Expression of transient receptor potential channels and two-pore potassium channels in subtypes of vagal afferent neurons in rat

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Zhao H, Sprunger LK, Simasko SM. Expression of transient receptor potential channels and two-pore potassium channels in subtypes of vagal afferent neurons in rat. Am J Physiol Gastrointest Liver Physiol 298: G212–G221, 2010. First published December 3, 2009; doi:10.1152/ajpgi.00396.2009.—Vagal afferent neurons relay important information regarding the control of the gastrointestinal system. However, the ionic mechanisms that underlie vagal activation induced by sensory inputs are not completely understood. We postulate that transient receptor potential (TRP) channels and/or two-pore potassium (K2p) channels are targets for activating vagal afferents. In this study we explored the distribution of these channels in vagal afferents by quantitative PCR after a capsaicin treatment to eliminate capsaicin-sensitive neurons, and by single-cell PCR measurements in vagal afferent neurons cultured after retrograde labeling from the stomach or duodenum. We found that TRPC1/3/5/6, TRPV1-4, TRPM8, TRPA1, TWIK2, TRAAK, TREK1, and TASK1/2 were all present in rat nodose ganglia. Both lesion results and single-cell PCR results suggested that TRPA1 and TRPC1 were preferentially expressed in neurons that were either capsaicin sensitive or TRPV1 positive. Expression of TRPM8 varied dynamically after various manipulations, which perhaps explains the disparate results obtained by different investigators. Last, we also examined ion channel distribution with the A-type CCK receptor (CCK-RA) and found there was a significant preference for neurons that express TRAAK to also express CCK-Rα, especially in gut-innervating neurons. These findings, combined with findings from prior studies, demonstrated that background conductances such as TRPC1, TRPA1, and TRAAK are indeed differentially distributed in the nodose ganglia, and not only do they segregate with specific markers, but the degree of overlap is also dependent on the innervation target.

Information carried by vagal afferent nerves is required for proper function of cardiac reflexes, airway dynamics, and gastrointestinal (GI) regulation. These afferent neurons are multimodal, sensing various inputs such as temperature, osmotic stress, chemical, and mechanical stimuli (3). Not only do they contain a broad spectrum of receptive elements, but also individual neurons express multiple neurotransactive substances (57). This suggests that rather than transmitting a simple sensory message at their central terminals, they may be signaling complex responses that integrate numerous peripheral events (57). A deeper understanding of how these signals are generated would be a significant help in understanding the autonomic control of visceral function. A major limitation in understanding the generation of these signals is that the underlying ionic events that lead to activation of these afferent neurons by stimuli that are relevant to particular sensory modalities are not yet completely clear.

Vagal afferent neurons can be broadly categorized as A-type and C-type on the basis of their axonal conduction velocities (17). In addition, A-type and C-type neurons also differ in their expression of tetrodotoxin-sensitive sodium currents (5, 43) and the amount of I_h current they express (14, 45). Closer study of electrophysiological behavior further indicates that there are subgroups within the broad A-type and C-type categories (29, 49). Although the characterization of these voltage-dependent ion currents has led to deep insights into action potential behavior (29), it does not always reveal the mechanisms by which these neurons are activated by sensory relevant stimuli. In the latter case it is usually modification of background or resting conductances that leads to the neuron achieving action potential threshold.

Prime candidates for ion channels that mediate these background conductances are the members of the transient receptor potential (TRP) ion channel family and the two-pore potassium (K2p) channel family. TRP channels are nonselective cation channels that can be activated directly by a wide range of stimuli (e.g., temperature, osmotic, pH, mechanical) or indirectly through events downstream of receptor activation (38). K2p channels carry background potassium currents that are critical for the excitability of neurons and are broadly distributed in the nervous system (47). Both families have been implicated in numerous sensory processes with diverse modes of regulation (7, 13, 30); thus it is reasonable to postulate that they also mediate conductance(s) that are important for activating vagal afferent neurons. Indeed, vagal afferents have long been known to respond to capsaicin (10), and immunohistochemical studies have demonstrated that TRPV1 is expressed in the nodose ganglia, although not at the density observed in the jugular ganglia or dorsal root ganglia (DRG) (35). Other members of the TRP family of channels, such as TRPV1-4 (33, 56), TRPA1 (32, 54), TRPC1 and TRPC3-7 (19), and TRPN1 and TRPM8 (56) have been found in vagal afferents or the nodose ganglia at either the mRNA or protein level. On the other hand, although subtypes of K2p channels have been observed in DRG (11, 26, 39), no studies to our knowledge have documented their presence in vagal afferent neurons. Furthermore, only limited attempts have been made to determine whether subtypes of TRP or K2p channels are segregated into selected subpopulations of vagal afferent neurons.

An increasing body of studies has revealed various functions of TRP and K2p channels in the physiology of the GI tract and colon. TRPV1 has been suggested to play a role in satiety (1), GI motility, and inflammation (18). TRPV4 is mostly relevant to the regulation of acid secretion, foregut motility, and mu-
cosal protection (20). Expression of TRPV5 and TRPV6 in the small intestine and kidney is important for vitamin D-stimulated calcium uptake (50). TRPA1 is required for normal mechanosensation in the GI tract (6), and TRPA1 agonists have been shown to induce contractions in the proximal and distal colon (36). The TRPC family is mostly mechanosensitive, and thus it is not surprising that TRPC4 and TRPC5 are likely to be involved in GI smooth muscle contraction and intestinal motility (48). Relatively less is known about K2p channels, but their roles as sensors for mechanical or chemical stimuli in the GI tract are just emerging (20, 22, 42).

In the present study, we employed two strategies to gain deeper insights into the expression and distribution of TRP and K2p channels in the vagal afferent system. First, we used quantitative reverse transcription (RT)-PCR to compare mRNA abundance of selected TRP and K2p subtypes in the whole nodose ganglia before and after systemic capsaicin treatment. In the second approach, we used single-cell PCR measurements to determine whether specific channel subtypes segregated with TRPV1 or the CCK type-A receptor (CCK-Rₐ) in cells isolated from the nodose ganglia. We further refined this latter technique by selecting cells that were retrogradely labeled from either the stomach or duodenum. We found that TRPC1, TRPC3, TRPC5, TRPV1, TRPM8, TRPA1, TWIK2, TRAAK, TREK1, and TASK1–2 were all present in rat nodose ganglia. Both our lesion results and single-cell PCR results suggested that TRPA1 and TRPC1 were preferentially expressed in neurons that were either capsaicin sensitive or TRPV1 positive. Distribution of TRPM8 was complicated by observations that its expression changed substantially with time after the various manipulations. Finally, we found that the CCK-Rₐ had a slight preference for capsaicin-sensitive or TRPV1-positive neurons and that there was a significant preference for TRAAK expression in gut-innervating neurons that also expressed the CCK-Rₐ receptor, but, otherwise, no specific TRP or K2p channel significantly segregated with the CCK-Rₐ.

**MATERIALS AND METHODS**

*Animals.* We used adult male Sprague-Dawley rats (200–240 g) purchased from Simonsen Laboratories as subjects for all experiments. We housed the animals in Association for Assessment and Accreditation of Laboratory Animal Care-accredited quarters under a 12:12-h light-dark cycle, and they had ad libitum access to pelleted chow and water. The Washington State University Institutional Animal Care and Use Committee approved all procedures performed.

*Capsaicin application.* We performed all capsaicin injections under isoflurane anesthetic (5% induction/2%–3% maintenance) and positive-pressure ventilation. We pretreated animals with 0.1 ml of atropine (Phoenix Pharmaceuticals, St. Joseph, MO), followed by three consecutive intraperitoneal injections of capsaicin (90% grade; Sigma St. Louis, MO) or vehicle control: first injection (25 mg/kg) in the morning, the second injection (50 mg/kg) in the afternoon, and the last injection (50 mg/kg) the following morning. We used the corneal chemosensitivity test to assess functional deafferentation. Briefly, a drop of 0.1% NH₄OH is placed in the eye. Intact rats wipe the eye one to five times within 5 s of placement, whereas capsaicin treated rats do not. We collected both left and right nodose ganglia in successfully deafferented (capsaicin treated) rats 3 days or 2 mo after the last injection, and stored the tissue in RNAlater solution (Ambion, Austin, TX) for future use.

*Retrograde tracer application.* We used the fluorescent dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (30–50 mg/ml; Invitrogen, Carlsbad, CA) to retrogradely label vagal afferent neurons innervating the stomach and proximal duodenum as previously described (44). Briefly, in each tissue we injected DiI in 20 sites (0.5 µl/site) using a microsyringe (Hamilton, Reno, NV). The injection sites formed a grid pattern across the dorsal and ventral surfaces of the stomach and circumferentially around the proximal duodenum, beginning 1–2 mm from the pylorus and extending 20 mm aborally. We always immediately removed any spillage of tracer from the injection site with a cotton-tipped swab. We allowed a minimum of 4 wk before we collected nodose ganglia for culture. When we injected DiI into the abdominal wall in a pattern similar to our stomach and duodenal injections, we never observed cells that expressed fluorescence significantly above background levels of fluorescence, which suggests that our injection protocol does not lead to nonspecific labeling.

*Dissociation and culture of vagal afferent neurons.* We obtained vagal afferent neurons from the nodose ganglia as previously described (44). Briefly, we anesthetized rats with ketamine 25 mg/100 g and xylazine 2.5 mg/100 g and isolated nodose ganglia under aseptic conditions. We then placed the ganglia in HBBS, cleaned the connective tissue, and desheathed the ganglia. We dissociated neurons in 3 ml of digestion buffer (1 mg/ml dispase II and 1 mg/ml collagenase type Ia in HBBS) for 120 min in an incubator at 37°C, then washed dispersed cells with HEPES-buffered DMEM (HDMEM) and plated neurons onto poly-L-lysine-coated coverslips (200 µg/ml poly-lysine for 30 min). We maintained plated neurons in HDMEM supplemented with 10% fetal calf serum and always collected cells for PCR within 24 h of isolation.

*RT-PCR.* We pooled the right and left nodose ganglia and extracted total RNA using RNeAqueous kit (Ambion, Austin, TX) according to the manufacturer’s instructions. After treatment with DNase (Turbo DNA-free Kit, Ambion), we reverse transcribed RNA samples using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA), then amplified cDNA fragments using a Taq kit (Invitrogen, Carlsbad, CA) with sequence-specific oligonucleotide primers designed to reveal genes of interest. Primer sequences are listed in Table 1. We ran PCR products on a 2% agarose gel at 100 V for 30–40 min and then examined results under UV light.

*Quantitative PCR.* In the lesion study we performed probe-based quantitative real-time PCR (qPCR) reactions using Bio-Rad iCycler with the following protocol: polymerase activation at 95°C for 5 min and 45 cycles at 95°C for 20 s followed by incubation at 60°C for 45 s and then 72°C for 1 min. Primers and probes (Roche, Indianapolis, IN) were sequence-specific to each gene. For genes without proper probes, we employed a SYBR Green (Invitrogen) assay using conditions as follows: 50°C for 2 min, 8 min 30 s at 95°C, 40 cycles of 95°C for 15 s and 60°C for 1 min, 1 min at 95°C, 1 min at 55°C, and 80 cycles of increasing temperature by 0.5°C every 10 s to obtain a melt curve to check for primer-dimer artifacts or contamination present in the reaction. We used PGP9.5 (Protein Gene Product 9.5, also known as ubiquitin C-terminal hydrolase 1), a neuronal marker, as the reference gene for all experiments.

*Single-cell PCR.* We collected individual cultured nodose neurons into 10 µl of lysis buffer using sterile glass pipettes and immediately treated single-neuron lystate with DNase I (Cell-to-cDNA II kit, Ambion). After deactivating DNase, we then initiated reverse transcription according to the instruction manual. We prepared single-cell cDNA for conventional PCR reactions as described above. We repeated each run at least twice on different days and only treated assays that were consistently positive as positive observations.

*Statistical analysis.* For the lesion study, we collected real-time PCR results from 11 animals (5 capsaicin-treated rats and 6 controls) and then used GENEX software (Bio-Rad) to analyze the relative gene abundance. We performed Student’s t-test to determine whether the gene expression levels were different between the experimental
Table 1. Primers for quantitative RT-PCR reaction

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<th>Gene</th>
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Fig. 1. Detection of transcripts of selected transient receptor potential (TRP) and two-pore potassium (K2p) channels in the nodose ganglia. Electrophoresis clearly revealed amplification products of the predicted size for TRPC1, TRPC3, TRPC5, TRPC6, TRPV1, TRPV2, TRPV3, TRPV4, TRPM8, TRAAK, TREK1, TASK1, TASK2, TRPA1, and TWIK2 from whole nodose ganglia preparations (NG) but not controls (Neg: parallel prepared without added reverse transcriptase).

and control groups. Data are presented as means ± SE. For the single-cell study, we collected data from ~100–200 cells for each gene and used χ² analysis to determine whether a gene of interest was preferentially distributed to a particular subgroup of neurons. P values < 0.05 are considered significant.

RESULTS

Detection of TRP and K2p transcripts in rat nodose ganglia.
To determine the presence of selected TRP and K2p channels in rat nodose ganglia, we performed RT-PCR on whole nodose ganglia using primers designed specifically against each gene of interest (Table 1). As shown in Fig. 1, electrophoresis of the PCR products from whole nodose ganglia clearly revealed the presence of mRNAs of all channels tested (including TRPC1, TRPC3, TRPC5, TRPC6, TRPV1-4, TRPM8, TRPA1, TWIK2, TREK1, TRAAK, and TASK1-2), whereas none of the negative controls (prepared in parallel without reverse transcriptase) yielded any visible bands, arguing against nonspecific amplifications of mRNAs. Electrophoresis clearly revealed amplification products of the predicted size for TRPC1, TRPC3, TRPC5, TRPC6, TRPV1, TRPV2, TRPV3, TRPV4, TRPM8, TRAAK, TREK1, TASK1, TASK2, TRPA1, and TWIK2 from whole nodose ganglia preparations (NG) but not controls (Neg: parallel prepared without added reverse transcriptase).

Changes in ion channel expression after systemic capsaicin treatment. Capsaicin is a neurotoxin that destroys unmyelinated C-type and thinly myelinated Aδ-type fibers (21). Intraperitoneally administered capsaicin in the manner we used has been previously shown to eliminate significant portions of intestinal innervations (4, 24), as well as the neuronal bodies of the vagal afferent nerves in the nodose ganglia (12). This action is mediated via the TRPV1 ion channel (9). Therefore, in this study after the capsaicin treatment we have compared the changes in mRNA abundance for the genes of interest to the loss of TRPV1 mRNA.

We first compared mRNA abundance for TRPV1, normalized to the neuronal reference gene PGP9.5, between capsaicin- and vehicle-treated animals. As expected, the relative mRNA level of TRPV1 was substantially lower in the capsaicin-treated group; however, ~30% of the mRNA for TRPV1 remained after the capsaicin treatment (Fig. 2a). The loss of 70% of the relative amount of mRNA was the most extreme loss of message observed for any channel following the capsaicin treatment. Other ion channels with a similar loss of message included TRPA1 (Fig. 2b), TRPV2 (Fig. 2c), and TASK1 (Fig. 2d), which suggests that these channel types are expressed predominantly in capsaicin-sensitive neurons.

On the other hand, if the expression of a particular channel relative to PGP9.5 did not change after the lesion, it suggests that the channel was just as likely to be expressed in the neurons lost because of the capsaicin treatment as in the neurons that remained after the capsaicin treatment. Channels that fell into this category include TRPV2 (Fig. 2b), TRPC5 (Fig. 2h), TREK1 (Fig. 2m), TRAAK (Fig. 2n), and TWIK2 (Fig. 2o). Some channels showed a significant decrease in relative expression, but the decrease was statistically less than that observed for TRPV1. We conclude that these channels have an expression pattern slightly preferential for capsaicin-sensitive neurons. They include TRPC1 (Fig. 2f), TRPC3 (Fig. 2g), and TASK2 (Fig. 2f). Finally, there was a group of channels in which the relative expression increased after the capsaicin treatment. These include TRPV3 (Fig. 2c), TRPV4 (Fig. 2d), and TRPM8 (Fig. 2f). An increase in relative abun-
dance would suggest that the channel is preferentially expressed in capsaicin-resistant neurons.

**Distribution of ion channels determined by single-cell PCR.**

We also examined the distribution of various ion channels within subpopulations of nodose neurons through single-cell PCR measurements on isolated neurons. In each neuron tested we always ran two marker genes, TRPV1 and the CCK-RA. We observed evidence for all genes examined in the whole nodose ganglia to be present in single cells (data not shown). However, for several ion channel genes, either the expression level was near the lower limit of detection or the number of positive observations was too few to make reliable conclusions regarding distribution. Thus results for these channels (TRPV2, TRPV3, TRPV4, TRPC5, TREK1, and TWIK2) were excluded from this portion of the study.

**Distribution of TRP channel subtypes in relation to TRPV1.**

In agreement with our lesion study, in neurons that expressed TRPV1, there was a significant coexpression of both TRPA1 and TRPC1 mRNAs (Fig. 3, a and b). TRPA1 message was the most differentially expressed message with 65% of the TRPV1-positive neurons expressed mRNA for TRPA1 whereas only 20% of the TRPV1-negative neurons expressed mRNA for TRPA1. For TRPC1 the segregation was not as great (42% of TRPV1-positive neurons vs. 20% of TRPV1-negative neurons), which is consistent with the less extreme loss of relative expression of TRPC1 after the capsaicin lesion. In addition, we also found that TRPC1 was preferentially found in neurons that innervated the duodenum (Fig. 3, f).

Within this population, there was an even greater tendency for TRPC1 to be found in TRPV1-positive neurons (60% of duodenal TRPV1-positive neurons expressed TRPC1 vs. only 18% of TRPV1-negative neurons; data not shown); however, for TRPA1 the distribution in relation to TRPV1 was the same in neurons innervating the duodenum or stomach as was found for the general population of nodose neurons (data not shown). Finally, the single-cell study revealed an equal distribution of

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**Fig. 2.** Changes in ion channel expression after systemic capsaicin treatments. TRPV1 (a), TRPA1 (e), TRPC6 (i), and TASK1 (k) exhibited the greatest reduction in relative mRNA abundance after capsaicin. The expression levels in experimental group (Cap) relative to control group (Con) were 30 ± 3.9, 29.9 ± 2.8, 29.2 ± 9.2, and 29.0 ± 4.2%, respectively. Genes that decreased but to a lesser extent included TRPC1 (f), TRPC3 (g), and TASK2 (l), which were reduced to 67.8 ± 0.9, 62 ± 2.8, and 72.6 ± 2.3% of control levels after capsaicin, respectively. In contrast, the relative expression levels of TRPV3 (c), TRPV4 (d), and TRPM8 (j) were increased to 300 ± 104, 262 ± 92, and 331 ± 56% of control levels after capsaicin, respectively. Finally, TRPV2 (b), TRPC5 (h), TREK1 (m), TRAAK (n), and TWIK2 (o) were not significantly altered by the treatment. Symbols over data indicate $P$ values of comparisons (t-tests): *significantly different than control, $P < 0.05$; †significantly different than control, $P < 0.01$; ‡significantly less than control but significantly greater than TRPV1, $P < 0.05$. The decrease in TREK1 (m) was of borderline significance, $P = 0.06$. The decrease in TREK1 (m) was of borderline significance, $P = 0.06$. 

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The results for TRPM8 also revealed a discrepancy between the lesion study and the single-cell PCR study data. Rather than a preferential localization to TRPV1-negative neurons as expected from the lesion study, TRPM8 was found to be equally distributed between TRPV1-positive and -negative neurons (Fig. 3d). This suggests that the large increase in TRPM8 after the capsaicin treatment may have been due to upregulation of the gene as a result of tissue reaction to the lesion rather than reflecting the baseline distribution of the channel. To investigate this we examined the TRPM8 distribution in cells 5 h after isolation rather than the usual 24 h. We again found no differences in the percentages of TRPV1-positive and TRPV1-negative neurons expressing TRPM8 (63% of TRPV1-positive vs. 64% of TRPV1-negative at 5 h; 42% of TRPV1-positive vs. 48% of TRPV1-negative at 24 h, data not shown); however, the difference in overall percentage of cells expressing TRPM8 at 5 h vs. 24 h was significant (Fig. 3e). We further explored TRPM8 by comparing the relative expression of TRPM8 in whole nodose ganglia 3 days and 60 days after a capsaicin treatment. In this case, the large increase in relative TRPM8 expression observed at 3 days was not maintained at 60 days, whereas the decrease in TRPV1 was preserved (Fig. 3g). Thus, in both cases, the closer the time of the measurement was to tissue disruption (capsaicin treatment or cell isolation), the greater the amount of TRPM8 expression.

Distribution of K2p channel subtypes in relation to TRPV1. In the single-cell PCR study we obtained reproducible results for three K2p channels: TASK1, TASK2, and TRAAK. Unlike the capsaicin study in which TASK1 and TASK2 were decreased by the capsaicin lesion, the single-cell PCR study did not reveal significant differences in distribution for TASK1 or TASK2 (Fig. 4, a and b). However, there was a significant clustering of TRAAK channels in TRPV1-positive neurons (Fig. 4c), although in the capsaicin lesion study TRAAK had a nonsignificant decrease in relative expression after the lesion (Fig. 2n). Finally, we found a significant preferential expression of TRAAK in neurons that innervated the gastrointestinal tract (Fig. 4d; we found no difference between stomach and duodenum so results from these two targets have been combined).

Ion channel distribution in CCK receptor-positive cells. Another question of interest to us was ion channel distribution in cells that expressed the CCK-R_A. First, we found that CCK-R_A expression was significantly greater in neurons that innervated the stomach compared with those from the duodenum or to random neurons from the nodose ganglia (Fig. 5a). On the other hand, there was only a slight, nonsignificant preference for CCK-R_A expression in TRPV1-positive vs. TRPV1-negative neurons (Fig. 5b). Of all the ion channels examined in the single-cell study (TRPV1, TRPC1, TRPC3, TRPA1, TRPM8, TASK1, TASK2, TRAAK), we only found one, TRAAK, that demonstrated a significantly greater coexpression with CCK-R_A (Fig. 5c). We found that a high percentage of TRAAK-expressing cells also express the CCK-R_A (79%, 44 of 56); however, the proportion of TRAAK-positive cells among CCK-R_A-expressing cells is not as great (47%). When only neurons innervating the duodenum were examined, there was an even higher percentage of TRAAK-expressing cells that also expressed the CCK-R_A (Fig. 5d; 94% of TRAAK expressing neurons also expressed CCK-R_A).
treatment and has been widely used as a marker for C-type cells also express the CCK-RA, especially in neurons that express TRPV1, and that a high percentage of TRAAK-expressing neurons was preferentially found in neurons that express CCK-RA. Moreover, we conclude that TRPC1 is preferentially expressed in capsaicin-sensitive or TRPV1-positive neurons sensitive to capsaicin treatment or those that express TRPA1. Furthermore, we observed a recovery in the number of nodose neurons immunoreactive for TRPV1 by 60%–60 days, whereas in our measurements TRPV1 message continued to be reduced at 60 days. The reason for the difference in these results is not readily apparent. Czaja et al. documented a complex series of events that included both degeneration and neurogenesis. How abundance of TRPV1 message might change within individual cells during these processes is unknown. Nevertheless, the complex degeneration

**DISCUSSION**

In the present study we examined the expression of subtypes of TRP and K2p channels in the nodose ganglia since these channels are promising candidates to carry conductances that are important for vagal afferent functions. In whole nodose ganglia we confirmed that TRPV1-4, TRPA1, TRPC1, TRPC3, TRPC5, TRPC6, and TRPM8 were expressed in this tissue, and we report for the first time that several K2p channels, specifically TASK1, TASK2, TREK1, TRAAK, and TWIK2, are also present in the nodose ganglia. Our results with channel expression after capsaicin lesions or through single-cell PCR measurements indicate that TRPA1 is predominately found in neurons sensitive to capsaicin treatment or those that express TRPV1. Furthermore, we conclude that TRPC1 is preferentially expressed in capsaicin-sensitive or TRPV1-positive neurons, but not to the same extent as TRPA1. TRPM8 had a complicated expression profile that indicated its transcript is likely to be altered by the different manipulations to which the tissue was subjected. Among the K2p channels, we found that TRAAK was preferentially found in neurons that express TRPV1, and that a high percentage of TRAAK-expressing cells also express the CCK-RA, especially in neurons that innervated the duodenum.

**TRPV1 expression.** TRPV1 is the target of the capsaicin treatment and has been widely used as a marker for C-type afferent neurons (29). However, contrary to our expectation, a significant portion of TRPV1 mRNA remained after the capsaicin lesion. Failure to eliminate all TRPV1 expression after capsaicin treatments has also been reported by Czaja et al. (12). They observed that 11% of nodose neurons remaining after capsaicin treatments were TRPV1 immunoreactive, whereas in control conditions ~60% of nodose neurons were immunoreactive for TRPV1. Although the decrease they saw (11 of 60; or to ~17% of control levels) was not in exact concordance with our decrease to 30% of control, these values are not directly comparable because of the differences in techniques (immunohistochemical vs. PCR), and the manner in which values are quantified (percent positive cells vs. total message abundance). Czaja et al. also observed a recovery in the number of nodose neurons immunoreactive for TRPV1 by 30–60 days, whereas in our measurements TRPV1 message continued to be reduced at 60 days. The reason for the difference in these results is not readily apparent. Czaja et al. documented a complex series of events that included both degeneration and neurogenesis. How abundance of TRPV1 message might change within individual cells during these processes is unknown. Nevertheless, the complex degeneration

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**Fig. 4.** Distribution of K2p channels in the nodose ganglia. Single cell PCR did not reveal any preferred expression of TASK-1 (a) or TASK-2 (b) to either TRPV1-positive or TRPV1-negative neurons. Black, cells positive for the gene of interest; gray, cells negative for the gene of interest. a: 94 TRPV1-positive (VI+) neurons tested, 30 TASK1 positive and 64 negative; 33 TRPV1-negative (VI–) neurons tested, 8 TASK1 positive and 25 negative. b: 95 TRPV1-positive neurons tested, 17 TASK2 positive and 78 negative; 32 TRPV1-negative neurons tested, 6 TASK2 positive and 26 negative. c: 102 TRPV1-negative neurons tested, 48 TRAAK positive and 54 negative; 45 TRPV1-negative neurons tested, 8 TRAAK positive and 37 negative. d: A higher percentage of GI-innervating (GI) vagal afferent neurons expressed TRAAK mRNA (30 of 60) than the randomly selected (Ran) neurons (26 of 87). Symbols over data indicate P values of comparisons (χ² tests): *P < 0.05; #P < 0.01.

**Fig. 5.** Distribution of CCK A-type receptor (CCK-Ra) in the nodose ganglia and its coexpression with TRAAK. a: CCK-Ra was preferentially expressed by the stomach-innervating vagal afferent neurons (Sto; 34 of 48 positive), compared with randomly selected neurons (Ran; 61 of 118 positive) or duodenal-innervating neurons (Duo; 20 of 36 positive). b: 141 TRPV1-positive neurons tested, 85 CCK-Ra positive and 56 negative; 60 TRPV1-negative neurons tested, 31 CCK-Ra positive and 29 negative. Black, cells positive for the gene of interest; gray, cells negative for the gene of interest. c: TRAAK coexpression with CCK-Ra: 44 of 94 neurons expressing CCK-Ra expressed TRAAK; whereas only 12 of 41 neurons that did not express CCK-Ra expressed TRAAK. d: Segregation of TRAAK with CCK-Ra appeared to be even greater when restricted to neurons projecting to the duodenum: 16 of 26 CCK-Ra-positive neurons coexpressed TRAAK but only 1 of 10 CCK-Ra-negative neuron coexpressed TRAAK. Symbols over data indicate P values of comparisons (χ² tests): *P < 0.05; #P < 0.01.
events observed by Czaja et al. may indicate that changes after capsaicin treatment need to be interpreted with caution and may underlie disagreements between the lesion study and the single-cell PCR study we observed regarding the expression preference of TRPC3, TASK1, and TASK2.

**TRPA1 expression.** A TRP channel that has received much attention in vagal afferents is the TRPA1 channel. Fajardo et al. (15) reported that ∼50–60% of neurons in the rat nodose ganglia are sensitive to noxious cold and this cold sensitivity can be blocked by HC030031, a TRPA1-selective antagonist. This observation is in agreement with our observation that 53% (99 of 186) of nodose neurons tested expressed TRPA1 mRNA. In addition, Nassenstein et al. (32) reported that 86% of mouse nodose neurons responsive to cinnamondehyde (a TRPA1-selective agonist) also responded to capsaicin, which is in excellent agreement with our finding that 89% of rat nodose neurons that express TRPA1 mRNA also express TRPV1 mRNA. Furthermore, when viewed from the perspective of TRPV1, Nassenstein et al. found that ∼60% of capsaicin-responsive neurons responded to cinnamaldehyde, which agrees closely with our finding that 64% of neurons expressing TRPV1 mRNA express TRPA1 mRNA. On the other hand, in some specific targets innervated by vagal afferents the percentage of C-type neurons that express TRPA1 is much greater than 60–66%. For example, in a study of vagal afferents that innervate the esophagus in guinea pig, Yu and Ouyang (55) found that 15 of 18 (83%) C-type neurons identified by conduction velocity also responded to AITC (a TRPA1 agonist). Nassenstein et al., using a single-cell PCR approach, found that all TRPV1-expressing vagal afferents identified by retrograde tracers as airway innervating also expressed TRPA1. However, in our observations of vagal afferents identified as GI innervating, the percentage of TRPA1-positive neurons was not significantly different from that observed among random nodose neurons. This is consistent with the fact that a majority of vagal afferents innervate GI structures and thus would not expect a large difference between random and GI-specific vagal afferents. In total, these results demonstrate that a high percentage of C-type vagal afferents (or TRPV1-expressing neurons) that innervate structures near the oral end of the alimentary tract or the airways, where exposure to cold is more likely, express TRPA1 and thus imply almost all C-type neurons innervating these structures are responsive to noxious cold. On the other hand, in the GI tract, where thermal homeostasis is well kept under normal circumstances, a significant number of TRPV1-positive neurons do not express TRPA1, a hint that in this area sensing painful cold is less likely to be the primary function of TRPA1. Indeed, Nozawa et al. (34) argues that TRPA1 regulates gastrointestinal motility through serotonin release from enterochromaffin cells.

**TRPC1 expression.** TRPC1 was the first member of the TRPC family to be cloned (51), but knowledge about its distribution and function in the primary sensory system has been rather limited. A prior study using immunohistochemical approach to examine the cell bodies of rat vagal afferent neurons and the peripheral axons targeting the aortic arch revealed that TRPC1 is present in the nodose ganglia, as well as both myelinated and unmyelinated fibers (19). The fact that not all fibers express TRPC1 and/or other TRPC channels examined suggests that specific TRPC proteins might be aligned with subclasses of vagal afferent neurons (19). More recently, Kress et al. (28) reported that TRPC1 immunoreactivity could be detected in a subpopulation of rat DRG neurons. Staining for TRPC1 was reported to be stronger in TRPV1 expressing neurons, but it was also found in neurons that did not express TRPV1. Our data from both the lesion study and the single-cell study are in excellent agreement with these previous studies. We found TRPC1 was expressed in a subpopulation of vagal afferents (∼30%), with a moderate but significant segregation with TRPV1. We also provide novel evidence that TRPC1 mRNA is preferentially expressed by the duodenum-innervating vagal afferents, where the clustering with TRPV1 mRNA was significantly greater than in the general population of nodose neurons. Emerging data have demonstrated diverse roles for TRPC1, such as a mechanosensitive ion channel (23), a store-operated calcium channel (52), or a signal transducer secondary to receptor activation (19). However, more investigations are needed to demonstrate how TRPC1 channels function in vagal afferent neurons. Of special note would be whether TRPC1 has any special role in sensing duodenal signals since we found it is more highly expressed in neurons that innervate the duodenum.

**TRPM8 expression.** Commonly known as the menthol- and noxious cold-sensitive channel, TRPM8 has been the focus of several studies. However, discrepancies exist with regard to its distribution profiles. Story et al. (46), using in situ hybridization, reported that in mouse DRG none of the 95 neurons that expressed TRPM8 mRNA coexpressed TRPV1 mRNA. Kobayashi et al. (27) employed the same technique in rat DRG and found that only 8% of TRPM8-positive neurons were TRPV1 positive, leading to the conclusion that TRPM8 rarely overlaps with TRPV1. In contrast, Babes et al. (2) reported that menthol, a TRPM8 activator, stimulated ∼16% of DRG neurons in culture and that a large fraction of the menthol-sensitive DRG neurons (∼60%) were also sensitive to capsaicin. In lung-innervating jugular/nodose neurons labeled by retrograde markers, Nassenstein et al. (32) found that only 1 of 22 cells examined expressed TRPM8, but that neuron was TRPV1 positive. In contrast to the poor expression of TRPM8 in lung-innervating vagal afferents, we found that a significant percentage of random vagal afferents expressed TRPM8 mRNA. Taken together, these two results suggest that TRPM8 is preferentially found in vagal afferents that do not innervate the lungs. In agreement with Story et al. and Kobayashi et al., our lesion study indicated that TRPM8 was preferentially expressed in capsaicin-insensitive neurons. However, our single-cell PCR study did not agree with our lesion study, and by this approach we found that TRPM8 was just as likely to be present in TRPV1-expressing cells as those that did not express TRPV1. This agrees with the conclusion obtained in DRG neurons by Babes et al. that there is a large overlap between the expression of TRPM8 and TRPV1. Furthermore, we also confirm the findings of Babes et al. that with time in culture the percentage of cells expressing TRPM8 decreased. Our finding that TRPM8 expression varied depending on the conditions under which the tissue was isolated suggests that TRPM8 expression is labile and easily changes under different conditions. This perhaps helps to explain the disparate results obtained by different investigators. One condition that is known to alter TRPM8 expression is the presence of nerve growth factor (2). However, whether nerve growth factor contributes
to the difference we observed between 3 and 60 days after capsaicin treatment remains unknown.

**CCK-R\(_A\) expression.** Many effects of CCK on food intake appear to be mediated by capsaicin-sensitive C-type vagal afferent fibers (40, 41). However, both capsaicin-sensitive and capsaicin-insensitive nodose neurons can be directly activated by CCK (44). Consistent with this previous study, we found CCK-R\(_A\) message in both neurons that express TRPV1 mRNA as well as those do not. Although our lesion study did indicate a significantly greater probability of finding CCK-R\(_A\) in capsaicin-sensitive neurons, the single-cell PCR study indicated only a statistically nonsignificant trend toward expression in a greater percentage of TRPV1-positive neurons. A statistically nonsignificant trend toward more frequent alignment of CCK responses with C-type neurons was also found in a previous electrophysiological study (44). These studies reinforce that the CCK-R\(_A\) is more likely to be expressed in neurons sensitive to capsaicin or expressing TRPV1 but also indicate that a significant portion of neurons insensitive to capsaicin or TRPV1-negative also express CCK-R\(_A\).

On the other hand, we did find CCK-R\(_A\) to be more abundant in gastric vagal afferents, where \(-70\% of neurons tested expressed CCK-R\(_A\) mRNA. Our data are in close agreement with two prior studies. Burdyga et al. (8) reported that among NG neurons projecting to the stomach identified via retrograde labeling, 70–80\% of the population exhibited CCK-R\(_A\) immunoreactivity, and we previously reported by use of single-cell calcium measurements that 74\% of neurons labeled from the stomach responded to CCK (37). In our previous study we also reported that 71\% of duodenal innervating neurons responded to CCK, whereas in the present study we find a value of 60\%. Taken together, our data confirmed that CCK-R\(_A\) is expressed at an elevated level in vagal afferent neurons innervating the stomach, and to a lesser extent, in the duodenum. Whether this prevalence is preserved at the terminals is not known. In addition, although it is likely that the duodenal innervating neurons are responsive to the release of CCK from the I-cells in the mucosa of the duodenum, the physiological role of the CCK-R\(_A\) in gastric-innervating neurons remains unknown.

**K2p channel expression.** Although our lesion study suggested that TASK1 and TASK2 were selectively expressed in capsaicin-sensitive neurons, in the single-cell PCR study we did not find an alignment of these channels with TRPV1 message. However, by the use of single-cell PCR we did find TRAAK, a TWIK-related arachidonic acid-stimulated K channel, to be selectively expressed in TRPV1-positive neurons, and the lesion study also indicated a trend toward preference for expression in capsaicin-sensitive neurons. Although our report is the first to demonstrate TRAAK expression in the nodose ganglia, TRAAK has been characterized by immunohistochemistry in the trigeminal ganglia (53). Yamamoto et al. (53) reported that 60\% of the trigeminal neurons are positive for TRAAK, whereas we find TRAAK expression in 30\% of nodose neurons overall, increasing to 50\% among gastrointestinal innervating neurons. We find that 47\% of TRPV1 expressing neurons also express TRAAK, similar to the 41\% reported by Yamamoto et al. In contrast, we found that almost all TRAAK-positive neurons to also express TRPV1 (86\%), whereas Yamamoto reported that 53\% of TRAAK-positive neurons did not express TRPV1. Of all the channels we examined, TRAAK was the only one that demonstrated a marked segregation with CCK-R\(_A\) (79\% of TRAAK-positive neurons express CCK-R\(_A\), and the percentage increases to 94\% among neurons innervating the duodenum). The activity of TRAAK currents is strongly increased by arachidonic acid as well as other polyunsaturated fatty acids (16). In addition, as a member belonging to the TREK family, TRAAK is also mechano- and temperature gated (25, 31). Given these unique and versatile modes of activation, our finding that TRAAK tends to colocalize with CCK-R\(_A\), especially in the duodenum-innervating vagal afferents, raises the possibility that either it is involved in the signal transduction of CCK in the duodenum or it facilitates the peripheral integration of the information carried by CCK with other modalities.

**Conclusion.** Several important questions that need to be answered to understand the ionic mechanisms by which sensory inputs activate vagal afferent neurons include: 1) whether specific ionic conductance(s) are differentially distributed within different phenotypes of vagal afferent neurons, 2) whether certain ionic conductances align with particular sensory functions, and 3) whether specific ionic conductances are associated with specific innervation targets. In this study we used quantitative PCR in conjunction with capsaicin-induced lesions as well as expression of TRPV1 mRNA by single-cell PCR to investigate differential ion channel expression in subtypes of nodose neurons, and we also investigated coexpression of specific channels with the CCK-R\(_A\) since CCK is an intestinal hormone in which activation of vagal afferents is an important component of its physiological actions. Our lesion study and single-cell PCR study confirmed several prior studies (32, 46) that TRPA1 is predominantly expressed by neurons sensitive to capsaicin or expressing TRPV1; however, unlike prior studies in the esophagus and airways in which the concordance of TRPA1 and C-type neurons (or neurons that express TRPV1 or respond to capsaicin) is very high (\(>80\%\)), the degree of association of TRPA1 with this phenotype of neurons in the general nodose population, and also those innervating GI structures, is not as great (\(~66\%)\). Our data also revealed a moderate yet significant preferential expression of TRPC1 in TRPV1-positive neurons, especially in the duodenal vagal afferents. The only channel we found that was significantly associated with the CCK-R\(_A\) was TRAAK, with the most significant coexpression found in duodenum-innervating nodose neurons in which almost all TRAAK-expressing neurons also expressed the CCK-R\(_A\). This suggests a possible involvement of TRAAK in the physiological actions of CCK released in the duodenum. Finally, our data clearly indicated that expression of TPMR8 could be dynamically modified in response to trauma or tissue damage, which should be taken into consideration for future research involving in vitro manipulations. These findings demonstrated that background conductances such as TRPC1, TRPA1, and TRAAK are indeed differentially distributed in subpopulations of nodose neurons, and not only do they segregate with specific markers, but the degree of overlap is also dependent on the innervation target. A limitation of our studies is that we only examine mRNA expression in the cell body. These studies need to be extended by immunohistochemical and functional approaches to assess whether and how differences in message expression result in significant differences in cellular actions, and, furthermore, whether the segregation we observed is restricted to the cell.
body or is also observed in the nerve terminals. Finally, the significance of these linkages needs to be confirmed in physiological studies with specific channel blockers or genetic manipulations of channel expression.

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DISCLOSURES
No conflicts of interest are declared by the author(s).

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