Capsaicin-Induced Glutamate Release Is Implicated in Nociceptive Processing Through Activation of Ionotropic Glutamate Receptors and Group I Metabotropic Glutamate Receptor in Primary Afferent Fibers

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Received September 24, 2008; Accepted December 12, 2008

Abstract. Glutamate (Glu) is the major excitatory neurotransmitter in the central nervous system. The role of peripheral Glu and Glu receptors (GluRs) in nociceptive transmission is, however, still unclear. In the present study, we examined Glu levels released in the subcutaneous perfusate of the rat hind instep using a microdialysis catheter and the thermal withdrawal latency using the Plantar Test following injection of drugs associated with GluRs with or without capsaicin into the hindpaw. The injection of capsaicin into the rat hind instep caused an increase of Glu level in the s.c. perfusate. Capsaicin also significantly decreased withdrawal latency to irradiation. These effects of capsaicin were inhibited by pretreatment with capsazepine, a transient receptor potential vanilloid receptor 1 (TRPV1) competitive antagonist. Capsaicin-induced Glu release was also suppressed by combination with each antagonist of ionotropic GluRs (iGluRs: NMDA/AMPA receptors) and group I metabotropic GluR (mGluR), but not group II and group III mGluRs. Furthermore, these GluRs antagonists showed remarkable inhibition against capsaicin-induced thermal hyperalgesia. These results suggest that Glu is released from the peripheral endings of small-diameter afferent fibers by noxious stimulation and then activates peripheral iGluRs and group I mGluR in development and/or maintenance of nociception. Furthermore, the activation of peripheral NMDA/AMPA receptors and group I mGluR may be important in mechanisms whereby capsaicin evokes nociceptive responses.

Keywords: glutamate, peripheral, capsaicin, thermal hyperalgesia, microdialysis

Introduction

Glutamate (Glu) is a main excitatory neurotransmitter in the central nervous system. Moreover, Glu has been shown recently to play an important role in the peripherally nociceptive transmission (1). Concerning the existence of Glu in the small-diameter afferent fibers, we have reported that electrical stimulation of the sciatic nerve or noxious heat stimulation (50°C) caused an increase of the Glu level in subcutaneous perfusate (2). Glu receptors (GluRs) are classified into ionotropic GluRs (iGluRs: NMDA, AMPA/kainate receptor) and metabotropic GluRs (mGluRs: group I, II, and III mGlu receptor). There are evidences that virtually all DRG cells as well as their central and peripheral terminals are positively labeled for iGluRs (3) and mGluRs (4). Electron microscope studies demonstrate that Glu receptors are transported from the DRG cell bodies into central and/or peripheral primary afferent terminals (5). NMDA, AMPA, and kainate receptors (NMDA/AMPA-
kainate receptors) are localized on unmyelinated axons at the dermal–epidermal junction in the glabrous and hairy skin of the rat (6, 7) and in human skin hair (8).

In addition to these anatomical data, behavioral observations support a role for peripheral GluR in normal nociceptive transmission. Intraplantar injection of L-Glu or GluRs agonists into the hindpaw evokes thermal and mechanical hyperalgesia and allodynia, which can be blocked by appropriate antagonists (6, 9, 10).

Recently, there is some data supporting the modulation of vanilloid receptor function through GluRs. For example, group 1 mGluRs increase thermal sensitivity by enhancing vanilloid-receptor function (11). Furthermore, peripheral NMDA receptor modulates jaw muscle electromyographic activity induced by capsaicin injection into the temporomandibular joint of rats (12). These findings indicate that peripheral GluRs are responsible for nociceptive responses via transient receptor potential vanilloid receptor 1 (TRPV1) activation.

TRPV1 is located in a neurochemically heterogeneous population of small-diameter primary afferent neurons (13). A broad range of stimuli such as noxious heat, protons, lipid-derived endovanilloids, and inflammatory mediators either directly activated or modulate TRPV1 (14, 15). At the peripheral terminals of primary afferents, TRPV1-mediated Ca\(^{2+}\) influx triggers the release of neuropeptides and neurotransmitters, which is responsible for nociceptive processing (14).

In this connection, we have already reported that local application of capsaicin cream to the instep also evoked a marked increase in Glu level and this capsaicin-evoked Glu release was significantly decreased by daily high-dose pretreatment with capsaicin for three consecutive days (2). However, the interactions between TRPV1 and GluRs concerning nociceptive processing at the periphery are still poorly understood.

In this study, to determine how GluRs and Glu existing in the peripheral endings of small-diameter afferent fibers and their extracellular space, respectively, may be involved in development and/or maintenance of nociception evoked by capsaicin, the changes of Glu levels in the extracellular space and pain behavior in thermal withdrawal latency were measured following subcutaneous (s.c.) injection of drugs associated with GluRs with/without capsaicin.

Materials and Methods

All surgical and experimental procedures for animals were reviewed and approved by the Ohu University Intramural Animal Care and Use Committee and conformed to the guidelines of the International Association for the Study of Pain (16).

Experimental procedures

Adult male Sprague Dawley rats weighing 200–300 g (CLEA Japan, Inc. Tokyo) were used in all experiments. Rats were on a 12-h light/dark cycle and received food and water ad libitum.

Release of Glu into the subcutaneous space

Rats were anesthetized with urethane (1 g/kg, i.p.). A single loop catheter whose tip was covered with a dialysis membrane with a cut-off of 5000 Da (MS 0045, PSS® SELECT; PSS World Medical, Jacksonville, FL, USA) was introduced into the s.c. space of the instep using a 2.2-mm outer diameter polyethylene tube as a guide. Ringer’s solution was perfused at 15 μl/min through this catheter with a microsyringe pump (EP-60; Eicom, Kyoto), and perfusate was collected into the tubes placed in an ice bath at intervals of 20 min. The samples were kept at −80°C until analysis.

Amino acid analysis

Amino acids in the dialysate were analyzed by a high-performance liquid chromatography (HPLC) system for automated analysis of amino acids using o-phthalaldehyde derivatization and fluorescence detection. Amino acids were quantified by reverse-phase chromatography using a C\(_{18}\) octadecysilyl (ODS) silica-gel column (EICOMPAK SC-50DS, 2.1 mm × 150 mm; Eicom) with pre-column (EICOM PREPAKSET-AC, 3 mm × 4 mm; Eicom). This column was attached to an HPLC system (HTEC500, Eicom) consisting of a pump connected with a degasser, a sampling injector with a sample processor and a cool pump, a fluorescence HPLC monitor, and a personal computer with a data processor (Power Chrom, EPC-500; Eicom). The mobile phase consisted of 100 mM, pH 6 phosphate buffer containing 30% methanol and 10 μM EDTA. The flow rate was 0.23 ml/min. Peak areas of unknown substances were compared to those of control compounds for quantitation. To determine the effect of drugs on the level of Glu, the average amounts of Glu concentration in two 20-min fractions collected over periods of 40 min before local application of capsaicin and/or GluR-associated drugs were obtained and expressed as percentages of the control value before stimulation.

Drug administration

While animals were inside the small cage, drugs were administered s.c. into the left hind paw.

Except for capsazepine, other drugs were administered in the volume of 50 μl, s.c., into the plantar surface of the left hind paw using a 100-μl Hamilton syringe (Reno, NV, USA) with a 30-gauge needle without anesthesia while the animals remained in the small cage.
The needle was inserted into the plantar skin proximal to the midpoint of the hind paw. Capsazepine (30 mg/kg) at the volume of 50 μl was s.c. injected into the neck.

**Behavioral assessments**

The Plantar Test (model 7370; Ugo Basile, Verese, Italy) was performed according to the previously described method (21) to determine whether the rats were hyperalgesic. In brief, prior to testing, the animals were placed in a small cage on a glass plate. They were not restrained and could move about and explore freely. Radiant heat was beamed onto the plantar surface of the hind paw. The intensity of the beam was controlled and adjusted prior to the experiments, and the cutoff latency was set at 24 s. The beam was applied to the test and control foot in turn and the latency of the withdrawal reflexes recorded. The mean of four such responses was determined prior to the experiments, and the cutoff latency was set at 24 s. The beam was applied to the test and control foot in turn and the latency of the withdrawal reflexes recorded. The mean of four such responses was determined; and the ratio of the test foot latency divided by control foot latency, multiplied by 100, was calculated and termed the “percentage withdrawal latency”. The withdrawal latencies were determined at hourly intervals, from 1 h before injection of the drugs to 6 h after the injection, except for 15 min after the injection. 

**Drugs**

We used the following drugs and chemicals: as GluR agonist, L-glutamic acid; selective NMDA-receptor agonist, AMPA; selective group I mGluR agonist, (S)-3,5-dihydroxyphenylglycine [(S)-3,5-DHPG]; group II mGluR agonist, (2S,1'S,2'S)-2-(carboxycyclopropyl) glycine (L-CCG-I); selective group III mGluR agonist, L-(-)-2-amino-4-phosphonobutyric acid (L-AP4). We used the following GluR antagonists: selective non-competitive NMDA-receptor antagonist, (5S,10R)-(+) -5-methyl-10,11-dihydro-5'H-dibenzo[a,d]cyclo-hepten-5,10-imine hydrogende maleate [(+)-MK-801 hydrogende maleate]; competitive kainate/AMPA-receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX) and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt (NBQX); group I mGluR selective non-competitive mGluR antagonist, 7- (hydroxyimino) cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt); group I mGluR mGluR-subtype-selective antagonist, 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP); group II mGluR antagonist, (2S,3S,4S)-2-methyl-2-(carboxycyclopropyl)glycine (MCCG); selective group III mGluR antagonist, (R,S)-a-methylserine-O-phosphate (MSOP). These agonist and antagonists of the GluR were obtained from Tocris (Ballwin, MO, USA) and Cayman Chemical (Ann Arbor, MI, USA), respectively. All other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka).

In accordance with the product material safety data sheets, L-glutamate acid, L-CCG-I, and L-AP4 were diluted in NaOH; and MK801, NMDA, (S)-3,5,-DHPG, MCCG, and MSOP were diluted in water. CNQX, CPCCOEt, and MPEP were diluted in dimethyl sulfoxide. The other drugs except for these were dissolved in saline. Capsazepine was prepared as a 10 mg/ml solution in saline containing 10% ethanol and 10% Tween 80. The pH of all solutions was adjusted to 7.4. Capsazepine was dissolved in dimethyl formamide and then diluted with saline. o-Phthalaldehyde was dissolved in methanol and adjusted to 4 mM with 0.1 M, pH 9.5 carbonate buffer.

**Abbreviations**

Cap + MK801: capsazepine combined with MK801, Cap + CNQX: capsazepine combined with CNQX, Cap + NBQX: capsazepine combined with NBQX, Cap + CPCCOEt: capsazepine combined with CPCCOEt, Cap + MCCG: capsazepine combined with MCCG, Cap + MSOP: capsazepine combined with MSOP.

**Statistical analysis**

All data are expressed as the mean ± S.E.M. In the study of Glu release, statistical analyses were performed using the posthoc test of Fisher’s protected least significant difference. A level of P<0.05 was considered to be statistically significant. In the behavioral study, statistical analyses were performed with Dunnett’s test for multiple comparison subsequent to analyses of variance (ANOVA). P<0.05 was accepted as statistically significant.

**Results**

**Basal Glu release**

The concentration of Glu in the perfusate was initially high, but gradually decreased with time reaching a stable level after 2 h of perfusion, which was then continued for at least 4.5 h. Glu was present at 1.95 ± 0.25 μM (n = 10, S.E.M.) in the resting state, which is defined here as the mean of the three 20-min fractions collected from 60 min after starting perfusion to 120 min (fractions 4 – 6 in the capsazepine group, Fig. 1).

**Effects of capsazepine on capsazepine-evoked Glu release**

The s.c. injection of capsazepin (3 mM) in the vicinity
of the perfusion side evoked a significant increase in Glu release (Fig. 1). The average concentration of the released Glu was 4.86 ± 0.48 μM/20 min in 2 fractions collected after the injection of vehicle or capsaicin, respectively. Injections (s.c.) of vehicle or capsaicine alone did not produce any significant changes in the levels of Glu in the perfusates.

**Effects of iGluRs antagonists injection on capsaicin-evoked Glu release**

The combined injection of capsaicin with MK801 (1 mM) (Cap + MK-801) or NBQX (5 mM) (Cap + NBQX) into the perfusion region showed far less Glu release than injection of capsaicin alone (Fig. 2A). The average concentration of the released Glu was 1.20 ± 0.1 or 1.70 ± 0.1 μM/20 min in 2 fractions collected after the co-injection of MK-801 or NBQX with capsaicin, respectively. These inhibitory effects of iGluRs antagonists sustained over 2.5 h.

**Effects of mGluRs antagonists injection on capsaicin-induced Glu release**

At the doses employed, CPCCOEt (5 mM) (Cap + CPCCOEt) showed remarkable inhibition in capsaicin-evoked Glu release. The average concentration of the released Glu was 1.46 ± 0.1 μM/20 min after the co-injection of CPCCOEt with capsaicin. Combined s.c. injection of MCCG (5 mM) (Cap + MCCG) or MSOP (5 mM) (Cap + MSOP) with capsaicin caused no significant decrease in Glu release compared to capsaicin injection alone. The average concentration of the released Glu was 3.68 ± 0.38 or 4.31 ± 0.60 μM/20 min in 2 fractions collected after the co-injection of MCCG.
or MSOP with capsaicin, respectively (Fig. 2B).

Effects of capsaizepine on capsaicin-induced thermal hypersensitivity

The mean withdrawal latencies to stimulation with radiant heat at pre-injection were 11.2 ± 0.3 and 11.2 ± 0.3 s (n = 40) on the left and right side, respectively (Fig. 3A). The withdrawal latency did not significantly change after injection of vehicle or low dose of capsaicin (0.6 mM). At 15 and 60 min after injection of capsaicin (3 and 6 mM), withdrawal latency to irradiation decreased to much shorter than that of vehicle injection, which was recorded at the same interval (P < 0.05); and then it recovered gradually to the level of vehicle injection by 4 h after injection of capsaicin. Pretreatment with capsaizepine (30 mg/kg, s.c.) produced a marked inhibition against capsaicin-induced thermal hyperalgesia (Fig. 3B).

Thermal sensitivity after injection of iGluRs agonists

Subcutaneous injections of Glu, NMDA, or AMPA produced dose-dependent decreases in withdrawal latency on the ipsilateral side 15 min after injection, which lasted for a few hours (Fig. 4). Subcutaneous injection of the vehicle produced no changes in thermal withdrawal latency.

Subcutaneous injection of (S)-DHPG caused a dose-dependent decrease in withdrawal latencies on the ipsilateral side from 15 min to 6 h, but L-CCG-I and L-AP4 caused no significant changes (Fig. 5).

Effect of iGluRs antagonists injection on capsaicin-induced thermal hypersensitivity

When MK801 or CNQX were injected together with capsaicin (Cap + MK801 or Cap + CNQX), a dose-dependent increase in withdrawal latency was observed. These analgesic effects of MK801 or CNQX on
capsaicin-induced thermal hyperalgesia lasted for more than 6 h (Fig. 6). The single injection of MK801 or CNQX into the hindpaw produced no changes in withdrawal latencies compared to vehicle injection.

**Effect of mGluRs antagonists injection on capsaicin-induced thermal hypersensitivity**

Following s.c. injection of CPCCOEt (5 mM), MPEP (30 mM), MCCG (5 mM), and MSOP (5 mM) into hindpaw, there was no changes in withdrawal latencies compared to vehicle injection (Figs. 7 and 8). When CPCCOEt or MPEP were injected together with capsaicin (Cap + CPCCOEt or Cap + MPEP), withdrawal latencies dose-dependently increased from 15 min to 2–3 h after the injection compared with injection of capsaicin alone \( P < 0.05 \) (Fig. 7). The heat insensitivity evoked in the ipsilateral side following Cap + CPCCOEt and Cap + MPEP injection continued for 5 h or more. Subcutaneous injection of MCCG or MSOP combined with capsaicin caused no significant changes in withdrawal latencies compared to capsaicin injection alone (Fig. 8).

**Discussion**

Glutamate (Glu) is the principal excitatory neurotransmitter and plays a key functional role to the central nervous system. With regard to existence of Glu in the small-diameter afferent fibers, it has been clarified that their central (17, 18) and peripheral (18, 19) processes as
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well as dorsal root ganglion (DRG) cells (18, 20) contain Glu, and Glu is also an important transmitter in peripheral nociception (1).

We have already reported that Glu was released from peripheral nerve endings by electrical stimulation of the sciatic nerve, heat stimulation (50°C), or local application of capsaicin cream to the hind instep (2). In the present study, the s.c. injection of capsaicin in the vicinity of the perfusion region caused a marked increase in Glu release (Fig. 1). This increase of Glu release was significantly inhibited by capsazepine, a TRPV1 competitive antagonist (Fig. 1). In the behavioral study shown in Fig. 3, the pretreatment with capsazepine (s.c.) also inhibited capsaicin-induced thermal hypersensitivity.

TRPV1 is located in a neurochemically heterogeneous population of small diameter primary afferent neurons (13). Repeated exposure to high-dose capsaicin, furthermore, selectively produces a prolonged influx of cations leading to desensitization of small-diameter sensory neurons to subsequent noxious stimulation (14, 21 – 23), while myelinated Aβ fibers are insensitive to capsaicin (24 – 26).

Considering these findings, the present results suggest...
that the activation of capsaicin-sensitive afferent fibers by capsaicin causes release of Glu from the peripheral endings via activation of peripheral TRPV1, particularly from those of small-diameter fibers possibly through a mechanism such as the axon-reflex pathway or an autocrine and/or paracrine mechanism. As it has now been shown clearly that TRPV1 exists in the skin (27), it is undeniable that capsaicin should be able to cause a release of Glu from skin. However, there is no such finding so far. Therefore, it is reasonable to speculate that the axon-reflex mechanism may be involved in the capsaicin-induced Glu release observed in Figs. 1 and 2, as only nociceptive afferent fibers, localized on the superficial tissues exposed to noxious influences, have the axon-reflex mechanism (28).

The amount of capsaicin-evoked Glu release was remarkably decreased by concomitant injection of the iGluRs antagonists MK801 and NBQX or the group I mGluR antagonist CPCCOEt in the hindpaw, but not by injection of the group II mGluR antagonist MCCG or the group III mGluR antagonist MSOP (Fig. 2). These results suggest that iGluRs and group I mGluR among all peripheral GluR subtypes mainly play a role in mediating capsaicin-evoked increase in Glu release, and then the released Glu could further activate iGluRs and group I mGluR on the same neuronal terminal or adjacent neighboring peripheral terminals. In this connection, there were evidences supporting the colocalization of peripheral NMDA and TRPV1 receptors on the same primary afferent terminal (29, 30).

Activation of peripheral GluRs could lead to enhancement of the Glu release in the peripheral tissues and might alter TRPV1-receptor responsiveness to reinforce nociceptive responses. This idea is strongly supported by the results that the intraplantar injection of iGluR or group I mGluR agonists evoked dose-dependent thermal hyperalgesia. Moreover, it is very interesting to note that injection of GluRs antagonists alone did not produce any changes on withdrawal latency, and intraplantar co-injection of iGluR or group I mGluR antagonists with capsaicin not only antagonized capsaicin-induced hyperalgesia, but also resulted in remarkably longer withdrawal latency to heat irradiation. Therefore, these findings suggest that peripheral GluRs may play an important role in mediating the enhanced nociceptive responses elicited by the activation of TRPV1.

Concerning the interaction between GluRs and TRPV1 in development and maintenance of hyperalgesia, besides our results, there are other supporting data. For example, group I mGluRs increase thermal sensitivity by enhancing vanilloid receptor function (11). Furthermore, peripheral NMDA receptor modulates jaw muscle electromyographic activity induced by capsaicin injection into the temporomandibular joint of rats (12). These findings and our present results indicate that the activation of peripheral GluRs, especially iGluRs and group I mGluR, could be indispensable in the mechanisms whereby capsaicin evokes enhanced nociceptive responses.

With regard to the mechanisms of Glu release evoked by TRPV1 activation, activation of TRPV1 triggers a huge amount of Ca\(^{2+}\) influx, which induces release of neurotransmitters and neuropeptides at the central and the peripheral terminals of primary afferents (31–36). Recently it has been shown that the elevation of [Ca\(^{2+}\)]\(_{\text{cyt}}\), in presynaptic mitochondria plays important role in TRPV1-mediated Glu release (37). Although these findings suggest the interaction of TRPV1 with GluR through intracellular change of Ca\(^{2+}\) in the peripheral terminals of sensory neurons, so far the mechanisms through which co-injection of iGluR or group I mGluR antagonists with capsaicin not only antagonized capsaicin-induced hyperalgesia, but also produced remarkably longer withdrawal latency to heat irradiation are still unknown.

Furthermore, several groups demonstrated that activation of peripheral group II and III mGluRs are responsible for analgesia (38). As shown in Fig. 5, however, a significant analgesic effect was not observed with the agonists of these receptor subtypes. It is necessary, therefore, to investigate the detailed mechanisms of modulation of TRPV1 function by GluRs including in the peripheral role of group II and III mGluRs in the development and maintenance of nociception.

Our present study strongly support the view that GluRs, in particular, iGluRs and group I mGluR existing in peripheral endings of capsaicin-sensitive afferent fibers, play an important role in the development and maintenance of hyperalgesia following excitation of TRPV1. In addition, the formulation of the peripheral iGluRs and group I mGluR antagonists that do not cross the blood–brain barrier may be of potential benefit by reducing peripheral nociceptive excitability, and therefore they could provide a new therapeutic target for pain control in the periphery.

**Acknowledgments**

The authors thank Mr. Toshinori Ito of Ohu University School of Pharmaceutical Science for reviewing our manuscript.

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