Abstract—Sensory neurons that innervate the heart sense ischemia and mediate angina. To use patch-clamp methods to study ion channels on these cells, we fluorescently labeled cardiac sensory neurons (CSNs) in rats so that they could later be identified in dissociated primary culture of either nodose or dorsal root ganglia (DRG). Currents evoked by a variety of different agonists imply the importance of lowered pH (≤7.0) in signaling ischemia. Acidic pH evoked extremely large depolarizing current in almost all cardiac afferent neurons from the DRG (CDRGNs). In contrast, only about half of the unlabeled DRG neurons responded to acid, and their current amplitudes were much less than that in CDRGNs. In all respects tested—kinetics, selectivity, and pharmacology—the acid-evoked current was similar to that of previously described native and cloned acid-sensing ion channels. Cardiac afferents from the nodose ganglia differed from CDRGNs in having smaller acid-evoked currents but clearly larger currents evoked by ATP. Serotonin, acetylcholine, bradykinin, and adenosine elicited currents in fewer CSNs than did ATP or lowered pH, and the currents were relatively small. Capsaicin, an activator of small nociceptive sensory neurons that innervate skin, evoked only small and rare currents in CDRGNs. The extremely large amplitude and prevalent expression of acid-evoked current in CSNs imply a critical role for acidity in sensation associated with myocardial ischemia. (Circ Res. 1999;84:921-928.)

Key Words: myocardial ischemia • cardiac sensory neuron • proton • whole-cell patch clamp

Although much is understood about the effect of autonomic nervous system input to the heart, the cardiac sensory, or afferent, system and its role in physiological and pathological conditions are less well understood. Historically, the major impetus for research on the cardiac sensory system has been to find the source of cardiac pain, or angina. During the first half of this century, the neuroanatomical pathways of the cardiac sensory system were defined by clinical reports of surgical attempts to relieve angina and by experimental studies. These studies revealed that the cardiac sensory neurons (CSNs) follow the sympathetic and vagal nerve tracts en route to the central nervous system (Figure 1). The cell bodies of those sensory axons following the sympathetic tracts are found in the upper thoracic dorsal root ganglia (DRG); those following the vagal tracts are located in the nodose ganglia. The sensory innervation of the fibrous and serous parietal pericardium, separate from that of the heart and epicardium, follows the phrenic nerves to the cervical DRG (C3-C5).4

It has long been understood that cardiac pain is associated with myocardial ischemia, which causes oxygen supply/demand insufficiency.5 In various whole-animal preparations, occlusion of a coronary artery activates the cardiac afferent nerve fibers in the sympathetic and vagal tracts.9,10 Various substances released during myocardial ischemia have been implicated as chemical mediators of myocardial ischemic sensation. Several of these substances have been shown to activate CSNs: ATP,8,10 serotonin (5HT),11 bradykinin (BK),12,13 and adenosine.8,10 In turn, stimulation of sensory fibers elicits reflexes specifically mediated by the sympathetic tract14 and by the vagal tract.15 Still, the precise stimuli that are sensed during myocardial ischemia are incompletely understood (see Reference 16 for review).

A likely contributor is acid. The heart is an organ of high metabolic activity and is susceptible to drops in pH during ischemia or hypoxia. It has been demonstrated that pH is lowered intracellularly17 and extracellularly18,19 in ischemic heart models and clinically in patients with coronary artery disease.20 In dogs, lowered pH stimulates afferent cardiac sympathetic nerve fibers.21 In another organ system, rat skin, acid plays a dominant role in exciting sensory neurons when compared with other potential chemical mediators of inflammation.22

Acid evokes depolarizing currents in sensory neurons studied in primary dissociated culture,23-26 and a variety of different components are distinguished by kinetic criteria.27 Most components activate somewhere between pH 7 and pH 6 and desensitize in response to a maintained stimulus. These desensitizing currents all have the unusual property of selectively passing Na⁺ over K⁺ about as effectively as voltage-gated Na⁺ channels. In addition to the desensitizing, Na⁺-selective currents in DRG neurons, there is a sustained,
nonselective current that is evoked by pH below 6.0. The channels underlying these currents are believed to be the recently cloned acid-sensing ion channels (ASICs), which are members of the amiloride-sensitive Na\(^+\) channel/degenerin family of cation channels. These acid-evoked currents may play a role in mediating the pain of cardiac and skeletal muscle ischemia and perhaps also of inflammation. It is difficult to explore this possibility in culture, because the sensory modality and the site of innervation of individual neurons are not known. The first goal of the present study was to fluorescently label CSNs in the rat so that they can later be distinguished from other sensory neurons in dissociated culture; we accomplished this using a retrogradely transported dye placed in the pericardial space. We found that acid evoked extraordinarily large currents in the cardiac afferent neurons from the DRG (CDRGNs) compared with other, unlabeled DRG neurons (UDRGNs). The very high expression of these currents in cells thought to be specialized for sensing ischemia suggests an important role of acid in mediating cardiac pain.

Materials and Methods

In Vivo Labeling of CSNs

Surgical Preparation

Sprague-Dawley rats (200 to 300 g) were anesthetized by intramuscular injection of 1 mL/kg rat anesthetic (in mg/mL, ketamine 55, xylazine 5.5, and acpromazine 1.1). Each animal was intubated and respiration maintained with a rodent ventilator (Harvard model 683). The heart and thymus were exposed through a left lateral thoracotomy at the fifth intercostal space. The thymus, with the anterior superior portion of the pericardium adherent to its undersurface, was gently retracted cephalad to better delineate the pericardium and pericardial space. Axons of other CSNs travel within the vagal nerve to their cell bodies in the nodose ganglia. Afferent neurons from the pericardium follow the phrenic nerves to their cell bodies in the DRG of the upper cervical region (C\(_2\)-C\(_5\)).

Tissue Culture and Identification of Labeled CSNs

During the 2- to 4-week postoperative period, DiI was carried through retrograde transport back to the cell bodies of the CSNs. The rats were then sacrificed, and the right and left dorsal root (C\(_8\)-T\(_3\)) and nodose ganglia were collected. The ganglia were dissociated and cultured as previously described, except that the Percoll spin was omitted. In brief, the ganglia underwent enzymatic dissociation successively in papain and collagenase/dispase solutions; this was followed by trituration in Hanks solution. The cells were then plated on polylysine- and laminin-coated plastic in F12 medium plus nerve growth factor (50 ng/mL) at 37°C in 5% CO\(_2\). After several hours, the medium was changed to L15 plus nerve growth factor, and the cells were maintained at 22°C in air. CSNs were identified by fluorescence microscopy (Figure 2C and 2D). From the animals that underwent the pericardial space injection preparation, the following
whole-cell patch-clamp recording

Whole-cell currents were recorded with an EPC-9 amplifier (HEKA Elektronik). For most experiments, pipettes of 2- to 4-MΩ resistance were filled with KCl internal solution containing (in mmol/L) KCl 140, EGTA 5, HEPES 5, MgCl₂ 5, Na₂ATP 1, and Na₃GTP 0.3, adjusted to pH 7.4 with KOH unless otherwise stated. For the monovalent permeability experiments, NaCl replaced KCl in the internal solution, and pH was adjusted with NaOH; high internal Na⁺ permeability experiments, NaCl replaced KCl in the internal solution, and pH was adjusted with NaOH, KOH, or CsOH. For the Ca²⁺ permeability experiments, external solutions consisted of (in mmol/L) NaCl (or KCl, NaCH₃SO₃, or CsCl) 130, MgCl₂ 2, HEPES 10, and MES 10; NaCl replaced KCl in the internal solution, and pH was adjusted with NaOH, KOH, or CsOH. for the Ca²⁺ block, external solutions consisted of (in mmol/L) NaCl 130; HEPES 10; MES 10; and CaCl₂ 1, 2, or 10. The series resistance ranged from 3 to 7 MΩ, and it was compensated by ~50%.

All dose-response curves were made by random-order application of various concentrations at 30-second intervals. Solutions were applied through an array of 1- or 10-μL pipettes positioned ~50 μm from the cell under 40 cm of water pressure. Rapid solution exchanges were controlled via computer-driven solenoid valves and were accomplished within 5 ms as measured by an osmotically induced change in current (Figure 5A). Cells were held at ~70 mV unless otherwise stated. Experiments were performed at room temperature (~22°C). We studied most cells after 1 to 2 days in culture; however, some experiments were done on cells cultured up to 7 days. We saw no obvious difference in the responses of cells cultured for longer times.

Data Analysis

The equation \( I(\text{H}^+) = I_0(1 + (K_d / [\text{H}^-]))^n \), where pH at half-maximal response is \( -\log K_d \), and \( I \) is the current at a given proton concentration, \([\text{H}^-] \), was best-fit to the dose-response data using the program NFIT (University of Texas Medical Branch, Galveston, TX), a least-squares algorithm. PulseFit (HEKA Elektronik) was used to determine the time constants of current activation and desensitization, fit to a single exponential. Igor software (WaveMetrics, Inc) was used to curve-fit the time of recovery from desensitization. Permeability ratios were calculated from reversal potentials using the Goldman-Hodgkin-Katz equation. \( P_N/P_K \) was calculated from the change in reversal potential \( (\Delta E_{rev}) \) when \( K^+ \) replaced \( Na^+ \) in the external solution: \( \Delta E_{rev} = (RT/F) \ln \left[ P_N / (P_N + P_K - 1) \right] \), where \( T \) is absolute temperature, \( R \) and \( F \) are gas and Faraday constants, respectively, and \( P_K, P_N \) are permeabilities.

Results

Response of CSNs to Chemical Stimuli

To study the response of CSNs to potential chemical mediators of ischemia, the following chemicals were each dissolved in an external solution of pH 7.4: in mmol/L, ATP 30, 32 5HT 30, 33 capsaicin 1, 34 acetylcholine (ACh) 200, 35 and adenosine 200, 37 as well as 500 nmol/L BK. 36 The concentrations chosen for each compound produced maximal responses in our experiments and in the references cited. The largest measured currents (8.58±1.44 nA) were consistently evoked (93%) by acid applied to CDRGNS (Figure 3). A smaller percentage of UDRGNS (54%) and CNDNs (74%) responded to acid, and the cells that responded displayed significantly smaller currents (3.48±1.10 nA in UDRGNS and 1.15±0.23 nA in CNDNs; \( P<0.01 \) versus pH-evoked current in CDRGNS) (Figure 3B and 3C). These differences between cell populations refer to the amplitude of transient acid-evoked current (see below); the sustained component evoked by very low pH was seen in virtually every neuron and did not distinguish different cell populations.

Another consistent and large response (4.87±0.59 nA) was evoked by ATP acting on ion channels called P2X receptors in CNDNs. The current was slow activating and only partially desensitized (data not shown), which is indicative of
Acid-Evoked Currents in Cardiac Sensory Neurons

The exceptionally large amplitude and prevalence of the transient acid-evoked current in CDRGNs suggests its significance in sensing cardiac ischemia. Therefore, we characterized its biophysical and pharmacological properties to see how these compared with the variety of acid-evoked currents seen in sensory neurons. In contrast, the ATP-evoked currents in CDRGNs and UDRGNs were substantially smaller and consisted primarily of a fast-activating and fast-desensitizing current (Figure 3A), which suggests either the P2X1 or P2X3 receptor subtypes.39 In contrast, the ATP-evoked currents in CDRGNs and UDRGNs were substantially smaller and consisted primarily of a fast-activating and fast-desensitizing current (Figure 3A), which suggests either the P2X1 or P2X3 receptor subtypes.39

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Figure 3. CSNs respond to a variety of chemical activators. A, Representative currents evoked by application of various chemicals to CDRGNs. Note the different scale bars and application times. Each of the test solutions was applied to cells, in random order, for a minimum of 3 seconds; a longer application was made if needed to see the peak current amplitude. Control solution flowed onto the cells for 30 seconds between chemical applications. B, Percentages of CDRGNs, UDRGNs, and CNodNs that responded to the following: pH 5.0; (in μmol/L) ATP 30, 5HT 30, capsaicin 1, ACh 200, and adenosine 200; and 500 nmol/L BK. A positive response was defined as an evoked current >50 pA. For pH response, the number of CDRGNs studied was 29; UDRGNs, 22; and CNodNs, 19. Not all of the cells studied were tested for all chemicals; each bar represents at least 12 cells. C, Mean current amplitudes of the responding neurons. Data are mean ± SEM. *P<0.01 vs pH-evoked current (transient desensitization). Boxed inset magnifies the region where the transient activation and desensitization curves overlap. Points represent mean ± SEM.

A drop in pH to 7.0 reproducibly evoked a transient, rapidly activating and rapidly desensitizing current in CDRGNs (Figure 4A and 4C). This transient current was half-activated by a pH step to 6.6 and half-desensitized by preincubation at pH 7.2 (Figure 4C). The Hill coefficient of the activation curve was 2.5. A smaller, nondesensitizing current was evoked by more extreme decreases in pH (≤6). The activation curve for this current is fitted with a pH0.5 of 3.7. The combination of transient and sustained components at low pH has previously been seen in unlabeled rat sensory neurons.26,27

Varying the pH before a test stimulus of pH 5 revealed that a significant fraction of the transient current is desensitized at a resting pH of 7.4 (Figure 4B). This is consistent with the previous demonstration that acid-evoked channels need not open to desensitize.25 The steady-state desensitization and activation curves show clear overlap in the vicinity of pH 7 (Figure 7C and inset), suggesting that the channel can generate a standing current at pH 7.

A closer look at the time constants of activation and desensitization of the transient currents in CDRGNs revealed multiple transient components (Figure 5), as noted by Krish-atal and Pidoplichko.27 Most cells exhibited a rapidly activating and desensitizing current, but some had a current that was 10-fold slower. The fast and slow transient currents were not

Biophysical and Pharmacological Properties of Acid-Evoked Currents in CDRGNs

The exceptionally large amplitude and prevalence of the transient acid-evoked current in CDRGNs suggests its significance in sensing cardiac ischemia. Therefore, we characterized its biophysical and pharmacological properties to see how these compared with the variety of acid-evoked currents seen in sensory neurons.23–26 In short, we found no properties unexpected from those described in the literature.
Figure 5. Kinetics of acid-evoked transient currents in CDRGNs. A, Typical transient current evoked by pH 6.8. Trace on the right is a shorter pH application to the same cell, displayed on a faster time scale to demonstrate the fast activation. Top right trace shows the measured time course of pH application (see Materials and Methods). B, In a small subpopulation of CDRGNs, acid evoked a different transient current with slower activation and desensitization. Left and right scale bars correspond to left and right traces for both panels A and B. C, Activation, desensitization, and recovery from desensitization are each faster in the fast transient (○) than the slow transient (□) current. Time constants for activation and desensitization at various pH solutions are from exponential fits to the rising and falling phase of currents, as in panels A and B. Recovery times are at pH 7.4. Horizontal axis indicates interval at pH 7.4 spent between 2 pulses to pH 6.8. The first pulse completely desensitizes the current, and the second tests the extent of recovery. Vertical axis is the relative amplitude of currents evoked by the second and first pulses. Time constants of the exponential fits are 0.44 and 6.8 seconds for the fast and slow currents, respectively.

The fast transient current is Na⁺ selective but Ca²⁺ permeable (Figure 6). With the usual internal (high K⁺) and external (high Na⁺) solutions, the reversal potential was in the vicinity of +50 mV (data not shown). To quantify the evident preference for Na⁺ over K⁺, we measured reversal potentials with Na⁺ as the internal ion (see Materials and Methods). The reversal potential shifted –50±1.2 mV when K⁺ replaced Na⁺, corresponding to a PNa/PK of 6.8.

Current-voltage relationships measured in 10 mmol/L extracellular Ca²⁺ with 15 mmol/L intracellular Na⁺ displayed an average reversal potential of –47.2±1.2 mV (n=7), corresponding to a PNa/PCa of 105 (Figure 6C). In all cells, current amplitude increased when [Ca²⁺] was increased from 10 to 30 mmol/L (n=7; amplitude increase varied from 28% to 725%; data not shown). Thus, as previously shown,⁴⁰ the current can be carried by Ca²⁺. To determine whether the acid-evoked currents are blocked by millimolar extracellular Ca²⁺, as seen in some native ²⁴,²⁵ and cloned ⁴¹ channels, we measured Na⁺ currents elicited by pH 6.0 in 1, 2, and 10 mmol/L Ca²⁺. There was no change in amplitude (n=5; data not shown); therefore, the current was not blocked by millimolar Ca²⁺ concentrations.

The fast transient and sustained current components differ in 2 respects: ion selectivity (Figure 7A) and pharmacology (Figure 7B). Both components are cation selective, because they were unchanged when extracellular Cl⁻ was replaced with the impermeant anion CH₃SO₃⁻. Replacement of extracellular Na⁺ with Cs⁺ revealed that the transient current does not readily pass Cs⁺, whereas the sustained current does. Thus, as previously described in UDRGNs, ²⁶ the transient current is selective for Na⁺, whereas the sustained current is a nonselective cationic current.

Amiloride, a K⁺-sparing diuretic that has been shown to block proton-activated currents in mouse neuroblastoma cells, ⁴² blocked the fast transient current (IC₅₀=9.2 μmol/L) but not the sustained current (Figure 7B). This difference is consistent with one of the ASICs. ⁴³ The slow transient current was similarly inhibited by amiloride (data not shown). The amiloride derivative ethylisopropylamiloride inhibited the fast transient current with an IC₅₀ of 37.6 μmol/L (data not shown). These blocking concentrations are ~100-fold greater than those needed to block the epithelial amiloride-sensitive Na⁺ channel ⁴⁴ and are high enough to block unrelated ion channels. ⁴²
Acid-Evoked Currents in Cardiac Sensory Neurons

Discussion

There are 4 key findings in this study, as follows. (1) We describe a method to distinguish CSNs from neurons of other sensory modalities in primary dissociated tissue culture. (2) Compared with other sensory neurons, CDRGNs have extremely large currents evoked when pH drops to 7.0 or below. (3) CNodNs have smaller acid-evoked currents, but larger ATP-evoked currents than CDRGNs. (4) Other than its large amplitude, the acid-evoked current in CSNs has no biophys-

Isolation of CSNs

As described in Materials and Methods, considerable effort was made to validate this preparation. Each of the control injection experiments resulted in significantly fewer labeled neurons compared with the pericardial space injections; this lends support to our assertion that we have specifically labeled and isolated CSNs. Finally, the different responses between the labeled DRG neurons and UDRGNs in our whole-cell patch-clamp experiments provide further evidence that we have isolated a distinct subgroup of sensory neurons from the DRG population at large.

Although neuroanatomy was not the primary focus of this study, some anatomical information can be gleaned from our data. The number of labeled neurons we obtained was consistent with previous neuroanatomical studies in the rat in which fluorescent tracers were injected into the pericardial space. In contrast to the large number of labeled neurons appearing after pericardial space injections, we found relatively few labeled neurons after intramural injections. This may reflect either less tissue exposure to dye compared with the pericardial space injection or a true paucity of nerve terminals within the rat intramural myocardium. The endocar- dial layers were not labeled in our study. Interestingly, in a dog preparation, myocardial ischemia caused cardiac sympathetic afferent firing only if the ischemia was transmural and involved the superficial epicardial layers.

Chemical Activation of CSNs

Several insights arise from a comparison of responses to different agonists in the different cell populations that we isolated. Most importantly, modest decreases in extracellular pH (ie, to pH 7.0 or below) evoke exceptionally large currents in almost all epicardial CDRGNs. In contrast, and as reported previously, only ~50% of UDRGNs respond to acid; those that did respond had much smaller average currents than CDRGNs.

Cardiac afferents with cell bodies in the DRG differed from those in the nodose ganglia. CNodNs had significantly smaller acid-evoked currents and larger ATP-evoked currents than CDRGNs. This raises the possibility that the 2 cell populations sense different chemical signals during cardiac ischemia.

There are 2 classes of molecules that are proposed to sense changes in extracellular pH in sensory neurons: ASICs and vanilloid (capsaicin) receptors. Vanilloid receptors are activated by noxious heat and by capsaicin (the compound in pepper that tastes "hot"); also, current through vanilloid receptors is strongly increased by acidic pH. Therefore, it is considered that vanilloid receptors, in addition to sensing heat, may mediate sensory responses to acidity caused by inflammation and ischemia. The neurons we isolated detect cardiac ischemia, yet only a small fraction exhibit capsaicin-activated current, and those that do respond have small currents compared with UDRGNs. In contrast, almost all exhibit grossly large currents through ASICs. These results argue that ASICs are more important than vanilloid receptors for sensing myocardial ischemia.

5HT evoked currents that were substantial in cardiac afferents, but they were still smaller than the acid- or ATP-evoked currents. Various cells that contribute to the immune response to tissue damage release 5HT, so these currents may provide a means of communication between immune cells and CSNs.

Of the chemicals tested, protons, ATP, 5HT, and capsaicin activate ion channels (the ASICs, P2X receptors, 5HT3 receptor, and vanilloid receptors, respectively) that are presumed to serve as sensory transducers in sensory neurons. No current was ever evoked by ACh, but currents were occasionally seen in response to BK or adenosine. We do not infer anything from the relatively rare and small currents evoked by these compounds, because they do not directly gate ion channels in sensory neurons; in fact, adenosine inhibits a Ca2+-activated K+ channel. The current we saw presumably arose from modulation of a channel by an

Figure 7. Fast transient and sustained acid-evoked currents in
CDRGNs differ in ion selectivity and in amiloride sensitivity. A, Superimposed acid-evoked currents in NaCl and NaCH3SO4 (left). Both the transient and sustained acid-evoked currents were unchanged when extracellular Cl– was replaced with the impermeable anion CH3SO4. Thus, neither current is carried by anions. Right, Currents evoked in extracellular solutions containing either NaCl or CsCl. Transient current is more permeable to Na+ than Cs+, whereas the sustained current is more permeable to Cs+ than Na+. B, Left, currents evoked by pH 5.0 and by pH 5.0 plus 100 μmol/L amiloride (*). The transient current was significantly reduced by amiloride, whereas the sustained current was unchanged. Right, dose-response curve for amiloride on current evoked by a pH change from 7.4 to 6.8. IC50=9.2 μmol/L (n=5).

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intracellular signaling cascade; the importance of this may be understated by simply comparing the amplitude with those of channels directly gated by protons, ATP, or 5HT.

Physiological and Pathophysiological Significance

CSNs respond to lowered pH (in the range produced by myocardial ischemia) with consistent and robust depolarizing currents; this suggests that acid is a potential mediator of myocardial ischemic sensation.

In humans, the only conscious sensation from the heart is pain or angina, which most commonly occurs during myocardial ischemia. However, objective measurements of myocardial ischemia often do not correlate with the presence or severity of chest pain. In fact, ambulatory electrocardiographic monitoring in patients with myocardial ischemia has revealed that the majority of ischemic episodes are not reported as painful.48 Attempts to model ischemic cardiac pain in animals have produced variable results. The pseudoaffective measures of pain in these behavioral studies correlate poorly with sensory neuronal activation.13,15,16 Thus, it is reasonable to conclude that the activation of CNSs with acid does not equate with nociception. For example, in patients with “silent” ischemia (defined as objective myocardial ischemia that is not equated with nociception), there is evidence of sensory activation to the level of the thalamus, whereas patients with typical angina have additional activation of the cerebral cortex.50 Thus, the conscious perception of chest pain most certainly involves complex central processing and integration at multiple levels, conscious perception of chest pain most certainly involves complex central processing and integration at multiple levels, and activation of CNSs is probably necessary but not sufficient to produce pain. Regardless, ischemia- or acid-induced activation of CNSs, whether painful or not, may be an important initiator of cardiovascular reflexes in pathological cardiac conditions.

A broad range of cardiovascular disease processes, including myocardial ischemia,51 congestive heart failure,52 and arrhythmias,53 are precipitated or worsened by perturbations in the autonomic nervous system. Much of the current pharmacological therapies are directed toward blocking the compensatory, but often deleterious, neurohormonal systems that are activated in these diseases. In human skeletal muscle, ischemia-induced acidic pH is coupled with sympathetic efferent nerve discharge.54 Also, abdominal visceral ischemia leads to profound cardiovascular reflex changes, the degree of which appears related to the level of the resulting acidosis.55 A similar acid-evoked reflex loop may exist in the heart and contribute to the detrimental effect of sympathetic activation in myocardial ischemic conditions. Specific blockade of acid-evoked activation of CNSs presents a potential new therapeutic management strategy in the treatment of ischemic heart disease.

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