

Extracellular Protons Both Increase the Activity and Reduce the Conductance of Capsaicin-Gated Channels

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Capsaicin evokes a membrane current in trigeminal ganglion neurons that is increased substantially in a moderately acidic extracellular environment. Using excised outside-out membrane patches, we studied the mechanism by which protons enhance the sustained response to capsaicin. In the absence of capsaicin, extracellular exposure to a moderately acidic physiological solution (pH 6.6) did not result in sustained channel openings in any capsaicin-sensitive outside-out patches. When co-applied with capsaicin, the acidic extracellular solution greatly increased the probability of capsaicin-gated channels

being in the open state. In addition, acidic extracellular solution appeared to increase the number of channels available to be opened by capsaicin. The amplitude of the unitary currents was reduced by the acidic extracellular solution. These results show that the proton enhancement of the capsaicin-evoked whole-cell excitatory current is attributable to proton-receptive site(s) causing a marked increase in the activity of capsaicin-gated channels.

Key words: vanilloid; pH; sensory; irritant; patch-clamp; trigeminal ganglion; pain; hyperalgesia

Capsaicin, the active principle in hot (*Capsicum*) peppers, is eaten as a spice daily by approximately one-fourth of the world's population (Rozin, 1990) and elicits a painful burning sensation in the mouth (Lawless, 1984; Lawless and Gillette, 1985; Green, 1991). Capsaicin excites nociceptive neurons (Szolcsányi et al., 1988; Baumann et al., 1991; LaMotte et al., 1992). The mechanism by which capsaicin excites nociceptive neurons has been studied in dissociated cell cultures of trigeminal ganglion (TG) and dorsal root ganglion (DRG) neurons (Bevan and Forbes, 1988; Ingram et al., 1993; Vlachová and Vyklický, 1993; Liu and Simon, 1994; Martenson et al., 1994; Baumann et al., 1996; Oh et al., 1996). Many cultured TG neurons respond to capsaicin with a slowly activating, prolonged current (Ingram et al., 1993; Liu and Simon, 1996), whereas others show rapidly inactivating membrane currents (Liu and Simon, 1994). Studies in DRG neurons indicate that the sustained currents evoked by capsaicin are caused by the opening of nonselective cation channels (Bevan and Forbes, 1988) with a slope conductance of ~ 45 pS (at -60 mV) (Oh et al., 1996). A channel that is believed to be responsible for the response of DRG and TG neurons to capsaicin has been cloned and named VR1 (Caterina et al., 1997).

Previous whole-cell patch-clamp studies (Petersen and LaMotte, 1993; Martenson et al., 1994; Baumann and Martenson, 1995; Kress et al., 1996) demonstrated that moderately acidic solutions strongly enhance the response of TG and DRG neurons to capsaicin. We also observed (Baumann and Martenson, 1995)

that the low-pH-enhanced, capsaicin-gated currents are blocked by capsazepine, a competitive capsaicin antagonist (Walpole et al., 1994), even though proton gating (pH ≤ 6.2 , in the nominal absence of capsaicin) of a channel believed to be linked to the capsaicin receptor is not blocked by capsazepine (Bevan and Yeats, 1991). Furthermore, a study using VR1-transfected HEK293 cells demonstrated that protons shift the concentration-response relationship for capsaicin-evoked whole-cell currents to the left (Tomimaga et al., 1998). Taken together, these observations strongly suggest that moderate acidity may enhance capsaicin-evoked currents by modulating transduction through the capsaicin receptor-channel complex.

In the present study we examined unitary currents of capsaicin-gated channels to elucidate how acidic conditions potentiate the response of TG neurons to capsaicin. We tested the possibility that protons increase the unitary conductance of the capsaicin-gated channel, the proportion of time the channel spends in the open state, or the number of channels available to be opened by capsaicin. The results of this study show that potentiation in a moderately acidic extracellular environment is not explained by an increase in the conductance of channels gated by capsaicin.

Preliminary results have been published previously in abstract form (Baumann and Martenson, 1997, 1999).

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MATERIALS AND METHODS

Preparation. Three adult male Sprague Dawley rats were killed with an intraperitoneal injection of 1.5 ml of sodium pentobarbital (Abbott, Chicago, IL). The procedure was approved by the Institutional Animal Care and Use Committee. The trigeminal ganglia were dissected, and dispersed cultures were prepared as described previously (Baumann, 1993). Briefly, the ganglia were minced and allowed to dissociate in a mixture of 0.1% (w/v) trypsin (type III; Sigma, St. Louis, MO), 0.1% collagenase (type IIS; Sigma), and 0.01% DNase (type IV; Sigma) for 30–60 min at 37°C. The dispersed cells were collected in a solution containing 0.2% soybean trypsin inhibitor (type IIS; Sigma), 0.1% bovine serum albumin (fraction V; Sigma) and 10% fetal calf serum (Hyclone, Logan, UT), washed by centrifugation, and suspended in L-15/air growth medium (Life Technologies, Grand Island, NY) containing supplements, nerve growth factor (mouse, 7S; Chemicon, Temecula, CA), and equine serum (Hyclone). The cell suspension was plated onto poly-L-lysine-coated glass coverslips, and the cultures were maintained in an incubator at 37°C.

Recording. Standard methods (Hamill et al., 1981) were used to establish gigaseals and to record unitary currents in outside-out membrane patches. Microelectrodes were pulled from thick-wall borosilicate glass (Sutter Instruments, Novato, CA) using a two-stage puller (PB-7; Narishige, Tokyo, Japan) and had an average resistance of 18 M Ω when filled with the recording solution. Electrode tips were coated with Sylgard (Dow-Corning, Midland, MI). All recordings were made using an amplifier with capacitive feedback (Axopatch 200A; Axon Instruments, Foster City, CA; or Dagan 3900; Dagan Corp., Minneapolis, MN). Responses to chemical stimulation were measured under voltage-clamp conditions (at a holding potential of -50 mV). Amplified signals were low-pass-filtered at 2 kHz with an eight-pole Bessel filter (model 902; Frequency Devices, Haverhill, MA), digitized at 10 kHz (using a TL-1 interface and pClamp 6.0.3 software; Axon Instruments), and stored directly on the hard disk of a laboratory computer.

Bath solutions (temperature 20–23°C) were supplied by gravity via a glass pipe placed ~ 100 μ m from the patch under study. The solutions ran continuously (flow rate, ~ 0.4 ml/min) and were changed by manually operating a six-way valve connected to different supply barrels. A remote suction device was used to maintain the fluid volume in the recording chamber at ~ 0.6 ml. The possibility of inadvertent desensitization or sensitization by previous application of chemical stimuli was avoided by studying only one patch per coverslip. The recording chamber was flushed thoroughly between experiments.

Solutions. All recording solutions were made from distilled, deionized water and filtered through 0.2- μ m-pore size filters (Millipore, Bedford, MA). Electrodes were filled with internal solution of the following composition (in mM): 119 potassium gluconate, 9.3 NaCl, 4.6 MgCl₂, 4.6 EGTA, 4.6 Na₂ATP, 0.4 Na₃GTP, and 18.5 HEPES, pH adjusted to 7.2 with 1N KOH. Tight seals were established on neurons bathed in balanced salt solution (BSS) of the following composition (in mM): 127.7 NaCl, 4.6 KCl, 0.9 CaCl₂ (free Ca²⁺ = 0.8), 1.1 MgSO₄, 1.9 H₃PO₄, 5.1 glucose, 14.8 HEPES, and 14.8 [2-(N-morpholino)-ethanesulfonic acid], pH adjusted to 7.35 with 1N NaOH. Acidified BSS solutions, pH 6.6, were prepared by adding 1N HCl to BSS. Sodium activities of the BSS and acidified BSS solutions were 145 and 144 mM, respectively. The osmolarities of the BSS and internal solutions were 295 and 285 mOsm, respectively.

Capsaicin (Fluka, Buchs, Switzerland; natural capsaicin) stock solutions were made in 98% ethanol and stored at -20°C . Capsaicin-containing BSS solutions (100 nM capsaicin) were prepared by dissolving 9.2 μ l of capsaicin stock solution (0.545 mM) in 50 ml of BSS or acidified BSS. HPLC was used to verify capsaicin concentrations in BSS. Dihydrocapsaicin accounted for 34% of total capsaicin, in accordance with the supplier's specifications. Nominally 100 nM capsaicin solution contained 92 nM total capsaicin. Samples collected during a simulated experiment indicated that the total capsaicin concentration that reached the recording chamber was even lower (83.5 nM). Thus capsaicin appeared to adhere to the plastic vessels and tubing used in the perfusion setup. However, control experiments showed that acidified BSS solutions did not leach enough capsaicin from the perfusion setup to account for the strong increase in open channel probability. Capsaicin concentrations presented in the text and figures are nominal concentrations.

Data analysis. Digitized records of unitary channel activity were scanned visually, and artifacts attributable to resetting of the capacitive feedback integrator of the patch-clamp amplifier were removed using the program Clampfit (pClamp 6.0.3; Axon Instruments). Mean-variance

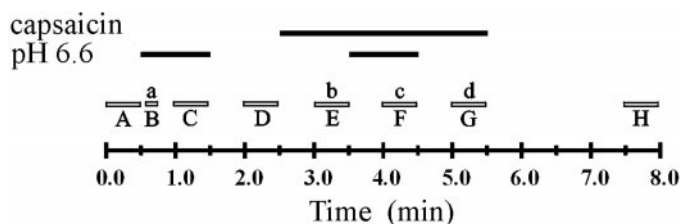


Figure 1. Stimulus presentation paradigm. Chemical stimuli were balanced salt solutions of physiological pH (7.35) and increased acidity (pH 6.6) with or without capsaicin. Dark horizontal bars show the timing of stimulus application. Open bars A–H indicate periods for which mean-variance histograms (Fig. 3) were constructed. Open bars a–d give the timing of raw data traces in Figure 2.

histograms (Patlak, 1993) were generated, and unitary current amplitudes were measured using a program (mvMachine, version 3.0; kindly provided by Dr. J. B. Patlak, University of Vermont, Burlington, VT). Mean-variance histograms were also used to measure the proportion of time that a certain number of channels were open simultaneously in the presence of a given stimulus.

The “most likely” number (N) of capsaicin-gated channels present in a given patch and their open probability (p_o) were estimated by maximizing the logit function $\ln[p_o(1 - p_o)^{-1}]$ separately for each patch and stimulus condition (capsaicin at pH 7.35 and 6.6) using the program O-matrix (Harmonic Software, Seattle, WA). For each particular patch and stimulus condition, N was set greater than or equal to the maximum observed number of simultaneously open channels, and p_o was allowed to be the free parameter. Optimization was done under the assumptions that the proportions of time spent at the different open levels conformed to a binomial distribution and that p_o was identical for all capsaicin-gated channels in a given patch under the given stimulus condition.

Descriptive statistics are reported as means \pm SD. Statistical comparisons of unitary current amplitudes were made using the t test for related measures (Zar, 1984).

RESULTS

Moderate acidity facilitates the sustained response to capsaicin

Experiments were performed on 41 outside-out patches taken from cultured adult rat TG neurons (one patch per neuron) after 1–14 d in culture (5 ± 4 d). The low-pH and capsaicin stimuli were applied first in isolation, and then the two stimuli were combined, according to the experimental paradigm illustrated in Figure 1.

Figure 2 shows the response of an outside-out patch to a moderately acidic stimulus (pH 6.6) and capsaicin (100 nM). As expected, based on previous studies (Krishtal and Pidoplichko, 1981; Kovalchuk et al., 1990), the acidic stimulus alone caused a transient activation of ion channels. The amplitude of the transient unitary current was relatively small (approximately -1.1 pA). The activity subsided within 500 msec (Fig. 2, trace a). Subsequent application of capsaicin at physiological pH (7.35) evoked several brief openings of channels with a larger unitary amplitude (-2.3 pA) and occasional bursts of channel openings, which occurred throughout the application of the capsaicin stimulus (Fig. 2, trace b). Lowering the pH of the bathing solution to 6.6 during continued application of the capsaicin stimulus caused a dramatic increase in the activity of the larger-amplitude channels (trace c). The increase was sustained for the duration of the combined stimulus (60 sec) but subsided quickly (within 1–2 sec; not illustrated) after removal of the low-pH stimulus (trace d).

Mean-variance histograms (Patlak, 1993) were used to show the trajectories of transitions between states and to determine the proportion of time one or more channels were in the open state (Fig. 3). Before the application of chemical stimuli there was

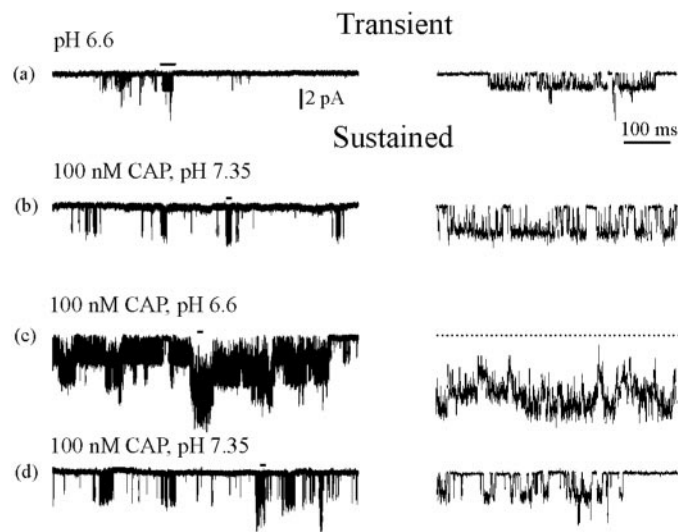


Figure 2. Activation of unitary currents by capsaicin (CAP) and low pH. Voltage-clamp recording from an outside-out patch taken from a cultured adult rat trigeminal ganglion neuron (membrane voltage = -50 mV) is shown. Traces on the left were collected during the times indicated by corresponding open bars (a–d) in Figure 1. Note the difference in the time scale for trace a (transient current evoked by low pH) and traces b–d (sustained currents evoked by capsaicin at normal and low pH). All horizontal bars on the left represent 500 msec. Traces in the right column are expanded sections of the data (corresponding to the short horizontal bars above the traces on the left). The horizontal bar on the right represents 100 msec for all traces on the right. The dashed line denotes zero current level. The vertical bar indicates 2 pA and applies to all traces.

virtually no background activity in the patch. Acidified solution (pH 6.6) caused the transient appearance of unitary currents with an amplitude of -1.1 pA. One or occasionally two channels were fully open at the same time (Fig. 3B). There was no sustained channel activity during the latter portion of the low-pH stimulus (Fig. 3C) or during the wash period (Fig. 3D). Capsaicin at physiological pH evoked unitary currents with an amplitude of -2.3 pA, but the channel activity was relatively low (Fig. 3E; $N \cdot p_o = 0.03$). Activity increased drastically when the acidity of the capsaicin solution was augmented (Fig. 3F; $N \cdot p_o = 0.91$). Although only one channel was open (at any given time) at physiological pH, up to four channels were open simultaneously at the lower pH. The amplitude of the unitary current, however, was reduced to -1.8 pA. With return to capsaicin at pH 7.35, channel hyperactivity subsided (Fig. 3G; $N \cdot p_o = 0.05$), and the amplitude of the unitary current returned to the level seen in Figure 3E. All remaining activity returned to prestimulus levels after subsequent washout of capsaicin (Fig. 3H).

Identical experiments were performed with all 41 outside-out patches. Most patches (30 of 41) did not respond to capsaicin at either pH. Strong upmodulation of single-channel activity by the low pH solution was evident in 8 of 11 patches that were excited by 100 nM capsaicin at pH 7.35. (In two patches, the response to capsaicin desensitized too quickly to allow upmodulation to be observed; response in the remaining capsaicin-sensitive patch was too complex for interpretation.) In six of eight patches that showed unambiguous upmodulation of the capsaicin response at low pH, background activity of other channels prevented quantitative determination of the proportion of time during which a given number of capsaicin-gated channels were open simultaneously. Thus, quantitative estimates of p_o and N were possible in

only two of the patches that responded to 100 nM capsaicin and showed potentiation of the response at pH 6.6.

Estimates of N and p_o

Table 1 shows the log-likelihood estimates of the number of capsaicin-gated channels and their open probability at different pH levels. Low pH increased the maximum number of sustained unitary current levels observed, and the most likely estimate of the number of channels in a patch during the stimulus was equal to the maximum number of sustained open levels observed for that particular stimulus and patch. Thus a moderately acidic extracellular environment increased the number of channels available to be gated by capsaicin. In addition, log-likelihood calculations showed that protons increased p_o substantially.

In summary, capsaicin applied alone evoked unitary currents with an amplitude of -2.5 ± 0.2 pA (mean value for those patches in which the unitary amplitude could be measured reliably; $n = 6$). The openings of such channels were observed during the entire duration of the capsaicin stimulus. Acidified physiological solution (pH 6.6) applied alone caused only a transient appearance of unitary currents in 17 of 41 patches (-1.3 ± 0.2 pA in amplitude; $n = 5$) and, in the present experiments, never elicited opening of ion channels during the later stage of the stimulus. The same acidic solution caused a substantial increase in the open probability of capsaicin-gated channels and the number of channels gated by capsaicin but at the same time significantly reduced the apparent amplitude of the unitary currents to -1.9 ± 0.2 pA ($n = 6$; $p < 0.005$, one-tailed) (Baumann and Martenson, 1997).

DISCUSSION

Previous whole-cell recordings demonstrated that acidic solutions strongly enhance the sustained response of TG and DRG neurons to capsaicin (Petersen and LaMotte, 1993; Martenson et al., 1994; Baumann and Martenson, 1995; Kress et al., 1996). A similar increase of the response to capsaicin by protons has also been observed in non-neuronal cells transfected with the VR1 vanilloid receptor (Caterina et al., 1997; Tominaga et al., 1998). The present study demonstrates that the sustained increase in capsaicin-evoked currents observed in whole-cell recordings is attributable to a proton-dependent increase in the activity of capsaicin-gated channels. Concomitant with the large increase in activity, an increase in the extracellular concentration of protons also causes a decrease in unitary conductance of the capsaicin-gated channels. Because the increase in channel activity is substantial ($N \cdot p_o$ increased by an average factor of 24 in the two patches where it could be measured), whereas the decrease in unitary conductance is relatively small ($\sim 24\%$), the overall effect of extracellular protons is to increase the total amount of charge carried by the capsaicin-gated channel, thus explaining the effect of protons seen at the whole-cell level.

A mechanistic explanation of the observed changes in activity will have to await further experiments. A change in channel availability could result from either greater accessibility of capsaicin receptor sites, as a result of protonation-induced conformational changes, or from pH-dependent insertion or aggregation of capsaicin receptor–channel subunits in the membrane. An increase in open probability (i.e., change in the kinetics) is likely to be caused by protonation of the channel protein.

Reduction of unitary current amplitude

The amplitude of capsaicin-evoked single-channel current (-2.5 ± 0.2 pA) is in agreement with the values reported by

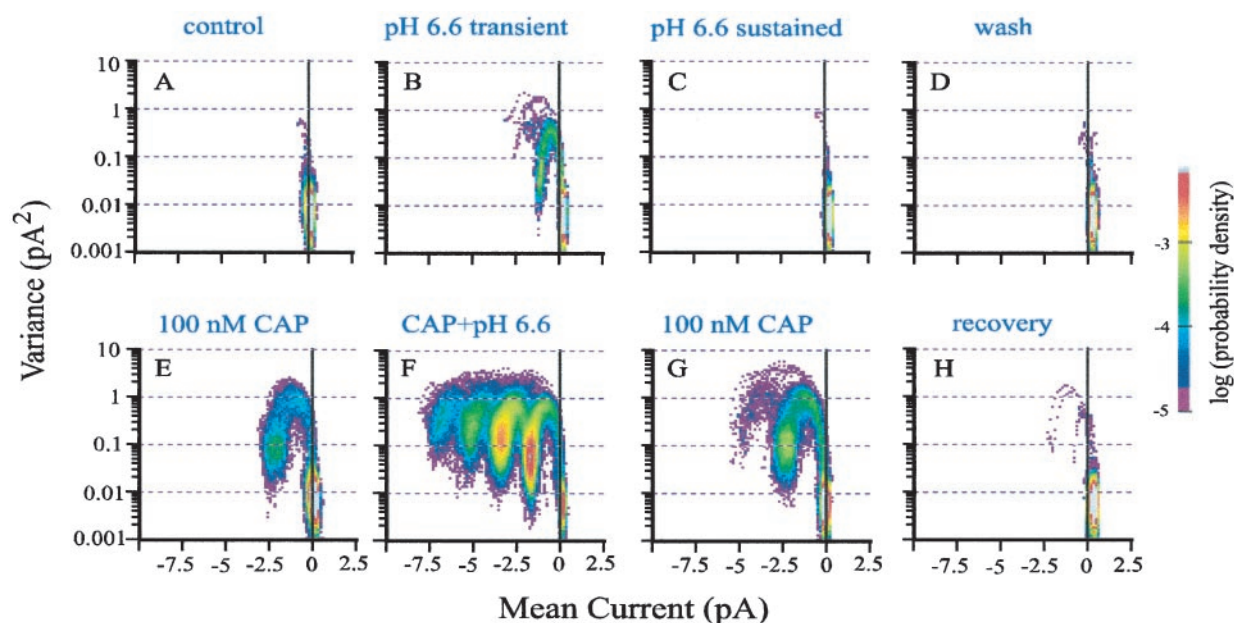


Figure 3. Mean-variance histograms showing modulation of open-channel probability and unitary current amplitude. *A*, Control. *B*, Transient response to the acid stimulus (pH 6.6). *C*, Absence of a sustained response to the low-pH stimulus. *D*, Wash. *E*, Currents evoked by 100 nM capsaicin, pH 7.35. *F*, Response to capsaicin, pH 6.6 (note multilevel openings). *G*, Response to capsaicin after removal of the low-pH stimulus. *H*, Recovery after the removal of both the low-pH and capsaicin stimuli. Each histogram (*A–H*) contains data collected during the times indicated by the corresponding *open horizontal bars* in Figure 1 (membrane voltage = -50 mV). Histograms *B* and *E–G* contain the same data as raw *traces a–d* in Figure 2.

Table 1. Estimates of the number of channels and their open probability

Stimulus		Patch no.	Max. no. of levels observed	N assumed	p_o estimated \pm ASE	$L(\theta)$
Capsaicin (100 nM)	pH					
7.35	1 ^a	1	1	1	0.031 ± 0.012	$-26,208$
			4	4	0.008 ± 0.013	$-34,455$
			5	5	0.006 ± 0.013	$-35,775$
6.6	4	4	4	4	0.333 ± 0.001	$-501,383$
			5	5	0.267 ± 0.001	$-571,100$
7.35	2	2	2	2	0.062 ± 0.005	$-104,580$
			3	3	0.041 ± 0.006	$-116,184$
			4	4	0.031 ± 0.006	$-124,353$
6.6	3	3	3	3	0.260 ± 0.002	$-339,853$
			4	4	0.195 ± 0.002	$-390,095$

Estimates were made during the 30 sec periods marked *E* and *F* in Figure 1. $L(\theta)$, Log likelihood; ASE, asymptotic SE.

^a Same patch as in Figures 2 and 3.

others (Vlachová and Vyklický, 1993; Oh et al., 1996; Lopshire and Nicol, 1998). The reduction in amplitude of the unitary current by the acidified extracellular solution was not entirely unexpected, because capsaicin-gated channels are nonselective cation channels (Oh et al., 1996), and protons are known to block cation channels (Hille, 1992; Root and MacKinnon, 1994). Because sodium activities of the BSS and acidified BSS solutions were nearly identical, the reduction in the unitary current amplitude was not simply a reflection of a reduced concentration gradient of a permeant ion as a result of buffer titration.

Activation of channels by protons in the absence of capsaicin

Acidified physiological solution (pH 6.6) applied alone caused only a transient appearance of unitary currents in outside-out patches. Such currents were observed in 17 of 41 outside-out patches. Although we did not perform any pharmacological or ion substitution experiments to further characterize these channels, the amplitude of unitary currents and the presence of mRNA for acid-sensing ion channel 1 (ASIC1) and ASIC3 in intact trigeminal ganglia (Liu et al., 1998; P. Chaudhary and T. K. Baumann, unpublished data) are consistent with the idea that these channels are related to the recently cloned (Waldmann et al., 1999) ASICs.

Functional relevance of increase in capsaicin-gated channel activity by protons

The proton enhancement of vanilloid channel open probability and availability shown in the present study is of considerable interest, because it takes place at moderate levels of acidity, well above the pH level that would interfere significantly with neuronal excitability (Baumann et al., 1996) by blocking voltage-gated sodium currents (Drouin and Neumcke, 1969; Woodhull, 1973; Iijima et al., 1986; Tombaugh and Somjen, 1996). It has been reported (Kress et al., 1997) that inflammatory mediators and protons interact to activate the same ion channels as capsaicin; therefore, the increase in vanilloid channel open probability by protons is likely to play a significant role in sustained pain and hyperalgesia caused by acidosis in inflamed or ischemic tissues.

The proton enhancement of trigeminal responses to vanilloids may also be of culinary relevance, because many ethnic foods contain capsaicin and/or other vanilloids in combination with acidic ingredients such as lemon juice and vinegar (Baylón, 1960; Creen, 1994). Based on the results of the present study, one would expect capsaicin-induced oral irritation to be more pronounced

in the presence of the acidic ingredients, provided that the acids manage to penetrate the oral mucosa and reduce the extracellular pH near capsaicin-sensitive trigeminal nerve endings.

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