Neuroscience 164 (2009) 1833–1844

EFFECT OF RESINIFERATOXIN ON GLUTAMATERGIC SPONTANEOUS EXCITATORY SYNAPTIC TRANSMISSION IN SUBSTANTIA GELATINOSA NEURONS OF THE ADULT RAT SPINAL CORD

C.-Y. JIANG, T. FUJITA, H.-Y. YUE, L.-H. PIAO, T. LIU, T. NAKATSUKA AND E. KUMAMOTO*

Department of Physiology, Saga Medical School, 5-1-1 Nabeshima, Saga 849–8501, Japan

Abstract—The transient receptor potential (TRP) vanilloid type 1 (TRPV1) agonist, capsaicin, enhances glutamatergic spontaneous excitatory synaptic transmission in CNS neurons. Resiniferatoxin (RTX) has a much higher affinity for TRPV1 than capsaicin, but its ability to modulate excitatory transmission is unclear. We examined the effect of RTX on excitatory transmission using the whole-cell patch-clamp technique in substantia gelatinosa (SG) neurons of adult rat spinal cord slices. Bath-applied RTX dose-dependently increased the frequency, but not the amplitude, of spontaneous excitatory postsynaptic current (sEPSC), independent of its application time. In about a half of the neurons tested, this effect was accompanied by an inward current at −70 mV that was sensitive to glutamate-receptor antagonists. Repeated application of RTX did not affect excitatory transmission. RTX was more potent than capsaicin but showed similar efficacy. RTX activity could be blocked by capsazepine or SB-366791, a TRPV1 antagonist, but not tetrodotoxin, a Na^+-channel blocker, and could be inhibited by pretreatment with capsaicin but not the TRPA1 agonist, allyl isothiocyanate. RTX enhances the spontaneous release of L-glutamate from nerve terminals with similar efficacy as capsaicin and produces a membrane depolarization by activating TRPV1 in the SG, with fast desensitization and slow recovery from desensitization. These results indicate a mechanism by which RTX can modulate excitatory transmission in SG neurons to regulate nociceptive transmission.

Key words: spinal dorsal horn, TRPV1, whole-cell patch-clamp, pain.

Transient receptor potential vanilloid type 1 (TRPV1) is a molecular integrator of painful stimuli such as vanilloids, noxious heat and protons on the peripheral terminals of primary-afferent neurons (Caterina et al., 1997; Caterina and Julius, 2001). TRPV1 is also expressed in several brain nuclei such as the hypothalamus, substantia nigra and periaqueductal gray (PAG) (Sasamura et al., 1998; Mezey et al., 2000; Szallasi and Di Marzo, 2000; McGaraughty et al., 2003; Cristino et al., 2006; Starowicz et al., 2007a) and plays a role in various physiological functions including motor control, cognition and antinociception (Marinelli et al., 2003; McGaraughty et al., 2003; Starowicz et al., 2008). At central synapses, TRPV1 activation by capsaicin, the pungent ingredient of hot chili peppers, increases the spontaneous release of L-glutamate from nerve terminals (Sasamura et al., 1998; Marinelli et al., 2003; McGaraughty et al., 2003; Xing and Li, 2007; Starowicz et al., 2007a).

TRPV1 activation in the central terminals of primary-afferent neurons increases the spontaneous release of L-glutamate to substantia gelatinosa (SG) neurons of the spinal dorsal horn (Yang et al., 1998; Guo et al., 1999; Valtschanoff et al., 2001; Morisset and Urban, 2001; Hwang et al., 2004), which play a pivotal role in regulating nociceptive transmission (Willis and Coggeshall, 1991). TRPV1 activation also potentiates L-glutamate release in rat superficial medullary dorsal horn neurons (Jennings et al., 2003). Endogenous agonists for TRPV1 include endocannabinoids and lipoxygenase metabolites, which have similar structures to capsaicin, which is not produced endogenously (Zygmunt et al., 1999; Hwang et al., 2000; for review see Caterina and Julius, 2001; Starowicz et al., 2007b). Tonic TRPV1 activation by endogenous agonists in the PAG may activate descending antinociceptive pathways in rats (Starowicz et al., 2007a).

Resiniferatoxin (RTX) is an ultrapotent TRPV1 agonist, a capsaicin analog isolated from the dried latex of the cactus-like plant, *Euphorbia resinifera* (Hergenhahn et al., 1975; Schmidt and Evans, 1979). RTX binds to the same recognition site as capsaicin in the cytosolic tails of TRPV1 (Jung et al., 2002). RTX has higher binding affinity and is three to four orders of magnitude more potent than capsaicin at producing current responses in oocytes expressing TRPV1, in inhibiting twitch contraction of the vas deferens and in thermoregulation and neurogenic inflammation (Szallasi and Blumberg, 1989; Maggi et al., 1990; Szallasi et al., 1993, 1995; Szallasi, 1994). [^{3}H]RTX is widely used to examine the distribution of TRPV1 in the nervous system (Szallasi and Blumberg, 1990; Szallasi, 1994; Acs et al., 1996). RTX may also be useful in treating disorders such as neuropathic pain and lower urinary tract dysfunction that involve excessive TRPV1 activity (Szallasi, 2002). In dorsal root ganglion (DRG) neurons or heterologous cells expressing TRPV1, RTX produces a whole-cell current response and single-channel current activity that persist after its washout (Raisinghani et al., 2005). It has not been fully examined yet how RTX affects excitatory trans-

*Corresponding author. Tel: +81-952-34-2273; fax: +81-952-34-2013.
E-mail address: kumamote@cc.saga-u.ac.jp (E. Kumamoto).

Abbreviations: AITC, allyl isothiocyanate; APV, DL-2-amino-5-phosphonovaleric acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DRG, dorsal root ganglion; EC_{50}, effective concentration producing half-maximal response; NMDA, *N*-methyl-D-aspartate; RTX, resiniferatoxin; sEPSC, spontaneous excitatory postsynaptic current; SG, substantia gelatinosa; TRPA1, transient receptor potential A1; TRPV1, transient receptor potential vanilloid type 1; TTX, tetrodotoxin; V_H, holding potential.

0306-4522/09 $ - see front matter
doi:10.1016/j.neuroscience.2009.09.033

mission. Here, we examined the effects of RTX on glutamatergic spontaneous excitatory synaptic transmission in SG neurons of adult rat spinal cord slices using the whole-cell patch-clamp technique.

EXPERIMENTAL PROCEDURES

All animal experiments were approved by the Animal Care and Use Committee of Saga University. All efforts were made to minimize animal suffering and the number of animal used.

Spinal cord slice preparation

Spinal cord slices from adult rats were prepared as described previously (Yue et al., 2005; Fujita and Kumamoto, 2006; Liu et al., 2008). Male Sprague–Dawley rats (6–8 weeks old) were anesthetized with urethane (1.2 g/kg i.p.), and a lumbosacral segment (L_1–S_3) of the spinal cord was extracted and placed in preoxygenated cold Krebs solution (2–4 °C) preequilibrated with 95% O_2 and 5% CO_2. The composition of Krebs solution used was (in mM): NaCl, 117; KCl, 3.6; $CaCl_2$, 2.5; $MgCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 25; and glucose, 11 (pH=7.4 when saturated with the gas). After cutting all ventral and dorsal roots, the pia-arachnoid membrane was removed. The spinal cord was mounted on a microslicer (DTK-1000, Dousaka, Kyoto, Japan) and several 650 μm-thick transverse slices were cut. One of the slices was transferred to a recording chamber (0.5 ml in volume), then completely submerged and superfused at 12–15 ml/min with Krebs solution saturated with 95% O_2 and 5% CO_2 and maintained at 36.0±0.5 °C. The remaining slices (at most five) were stored under similar conditions until use.

Patch-clamp recordings from SG neurons

The SG can be identified under a stereomicroscope as a translucent band across the dorsal horn. Whole-cell voltage-clamp recordings from SG neurons were made at a holding potential (V_H) of −70 mV using a patch-pipette. The recorded neurons were located at the center of SG to avoid recordings from laminae I and III neurons, as reported previously (Yue et al., 2005; Fujita and Kumamoto, 2006). The patch-pipette solution used was composed of (in mM): K-gluconate, 135; $CaCl_2$, 0.5; $MgCl_2$, 2; KCl, 5; ethyleneglycol-bis(aminoethylether) tetraacetate, 5; *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonate, 5; and Mg–ATP, 5 (pH=7.2). Signals were acquired using an Axopatch 200 B amplifier (Molecular Devices, Sunnyvale, CA, USA). Data were low-pass filtered at 5 kHz, and digitized at 333 kHz with an A/D converter. The data were stored and analyzed with a personal computer using pCLAMP 8.1 software (Molecular Devices) and Mini Analysis Program (Synaptosoft, Decatur, GA, USA); detection criteria for spontaneous excitatory postsynaptic currents (sEPSCs) included a 4.5 pA event threshold, with a fast rise time and a decay curve that approximated exponential decay.

Drug application

Drugs were applied by superfusing a solution containing drugs without altering the perfusion rate or temperature. The drugs used were RTX, SB-366791, DL-2-amino-5-phosphonovaleric acid (APV; Sigma Aldrich, St. Louis, MO, USA), allyl isothiocyanate (AITC), capsaicin, capsazepine, tetrodotoxin (TTX; Wako, Osaka, Japan) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Tocris Cookson, Bristol, UK). These drugs (except for capsaicin, TTX and APV where distilled water was used as a solvent) were first dissolved in dimethyl sulfoxide at 1000x stocks and then stored at −20 °C. These drugs were then diluted to the final concentration in Krebs solution immediately before use.

Statistical analysis

Numerical data are given as the mean±standard error of the mean (SEM). Statistical significance is determined as $P<0.05$ using a Student's *t*-test unless otherwise mentioned. In all cases, *n* refers to the number of neurons studied.

RESULTS

Whole-cell patch-clamp recordings were made from a total of 128 SG neurons. Stable recordings could be obtained from slices maintained *in vitro* for more than 10 h, and recordings were made from single SG neurons for up to 3 h. All SG neurons tested had resting membrane potentials lower than −60 mV (when measured in current-clamp mode), and exhibited sEPSCs at a V_H of −70 mV, near the reversal potential for inhibitory postsynaptic currents (Iyadomi et al., 2000; Liu et al., 2008). The sEPSCs were completely blocked by a non-*N*-methyl-D-aspartate (NMDA) receptor antagonist, CNQX (10 μM), and were not affected by a voltage-gated Na^+-channel blocker, TTX (0.5 μM) (Iyadomi et al., 2000; Yue et al., 2005). Thus, sEPSCs were not contaminated by spontaneous inhibitory postsynaptic currents and occurred without spike propagation from the soma of presynaptic TTX-sensitive neurons.

Effects of RTX on glutamatergic spontaneous excitatory transmission in SG neurons

RTX (0.5 μM) superfused for 1 min enhanced spontaneous excitatory transmission in a SG neuron (Fig. 1A). The sEPSC frequency increased gradually over time, peaking around 4 min after RTX addition; this facilitation was accompanied by a small increase in sEPSC amplitude (Fig. 1B). This increase in sEPSC frequency did not subside for at least 10 min after RTX washout. RTX significantly increased the proportion of sEPSCs with a shorter inter-event interval and a larger amplitude (Fig. 1C); this effect was confirmed in three other neurons and accompanied by a small inward current.

Capsaicin-induced enhancement of spontaneous excitatory transmission in SG neurons shows slow recovery from desensitization (Yang et al., 1998). Similarly, a second RTX application 1 or 2 h later did not affect excitatory transmission (Fig. 2). The peak amplitude of inward current produced by the second application was smaller than that of the first application (Fig. 2A, B). For example, the initial RTX treatment produced an inward current with a peak amplitude of 6.4 or 15.5 pA, but a second application 1 h later did not change the holding currents. Overall peak current amplitudes were 14.1±3.6 pA initially and 2.3±1.2 pA ($n=3$) 2 h later, although some neurons did not produce any inward current after RTX. We therefore only added RTX once to a spinal cord slice in subsequent experiments.

At around 4 min after RTX addition, when the sEPSC frequency increase was maximal (see Fig. 1B), RTX increased sEPSC frequency to 236±20% ($n=23$; $P<0.05$) of control (11.9±1.6 Hz) in most (88%) of neurons examined ($n=26$). sEPSC frequency did not change in three