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TRPA1–expressing primary afferents synapse with specific morphological subtypes of substantia gelatinosa neurons in the adult rat spinal cord

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Abstract

The TRPA1-channel has been proposed to be a molecular transducer of cold and inflammatory nociceptive signals. It is expressed on a subset of small primary afferent neurons both in the peripheral terminals, where it serves as a sensor, and on the central nerve endings in the dorsal horn. The substantia gelatinosa (SG) of the spinal cord is a key site for integration of noxious inputs. The SG neurons are morphologically and functionally heterogeneous and the precise synaptic circuits of the SG are poorly understood. We examined how activation of TRPA1 channels affects synaptic transmission onto SG neurons using whole-cell patch-clamp recordings and morphological analyses in adult rat spinal cord slices. Cinnamaldehyde (TRPA1 agonist) elicited a barrage of EPSCs in a subset of the SG neurons that responded to allyl isothiocyanate (less specific TRPA1 agonist) and capsaicin (TRPV1 agonist). Cinnamaldehyde evoked EPSCs in vertical and radial, but not islet or central SG cells. Notably, cinnamaldehyde produced no change in IPSCs nor did it produce direct post-synaptic effects. In the presence of TTX, cinnamaldehyde increased the frequency but not amplitude of miniature EPSCs. Intriguingly, cinnamaldehyde had a selective inhibitory action on monosynaptic C (but not Aδ) fiber-evoked EPSCs. These results indicate that activation of spinal TRPA1 presynaptically facilitates miniature excitatory synaptic transmission from primary afferents onto vertical and radial cells to initiate action potentials. The presence of TRPA1 channels on the central terminals raises the possibility of a novel mechanism for a cell type-specific bidirectional modulatory action on the SG.

Keywords

Noxious cold; synaptic transmission; spinal dorsal horn; TRP channel; C fiber

Introduction

The substantia gelatinosa (SG; lamina II of Rexed, 1952) of the spinal dorsal horn is the first site of synaptic processing of nociceptive information and is considered to have sophisticated circuits to modulate synaptic transmission. The SG receives a dense innervation of finely myelinated A δ and unmyelinated C primary afferent fibers (Kumazawa & Perl, 1978; Light *et al.*, 1979; Brown, 1982; Woolf & Fitzgerald, 1983; Yoshimura & Jessell, 1989b; Furue *et al.*, 1999). SG neurons have been morphologically classified into at least four types; vertical, radial, islet and central cells, based on the location and orientation of their dendrites and axons (Todd & Spike, 1993; Grudt & Perl, 2002; Lu & Perl, 2005; Yasaka *et al.*, 2007). All these different types of neurons receive monosynaptic excitatory synaptic inputs from C fibers (Grudt & Perl, 2002; Yasaka *et al.*, 2007). Strikingly, although the nociceptive afferent fibers are functionally heterogeneous reflected in the differential expression of molecular markers such as peptides and receptors (Willis & Coggeshall, 2004), it is unknown how such “labeled lines” of primary afferent information connect to the different types of SG neurons.

TRPA1, a member of the TRP family of cation channels, is a molecular marker for a subgroup of small dorsal root ganglion (DRG). TRPA1 is expressed in peptidergic neurons which are a subset of the TRPV1 (Capsaicin-sensitive noxious thermal-sensing TRP channel)-expressing neurons and they do not express TRPM8 (cool sensing TRP channel) (Story *et al.*, 2003; Bandell *et al.*, 2004; Jordt *et al.*, 2004). TRPA1 is activated by noxious cold stimuli, endogenous substances including the inflammatory mediator bradykinin and also by intracellular Ca²⁺ (Bandell *et al.*, 2004; Bautista *et al.*, 2006; Zurborg *et al.*, 2007). The natural pungent compounds; cinnamaldehyde and allyl isothiocyanate (AITC) also activate TRPA1 to provoke their burning sensation (Bandell *et al.*, 2004; Jordt *et al.*, 2004; Bautista *et al.*, 2005; Macpherson *et al.*, 2005). In addition to their peripheral role as receptors for noxious stimuli, several lines of evidence indicate that TRP channels including TRPA1 have a role in central synaptic modulation (Engelman & MacDermott, 2004). Activation of spinal TRPA1 and other TRP channels located on presynaptic afferent terminals enhances the spontaneous release of glutamate (Yang *et al.*, 1998; Baccei *et al.*, 2003; Tsuzuki *et al.*, 2004; Kosugi *et al.*, 2007; Suzuki *et al.*, 2007; Wrigley *et al.*, 2009). However, it is unknown which classes of SG neurons are contacted by TRP-expressing afferent fibers, and how sensory information conveyed by the fibers is modulated by central TRPA1 activation. To address this, we investigated the effect of TRPA1 agonists on spontaneous and evoked synaptic inputs to morphologically identified SG neurons using whole-cell patch-clamp recordings in conjunction with neurobiotin labeling.

Methods

All of the experimental procedures were conducted in accordance with Guidelines of Kyushu University and National Institute for Physiological Sciences for Animal Experimentation and the Guiding Principles for the Care and Use of Animals recommended by the Physiological Society of Japan. All efforts were made to minimize animal suffering and the number of animals used for the studies.

Preparation of spinal cord slices

The methods used for obtaining transverse or parasagittal slice preparations of the adult rat spinal cord with an attached dorsal root have been described elsewhere (Yoshimura & Nishi, 1993; Yang *et al.*, 2001). Briefly, male adult Sprague–Dawley rats (6–9 weeks old, 200–350 g) were obtained from Kyudo (Fukuoka, Japan). They were anesthetized with urethane ($1.2\text{--}1.5\text{ g kg}^{-1}$, intraperitoneally), and a thoracolumbar laminectomy was performed. The lumbosacral segments of the spinal cord (L1–S1) with ventral and dorsal roots were removed, and placed in an ice-cold Krebs solution equilibrated with 95% O₂–5% CO₂. The Krebs solution contained (in mM): NaCl 117, KCl 3.6, CaCl₂ 2.5, MgCl₂ 1.2, NaH₂PO₄ 1.2, NaHCO₃ 25 and glucose, 11. Immediately after the removal of the spinal cord, the rats were killed by exsanguination under urethane anesthesia. The pia-arachnoid membrane was removed after cutting all the ventral and dorsal roots, except for the L4 or L5 dorsal root on one side. The spinal cord was mounted on a vibratome and a 500 μm thick transverse or parasagittal slice with an attached dorsal root was cut. The slice was placed on a nylon mesh in the recording chamber with a volume of 0.5 ml, and was completely submerged and perfused with Krebs solution saturated with 95% O₂ and 5% CO₂ at $36 \pm 1^\circ\text{C}$ at a flow rate of 10–15 ml/min. The dorsal root was stimulated using suction electrode.

Whole-cell patch-clamp recordings from SG neurons

The SG was easily discernible with transmitted illumination as a relatively translucent band across the dorsal horn in the transverse or parasagittal slice preparations. Blind whole-cell voltage-clamp recordings were made from SG neurons, as previously described (Yoshimura & Nishi, 1993; Yang *et al.*, 2001). The patch pipettes were filled with a solution containing (mM): potassium gluconate solution (K-gluconate 135, KCl 5, CaCl₂ 0.5, MgCl₂ 2, EGTA 5, HEPES 5, ATP-Mg 5; pH 7.2) or cesium solution (Cs₂SO₄ 110, tetraethylammonium 5, CaCl₂ 0.5, MgCl₂ 2, EGTA 5, HEPES 5, ATP-Mg 5; pH 7.2). The tip resistance of the patch pipettes was 6–12 M Ω . Series resistance was assessed according to the response to a 5 mV hyperpolarizing step. This value was monitored during the recording session, and data were rejected if values changed by >15%. Signals were acquired with a patch clamp amplifier (Axopatch 700A, Molecular Devices, Union City, CA, USA). The data were digitized with an analog-to-digital converter (Digidata 1321A, Molecular Devices), stored on a personal computer using a data acquisition program (Clampex version 9.0, Molecular Devices), and analyzed using a software package (Clampfit version 9.0, Molecular Devices).

Cell recordings were made in voltage-clamp mode at holding potentials of -70 mV to record EPSCs and 0 mV for IPSCs, at these potentials the GABA-/glycine-mediated IPSCs, and glutamate-mediated EPSCs, were negligible, respectively (Yoshimura & Nishi, 1993). The dorsal roots were stimulated at a frequency of 0.2 Hz (duration, 100 μs) to elicit EPSCs. The A δ - or C-afferent-mediated responses were distinguished on the basis of the conduction velocity of the afferent fibers and stimulus threshold. The conduction velocity was calculated from the latency of synaptic responses and the length of the dorsal root. The A δ and C fiber-evoked responses were considered monosynaptic if the latency remained constant when the root was stimulated at 20 Hz for the A δ fiber-evoked EPSCs, and there was no failure regardless of the constancy of the latency stimulated at 2 Hz for the C fiber-evoked EPSCs, respectively (Nakatsuka *et al.*, 1999). Dorsal root stimuli were not applied

during examination of the effects of drugs on the spontaneous or miniature synaptic responses, to avoid the influence of evoked release on the presynaptic terminals. We arbitrarily defined neurons as being sensitive to a particular drug when the frequency or amplitude of the synaptic responses was altered by more than $\pm 20\%$ of control. The firing patterns of SG neurons were determined in current-clamp mode by passing 0.5 s depolarizing current pulses through the recording electrode from a membrane potential of -60 mV. In each cell the depolarizing pulses were graded from subthreshold to considerably supraliminal. In some instances the firing pattern altered as the magnitude of injected current was increased. Therefore, we analyzed the firing patterns observed with near threshold depolarizations. We classified the recorded neurons on the basis of their firing patterns into five types, as proposed by previous studies (Grudt & Perl, 2002; Ruscheweyh & Sandkuhler, 2002). We used a holding membrane potential of -70 or -80 mV when the firing pattern could not be correctly classified at -60 mV.

Morphological identification of SG neurons

The recorded neurons were anatomically identified post hoc as SG neurons by the intracellular injection of neurobiotin (0.2% in the pipette solution; Vector Laboratories, Burlingame, CA, USA). After electrophysiological recording, the spinal cord slices were fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) at 4°C and then rinsed in PB. One or two neurons were recorded in each slice using the neurobiotin containing pipette solution for post-hoc morphological analysis. Neurobiotin-filled neurons were visualized using diaminobenzidine (DAB) staining or fluorescence histochemistry. For DAB staining, free-floating sections were incubated overnight in Vectastain (Elite kit; Vector Laboratory) with 0.1% Triton X-100. The peroxidase activity was revealed with DAB in the presence of hydrogen peroxide, and sections were mounted on gelatinized slides. For fluorescence histochemistry, the spinal cord slices were re-sectioned ($50\text{ }\mu\text{m}$ thick) using a vibratome. Free-floating sections were incubated overnight at 4°C in phosphate-buffered saline (PBS) with 0.3% Triton X-100 containing streptavidin-Texas Red (diluted 1: 750; Jackson ImmunoResearch, West Grove, PA) and isolectin B4-FITC (IB4, diluted 1: 200; Sigma, St Louis, MO, USA) used for identification of the border between lamina II and lamina III. After further washes in PBS the sections were mounted in glycerol-based mounting medium (Vectashield, Vector).

Drug application

The drugs were dissolved in Krebs solution and applied by exchanging solutions via a three-way stopcock. The drugs used were capsaicin (Wako Pure Chemical Industries, Osaka, Japan), trans-cinnamaldehyde (Sigma), allyl isothiocyanate (Sigma), ruthenium red (Nacalai Tesque, Kyoto, Japan), capsazepine (Wako), 2-(1, 3-dimethyl-2, 6-dioxo-1, 2, 3, 6-tetrahydro-7H-prin-7-yl)-N-(4-isopropylphenyl)acetamide (HC-030031, Nacalai Tesque), 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX, Sigma), 7-(Hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt, Nacalai), (2S)-2-Amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495, Nacalai), strychnine (Sigma), tetrodotoxin (TTX, Wako), and bicuculline (Sigma). To determine whether neurons were sensitive to both cinnamaldehyde and capsaicin, we applied cinnamaldehyde both before and after capsaicin application. The

relative increase in the frequency of spontaneous EPSCs evoked by cinnamaldehyde was not different when tested before or after capsaicin application ($285 \pm 186\%$ of control for the first cinnamaldehyde application; $277 \pm 149\%$ of control for the second cinnamaldehyde application after capsaicin; $p = 0.75$, $t_{13} = 0.39$).

Statistical analysis

All data values were expressed as mean $\pm$ S.D. Statistical significance was determined as $p < 0.05$ using Student's paired and unpaired t -test. The Kolomogorov-Smirnov test was used to compare the cumulative distributions of synaptic responses. In all cases, n refers to the number of neurons studied.

Results

Stable whole-cell recordings were obtained from a total of 364 SG neurons with an average recording period of 40 min in slices maintained in vitro for more than 10 h. In 318 SG neurons, the effects of cinnamaldehyde (CA) on excitatory and inhibitory synaptic transmission were examined under voltage-clamp conditions, and in the remaining 46 neurons the effects of CA were examined under current-clamp conditions. They had input resistances between 150 and 850 MOhm and a resting membrane potential of -62.0 ± 4.7 mV ($n = 46$) consistent with our previous studies (Kato *et al.*, 2004). One or two neurons were recorded in each slice using the neurobiotin-containing pipette solution for post-hoc morphological analysis. A total of 60 SG neurons were successfully stained, allowing examination of the correlation between the morphological classification and the action of CA on the synaptic transmission.

All SG neurons exhibited spontaneous EPSCs (sEPSCs) at an average frequency of 11.2 ± 8.5 Hz (range, 0.5–36.4 Hz; $n = 318$) with an average amplitude of 13.5 ± 4.7 pA (range, 8.1–32.9 pA; $n = 318$, holding potential -70 mV). The sEPSCs were always completely suppressed by the addition of CNQX (10 μ M), indicating that they were glutamatergic as shown previously (Furue *et al.*, 1999; Kato *et al.*, 2004). TRPA1 (and TRPV1) agonists are known to have excitatory actions on sEPSCs (Yang *et al.*, 1998; Baccei *et al.*, 2003; Kosugi *et al.*, 2007; Suzuki *et al.*, 2007; Wrigley *et al.*, 2009). We used the selective TRPA1 agonist, CA which is thought to be the most specific activator for TRPA1 (Bandell *et al.*, 2004). Application of CA (300 μ M) reversibly evoked a barrage of EPSCs (*the left trace* in Fig. 1A) in 85 out of 269 (32%) of SG neurons. Note there was no evidence for direct actions of CA on any of the recorded cells.

Pharmacological profile of the action of CA on sEPSCs

We examined the pharmacological profile of this CA excitation of sEPSCs. CA dose-dependently increased the frequency of sEPSCs ($292 \pm 178\%$ of control for 300 μ M ($n = 85$) and to $388 \pm 221\%$ of control for 500 μ M ($n = 15$)) and amplitude of sEPSC ($132 \pm 44\%$ of control for 300 μ M ($p < 0.001$, $t_{84} = 7.30$) and to $172 \pm 54\%$ of control for 500 μ M ($p < 0.001$, $t_{14} = 5.63$)) (Fig. 1A and C). The perfusion of CA (500 μ M) produced a striking increase in the number of large amplitude sEPSCs (>30 pA) (Fig. 1B). Lower concentrations of CA (0.3–30 μ M) had no effect on the frequency and amplitude of sEPSCs ($n = 18$). The

excitatory action of CA (300 μ M) on sEPSCs (frequency increased to $230 \pm 91\%$ of control, $n = 8$) was completely blocked in the presence of ruthenium red (RR, 30 μ M, a TRP channel antagonist) ($108 \pm 11\%$ of control, $p < 0.001$, $t_7 = 5.41$). After washout of RR, CA again increased the frequency of sEPSCs ($246 \pm 119\%$ of control, $n = 8$; Fig. 1C). Moreover, the CA-evoked increase in sEPSC frequency ($319 \pm 118\%$ of control, $n = 5$) was inhibited in the presence of HC-030031 (100 μ M, a TRPA1 channel antagonist) ($120 \pm 39\%$ of control, $p = 0.005$, $t_4 = 4.64$; Fig. 1C). After washout of HC-030031, CA again increased the frequency of sEPSCs ($295 \pm 133\%$ of control, $n = 5$). However, a previous study has reported that HC-030031 also acts as a TRPV1 antagonist (Iwasaki *et al.*, 2009). Therefore, we examined the excitatory action of CA in the presence of TRPV1 antagonist, capsazepine (10 μ M). The CA-evoked increase in sEPSCs frequency ($276 \pm 68\%$ of control) was unchanged in the presence of capsazepine ($261 \pm 112\%$ of control, $p = 0.91$, $t_2 = 0.39$).

Extracellular Ca^{2+} was needed for the CA-elicited facilitation of sEPSCs as in a Ca^{2+} -free solution, CA (300 μ M) did not change the frequency or amplitude of sEPSCs ($110 \pm 18\%$ and $99 \pm 4\%$ of controls, respectively, $p = 0.0019$, $t_4 = 4.67$ and $p = 0.041$, $t_4 = 3.98$, respectively; Fig. 1C). By contrast, in the presence of a voltage-gated calcium channel blocker, cadmium (Cd^{2+} , 100 μ M), CA (300 μ M) still increased the frequency of sEPSCs (increased to $209 \pm 29\%$ of control before Cd^{2+} and $192 \pm 34\%$ in the presence of Cd^{2+} ($p = 0.52$, $t_4 = 1.46$)). There was no significant difference between the action of CA on the frequency of sEPSCs in the absence and presence of Cd^{2+} in SG neurons ($p = 0.745$, $t_4 = 0.75$). The sEPSCs were completely inhibited in the presence of CNQX (10 μ M) to block AMPA type ionotropic glutamatergic receptors. Under this condition, CA (300 μ M) did not elicit any barrages of EPSCs ($n = 6$).

CA increases the frequency of miniature EPSCs

We next investigated whether CA action was presynaptic or postsynaptic by analyzing miniature EPSCs (mEPSCs). In the presence of TTX (1 μ M), SG neurons exhibited mEPSCs at an average frequency of 12.2 ± 6.4 Hz with an average amplitude of 12.6 ± 7.6 pA ($n = 10$). Application of CA (300 μ M) evoked a barrage of mEPSCs and shifted the cumulative distribution curve of mEPSCs inter-event intervals to the left whereas no change was observed in the amplitude of mEPSCs ($p < 0.001$ and $p = 0.67$, respectively, Kolomogorov-Smirnov test). Across the group of cells CA (300 μ M) significantly increased the frequency ($248 \pm 42\%$ of control, $p < 0.001$, $t_9 = 5.83$), but not the amplitude of mEPSCs ($105 \pm 19\%$ of control, $p = 0.178$, $t_9 = 0.19$), suggesting that CA acts on presynaptic TRPA1 receptors to facilitate synaptic release.

Taken together these analyses of sEPSC and mEPSC suggest that CA presynaptically enhances glutamate release, probably by an increase of Ca^{2+} concentration in the presynaptic terminals through activation of TRPA1 non-selective cation channels.

CA increases spontaneous EPSCs in a subset of capsaicin-sensitive SG neurons

Since TRPA1 is expressed in a subset of the DRG neurons expressing TRPV1 (Bandell *et al.*, 2004; Obata *et al.*, 2005), we compared the number of neurons responding to CA with those responding to capsaicin (Cap, agonist for TRPV1). The application of Cap (1 μ M)

elicited a barrage of EPSCs (*left traces* in Fig. 2A) in 73% of SG neurons (36 out of 49), consistent with previous studies (Yang *et al.*, 2001; Baccei *et al.*, 2003; Suzuki *et al.*, 2007). We examined the effects of CA on sEPSCs in the same neurons. Of the 49 SG neurons tested, 14 (29%) neurons were sensitive to both Cap and CA (Fig. 2A). Twenty two neurons were sensitive to Cap but not CA (Fig. 2B). In the remaining 13 neurons, neither Cap nor CA had detectable effects on sEPSCs (Fig. 2C). No neurons were found that were sensitive to CA but not to Cap. These results indicate that TRPA1 agonist CA has a similar excitatory action on sEPSCs as TRPV1 agonists in a subset of the neurons that responded to Cap (Fig. 2D).

CA increases spontaneous EPSCs in a subset of AITC-sensitive SG neurons

AITC is also a TRPA1 agonist, however, it has been reported to activate a larger population of DRG neurons than CA including cold-insensitive cells (Bandell *et al.*, 2004). Therefore we compared the actions of CA (300 μ M) and AITC (10 or 100 μ M) in the same SG neurons. Applications of AITC also elicited barrages of EPSCs in some SG neurons, consistent with previous studies (Kosugi *et al.*, 2007; Wrigley *et al.*, 2009). Some 28% of SG neurons (7 out of 25) showed increased sEPSCs in response to both AITC and CA (Supporting Information Fig. S1, A). As we anticipated, AITC also had an excitatory action on neurons ($n = 6$, 24%) that were insensitive to CA (Supporting Information Fig. S1, B). In the remaining 12 neurons there were no detectable effects of CA and AITC (Supporting Information Fig. S1, C). No neurons were found that were sensitive to CA but not to AITC. Thus AITC had an excitatory effect on a larger population of SG neurons than CA (Supporting Information Fig. S1, D). These results indicate that CA has a more selective action on excitatory synaptic transmission than AITC. The excitatory action of AITC (100 μ M) on sEPSCs (frequency increased to $292 \pm 50\%$ of control, $n = 5$) was suppressed (but not completely blocked) in the presence HC-030031 (100 μ M) ($162 \pm 60\%$ of control, $p = 0.036$, $t_4 = 4.04$). After washout of HC-030031, AITC again increased the frequency of sEPSCs ($280 \pm 70\%$ of control, $n = 5$).

Firing pattern of SG cells receiving TRPA1-expressing afferents—Previous studies have classified SG neurons on the basis of their firing patterns in response to depolarizing current pulses (Thomson *et al.*, 1989; Grudt & Perl, 2002; Ruscheweyh & Sandkuhler, 2002; Lu & Perl, 2003; Daniele & MacDermott, 2009). We examined the firing patterns of SG neurons receiving CA-sensitive afferent fibers. In current-clamp mode, SG neurons discharged action potentials in response to a just supra-threshold current pulse. SG neurons tested ($n = 48$) were classified into five types: delayed firing ($n = 18$, Fig. 3A), sustained repetitive firing ($n = 12$, Fig. 3B), phasic firing ($n = 4$, Fig. 3C), initial firing ($n = 11$, Fig. 3D) and other firing (e.g. single; $n = 3$). In this study using adult rats, the sustained repetitive firing type showed regular firing throughout the current pulses without a long delay to initiate spikes as seen in the delayed firing type. This type of firing pattern has also been seen in SG neurons of adult mice and young (3-5 weeks old) hamsters (Grudt & Perl, 2002; Schoffnegger *et al.*, 2006), but not of young (2-4 weeks old) rats (Ruscheweyh & Sandkuhler, 2002).

CA (300 μ M) increased the frequency of sEPSCs in 23 out of 48 SG neurons tested. Most (78%) of the CA-sensitive SG neurons were the delayed ($n = 11$) and sustained repetitive ($n = 7$) firing types, although CA-insensitive cells were also found in these two types of SG neurons (Figs. 3A, B). In the phasic firing type, 2 out of 4 cells were sensitive to CA (Fig. 3C). In the initial firing type, CA-sensitive neurons were not detected except for one cell (1 out of 11) (Fig. 3D). In other firing type, one out of 3 cells was CA-sensitive. The CA-evoked sEPSC frequency increase in each type was $290 \pm 70\%$ ($n = 11$) for delayed firing, $296 \pm 48\%$ ($n = 7$) for sustained repetitive firing, $312 \pm 80\%$ ($n = 2$) for phasic firing, 152% ($n = 1$) for initial firing and 267% ($n = 1$) for other firing type. The CA-evoked sEPSC amplitude increase was $151 \pm 59\%$ ($n = 11$) for delayed firing, $132 \pm 15\%$ ($n = 7$) for sustained repetitive firing, $206 \pm 136\%$ for phasic firing ($n = 2$), 117% ($n = 1$) for initial firing and 103% ($n = 1$) for other firing type.

Morphological characterization of SG cells receiving TRPA1- and TRPV1-expressing afferents

The SG neurons receiving excitatory synaptic inputs from CA-sensitive afferent fibers represented a subpopulation (32%) of SG neurons. To morphologically classify which SG neurons receive CA-sensitive synaptic inputs, we filled a sample of recorded cells with neurobiotin and performed post-hoc anatomical analysis. The laminar location of neurons (cell bodies) receiving CA-sensitive and insensitive synaptic inputs were determined. Of the cells with CA-sensitive inputs 58% (14 of 24) were in lamina IIo and the remaining 42% were in lamina Ii. Of the CA-insensitive cells studied ($n = 36$), 78% were located in lamina Ii and 22% in IIo.

A total of 60 SG neurons were successfully filled and reconstructed after the examination of CA action on their sEPSCs. The morphological features of the neurons were similar to those of SG neurons described previously using Golgi staining, horseradish peroxidase labeling or neurobiotin labeling. According to the classification scheme proposed by previous studies (Todd & Lewis, 1986; Grudt & Perl, 2002; Yasaka *et al.*, 2007), the neurobiotin-stained cells were classified into five types, vertical ($n = 15$), radial ($n = 14$), islet ($n = 6$), central ($n = 10$) and unclassified cells ($n = 15$) (see Table 1 and Fig. 4). Vertical cells were characterized by the pronounced ventral orientation of their dendritic tree (Fig. 4A). Even if the rostrocaudal extension of the dendritic tree was in many cases still larger than the dorsoventral extension, the dendritic tree clearly descended into lamina II-IV (some of these cells resembled stalked cells). Radial cells had dendrites that extended in several directions with roughly similar rostrocaudal and dorsoventral extent (Fig. 4B). The dendritic trees of islet cells were extremely elongated in the rostrocaudal direction (parallel to the layer borders) and were limited in the dorsoventral and mediolateral directions (Fig. 4C). Distinguishing between islet and central cells is often difficult (Heinke *et al.*, 2004), although it has been reported that the rostrocaudal extent of islet cell dendritic trees is typically $> 400 \mu\text{m}$, and that axons of islet cells are generally limited to the volume of their dendritic trees. Central cells displayed similar morphological characteristics to the islet cells, but had a shorter rostrocaudal dendritic spread (Fig. 4D). We could not assign 15 SG neurons to any of the major groups described above (Fig. 4E). Previous studies have

reported that a relatively high proportion of neurons do not fit any of the recognized morphological categories (Todd & Lewis, 1986; Yasaka *et al.*, 2007).

CA (300 μ M) increased the frequency of sEPSCs in 67% of the vertical cells (10 out of 15), 64% of the radial cells (9 out of 14) and 33% of unclassified cells (5 out of 15) (Fig. 4). However, CA had no effect on sEPSCs in islet ($n = 6$) or central cells ($n = 10$) (note all of these cells showed sEPSCs under basal conditions). In the CA-sensitive cells, there was no difference across the morphological groups in the magnitude of the CA-evoked increase in sEPSC frequency ($272 \pm 56\%$ - vertical, ($n = 10$); $282 \pm 50\%$ - radial, ($n = 9$); and $396 \pm 56\%$ - unclassified, ($n = 5$)) or in amplitude ($141 \pm 20\%$ - vertical, ($n = 10$); $132 \pm 16\%$ - radial, ($n = 9$); and $132 \pm 20\%$ unclassified cell, ($n = 5$)). These results indicate that subpopulations of vertical, radial and unclassified cells selectively receive synaptic inputs from CA-sensitive afferent fibers. The cell bodies of the morphologically classified CA-sensitive cells were located in both lamina Iii (one vertical, 7 radial and one unclassified cells) and Iio (10 vertical, 2 radial and 3 unclassified cells).

On the other hand, most (73%) of SG neurons received excitatory synaptic inputs from Cap-sensitive afferent fibers. Of these Cap-sensitive cells whose anatomy was examined ($n = 15$), 60% of the cell bodies (9 of 15) were in lamina Iii and the remaining 40% were in lamina Iio. Of the Cap-insensitive cells studied ($n = 4$), 50% each were located in lamina Iii and Iio. Cap (1 μ M) increased the frequency of sEPSCs in 83% of the vertical cells (5 out of 6), 80% of the radial cells (4 out of 5), all of the islet cells (2 out of 2), 66% of central cells (2 out of 3) and 66% of unclassified cells (2 out of 3). In the Cap-sensitive cells, Cap increased the frequencies of sEPSC in each cell type ($337 \pm 67\%$ - vertical ($n = 5$); $274 \pm 62\%$ - radial, ($n = 4$); $263 \pm 44\%$ - islet, ($n = 2$); $308 \pm 11\%$ - central, ($n = 2$); and $289 \pm 58\%$ - unclassified, ($n = 2$)). The amplitudes of sEPSCs under the action of Cap were $119 \pm 18\%$ for vertical ($n = 5$), $111 \pm 23\%$ for radial ($n = 4$), $115 \pm 6\%$ for islet ($n = 2$), $107 \pm 1\%$ for central ($n = 2$) and $112 \pm 8\%$ for unclassified ($n = 2$) cells.

Selective inhibitory action of CA on monosynaptic C fiber- but not A δ fiber-evoked EPSCs

Stimulation of the dorsal root attached to spinal cord slices evoked monosynaptic EPSCs with short and/or long latencies in 43 SG neurons tested. These two types of monosynaptic responses were considered as A δ or C fiber-mediated responses on the basis of the conduction velocity of the afferent fibers and stimulus threshold (Fig. 5A). The average conduction velocities of the afferent fibers were 3.6 ± 1.4 m/s for A δ fibers ($n = 21$) and 0.47 ± 0.13 m/s for C fibers ($n = 44$). The threshold stimulus intensities for the monosynaptic A δ fiber- and C fiber-evoked EPSCs were 0.06 ± 0.05 ($n = 21$) and 0.85 ± 0.53 mA ($n = 44$), respectively. These conduction velocities and stimulus intensities were comparable to those observed in previous studies (Yoshimura & Jessell, 1989b; Ito *et al.*, 2000).

CA inhibited the monosynaptic C fiber-evoked EPSCs in 14 of 44 (32%) of neurons tested (Fig. 5A and B). In 7 of those neurons, CA completely inhibited one of the components of the monosynaptic C fiber-evoked EPSC as shown in Fig. 5A. The peak amplitude of monosynaptic C fiber-evoked EPSCs in neurons sensitive to CA was decreased to $46 \pm 26\%$ of control from an average amplitude of 172 ± 64 pA ($n = 14$; Fig. 5C), and recovered to 89

$\pm 7\%$ of control after washout ($n = 14$). The conduction velocities of CA-sensitive and insensitive C fibers which made a monosynaptic input to SG neurons were 0.48 ± 0.11 m/s ($n = 14$) and 0.47 ± 0.11 m/s ($n = 30$), respectively. The stimulus intensities of CA-sensitive and insensitive ones were 0.88 ± 0.64 mA ($n = 14$) and 0.85 ± 0.49 mA ($n = 30$), respectively. There were no significant differences between the groups in the conduction velocities ($F_{13, 29} = 1.42$, $p = 0.14$, $t_{42} = 0.89$; unpaired t -test) and stimulus intensities ($F_{13, 29} = 1.87$, $p = 0.913$, $t_{42} = 0.153$; unpaired t -test). CA (300 μ M) had no effect on the amplitude of A δ fiber-evoked monosynaptic EPSCs in 20 of 21 neurons tested ($95 \pm 18\%$ of control with an average peak amplitude of 134 ± 82 pA, $n = 21$; Fig. 5A and B). The time courses of the inhibitory actions of CA on monosynaptic C fiber-evoked EPSCs and excitatory action on sEPSCs were remarkably similar (Fig. 5D). In SG neurons whose monosynaptic C fiber-mediated EPSCs were inhibited by CA, the frequency of sEPSCs was also increased by CA ($236 \pm 75\%$ of control, $n = 6$). These results suggest that CA mainly acts through TRPA1 receptors expressed on C fibers but not on A δ fibers to reduce the glutamatergic evoked EPSCs.

Lack of effect of CA on spontaneous IPSCs (sIPSCs)

All SG neurons tested exhibited sIPSCs at a holding potential of 0 mV. CA (300 μ M) had no effect on the frequency ($102 \pm 5\%$ of control, $n = 14$) and amplitude ($101 \pm 2\%$ of control, $n = 14$) of sIPSCs. In 5 neurons out of the same neurons tested, CA (300 μ M) still increased the frequency of sEPSCs ($256 \pm 54\%$ of control) at a holding potential of -70 mV. In the remaining 9 neurons, CA had no effect on the frequency of sEPSCs ($105 \pm 7\%$ of control). The sIPSCs were suppressed by either bicuculline (20 μ M, GABA_A antagonist) or strychnine (2 μ M, glycine antagonist), as shown in previous studies (Yoshimura & Nishi, 1993; Kato *et al.*, 2004). In the presence of strychnine, CA (300 μ M) had no effect on GABAergic sIPSCs (either frequency or amplitude, $n = 9$, $p = 0.31$, $t_8 = 0.31$ and $p = 0.72$, $t_8 = 0.59$, respectively) which were completely inhibited by the supplemental addition of bicuculline. Similarly in the presence of bicuculline, there was no effect of CA on the remaining strychnine-sensitive glycinergic sIPSCs (either frequency or amplitude, $p = 0.63$, $t_8 = 0.77$ and $p = 0.23$, $t_8 = 1.89$, respectively; Fig. 6). These results show that CA does not affect inhibitory synaptic transmission onto SG neurons.

CA increase in sEPSPs evokes action potentials in SG neurons

The preceding results show that CA had an excitatory effect on sEPSCs but an inhibitory action on monosynaptic C fiber-evoked EPSCs without affecting inhibitory synaptic transmission. The action of CA was examined under current-clamp conditions in 46 SG neurons to determine the net effect on SG neuronal excitability. All SG neurons exhibited sEPSPs at their resting membrane potential (-62.0 ± 4.7 mV) but did not exhibit spontaneous action potential discharge as shown in our previous study (Yoshimura & Jessell, 1989a). In 33% of neurons tested (15 of 46), CA (300 μ M) reversibly increased the frequency of EPSPs. In 5 of these 15 CA-sensitive neurons, the sEPSPs summated to initiate action potential discharge (Fig. 7A). The average maximum frequency of APs elicited by CA (300 μ M) was 0.94 ± 0.31 Hz ($n = 5$) (Fig. 7B). This indicates that the presynaptic facilitatory action of CA on excitatory synaptic transmission can generate spike discharge in SG neurons.

Discussion

The present study demonstrates for the first time that activation of spinal TRPA1 channels, by CA, presynaptically increases excitatory, but not inhibitory, synaptic transmission onto a restricted subgroup of SG neurons to cause a robust excitation. Dorsal root stimulation revealed that the TRPA1 receptors are located on a subpopulation of C-fiber (but not A δ -) primary afferents. Post-hoc morphological analysis showed that the TRPA1 afferents contact vertical and radial, but not islet or central cells. This means that the sensory information conveyed by CA-sensitive (TRPA1-expressing) afferent fibers is conveyed to a limited group of neurons in the SG, in contrast to more diffuse transmission of the sensory information mediated through Cap-sensitive (TRPV1-expressing) afferent fibers.

Selective presynaptic excitatory effect of CA on synaptic transmission in SG neurons

Application of CA enhanced the frequency of sEPSCs in ~30% of SG neurons, whereas AITC had an excitatory action in ~50% of SG neurons (Fig. 1 and Supporting Information Fig. S1). A previous report studying the actions of these TRPA1 agonists on DRG neurons demonstrated that CA preferentially excites cold-sensitive DRG neurons while AITC activates both cold-sensitive and -insensitive DRG neurons (Bandell *et al.*, 2004). Our data similarly indicate that AITC has effects on a wider group of afferents than CA to alter synaptic transmission onto SG neurons. Indeed, Kosugi *et al.* showed that AITC altered excitatory synaptic transmission to 65% of SG neurons in adult rats, and they showed it also increased the amplitude and frequency of spontaneous IPSCs without affecting miniature IPSCs, suggesting that AITC also excites inhibitory interneurons (Kosugi *et al.*, 2007) in contrast our study showed CA to have no effect on sIPSCs. In addition, CA did not have any excitatory action on islet and central cells, which are thought to be inhibitory interneurons (Maxwell *et al.*, 2007). This more selective action of CA on sEPSCs was consistent with observations made in neonatal spinal cord slices where CA had an excitatory action on sEPSCs in a subpopulation (46%) of lamina I and II (SG) dorsal horn neurons, whereas, a similar action of AITC was detected in all neurons tested (Wrigley *et al.*, 2009).

In the present study analysis of miniature synaptic currents showed CA increased the frequency but not amplitude of mEPSCs, indicating that it has a presynaptic action. This raises the question of which terminals are selectively activated by CA, afferent fibers or spinal interneurons? SG neurons receive excitatory synaptic inputs from both spinal interneurons and afferent fibers. However, the mRNA for TRPA1 is present in small-sized DRG neurons but not in spinal neurons (Kobayashi *et al.*, 2005). Pertinently, TRPA1 is reported to be expressed in the central process of the DRG neurons (Nagatomo & Kubo, 2008). We show that the CA excitatory action was inhibited by the non-selective cation channel blocker, RR, and more specific TRPA1 antagonist (see *Results*), HC-030031, was suppressed in Ca²⁺ free extracellular solution and was not blocked by Cd²⁺ which blocks voltage-activated Ca²⁺ channels. These results support the idea that the presynaptic action of CA is mediated directly via TRPA1 expressed on afferent fibers. Our present results also show that SG neurons receiving CA-sensitive EPSCs were also sensitive to Cap (Fig. 2D); the percentage of CA-sensitive SG neurons (32%) in this study is also comparable to that (30%) of cells in the CA-sensitive DRG neurons in the previous study (Bandell *et al.*, 2004),

suggesting that CA acts on TRPA1-expressing afferent fibers which co-expressed with TRPV1.

Relationship between CA action and morphological features of SG neurons

In our present study, we classified SG neurons into four morphological groups (islet, central, vertical and radial cells) according to the classification scheme proposed by previous studies (Grudt & Perl, 2002; Yasaka *et al.*, 2007). The categorization for SG neurons resembled those described in other previous studies in the rat, cat and hamster (Gobel, 1978; Bicknell & Beal, 1984; Todd & Lewis, 1986). We also classified SG neurons into four firing types (delayed, sustained repetitive, phasic and initial firing types) according to the firing patterns of SG neurons shown previously (Thomson *et al.*, 1989; Grudt & Perl, 2002; Ruscheweyh & Sandkuhler, 2002; Lu & Perl, 2003; Daniele & MacDermott, 2009). The excitatory action of CA was detected in vertical and radial cells, and mostly in delayed and sustained repetitive firing types in addition to phasic firing type. Consistent with the present observations, a previous study exploring the relationship between the morphological class of SG neurons and their firing pattern, has shown that vertical cells exhibit the delayed or sustained firing type, and radial cells exhibit the phasic firing type (Grudt & Perl, 2002). Furthermore, in accordance with the present findings that there was a lack of effect of CA on sEPSCs elicited in all the central cells and most of the initial firing cells, central cells are reported to exhibit the initial firing pattern of discharge. The vertical (so called stalked) and radial cells have been considered to be excitatory interneurons, and islet and central cells as inhibitory interneurons (Todd & Sullivan, 1990; Spike & Todd, 1992). However, recent immunohistochemical studies showed that morphologically identified vertical and radial cells contains not only the vesicular glutamate transporter 2-positive (for glutamatergic) cells, but also glutamate decarboxylase-positive (for GABAergic) cells (Heinke *et al.*, 2004). A study using a transgenic mouse strain co-expressing enhanced green fluorescent protein, eGFP and GABA-synthesizing enzyme, GAD67 has also demonstrated that some of vertical neurons are GABAergic cells (Heinke *et al.*, 2004). On the other hand, all islet cells tested were reported to be GABAergic cells in these immunohistochemical and transgenic animal studies. It remains to be elucidated in detail whether the present CA-sensitive vertical and radial cells are glutamatergic or GABAergic neurons.

Inhibitory effects of CA on C fiber-evoked EPSCs and physiological role of the spinal TRPA1 receptors

In this study, we examined that actions of the selective TRPA1 agonist CA on dorsal root-evoked EPSCs with short and long latencies (classified as A δ and C-fiber-evoked responses, respectively) in adult SG neurons, and clearly show that CA depresses a subgroup of the C fiber-evoked synaptic excitations, but not those mediated through A δ fibers. In some instances, CA completely blocked one of the components of monosynaptic C fiber-evoked EPSCs. Therefore, similar to the case of TRPV1 as previously reported (Yang *et al.*, 1999), we propose that CA depolarizes the presynaptic terminals or axons of C (but not A δ) afferents by activation of TRPA1 expressed on the presynaptic membranes. This depolarizing action both has a facilitatory effect on mEPSCs while may also inhibit the evoked release from C fibers through the inactivation of voltage-gated Na⁺ channels or by shunting of the Na⁺ currents (Willis, 2006). Consistent with this proposal TRPA1 is reported

to be located on the axon of afferent fibers, just like TRPV1 (Tominaga *et al.*, 1998; Nagatomo & Kubo, 2008). Wrigley *et al.* have also reported in immature rat superficial dorsal horn neurons that high doses of icilin, that activates both TRPM8 and TRPA1, inhibited the short latency, dorsal rootlet-evoked EPSCs (Wrigley *et al.*, 2009). Activation of TRPM8 also enhanced the frequency of sEPSCs in SG neurons (Suzuki *et al.*, 2007; Wrigley *et al.*, 2009). These observations suggest that the activation of other TRP channels have similar reciprocal effects on sEPSCs and evoked EPSCs. Alternatively, it is possible that CA induces an increase in glutamate release from TRPA1-expressing C fibers and then the spillover of glutamate activates other receptors such as metabotropic glutamate receptors (mGluRs) to inhibit evoked release from the afferent fibers. We tested this hypothesis and showed that CA (300 μ M) still inhibited the amplitude of monosynaptic C fiber-evoked EPSCs in the presence of the group I mGluR antagonist CPCCOEt (10 μ M) (Park *et al.*, 2004) and group II/III mGluR antagonist LY341495 (100 μ M) (Fitzjohn *et al.*, 1998) ($31 \pm 26\%$ of control, $n = 4$) (Supporting Information Fig. S2). This inhibition did not differ from the control inhibitory action of CA on monosynaptic C fiber-evoked EPSCs ($27 \pm 20\%$ of control, $n = 4$) in the same neurons before examination of the mGluRs antagonists ($p = 0.93$, $t_3 = 0.22$).

In the present study, we cannot determine which sensory modality is conveyed by the CA-sensitive (TRPA1-expressing) afferent fibers. In experiments measuring single unit activities in peripheral nerves of adult rats, the most of the fibers responding to noxious cold were a subset (21%) of C fibers, only 5% of A δ fibers responded (Leem *et al.*, 1993). Although further experiments are needed to define the sensory modalities of TRPA1-expressing afferent fibers, they may functionally be C fibers conveying cold information. Intriguingly, the distribution of C and A δ fiber-evoked EPSCs inhibited by CA in this study (32% of C fiber-evoked and in 4% of A δ -evoked responses) was quite similar to those of the cold receptors *in vivo*.

Although the exact physiological role of the TRPA1 receptors at the spinal level is unknown, intracellular Ca^{2+} has been recently reported to be an endogenous ligand for TRPA1 (Zurborg *et al.*, 2007). Therefore, we speculate that elevation of intracellular Ca^{2+} in the presynaptic terminals might directly activate TRPA1 expressed in the terminals (or axons) in the SG. Intracellular Ca^{2+} concentration in the terminals is increased during presynaptic firing through activation of voltage-gated Ca^{2+} channels. Hence the basal $[\text{Ca}^{2+}]$ may be increased by repetitive presynaptic spike discharge. If action potentials are repetitively propagated to the central terminals of C fibers in response to intense peripheral noxious stimuli or under the condition of peripheral sensitization following inflammation (Dubner & Ruda, 1992), presynaptic TRPA1 in the terminals might be activated by the elevation of intracellular Ca^{2+} . If this is the case, central TRPA1 could serve as a spinal gate of noxious transmission like a low-pass filter; as such TRPA1 inhibits excessive noxious (presumably noxious cold) inputs to SG neurons by blocking the evoked responses, while it excites SG neurons at a lower frequency near 1 Hz by enhancement of miniature release. This modulation by TRPA1 activation in particular types of SG neurons would be a substrate for sophisticated circuits for noxious cold processing.

In conclusion, in the present report we show that the TRPA1 agonist, CA enhances excitatory, but not inhibitory synaptic transmission in specific populations of SG neurons probably through a direct action on the presynaptic terminals. This may constitute a modality specific “labeled line” for noxious cold transmission. Moreover, the blocking effects of TRPA1 activation on C afferent-evoked EPSCs suggests the substrate for a more advanced form of signal processing: a spinal gate of sensory transmission for intense noxious cold stimuli or in the case of peripheral sensitization following tissue damage. Further *in vivo* studies will be necessary to conclusively elucidate the physiological roles of the TRPA1 receptor channels in spinal noxious transmission.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AITC	allyl isothiocyanate
AP	action potential
CA	cinnamaldehyde
Cap	capsaicin
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CPCCOEt	7-(Hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester
DAB	diaminobenzidine
DRG	dorsal root ganglion
EPSC	excitatory postsynaptic current
eEPSC	evoked EPSC
GABA_A	γ-aminobutyric acid type A
HC-030031	2-(1, 3-dimethyl-2, 6-dioxo-1, 2, 3, 6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl)acetamide
IB4	isolectin B4
IPSC	inhibitory postsynaptic current
LY341495	(2S)-2-Amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl)propanoic acid
mEPSC	miniature EPSC
mGluR	metabotropic glutamate receptor

PBS	phosphate-buffered saline
RR	ruthenium red
SG	substantia gelatinosa
sEPSC	spontaneous EPSC
sIPSC	spontaneous IPSC
TTX	tetrodotoxin

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FIG. 1. Dose-dependent effects of cinnamaldehyde (CA) on spontaneous EPSCs (sEPSCs)
 Voltage clamp recordings (holding potential -70mV) of sEPSCs. (A) Representative effects of TRPA1 agonist CA (300 and $500\text{ }\mu\text{M}$) on sEPSCs in the same SG neuron. CA dose-dependently elicited barrages of EPSCs. (B) Histograms of the amplitude distribution of sEPSCs in this neuron during the control period and in the presence of CA ($500\text{ }\mu\text{M}$). Each histogram was constructed from 60 s of continuous recording. Note that the incidence of large amplitude EPSCs was greatly enhanced by CA. (C) Summary data of CA actions on sEPSC frequency and amplitude (** $P < 0.01$). Note that the significant increase in EPSC

frequency was blocked in the presence of RR, in Ca^{2+} free solution and in the presence of HC-030031, however, the Ca^{2+} channel blocker Cd^{2+} was without significant effect. In this and subsequent figures, numbers in parentheses indicate the number of neurons tested, vertical bars show SD.

FIG. 2. Actions of capsaicin (Cap) and CA on sEPSCs in the same SG neurons

(A) Representative SG neuron showing responses to TRPV1 agonist, Cap (1 μ M) and CA (300 μ M) both of which elicited barrages of EPSCs. The lower records are on an expanded time base showing sEPSCs during the control period and in the presence of each agonist. (B) An example of a Cap-sensitive but CA-insensitive SG neuron. (C) SG neuron which did not respond to either Cap or CA. (D) Percentage of neurons which were sensitive to Cap and CA (29%, $n = 14$), sensitive to Cap but insensitive to CA (45%, $n = 22$), and insensitive to both Cap and CA (26%, $n = 13$).

FIG. 3. Firing patterns of SG neurons and action of CA on each firing group

In response to the injection of a depolarizing current pulse the SG neurons exhibited distinctive firing patterns: (A) Delayed firing type. (B) Sustained repetitive firing type. (C) Phasic firing type. (D) Initial firing type. Pooled results under the firing traces in A-D showing the Effect of CA on sEPSC frequency relative to that of control. CA had an excitatory action on firing in all types of neurons.

FIG. 4. Examples of the morphology of neurobiotin-stained SG neurons and action of CA on sEPSCs in each cell type

(A) Vertical cells. (B) Radial cells. (C) Islet cells. (D) Central cells. (E) Unclassified cells. Lower drawings are the *camera lucida* representations of each neuron. Pooled results on the right hand side in A-E showing the frequency of sEPSCs in the presence of CA relative to that of control. Note that CA had an excitatory action on the majority of vertical, radial, and unclassified, but not on islet or central cells.

FIG.5. Actions of CA on monosynaptic A δ and C-fiber evoked EPSCs

(A) This example neuron showed one monosynaptic A δ fiber-evoked EPSC and two monosynaptic C fiber-evoked monosynaptic EPSCs which had distinct threshold stimulus intensities (TSI, shown overlaid in *left panel*) and conduction velocities (CV). As the stimulus intensity was increased, first an A δ fiber EPSC was evoked (TSI: 0.04 mA, CV: 4.3 m/s) followed by a first C fiber response (TSI: 0.27 mA, CV: 0.51 m/s) and then a second C-fiber EPSC at higher stimulus intensity (TSI: 0.68 mA, CV: 0.42 m/s). Application of CA (300 μ M) completely and selectively blocked the second monosynaptic C fiber-evoked

EPSCs (*right panel*). (B) Pooled data from all recordings showing the percentage of evoked EPSCs inhibited by CA showing that its actions were largely mediated on a subgroup of C fiber-afferents. (C) Summary showing proportional decrease in the amplitude of monosynaptic C fiber-evoked EPSCs by CA. (D) CA inhibited the amplitude of monosynaptic C fiber-evoked EPSCs and enhanced the frequency of sEPSCs in the same neuron with remarkably similar time courses. The CA action on C fiber-evoked EPSCs was first examined then after complete recovery the CA was reapplied and the effect on sEPSCs was examined.

FIG. 6. Effects of CA on GABAergic and glycinergic spontaneous IPSCs (sIPSCs)
 (A, B) GABAergic and glycinergic sIPSCs recorded in the presence of strychnine (2 μM) and bicuculline (10 μM), respectively. CA (300 μM) did not change the frequency or amplitude of GABAergic and glycinergic sIPSCs. These GABAergic and glycinergic IPSCs were inhibited by the supplemental application of bicuculline (10 μM) and strychnine (2 μM), respectively at the end of each recording session. (C) Summary showing effects of CA on the frequency and amplitude of GABAergic and glycinergic sIPSCs.

FIG. 7. CA evokes action potential discharge in SG neurons

(A) A continuous current clamp recording of the membrane potential showing the effect of CA (300 μ M) which evoked a barrage of EPSPs and resulted in the generation of action potentials (AP). Arrowhead shows an AP. (B) Time course of the frequency of APs elicited by CA. CA reversibly increased the frequency of APs ($n = 5$).

Table 1
Morphological dimensions of dendritic trees measured from neurons in classified categories

	RC	DV	SD	SV
Vertical	188 ± 35 (15)	99 ± 16 (15)	16 ± 4 (15)	84 ± 16 (15)
Radial	151 ± 34 (14)	92 ± 25 (14)	34 ± 16 (14)	58 ± 16 (14)
Islet	858 ± 325 (6)	55 ± 6 (6)	14 ± 4 (6)	40 ± 4 (6)
Central	236 ± 60 (10)	46 ± 21 (10)	15 ± 4 (10)	31 ± 20 (10)

Data reported as means ± S.D. (µm). Numbers of observations indicated in parentheses. RC, rostrocaudal; DV, dorsoventral; SD, dendritic spread from centre of soma to dorsal end; SV, dendritic spread from centre of soma to ventral end.