European Journal of Pharmacology 604 (2009) 20–26

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and Cellular Pharmacology

Effects of (−) epigallocatechin-3-gallate on Na^+ currents in rat dorsal root ganglion neurons

Tae Hoon Kim [a], Jae-Min Lim [a], Sung Su Kim [b], Jungho Kim [c], Mijung Park [d], Jin-Ho Song [a,*]

[a] Department of Pharmacology, College of Medicine, Chung-Ang University, Seoul 156-756, Republic of Korea
[b] Department of Anatomy, College of Medicine, Chung-Ang University, Seoul 156-756, Republic of Korea
[c] Laboratory of Molecular and Cellular Biology, Department of Life Science, Sogang University, Seoul 121-742, Republic of Korea
[d] Department of Visual Optics, Seoul National University of Technology, Seoul 139-743, Republic of Korea

ARTICLE INFO

Article history:
Received 9 October 2008
Received in revised form 20 November 2008
Accepted 9 December 2008
Available online 16 December 2008

Keywords:
Catechins
Dorsal root ganglion
(−) Epigallocatechin-3-gallate
Green tea
Na^+ current
Tetrodotoxin-resistant
Tetrodotoxin-sensitive

ABSTRACT

The natural product (−) epigallocatechin-3-gallate (EGCG) is the major polyphenolic constituent found in green tea. Dorsal root ganglion neurons are primary sensory neurons, and express tetrodotoxin-sensitive and tetrodotoxin-resistant Na^+ currents, which are both actively involved in the generation and propagation of nociceptive signals. Effects of EGCG on tetrodotoxin-sensitive and tetrodotoxin-resistant Na^+ currents in rat dorsal root ganglion neurons were investigated using the whole-cell variation of the patch-clamp techniques. EGCG inhibited both types of Na^+ currents potently and in a concentration-dependent manner. The apparent dissociation constant, K_d, was estimated to be 0.74 and 0.80 μM for tetrodotoxin-sensitive and tetrodotoxin-resistant Na^+ currents, respectively. (−) Epigallocatechin (EGC) was far less potent to inhibit Na^+ currents than EGCG, suggesting that gallate moiety of EGCG is an important functional group to modulate Na^+ currents. EGCG had little or no effect on the activation or steady-state inactivation voltage of either type of Na^+ current. EGCG simply reduced the availability of Na^+ channels for activation. Thus, EGCG appears to bind to resting Na^+ channels to inhibit them. EGCG slowed the recovery of tetrodotoxin-sensitive Na^+ current from inactivation. The property of EGCG to inhibit sensory Na^+ currents can be utilized to develop an analgesic agent.

1. Introduction

Flavonoids are polyphenolic compounds found in most plants. They have a variety of biological activities such as antibacteria, antivirus, antiallergy, anticancer, antithrombosis, and so forth. Strong antioxidant property is considered as the basis for many of their beneficial actions (Miller, 1996). Catechins are flavonoids present in green tea (*Camellia sinensis*) leaves in large quantity (Zaveri, 2006). Tea catechins include (−) epigallocatechin-3-gallate (EGCG), (−) epigallocatechin (EGC), (+) gallocatechin, (−) epicatechin-3-gallate, (−) epicatechin and (+) catechin, of which EGCG is the major and the most active constituent, accounting for more than 10% of the extract dry weight and 65% of the total catechin content (Mandel et al., 2004).

The potential of EGCG for use in human cancer prevention and treatment has been extensively studied in vitro and in vivo. Anticarcinogenic properties of EGCG include prevention of oxidative damages and angiogenesis, protection from UV radiation, smoking and heterocyclic amines, inhibition of the growth of carcinogenic bacteria, and promotion of apoptosis (Carlson et al., 2007). After oral administration, EGCG is widely distributed in various organs including brain (Suganuma et al., 1998), and exerts potent neuroprotection in neurodegenerative diseases (Mandel et al., 2004). In contrast to its proapoptotic effects on cancer cells, EGCG has antiapoptotic effects on neuronal cells usually at doses lower than those for anticancer activities (Zaveri, 2006). EGCG also chelates iron that enhances and promotes the generation of toxic reactive oxygen species, which play a pivotal role in the cognitive decline and neuronal loss in neurodegenerative diseases including Alzheimer's, Parkinson's, and Huntington's diseases (Mandel et al., 2006). Given that ion channels and their electrical activities serve neuronal function and their dysregulation is central to the pathophysiology of the neuronal diseases, EGCG may modulate them to have the beneficial effects. Indeed it has been shown that EGCG modulates ion channels including K^+ channels, Ca^{2+} channels, non-selective cation channels, ionotropic γ-aminobutyric acid ($GABA_A$) receptor and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor (Bae et al., 2002; Baek et al., 2005; Campos-Toimil and Orallo, 2007; Choi et al., 2001; Hossain et al., 2002).

Voltage-gated Na^+ channels are transmembrane proteins that are essential for the propagation of action potentials and play a critical role in electrical signaling between neurons. At least 9 homologous channel-forming α-subunits ($Na_V1.1$–$Na_V1.9$) have been discovered so far in mammals (Goldin, 2001). Dorsal root ganglion neurons transduce peripheral stimuli and transmit the sensory information

* Corresponding author. Department of Pharmacology, College of Medicine, Chung-Ang University, 221 Heuksuk-Dong, Dongjak-Gu, Seoul 156-756, Republic of Korea. Tel.: +82 2 820 5686; fax: +82 2 817 7115.
E-mail address: jinhos@cau.ac.kr (J.-H. Song).

0014-2999/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2008.12.015

to the central nervous system. They express tetrodotoxin-sensitive and tetrodotoxin-resistant Na^+ currents (Roy and Narahashi, 1992). Tetrodotoxin-sensitive Na^+ current is the composite current through $Na_V1.1$, $Na_V1.6$ and $Na_V1.7$, and tetrodotoxin-resistant Na^+ current through $Na_V1.8$ and $Na_V1.9$ (Black et al., 2004; Goldin, 2001). $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$ are expressed preferentially in small nociceptive neurons and play important roles in the pathogenesis of acute, inflammatory and neuropathic pains (Akopian et al., 1996; Cummins et al., 2007; Dib-Hajj et al., 1998; Djouhri et al., 2003). In the present study we found that EGCG potently inhibited tetrodotoxin-sensitive and tetrodotoxin-resistant Na^+ currents in rat dorsal root ganglion neurons, suggesting that EGCG has a potential for analgesic agent.

2. Materials and methods

2.1. Cell preparation

All experiments were carried out in accordance with the guidelines and the approval of the Institutional Animal Ethics Committee. Dorsal root ganglion neurons were isolated from Sprague–Dawley rats of 2–6 days old. Rats were decapitated and the spinal column was removed. Dorsal root ganglia in all spinal levels were harvested and axonal and connective tissue was cut away in ice-cold Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS, Sigma, St. Louis, MO). The isolated dorsal root ganglia were digested first with 0.125% collagenase (Type II-S, Sigma) for 15 min and then with 0.25% trypsin (Type XI, Sigma) for 10 min, both in PBS at 36 °C. After that, they were rinsed with a culture media composed of 10% fetal bovine serum in Dulbecco's Modified Eagle Medium (both from Gibco, Grand Island, NY). Individual cells were then mechanically dissociated by triturating dorsal root ganglia through fire-polished Pasteur pipettes. The dispersed cells were plated onto glass coverslips (12-mm diameter, Superior, Germany), previously coated with 0.1 mg/ml poly-L-lysine (Sigma) in 35-mm diameter tissue culture dishes. The dishes filled with the culture media were maintained in a humidified incubator at 36 °C under an atmosphere of 5% CO_2. All recordings were made within 2–7 h of dorsal root ganglia isolation.

2.2. Electrophysiological recording

Electrophysiological experiments were conducted at room temperature (22–24 °C). An individual glass coverslip was removed from the culture dish and placed into a perfusion chamber mounted on the stage of an inverted phase-contrast microscope. The chamber was continuously perfused with an external solution by gravity at a rate of 1 ml/min. The chamber volume was maintained at about 0.5 ml. Na^+ currents were recorded in the whole-cell voltage-clamp configuration of the patch-clamp technique. Voltage was controlled using an Axopatch 200B amplifier (Axon Instruments, Union City, CA) driven from a PC, from which pulse protocols were generated and into which data were stored for off-line analysis using Clampex 8 software (Axon Instruments) and a digital interface (Digidata 1322A, Axon Instruments). Recordings were made in solutions to select out Na^+ currents and Na^+ gradient was reduced in order to attain good voltage-clamp of the currents. The external solution contained (mM): NaCl 30, choline chloride 120, tetraethylammonium chloride 20, D-glucose 5, HEPES 5, $MgCl_2$ 1, $CaCl_2$ 1, and $LaCl_3$ 0.01 (pH titrated to 7.4 with tetraethylammonium hydroxide). The internal solution contained (mM): NaCl 10, CsCl 65, CsF 70 and HEPES 10 (pH titrated to 7.2 with CsOH). Patch pipettes were made from borosilicate glass capillaries (G150TF-4, Warner Instrument, Hamden, CT) with a two-step vertical puller (PP83, Narishige, Tokyo, Japan) and heat-polished with a microforge (MF83, Narishige). Pipette resistance was between 0.9 and 1.0 MΩ when filled with the internal solution. An Ag–AgCl pellet/3 M KCl–agar bridge was used for the reference electrode. Capacity transients were cancelled as much as possible. Voltage errors were minimized with a series resistance compensation of 70–90% with the amplifier circuitry. Linear leakage currents were digitally subtracted on-line with P/4 routines. The liquid junction potential of −4 mV measured between internal and external solutions was corrected. Recordings were always started at least 10 min after obtaining the whole-cell configuration to allow Na^+ current to stabilize. All salts were obtained from Sigma.

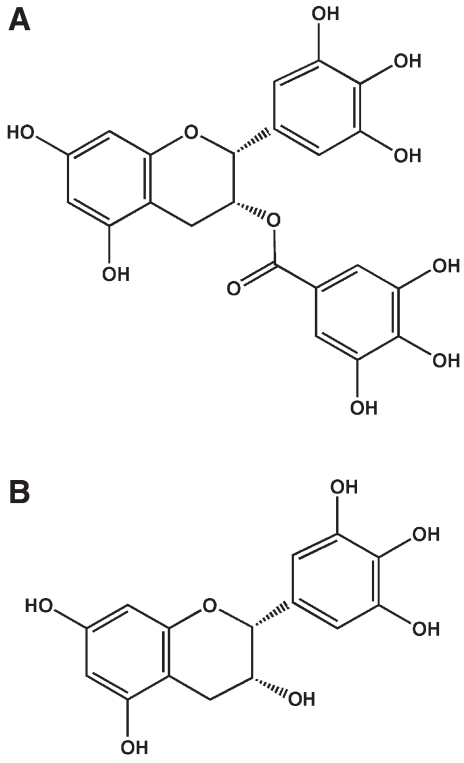

Fig. 1. Chemical structures of EGCG (A) and EGC (B).

A fast Na^+ current that inactivated within 2–3 ms at 0 mV depolarization in large-diameter neurons (>30 μm) was selected as tetrodotoxin-sensitive Na^+ current, and its tetrodotoxin sensitivity was confirmed with 100 nM tetrodotoxin (Sigma) at the end of each experiment. Tetrodotoxin-resistant Na^+ current was recorded from small-diameter neurons (<20 μm) and isolated by eliminating tetrodotoxin-sensitive Na^+ current by including 100 nM tetrodotoxin in the external solution in all experiments (Roy and Narahashi, 1992).

EGCG and EGC (Sigma) were dissolved in dimethylsulfoxide to form 1000-fold stock solutions. Stock solutions were stored frozen at −20 °C in small aliquots and diluted in the external solution to the desired concentrations immediately before use.

2.3. Data acquisition and analysis

Currents were low-pass filtered at 5 kHz and digitally sampled at 50 kHz. Data analysis was performed with a combination of Clampfit 8 (Axon Instruments) and SigmaPlot 9 (Jandel Scientific, San Rafael, CA) softwares. Data are presented as means±S.E.M. and n refers to the number of cells examined. Student's t-test was used for comparisons and statistical significance was assessed at $P<0.05$.

3. Results

3.1. EGCG inhibits Na^+ currents in dorsal root ganglion neurons

Effects of EGCG (Fig. 1) on Na^+ currents in rat dorsal root ganglion neurons were investigated. Tetrodotoxin-sensitive and tetrodotoxin-resistant Na^+ currents were separately recorded using the method

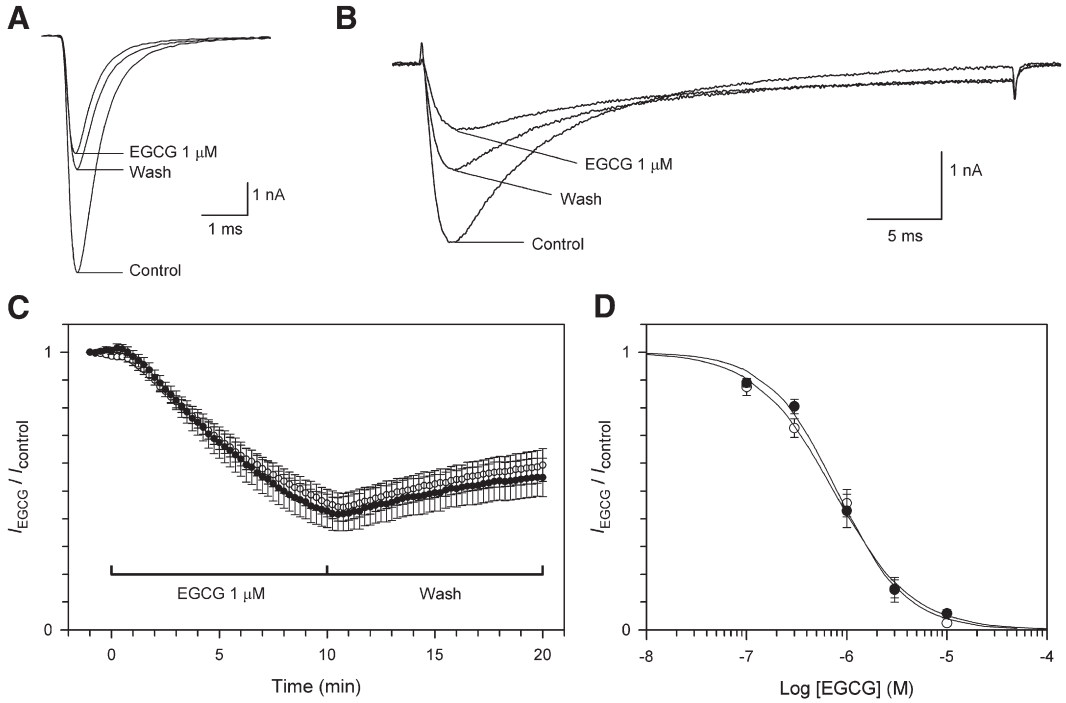

Fig. 2. EGCG inhibition of Na^+ currents in rat dorsal root ganglion neurons. (A, B) Typical tetrodotoxin-sensitive (A) and tetrodotoxin-resistant (B) Na^+ current traces before and after EGCG (1 μM) treatment for 10 min, and after washout for 10 min. Currents were elicited by depolarizing steps of 10-ms (A) or 40-ms (B) duration to 0 mV from a holding potential of −80 mV at 15-s intervals. (C) Time course of Na^+ current inhibition by EGCG (○, tetrodotoxin-sensitive, $n=8$; ●, tetrodotoxin-resistant, $n=8$). (D) Concentration–response relationship for EGCG inhibition of Na^+ currents. Peak current amplitude at 10 min application of EGCG (I_{EGCG}) was normalized to control current ($I_{control}$), and plotted against the concentration of EGCG. Every measurement was carried out in a different cell. The means of the data were fitted with the Hill equation to yield the smooth lines. ○, tetrodotoxin-sensitive; ● tetrodotoxin-resistant. $n=7$–8.

described in Materials and methods. After establishing a whole-cell configuration, neurons were voltage-clamped at −80 mV. Na^+ currents were evoked by depolarizing step pulses to 0 mV (tetrodotoxin-sensitive, 10 ms; tetrodotoxin-resistant, 40 ms) at 15-s intervals. Activation and inactivation kinetics of tetrodotoxin-sensitive Na^+ current were much faster than those of tetrodotoxin-resistant Na^+ current (Fig. 2A and B). After currents were stabilized EGCG (1 μM) was applied for 10 min and washed out for 10 min with EGCG-free external solution. EGCG gradually inhibited both Na^+ currents almost equally and the effects were partially reversible (Fig. 2C). Examples of tetrodotoxin-sensitive and tetrodotoxin-resistant Na^+ current traces before and after treatment with EGCG, and after washout are shown in Fig. 2A and B, respectively. EGCG (1 μM) for 10 min inhibited tetrodotoxin-sensitive Na^+ current to 46±5% of control ($n=8$), and tetrodotoxin-resistant Na^+ current to 43±6% of control ($n=8$). Upon washout for 10 min tetrodotoxin-sensitive and tetrodotoxin-resistant Na^+ currents were recovered to 59±6% and 55±7% of control, respectively.

The concentration–response relationship for EGCG inhibition of Na^+ currents was measured (Fig. 2D). Depolarizing step pulses to 0 mV from a holding potential of −80 mV were employed to generate Na^+ currents. Peak currents were measured in the absence and presence of EGCG for 10 min. Data were collected from 7–8 different cells for each concentration of EGCG. The mean values of the peak current fraction were fitted with the following Hill equation:

$$I_{EGCG}/I_{control} = 1/\left(1 + ([EGCG]/K_d)^h\right),$$

where I_{EGCG} is the peak current in the presence of EGCG, $I_{control}$ is the control peak current, [EGCG] is the concentration of EGCG, K_d is the apparent dissociation constant, and h is the Hill coefficient. Best fits were achieved when K_d and h were 0.74 μM and 1.12 for tetrodotoxin-sensitive Na^+ current, respectively, and 0.80 μM and 1.26 for tetrodotoxin-resistant Na^+ current, respectively.

EGC was far less potent to inhibit Na^+ currents than EGCG. At 3 μM EGC inhibited tetrodotoxin-sensitive Na^+ current by 15±3% ($n=8$), and tetrodotoxin-resistant Na^+ current by 6±3% ($n=8$), while at the same concentration EGCG suppressed both currents by around 85%.

3.2. Effects of EGCG on the activation of Na^+ currents

Current–voltage (I–V) relationships were constructed using a series of depolarizing pulses to a range of potentials between −55 mV and +50 mV in steps of 5 mV with 5-s intervals from a holding potential of −90 mV (tetrodotoxin-sensitive) or −80 mV (tetrodotoxin-resistant). Examples of the current families for tetrodotoxin-sensitive and tetrodotoxin-resistant Na^+ currents in the absence and presence of EGCG (1 μM) for 10 min are shown in Fig. 3A and B, respectively. The peak current at each potential was plotted to form I–V curves (Fig. 3C). The current activation threshold was approximately −50 mV for tetrodotoxin-sensitive Na^+ current and −30 mV for tetrodotoxin-resistant Na^+ current, and peak inward current occurred at approximately −15 mV and −10 mV, respectively. The reversal potential for both types of Na^+ currents was approximately +28 mV, which was close to the equilibrium potential calculated from intracellular and extracellular Na^+ concentrations. EGCG inhibited both types of Na^+ currents quite evenly at the range of potentials tested without noticeable change in the reversal potential, implying that EGCG inhibited Na^+ currents independently of the activation voltages.

To determine the voltage dependence of channel activation, the Na^+ conductance (G) was calculated. The peak current (I) for each test pulse potential (V_m) was divided by the driving force ($V_m - V_{rev}$), where V_{rev} is the membrane potential at which the peak current is reversed. Normalized Na^+ conductance was plotted against V_m and fitted with a Boltzmann function according to the equation:

$$G/G_{max} = 1/(1 + \exp((V_{g0.5} - V_m)/k_g)),$$

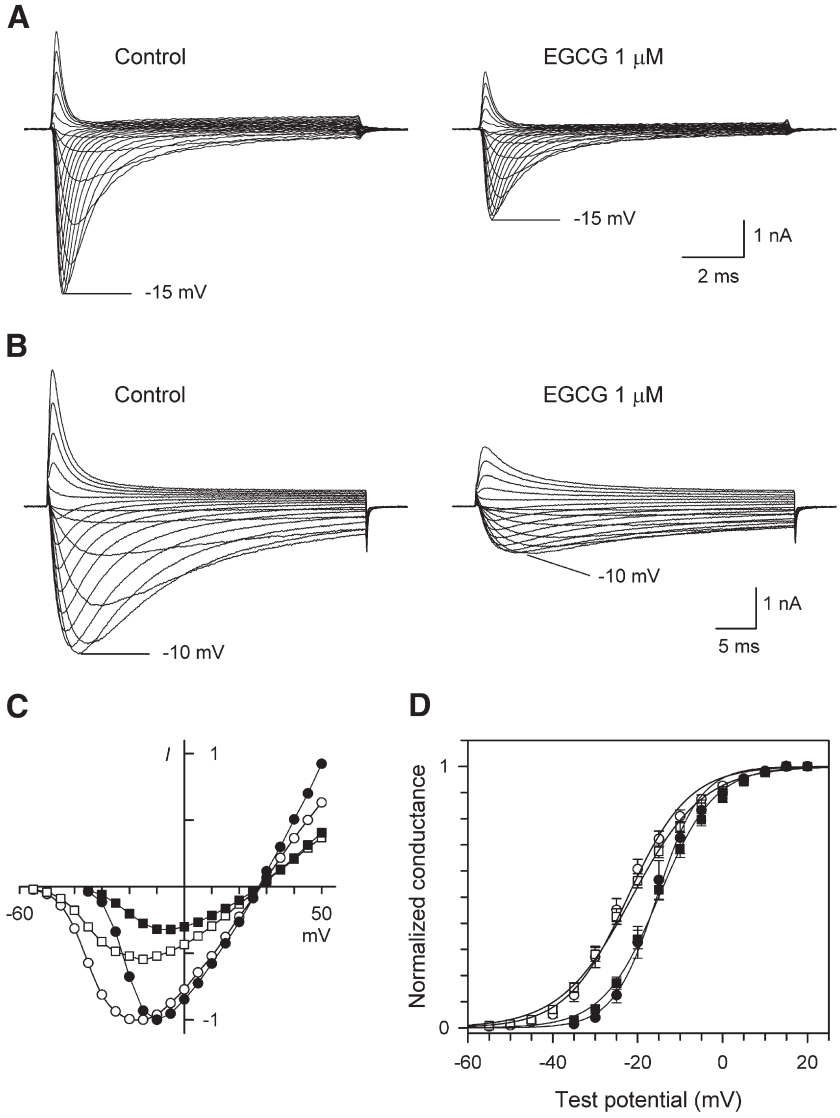

Fig. 3. Effects of EGCG on the activation of Na^+ currents. Membrane potential was held at −90 mV (A) or −80 mV (B), and currents were evoked with test pulses (A, 10 ms; B, 40 ms) to potentials ranging from −55 mV to +50 mV in 5-mV increments. (A, B) Examples of tetrodotoxin-sensitive (A) and tetrodotoxin-resistant (B) Na^+ current families before and after treatment with EGCG (1 μM) for 10 min. (C) Current–voltage (*I–V*) relationships for the currents shown in panels A and B. (D) Conductance–voltage (*G–V*) relationships derived from *I–V* data. Conductance was normalized with respect to the maximal conductance at +20 mV and plotted against the test potential (tetrodotoxin-sensitive, $n=10$; tetrodotoxin-resistant, $n=7$). Curves were drawn after fit with a Boltzmann function. ○, tetrodotoxin-sensitive, control; □, tetrodotoxin-sensitive, EGCG; ●, tetrodotoxin-resistant, control; ■, tetrodotoxin-resistant, EGCG.

where G_{max} is the maximal conductance at +20 mV, $V_{g0.5}$ is the membrane potential at which the half-maximal channel open probability occurs and k_g is the slope factor that describes the steepness of voltage dependent activation kinetics (Fig. 3D). The Boltzmann parameters are summarized in Table 1.

EGCG had a little influence on the activation voltages of both types of Na^+ currents. In tetrodotoxin-sensitive Na^+ current $V_{g0.5}$ and k_g were calculated to be −22.3±1.3 mV and 7.19±0.30 mV ($n=10$), respectively. EGCG (1 μM) treatment for 10 min produced a slight but significant depolarizing shift of $V_{g0.5}$ by 1.0±0.5 mV ($P<0.05$). In tetrodotoxin-resistant Na^+ current $V_{g0.5}$ and k_g were calculated to be −15.5±1.6 mV and 5.08±0.28 mV ($n=7$), respectively. The k_g was increased significantly by 1.46±0.27 mV ($P<0.01$) with 1 μM EGCG treatment for 10 min.

3.3. Effects of EGCG on the inactivation of Na^+ currents

EGCG slowed the inactivation of Na^+ currents. To assess current kinetics inactivation decay time constant was determined. Time constant was estimated by fitting the decay phase of the current trace to a single exponential function. EGCG increased the time constants of both Na^+ currents (Fig. 4).

Table 1
Boltzmann parameters for Na^+ current activation and steady-state inactivation

		Control (mV)	Shift by EGCG (mV)	Shift in solvent (mV)
Tetrodotoxin-sensitive current	$V_{g0.5}$	−22.3±1.3	+1.0±0.5[a] (10)	−1.0±0.4 (7)
	k_g	7.19±0.30	+1.21±0.28	+0.88±0.17
	$V_{h0.5}$	−79.1±1.7	−2.1±0.8 (8)	−3.0±0.4 (7)
	k_h	6.99±0.15	+0.85±0.16[a]	+0.23±0.16
Tetrodotoxin-resistant current	$V_{g0.5}$	−15.5±1.6	+0.4±1.0 (7)	−1.0±0.4 (7)
	k_g	5.08±0.28	+1.46±0.27[b]	+0.35±0.10
	$V_{h0.5}$	−53.6±2.2	+0.4±1.0 (7)	−2.1±0.7 (7)
	k_h	5.69±0.33	+2.13±0.38[b]	+0.46±0.23

Activation and steady-state inactivation curves were constructed as described in the text. $V_{g0.5}$ and $V_{h0.5}$ refer to the potential at which current is 50% activated or inactivated, respectively, and k_g and k_h are slope factors. Cells were treated with 1 μM EGCG for 10 min. [a]$P<0.05$, [b]$P<0.01$ compared with the shift in the solvent only (dimethylsulfoxide 0.1%, v/v). Parenthesis indicates the number of the cells examined.

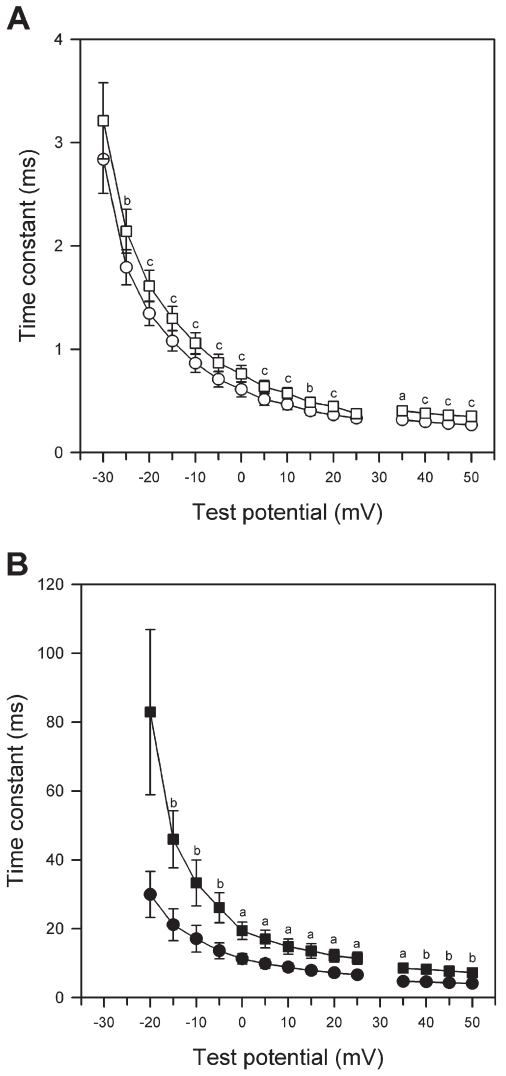

Fig. 4. Effects of EGCG on the inactivation of tetrodotoxin-sensitive (A) and tetrodotoxin-resistant (B) Na^+ currents. The decaying phase of the current traces obtained from *I–V* data was fitted with a single exponential function, and the time constant was plotted against the test potential. ○, tetrodotoxin-sensitive, control; □, tetrodotoxin-sensitive, EGCG; ●, tetrodotoxin-resistant, control; ■, tetrodotoxin-resistant, EGCG. $^aP<0.05$, $^bP<0.01$, and $^cP<0.001$ compared with the control. Tetrodotoxin-sensitive, $n=10$; tetrodotoxin-resistant, $n=7$.

The voltage dependence of steady-state inactivation was measured using a protocol consisted of a series of 10-s pre-pulses (from −120 mV to −40 mV in steps of 10 mV) from a holding potential of −100 mV, followed immediately by a test pulse to 0 mV. The protocol was run before and after treatment with 1 μM EGCG for 10 min. Typical families of tetrodotoxin-sensitive and tetrodotoxin-resistant Na^+ currents are shown in Fig. 5A and B, respectively.

For construction of the steady-state inactivation curve, normalized peak Na^+ current was plotted against the pre-pulse potential (Fig. 5C), and fitted with a Boltzmann function according to the equation:

$$I/I_{max} = A/(1 + \exp((V_h - V_{h0.5})/k_h)),$$

where I is the current amplitude, I_{max} is the maximal control current amplitude elicited at the most hyperpolarized pre-pulse, A is the maximum of I/I_{max}, V_h is the pre-pulse potential, $V_{h0.5}$ is the potential at which I/I_{max} reaches its half-maximum, and k_h is the slope factor. The Boltzmann parameters are summarized in Table 1.

The $V_{h0.5}$ for tetrodotoxin-sensitive Na^+ current was calculated to be −79.1±1.7 mV ($n=8$), which is about 26 mV lower than that for tetrodotoxin-resistant Na^+ current of −53.6±2.2 mV ($n=7$). EGCG at 1 μM for 10 min changed the $V_{h0.5}$ only by −2.1±0.8 mV and +0.4±1.1 mV, respectively, which were not different from the shift in the solvent only (dimethylsulfoxide 0.1%, v/v). The slope factor k_h, however, was changed by +0.85±0.16 mV ($P<0.05$) and +2.13±0.38 mV ($P<0.01$), respectively.

EGCG at 1 μM for 10 min reduced the maximal tetrodotoxin-sensitive Na^+ current at a holding potential of −120 mV by 49±4%, which was significantly different from the 1±3% decrease in the solvent only ($P<0.001$). It also reduced the maximal tetrodotoxin-resistant Na^+ current at a holding potential of −100 mV by 52±5%, which was significantly different from the 1±2% decrease in the solvent only ($P<0.001$).

3.4. Effects of EGCG on the recovery of Na^+ currents from inactivation

Recovery of Na^+ currents from inactivation was assessed using a two pulse protocol and varying an inter-pulse duration. Holding potential and inter-pulse potential were both −100 mV. The first pulse inactivates Na^+ currents and the current in response to the second

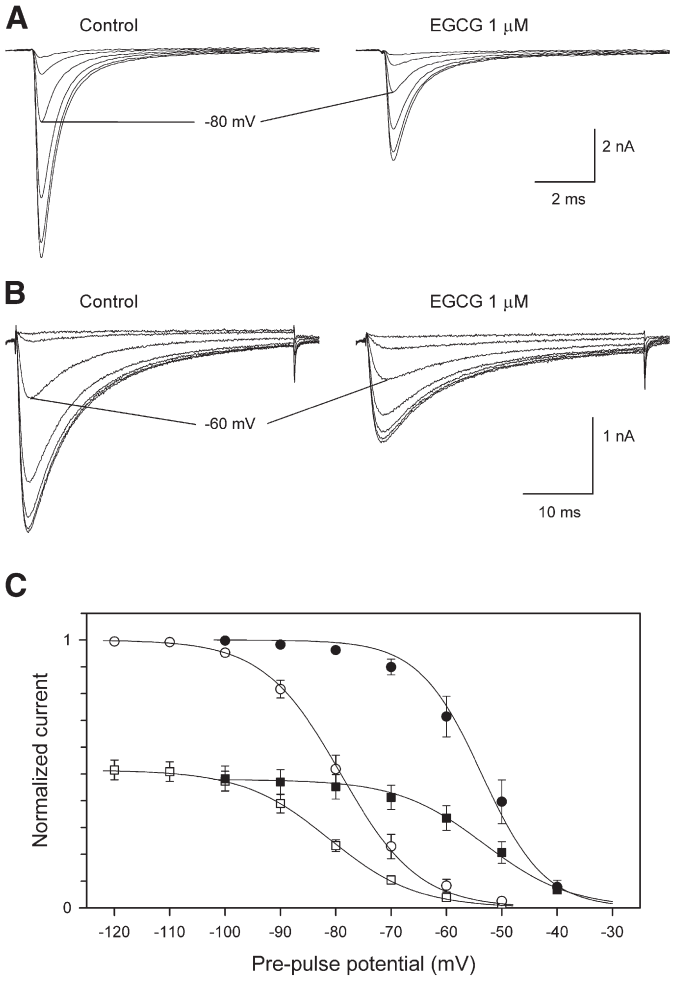

Fig. 5. Effects of EGCG on the steady-state inactivation of Na^+ currents. Membrane potential was held at −100 mV and an inactivating pre-pulse of 10 s was given between −120 mV and −40 mV in 10-mV increments, which was immediately followed by a depolarizing pulse (A, 10 ms; B, 40 ms) to 0 mV. (A, B) Examples of tetrodotoxin-sensitive (A) and tetrodotoxin-resistant (B) Na^+ current families obtained by the protocol before and after treatment with EGCG (1 μM) for 10 min. Pre-pulse potentials are indicated for comparison. (C) Current normalized to a maximal control current was plotted against the pre-pulse potential (tetrodotoxin-sensitive, $n=8$; tetrodotoxin-resistant, $n=7$). Curves were drawn after fit with a Boltzmann function. ○, tetrodotoxin-sensitive, control; □, tetrodotoxin-sensitive, EGCG; ●, tetrodotoxin-resistant, control; ■, tetrodotoxin-resistant, EGCG.

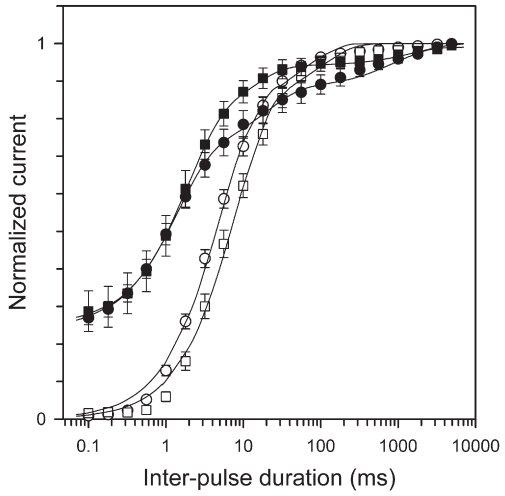

Fig. 6. Effects of EGCG on the recovery of Na^+ currents from inactivation. Two identical pulses of 10-ms (tetrodotoxin-sensitive) or 40-ms (tetrodotoxin-resistant) duration to 0 mV from a holding potential of −100 mV were separated by an inter-pulse (−100 mV) duration of increasing amount of time. The protocol was run before and after treatment with EGCG (1 μM) for 10 min. Current in response to the second pulse was normalized and plotted against the inter-pulse duration in logarithmic scale (tetrodotoxin-sensitive, $n=8$; tetrodotoxin-resistant, $n=7$). Curves are fits of the time course of recovery to the sum of two exponentials (tetrodotoxin-sensitive) or three exponentials (tetrodotoxin-resistant). ○, tetrodotoxin-sensitive, control; □, tetrodotoxin-sensitive, EGCG; ●, tetrodotoxin-resistant, control; ■, tetrodotoxin-resistant, EGCG.

pulse reflects the recovered fraction of Na^+ current that is dependent on the inter-pulse duration. The recovered fraction was fitted with the sum of two exponentials (tetrodotoxin-sensitive) or three exponentials (tetrodotoxin-resistant):

$$I = A_1(1-\exp(-t/\tau_1)) + A_2(1-\exp(-t/\tau_2)) + A_3(1-\exp(-t/\tau_3)) + C,$$

where I is the current normalized to the second pulse, A_1, A_2 and A_3 are the relative proportions of each exponential, τ_1, τ_2 and τ_3 are recovery time constants, t is the inter-pulse duration in ms, and C is the proportion of the current that is already recovered before the measurement limit of 0.1 ms.

The recovery time courses before and after treatment with EGCG (1 μM) for 10 min are shown in Fig. 6 and parameters for the exponential fit are summarized in Table 2. EGCG slowed the recovery of tetrodotoxin-sensitive Na^+ current from inactivation, which resulted from a significant increase in the τ_2 value. EGCG appeared to accelerate the recovery of tetrodotoxin-resistant Na^+ current from inactivation, which mainly resulted from the decrease of the τ_2 value. The decrease, however, was not statistically significant.

4. Discussion

In the present study we showed that EGCG potently inhibited tetrodotoxin-sensitive and tetrodotoxin-resistant Na^+ currents in rat dorsal root ganglion neurons in a concentration-dependent manner. EGCG slowed the inactivation kinetics of both types of Na^+ currents. However, EGCG had little or no effect on the activation or steady-state inactivation voltage of either type of Na^+ current. EGCG simply reduced the availability of Na^+ channels for activation. Thus, EGCG appears to bind to resting Na^+ channels to inhibit them. EGCG slowed the recovery of tetrodotoxin-sensitive Na^+ current from inactivation.

Recently it has been reported that EGCG inhibited Na^+ current in rat hippocampal neurons (Deng et al., 2008). Here EGCG reduced the available Na^+ channels and slowed the inactivation kinetics in line with our results. However, EGCG caused a hyperpolarizing shift of both activation and steady-state inactivation voltages, which were not observed in the present study. $Na_V1.1$, $Na_V1.2$ and $Na_V1.6$ are the major Na^+ channel isoforms found in hippocampus (Goldin, 2001). In dorsal root ganglia, however, $Na_V1.1$, $Na_V1.6$ and $Na_V1.7$ shape tetrodotoxin-sensitive Na^+ current, and $Na_V1.8$ and $Na_V1.9$ shape tetrodotoxin-resistant Na^+ current (Black et al., 2004; Goldin, 2001). The discrepancy of the EGCG effects between two neuronal types possibly stems from the different compositions of Na^+ channel isoforms.

Effects of EGCG on electrical activity in other tissues have been reported. EGCG (1–20 μM) depolarizes enteric neurons in the myenteric plexus of the small intestine possibly by directly activating cation channels (Homma et al., 2001). In the same preparation it (1–20 μM) also facilitates cholinergic ganglionic transmission by increasing the release of acetylcholine (Katayama et al., 2002). In vascular smooth muscle cells EGCG at 30 μM opens Ca^{2+}-permeable non-selective cation channels and EGCG at 100 μM potentiates and then inhibits voltage-gated Ca^{2+} channels (Campos-Toimil and Orallo, 2007).

In medial vestibular nuclear neurons EGCG (0.5–1 μM) lowers the spontaneous firing rate and hyperpolarizes the membrane without changing the amplitude of afterhyperpolarization (Jeong et al., 2005). On the contrary, in substantia nigra dopaminergic neurons, it (5–10 μM) increases the spike frequency, depolarizes the membrane and decreases the amplitude of afterhyperpolarization, possibly via inhibition of Ca^{2+}-dependent K^+ currents (Jeong et al., 2007). The increase in the spike frequency can enhance the dopamine release, and this may explain the usefulness of EGCG in Parkinson's disease.

Tea extract activates $GABA_A$ receptor, probably because tea contains GABA-like compounds (Hossain et al., 2002). However, EGCG inhibits the response of recombinant α1β1 or α1β2γ2L $GABA_A$ receptor expressed in *Xenopus* oocytes to GABA with IC_{50} values of 16 and 15 μM, respectively (Campbell et al., 2004; Hossain et al., 2002). Nevertheless at low concentration (0.1 μM) EGCG enhances the action of diazepam on the activation by GABA of α1β2γ2L $GABA_A$ receptor (Campbell et al., 2004). In hippocampal neurons EGCG up to 10 μM has no effect on $GABA_A$ current, but reverses $GABA_A$ receptor negative modulator methyl β-carboline-3-carboxylate inhibition on $GABA_A$ current (Vignes et al., 2006). Thus it is possible that EGCG displaces an endogeneous negative modulator of $GABA_A$ receptor. Besides EGCG (10 μM) attenuates the intracellular Ca^{2+} increase and excitotoxicity in hippocampal neurons induced by AMPA (Bae et al., 2002).

EGCG inhibits rat brain Kv1.5 K^+ channel with an IC_{50} of 101 μM with no influence on the voltage dependence of steady state activation or inactivation of the channel (Choi et al., 2001). EGCG also inhibits ATP-sensitive K^+ (K_{ATP}) channels, specifically Kir6.2/SUR1 and Kir6.2ΔC36, expressed in *Xenopus* oocytes with IC_{50} values of 142 and 19.9 μM, respectively (Baek et al., 2005). EGC is, however, less potent to inhibit K_{ATP} channels. A similar tendency was observed in the present study that EGC was far less potent to inhibit Na^+ currents than EGCG. Thus gallate moiety of EGCG appears to be an important functional group to modulate these channels.

The epidermal growth factor receptors are implicated in the abnormal growth of several types of human cancers. EGCG inhibits the

Table 2
Parameters for the recovery of Na^+ currents from inactivation

		Control	EGCG
Tetrodotoxin-sensitive current	A_1	0.83±0.04	0.83±0.03
	τ_1	5.1±0.4	8.2±0.9[b]
	A_2	0.17±0.04	0.17±0.03
	τ_2	129±43	343±182
Tetrodotoxin-resistant current	A_1	0.46±0.03	0.51±0.03
	τ_1	1.4±0.1	1.8±0.2[a]
	A_2	0.19±0.02	0.18±0.03
	τ_2	22±4	12±3
	A_3	0.11±0.02	0.07±0.02
	τ_3	873±163	1631±377
	C	0.23±0.02	0.25±0.05

Cells were treated with 1 μM EGCG for 10 min. τ is in ms. Tetrodotoxin-sensitive, $n=8$; tetrodotoxin-resistant, $n=7$. [a]$P<0.01$, [b]$P<0.001$ compared with the control.

activation of the receptors by altering the ordered membrane domain called lipid rafts, where the receptors are localized. EGCG also causes changes in the function of bilayer-incorporated gramicidin channels, which are used for a probe for measuring changes in mechanical properties of lipid bilayer (Adachi et al., 2007). Similarly EGCG may modulate Na^+ channels and other ion channels indirectly by altering the membrane organization.

Even though EGCG is abundant in green tea, its bioavailability is very low and only 0.1% of the ingested EGCG appears in the blood at the time to reach maximum plasma concentration. The peak plasma EGCG level after drinking 2 cups of green tea is around 0.17 μM (Lee et al., 2002). As listed above the concentration of ECGC to modulate electrical activities or ion channels is in the range of 1–100 μM. It would be difficult to attain this level of plasma concentration with ordinary green tea consumption. However, in the current study K_d values to inhibit tetrodotoxin-sensitive and tetrodotoxin-resistant Na^+ currents were estimated to be 0.74 and 0.80 μM, respectively. The figures may be even smaller considering that the EGCG inhibition still continued at 10 min, at which the measurement was made (Fig. 2C). This result suggests that, in vivo, EGCG can reach the physiological concentrations required for Na^+ current inhibition. Nonetheless it should be aware of the fact that EGCG inhibits hERG current in HEK293 cells with an IC_{50} of 6 μM (Kelemen et al., 2007). Inhibition of hERG current is related to QT interval prolongation and cardiac arrhythmia.

Na^+ channels in dorsal root ganglion neuron, especially $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$, are predominantly expressed in nociceptive dorsal root ganglion neurons and have been shown to be related to the pathogenesis of various pains (Akopian et al., 1996; Cummins et al., 2007; Dib-Hajj et al., 1998; Djouhri et al., 2003). Mutations in $Na_V1.7$ cause primary erythermalgia, paroxysmal extreme pain disorder, and channelopathy-associated insensitivity to pain (Cox et al., 2006; Fertleman et al., 2006; Yang et al., 2004). $Na_V1.8$ plays important roles in the formation of inflammatory, neuropathic and cold pains (Akopian et al., 1999; Dong et al., 2007; Zimmermann et al., 2007). $Na_V1.9$ is an effector of inflammatory pain hypersensitivity (Amaya et al., 2006). We showed that EGCG inhibited tetrodotoxin-sensitive and tetrodotoxin-resistant Na^+ currents in rat dorsal root ganglion neurons potently, and at concentrations that can be reached physiologically. Thus EGCG has a potential for use as an analgesic agent.

Acknowledgement

This work was supported by the Korean Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2007-314-C00194).

References

Adachi, S., Nagao, T., Ingolfsson, H.I., Maxfield, F.R., Andersen, O.S., Kopelovich, L., Weinstein, I.B., 2007. The inhibitory effect of (−)-epigallocatechin gallate on activation of the epidermal growth factor receptor is associated with altered lipid order in HT29 colon cancer cells. Cancer Res. 67, 6493–6501.

Akopian, A.N., Sivilotti, L., Wood, J.N., 1996. A tetrodotoxin-resistant voltage-gated sodium channel expressed by sensory neurons. Nature 379, 257–262.

Akopian, A.N., Souslova, V., England, S., Okuse, K., Ogata, N., Ure, J., Smith, A., Kerr, B.J., McMahon, S.B., Boyce, S., Hill, R., Stanfa, L.C., Dickenson, A.H., Wood, J.N., 1999. The tetrodotoxin-resistant sodium channel SNS has a specialized function in pain pathways. Nat. Neurosci. 2, 541–548.

Amaya, F., Wang, H., Costigan, M., Allchorne, A.J., Hatcher, J.P., Egerton, J., Stean, T., Morisset, V., Grose, D., Gunthorpe, M.J., Chessell, I.P., Tate, S., Green, P.J., Woolf, C.J., 2006. The voltage-gated sodium channel $Na_v1.9$ is an effector of peripheral inflammatory pain hypersensitivity. J. Neurosci. 26, 12852–12860.

Bae, J.H., Mun, K.C., Park, W.K., Lee, S.R., Suh, S.I., Baek, W.K., Yim, M.B., Kwon, T.K., Song, D.K., 2002. EGCG attenuates AMPA-induced intracellular calcium increase in hippocampal neurons. Biochem. Biophys. Res. Commun. 290, 1506–1512.

Baek, W.K., Jang, B.C., Lim, J.H., Kwon, T.K., Lee, H.Y., Cho, C.H., Kim, D.K., Shin, D.H., Park, J.G., Lim, J.G., Bae, J.H., Bae, J.H., Yoo, S.K., Park, W.K., Song, D.K., 2005. Inhibitory modulation of ATP-sensitive potassium channels by gallate-ester moiety of (−)-epigallocatechin-3-gallate. Biochem. Pharmacol. 70, 1560–1567.

Black, J.A., Liu, S., Tanaka, M., Cummins, T.R., Waxman, S.G., 2004. Changes in the expression of tetrodotoxin-sensitive sodium channels within dorsal root ganglia neurons in inflammatory pain. Pain 108, 237–247.

Campbell, E.L., Chebib, M., Johnston, G.A., 2004. The dietary flavonoids apigenin and (−)-epigallocatechin gallate enhance the positive modulation by diazepam of the activation by GABA of recombinant $GABA_A$ receptors. Biochem. Pharmacol. 68, 1631–1638.

Campos-Toimil, M., Orallo, F., 2007. Effects of (−)-epigallocatechin-3-gallate in Ca^{2+}-permeable non-selective cation channels and voltage-operated Ca^{2+} channels in vascular smooth muscle cells. Life Sci. 80, 2147–2153.

Carlson, J.R., Bauer, B.A., Vincent, A., Limburg, P.J., Wilson, T., 2007. Reading the tea leaves: anticarcinogenic properties of (−)-epigallocatechin-3-gallate. Mayo Clin. Proc. 82, 725–732.

Choi, B.H., Choi, J.S., Min, D.S., Yoon, S.H., Rhie, D.J., Jo, Y.H., Kim, M.S., Hahn, S.J., 2001. Effects of (−)-epigallocatechin-3-gallate, the main component of green tea, on the cloned rat brain Kv1.5 potassium channels. Biochem. Pharmacol. 62, 527–535.

Cox, J.J., Reimann, F., Nicholas, A.K., Thornton, G., Roberts, E., Springell, K., Karbani, G., Jafri, H., Mannan, J., Raashid, Y., Al-Gazali, L., Hamamy, H., Valente, E.M., Gorman, S., Williams, R., McHale, D.P., Wood, J.N., Gribble, F.M., Woods, C.G., 2006. An *SCN9A* channelopathy causes congenital inability to experience pain. Nature 444, 894–898.

Cummins, T.R., Sheets, P.L., Waxman, S.G., 2007. The roles of sodium channels in nociception: implications for mechanisms of pain. Pain 131, 243–257.

Deng, H.M., Yin, S.T., Yan, D., Tang, M.L., Li, C.C., Chen, J.T., Wang, M., Ruan, D.Y., 2008. Effects of EGCG on voltage-gated sodium channels in primary cultures of rat hippocampal CA1 neurons. Toxicology 252, 1–8.

Dib-Hajj, S.D., Tyrrell, L., Black, J.A., Waxman, S.G., 1998. NaN, a novel voltage-gated Na channel, is expressed preferentially in peripheral sensory neurons and down-regulated after axotomy. Proc. Natl. Acad. Sci. U. S. A. 95, 8963–8968.

Djouhri, L., Newton, R., Levinson, S.R., Berry, C.M., Carruthers, B., Lawson, S.N., 2003. Sensory and electrophysiological properties of guinea-pig sensory neurones expressing $Na_v1.7$ (PN1) Na^+ channel α subunit protein. J. Physiol. 546, 565–576.

Dong, X.W., Goregoaker, S., Engler, H., Zhou, X., Mark, L., Crona, J., Terry, R., Hunter, J., Priestley, T., 2007. Small interfering RNA-mediated selective knockdown of $Na_V1.8$ tetrodotoxin-resistant sodium channel reverses mechanical allodynia in neuropathic rats. Neuroscience 146, 812–821.

Fertleman, C.R., Baker, M.D., Parker, K.A., Moffatt, S., Elmslie, F.V., Abrahamsen, B., Ostman, J., Klugbauer, N., Wood, J.N., Gardiner, R.M., Rees, M., 2006. *SCN9A* mutations in paroxysmal extreme pain disorder: allelic variants underlie distinct channel defects and phenotypes. Neuron 52, 767–774.

Goldin, A.L., 2001. Resurgence of sodium channel research. Annu. Rev. Physiol. 63, 871–894.

Homma, T., Hirai, K., Hara, Y., Katayama, Y., 2001. Tea catechin, (−)-epigallocatechin gallate, causes membrane depolarizations of myenteric neurons in the guinea-pig small intestine. Neurosci. Lett. 309, 93–96.

Hossain, S.J., Hamamoto, K., Aoshima, H., Hara, Y., 2002. Effects of tea components on the response of $GABA_A$ receptors expressed in *Xenopus* Oocytes. J. Agric. Food. Chem. 50, 3954–3960.

Jeong, H.S., Kim, Y.S., Park, J.S., 2005. Modulation of neuronal activity by EGCG. Brain Res. 1047, 267–270.

Jeong, H.S., Jang, S., Jang, M.J., Lee, S.G., Kim, T.S., Tag, H., Lee, J.H., Jun, J.Y., Park, J.S., 2007. Effects of (−)-epigallocatechin-3-gallate on the activity of substantia nigra dopaminergic neurons. Brain Res. 1130, 114–118.

Katayama, Y., Homma, T., Hara, Y., Hirai, K., 2002. Tea catechin, (−)-epigallocatechin gallate, facilitates cholinergic ganglion transmission in the myenteric plexus of the guinea-pig small intestine. Neurosci. Lett. 319, 63–66.

Kelemen, K., Kiesecker, C., Zitron, E., Bauer, A., Scholz, E., Bloehs, R., Thomas, D., Greten, J., Remppis, A., Schoels, W., Katus, H.A., Karle, C.A., 2007. Green tea flavonoid epigallocatechin-3-gallate (EGCG) inhibits cardiac hERG potassium channels. Biochem. Biophys. Res. Commun. 364, 429–435.

Lee, M.J., Maliakal, P., Chen, L., Meng, X., Bondoc, F.Y., Prabhu, S., Lambert, G., Mohr, S., Yang, C.S., 2002. Pharmacokinetics of tea catechins after ingestion of green tea and (−)-epigallocatechin-3-gallate by humans: formation of different metabolites and individual variability. Cancer Epidemiol. Biomarkers Prev. 11, 1025–1032.

Mandel, S., Weinreb, O., Amit, T., Youdim, M.B., 2004. Cell signaling pathways in the neuroprotective actions of the green tea polyphenol (−)-epigallocatechin-3-gallate: implications for neurodegenerative diseases. J. Neurochem. 88, 1555–1569.

Mandel, S., Amit, T., Reznichenko, L., Weinreb, O., Youdim, M.B., 2006. Green tea catechins as brain-permeable, natural iron chelators–antioxidants for the treatment of neurodegenerative disorders. Mol. Nutr. Food Res. 50, 229–234.

Miller, A.L., 1996. Antioxidant flavonoids: structure, function and clinical usage. Altern. Med. Rev. 1, 103–111.

Roy, M.L., Narahashi, T., 1992. Differential properties of tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels in rat dorsal root ganglion neurons. J. Neurosci. 12, 2104–2111.

Suganuma, M., Okabe, S., Oniyama, M., Tada, Y., Ito, H., Fujiki, H., 1998. Wide distribution of [^{3}H](−)-epigallocatechin gallate, a cancer preventive tea polyphenol, in mouse tissue. Carcinogenesis 19, 1771–1776.

Vignes, M., Maurice, T., Lanté, F., Nedjar, M., Thethi, K., Guiramand, J., Récasens, M., 2006. Anxiolytic properties of green tea polyphenol (−)-epigallocatechin gallate (EGCG). Brain Res. 1110, 102–115.

Yang, Y., Wang, Y., Li, S., Xu, Z., Li, H., Ma, L., Fan, J., Bu, D., Liu, B., Fan, Z., Wu, G., Jin, J., Ding, B., Zhu, X., Shen, Y., 2004. Mutations in *SCN9A*, encoding a sodium channel alpha subunit, in patients with primary erythermalgia. J. Med. Genet. 41, 171–174.

Zaveri, N.T., 2006. Green tea and its polyphenolic catechins: medicinal uses in cancer and noncancer applications. Life Sci. 78, 2073–2080.

Zimmermann, K., Leffler, A., Babes, A., Cendan, C.M., Carr, R.W., Kobayashi, J., Nau, C., Wood, J.N., Reeh, P.W., 2007. Sensory neuron sodium channel $Na_v1.8$ is essential for pain at low temperatures. Nature 447, 855–858.