systat Software, Inc., U.S.A.).

**RESULTS**

**PIP Inhibited Intracellular Synchronized Ca$^{2+}$ Oscillations**  To examine the effects of PIP on synchronized Ca$^{2+}$ spikes, we bath-applied PIP to hippocampal cultures and recorded Ca$^{2+}$ imaging before and after the application. The stock solution was diluted 1000 times to a 2×working concentration with Krebs–Ringer’s solution just before application, then applied to the cells to achieve the desired final concentration through 1:1 dilution (v/v). Specifically, we first recorded Ca$^{2+}$ spikes for 2—4 min as control period in 1 ml of Krebs–Ringer’s solution, then added 1 ml of the 2×solutions, and recorded consecutively. For control, we performed the same procedure to apply 1 ml Krebs–Ringer’s solution or 2 μl DMSO diluted in 1 ml Krebs–Ringer’s solution. We found that calcium oscillations were completely inhibited after application of 15 μg/ml PIP (Fig. 2C). The inhibitory effect of PIP of lower concentration was time dependent. The frequencies reduced to 82% of the control period after 2 min of the application of 9.5 μg/ml PIP, while to 57% after 8 min of the application. Bath application of the control saline buffer and the diluted DMSO did not affect the frequency and amplitude of Ca$^{2+}$ spikes (Fig. 2B), indicating that no artifact was produced by the bath application method or by DMSO.

**The Inhibitory Effect of PIP Was Dose-Dependent**  We examined the effect of different concentrations of PIP on the frequencies of calcium oscillations. The data showed that inhibitory effect of PIP on calcium oscillations was dose-dependent (Fig. 3). Application of 9 μg/ml PIP almost had no effect on the frequencies and amplitude of calcium oscillations. The concentration-response curve could be fitted well with a logistic equation. The IC$_{50}$ value for the inhibitory effect of PIP was calculated 9.8 μg/ml.

**PIP Depressed Spontaneous Synaptic Currents (SSCs) and sEPSCs**  The former result simplified that the oscillatory activity was synaptically driven and relied on glutamatergic neurotransmission. We then performed electrophysiological recordings using the whole-cell patch clamp method in gap-free modes to record SSCs and sEPSCs. Healthy typical pyramidal hippocampal neurons were clamped at assumptive resting potential (~70 mV) and recorded for about 5 min as control. After application of 15 μg/ml PIP, we consecutively recorded for 10 min (Figs. 4Ba, b). The data indicated that the frequencies of SSCs were decreased to 56.2±7.8% after 2 min and to 33.5±9.2% after 10 min of 15 μg/ml PIP application (Fig. 4Ac). The sEPSCs were completely inhibited about 4—5 min after application (Figs. 4Ba, b).

**Effects of PIP Pretreatment on Glutamate-Induced Apoptosis of Hippocampal Neurons**  We also carried out morphological examinations for determining the type of cell death induced by glutamate. Hoechst 33342 staining showed that after excitotoxic insult of 125 μM glutamate, 44% of hippocampal neurons displayed apoptotic morphology characterized by condensation of chromatin, nuclear shrinkage, and formation of a few apoptotic bodies. Using one-way ANOVA followed by the Tukey test, we demonstrated that there were significant differences among groups ($p<0.001$). Compared with the negative control group, pretreatment with 15 μg/ml of PIP or 2 mM MK-801 both reduced the excitotoxic effect of glutamate on hippocampal neurons (Figs. 5A, B). However, the protective effect of PIP remarkably differed from

![Fig. 3. Concentration-Response Curve for Effects of PIP on the Intracellular Synchronized Calcium Oscillation Frequencies](image)

$IC_{50}=9.8 \mu g/ml$. Each point represents the average of 7—15 cells.
Effects of PIP Pretreatment on Glutamate-Induced Decrease of Cell Viability in Hippocampal Neurons

Glutamate stimulation significantly increased LDH release of hippocampal neurons to 22%. One-way ANOVA test followed by Tukey test illustrated that there were statistically significant differences among the treatments ($p < 0.001$). The protective effect of PIP was shown by the changes in LDH release (Fig. 5C). The glutamate receptor antagonist, MK-801, as positive control, also significantly inhibited glutamate-induced cytotoxicity according to LDH assay. Consistent with the Hoechst 33342 staining results, there was significant difference between the protective effects of PIP and MK-801 (Fig. 5C).

**DISCUSSION**

White pepper used as the Chinese traditional herbal medicine has been widely prescribed to treat epilepsy.\(^29\) PIP is known to be one of the effective components in the herb and was isolated many years ago. The chemical structure of PIP is different from that of prototype antiepileptic drugs. Its chemical structure places it in the group of cinnamamides, such as 3,4-DCPB, an analog of the cinnamamides.\(^30,31\) That the congeners of cinnamamides possess sedative, hypnotic, anticonvulsant, antidepressant, and skeletal muscle relaxing actions has been reported.\(^29\)

The results of previous experiments demonstrated that PIP significantly blocked convulsions induced by intracerebroventricular injection of threshold doses of kainate, but had no or only slight effects on convulsions induced by L-glutamate, N-methyl-D-aspartate, and guanidinosuccinate.\(^8\) We report here that appropriate concentration of PIP effectively inhibited the synchronized oscillation of intracellular calcium in rat hippocampal neuronal networks and repressed spontaneous synaptic activities in terms of SSCs and sEPSCs. Moreover, pretreatment with PIP had protective effect on glutamate-induced decrease of cell viability and apoptosis of hippocampal neurons. These data provide evidence underlying mechanisms of the antiepileptic effect of PIP.

Modulation of voltage-gated ion channels (including potassium, sodium, and calcium channels) is one of the main mechanisms of action of antiepileptic drugs. These channels shape the subthreshold electrical behavior of neurons and are integral to the generation of seizure discharges.\(^32\) However, our preliminary studies suggest that PIP dose not act through blocking voltage-gated channels (data not shown) and the inhibitory effect of PIP on synchronized calcium oscillations was also not due to the blocking of action potential generation and extracellular calcium influx through high-voltage-activated calcium channels.

Spontaneous and synchronous calcium oscillations are synaptically driven.\(^24\) Considering that the modulatory action of PIP on synaptic transmission may involve direct action on neurotransmitter release, we recorded SSCs and sEPSCs.
sEPSC. The sEPSCs comprised of an equal mixture of TTX-insensitive miniature EPSCs and EPSCs that appeared to result from spontaneous action potentials, which were all glutamate related. The effective inhibition of PIP on sEPSCs suggests that PIP may be involved in glutamate-mediated synaptic events. Although evidences from animal experiments suggest that PIP selectively blocks convulsions induced by kainate, our electrophysiological experiments showed that the whole-cell currents induced by application of kainate, NMDA, and NaATP were not affected by co-application of PIP (data not shown). These data are consistent with previous studies on spinal cord cells in primary dissociated cultures and indicate that PIP acts as antagonist presynaptically, but not as receptor antagonist postsynaptically.

Epileptogenesis is a process by which a normal brain develops epilepsy, a chronic condition in which seizures occur. Changes that occur during epileptogenesis are thought to include cell death, axonal sprouting, reorganization of neural networks, alterations in the release of neurotransmitters, and neurogenesis. These changes cause neurons to become hyperexcitable and could lead to spontaneous seizures. The hippocampus is one of the brain regions that is highly sensitive to insults and undergoes, epileptogenesis. Hyperexcitability was a characteristic feature of epileptogenesis in which the likelihood that neural networks will be activated is increased. Excessive release of the neurotransmitter glutamate is widely recognized as an important part of epileptogenesis early after brain injury, including in humans. The inhibitory effect of PIP on neuronal networks and glutamate release suggests its long-term neuroprotective action during the process of epileptogenesis.

Seizure-induced neuronal death results in large part from excitotoxic glutamatergic neurotransmission gating excessive Na+ and Ca2+ entry. Glutamate receptor antagonists protect against seizure damage, but their therapeutic value is unlikely to be realized because of neurotoxic side effects, particularly in the young, and the profound disturbance these drugs impart on normal brain function. Accordingly, alternative approaches to targeting seizure-induced neuronal death are required.

It was previously reported that cultured rat hippocampal neurons undergo morphological changes after 72-h incubation of 50 µM PIP. Cytoplasmic LDH release increased significantly after 96-h incubation of 50 µM PIP. In our study, however, 24-h pretreatment with PIP had protective effect on glutamate-induced decrease of cell viability and apoptosis of hippocampal neurons. Apoptosis is a morphologically distinct form of cell death characterized by cytoplasmic condensation, preservation and packaging of intracellular organelles, DNA fragmentation, and dispersal and phagocytosis of the cell as apoptotic bodies. Apoptosis may be triggered by two main pathways: activation of cell surface-expressed death receptors of the tumor necrosis factor TNF, superfamily and disruption to intracellular organelle homeostasis or DNA damage. Seizure-induced mitochondrial dysfunction and activation of Bcl-2 family are calcium dependent or at least partly of calcium dependent, whereas a role for death receptors contributing to seizure-induced neuronal death is less likely because there is no apparent requirement for calcium in the activation mechanism. Our results suggest that the neuroprotective effect of PIP should be calcium dependent and participate in protecting intracellular organelles, such as mitochondrion and endoplasmic reticulum (ER).

PIP was previously reported as an agonist of transient receptor potential vanilloid 1 (TRPV1). TRPV1 is abundant in hippocampus, cortex and cerebellum and can induce desensitization. Recent research has shown that TRPV1 receptors are necessary and sufficient for a form of long-term depression (LTD) at excitatory synapses onto hippocampal interneurons. The inhibitory effect was selective to excitatory synapses onto hippocampal interneurons, whereas neighboring excitatory synapses onto CA1 pyramidal cells were unaffected. The interneurons were essential in the precise control of firing of groups of principal cells as well as in network oscillations. The selective depression of these synapses was expected to increase the excitability of innervated pyramidal cells. One classical theory of epileptogenesis rested on the hypothesis that reduced inhibition within neuronal networks causes hyperexcitability thus leading to the occurrence of seizures. Indeed, not only on the membrane, extensive expression of TRPV1 was also revealed on ER, consistent with TRPV1 operating as a calcium release receptor. PIP is a weak base, highly lipophilic in nature with partial solubility in aqueous media, which suggests that PIP may also interact with TRPV1 on ER for calcium homeostasis and ER function regulation. We believe that the antiepileptic effects of PIP might, at least in part, act through TRPV1 pathway in vivo. Further work would help to ascertain whether hippocampal TRPV1 receptors could provide novel drug targets for epileptic disorder treatment.

In conclusion, our results suggest that the neuroprotective effects of PIP might be associated with suppression of synchronization of neuronal networks, presynaptic glutamate release, and Ca2+ overloading. However, whether the inhibitory effects of PIP on synchronized oscillation of intracellular calcium and spontaneous synaptic activities are reversible still needs to be investigated.

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