Properties of Mouse Cutaneous Rapidly Adapting Afferents: Relationship to Skin Viscoelasticity

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Submitted 27 October 2003; accepted in final form 10 March 2004

Grigg, P., D. R. Robichaud, and Z. Del Prete. Properties of mouse cutaneous rapidly adapting afferents: relationship to skin viscoelasticity. J Neurophysiol 92: 1236–1240, 2004. First published March 17, 2004; 10.1152/jn.01033.2003. When skin is stretched, stimuli experienced by a cutaneous mechanoreceptor neuron are transmitted to the nerve ending through the skin. In these experiments, we tested the hypothesis that the viscoelastic response of the skin influences the dynamic response of cutaneous rapidly adapting (RA) neurons. Cutaneous RA afferent neurons were recorded in 3 species of mice (Tsk, Pallid, and C57BL6) whose skin has different viscoelastic properties. Isolated samples of skin and nerve were stimulated mechanically with a dynamic stretch stimulus, which followed a pseudo Gaussian waveform with a bandwidth of 0–60 Hz. The mechanical response of the skin was measured as were responses of single RA cutaneous mechanoreceptor neurons. For each neuron, the strength of association between spike responses and the dynamic and static components of stimuli were determined with multiple logistic regression analysis. The viscoelastic material properties of each skin sample were determined indirectly, by creating a nonlinear (Wiener–Volterra) model of the stress–strain relationship, and using the model to predict the complex compliance (i.e., the viscoelastic material properties). The dynamic sensitivity of RA mechanoreceptor neurons in mouse hairy skin was weakly related to the viscoelastic properties of the skin. Loss modulus and phase angle were lower (indicating a decreased viscous component of response) in Tsk and Pallid than in C57BL6 mice. However, RA mechanoreceptor neurons in Tsk and Pallid skin did not differ from those in C57 skin with regard to their sensitivity to the rate of change of stress or to the rate of change of incremental strain energy. They did have a decreased sensitivity to the rate of change of tensile strain. Thus the skin samples with lower dynamic mechanical response contained neurons with a somewhat lower sensitivity to dynamic stimuli.

INTRODUCTION

Soft tissue structures contain the terminal processes of mechanically sensitive afferent neurons. When a soft tissue is stimulated mechanically, the stimulus to the neuron is some subset of the local tensile, compressive, and shear stresses and strains that may be present in the tissue. Because most soft tissues are viscoelastic, dynamic mechanical stimuli would result in there being time or rate dependence to the magnitude of those internal states of stress or strain. Because these states (or some subset of them) constitute the mechanical stimulus for mechanoreceptor neurons that innervate the soft tissue structure, it might be expected that any rate or time dependency in the mechanoreceptor response would reflect the viscoelastic mechanical response of the soft tissue structure. There is evidence both for (Bell and Holmes 1992; Damiano 1999; Loewenstein and Skalak 1966; Swerup and Rydqvist 1996) and against (Husmark and Ottoson 1971; Wilkinson and Fukami 1983) a potential role for tissue viscoelasticity in shaping the dynamic response properties of mechanoreceptors.

However, there have been limitations to the studies addressing this issue. First, in seeking relationships between spike responses and mechanical stimuli, it is necessary to know which of the many potential component(s) of a mechanical stimulus actually causes the spike response; in general, this is unknown. Also, previous investigations have centered mainly on transient responses (i.e., slow adaptation of responses evoked by step stimuli). In these studies, parallel behavior has been observed between the mechanical relaxation of input stimuli and the slow adaptation of a neuronal response. The potential problem with this approach is that the 2 processes may, in fact, be independent of each other.

In this communication, we used a new approach to address the question of whether tissue viscoelasticity influences the properties of mechanoreceptors. We used dynamic stimuli to study rapidly adapting (RA) cutaneous mechanoreceptor afferents in several strains of mice that have different skin viscoelasticities. RA afferents respond only to dynamic components of stimuli and are not sustained in response to static stimuli. If skin viscoelasticity is a determinant of the dynamic response of cutaneous mechanoreceptors, then afferents in the most viscoelastic samples should have a greater dynamic response than afferents in the least viscoelastic samples. In a recent paper (Del Prete et al. 2004) we showed significant differences in the viscous properties of skin from Tsk mice and wild-type (C57BL6) mice; Tsk skin had a lesser dynamic response of cutaneous mechanoreceptors, then afferents in the most viscoelastic samples should have a greater dynamic response than afferents in the least viscoelastic samples. In a recent paper (Del Prete et al. 2004) we showed significant differences in the viscous properties of skin from Tsk mice and wild-type (C57BL6) mice; Tsk skin had a lesser dynamic response to dynamic stretch stimuli than skin from C57BL6 mice. In the current experiment we used Tsk and C57BL6 mice; as an additional control we included Pallid mice, which are breeding colony controls for Tsk. We measured the viscoelastic properties of skin samples in each mouse phenotype by determining the complex compliance. The complex compliance includes measures of the loss modulus and the phase angle, both of which reflect the viscous component of response of a sample. In each sample we also determined the sensitivity of afferents to static versus dynamic components of stimuli.

Using mice also solves the problem of not knowing what components of a complex stimulus are acting on the neuron. In a recent paper from this laboratory (Del Prete et al. 2003) we identified the components of mechanical stimuli signaled by...
METHODS

The preparation, apparatus, and experimental methods for recording afferent neurons were, with a few exceptions, identical to those described in the recent communication from this laboratory (Del Prete et al. 2003). Adult (Fbn1tsk), Pallid (PldnPa), and wild-type (C57BL/6) mice were obtained from Jackson Laboratories (Bar Harbor, ME). Tsk mice were the experimental group; Pallid mice are controls from the colony in which the spontaneous Tsk mutation is maintained; and C57BL/6 mice are the background strain for Tsk.

Mice were anesthetized with IP Nembutal, 45 mg/kg, in a University of Massachusetts IACUC approved protocol. A specimen of skin and the sensory nerve innervating it were removed from the ventral surface of the hindlimb (Fig. 1A). The sample was fashioned as a “+” approximately 14 mm from end to end. A 5-mm-wide plastic tab was glued to each edge of the sample while the skin was in situ, and the specimen was then excised by cutting around its margins. The tabs were subsequently used to couple the skin to the apparatus. The skin was maintained in vitro, outside surface up, in a bath of HEPES-buffered artificial interstitial fluid (Koltzenburg et al. 1997), at pH 7.4 and at room temperature (20°C), in the apparatus depicted in Fig. 1B. Two tabs were coupled to the actuating arms of linear actuators (Aurora Scientific 300B lever systems) by means of strings with hooks on their ends engaged in holes in the tabs. The other 2 tabs were coupled to the sides of the apparatus. The tabs served to distribute the applied load over the entire width of the tab.

The nerve innervating the skin sample was pulled into a small oil-filled chamber and dissected into small filaments for recording. Individual filaments were placed on a platinum wire recording electrode; the indifferent electrode was placed in the bath. Neuronal activity was amplified and spike responses were discriminated using a template-matching algorithm (SPS; Prospect, S. Australia). The criteria for identifying single-neuron activity were the constant size and shape of the action potential. Individual afferents were classified as RA by their transient response (usually consisting of 1–2 action potentials) to manual stretch or to stroking the skin surface with a polished glass rod.

The skin was stretched following the procedure used by Grigg and Robichaud (2004). One actuator was used to stretch the sample; it was operated in force control mode with a pseudo Gaussian noise (PGN) command signal. The orthogonal actuator was operated in position control; loads were recorded along this direction but the length of the sample was fixed. In separate tests, each sample was actuated uniaxially along the X- and the Y-axes, where X and Y directions refer to along (X) and perpendicular to (Y) the long axis of the leg. Two amplitudes of stress stimuli were used: they had mean values of either 17 or 35 kPa. The range of values in the sequence was approximately twice the mean value. Stimulus bandwidth was 0–60 Hz, which matched the mechanical bandwidth of the actuators.

Data collection runs were 30 s in duration. Loads were measured along both directions, and displacements were measured along the actuated direction. They were sampled at 500/s, and stored in data files. Runs with fewer than 50 spikes were excluded from analysis.

Loads were converted to stresses (σ) by normalizing to the cross-sectional area of the sample, which was taken as the product of tab width and the thickness. We measured thickness by fixing skin samples in formalin while they were held at their in situ length, cutting 40-μm frozen sections normal to the skin surface, and measuring the thickness of the dermal layer using polarizing microscopy. The width of each specimen was measured from digital photographs of the preparations.

Strain could not be directly determined using actuator displacements in this experiment. When a skin sample is actuated along some direction, as depicted in Fig. 1B, local strain in the central region of the skin is smaller than that in the tabs. Displacements were converted to a pseudostrain variable (E) by using the expression \( E = \frac{\Delta L}{L_0} \), where \( \Delta L \) is the measured tab displacement and \( L_0 \) is the distance between the tabs in the unloaded state. E is not a true strain because the displacements are different along the length of the sample. Hence we use the symbol E rather than \( \epsilon \), which would denote a true strain. However, given that all our specimens had the same geometry, the variable E allowed us to make comparisons between them.

The relationship between spike responses and mechanical variables was determined using multiple logistic regression (MLR). MLR is a multiple correlation method used in models with multiple predictor variables and a binary outcome event (Hosmer and Lemeshow 1989). In this application there were 10 predictors: measured values of stress (σ), pseudostrain (E), their rates of change (dσ/dt and dE/dt), and the 6 first-order interactions between them. The interaction terms included \( \sigma \times E \), which would be proportional to strain energy density; and \( \sigma \times \frac{d\sigma}{dt} \) and \( E \times \frac{dE}{dt} \), which are proportional to the rate of change of strain energy density. The use of MLR in this application is described in detail in Del Prete et al. (2003). The outcome of MLR analysis is an odds ratio, whose magnitude reflects the strength of the association between the measured mechanical stimuli (predictors) and binary spike events. The predictors of interest in MLR analyses were stress and pseudostrain, their rates of change, and the first-order interactions between them, along the direction the skin was actuated. Loads measured along the orthogonal direction were ignored because of our finding (Grigg and Robichaud 2004) that there was no significant association between them and spike responses.

In RA cutaneous afferents, there are memory effects between stimuli and responses. A stimulus applied at a particular time has an effect that is observable later in time. Our prior report (Del Prete et al. 2003) shows in detail how memory effects can be quantified by “lag” analysis, in which the apparent time of occurrence of spikes is systematically shifted with respect to predictors, and the strength of association between predictors and spikes is determined. Odds ratios were calculated between spikes and all 10 predictors, for memory times from 0 to 50 ms. This required 26 MLR analyses: one for each 2-ms memory interval from 0 to 50 ms (Fig. 3A).

To characterize skin viscoelasticity, we measured the complex compliance of each skin sample. The complex compliance is a property that is measured using sinusoidal inputs. It shows the relationship between sinusoidal frequency and the storage compliance (inversely proportional to stiffness), the loss compliance (energy loss per cycle), and the phase angle between stress and strain. We determined the complex compliance indirectly, using a systems identification approach, described in detail in Hoffman and Grigg (2002). Stress and strain data collected in uniaxial data collection runs were used to create a constitutive model of the material properties of the tissue.
sample. An individual model took the form of a set of Wiener–Volterra kernels (Marmarelis 1994, 1993; Marmarelis and Marmarelis 1978). The model for a particular tissue specimen was calculated from PGN load–displacement data collected from that specimen, using Lysis 7 software (Biomedical Simulations Resource, Univ. Southern California). The Wiener–Volterra kernels, embodying the constitutive relationship for that specimen, were used to predict the specimen’s strain responses to a set of mathematically derived sinusoidal inputs with frequencies ranging from 0.1 to 25 Hz. The result of this process was an ideal (mathematically generated) sinusoidal stress input, and a strain output predicted by the Wiener–Volterra model. These were used to make a strain–stress Lissajous figure that was used, in turn, to compute the complex compliance (storage and loss compliances and the phase angle) at each frequency.

Differences in viscoelastic properties between the 3 mouse phenotypes were analyzed using a repeated-measures ANOVA, in which frequency was the repeated measure. Comparisons of odds ratios between mouse phenotypes were done with a repeated-measures ANOVA, in which loading treatments were the repeated measures. Homogeneity of variance was tested using Levene’s test of equality of error variances. In analyses where the data suffered from inhomogeneity of variance, we performed a logarithmic transformation on the data. All analyses were done using SPSS version 11.5.

RESULTS

Twenty neurons were recorded in 7 specimens taken from 6 Tsk mice, 18 neurons were recorded in 7 samples from 7 C57BL/6 mice, and 25 neurons were recorded in 5 specimens from 5 Pallid mice. Although all the animals were of approximately the same age, they differed with respect to body mass. At the time of use their approximate body masses were: C57BL/6: 28 g; Tsk: 20 g; Pallid: 22 g.

Skin thickness was measured in several specimens for each phenotype and was approximately 0.2 mm in each phenotype.

Load and displacement data were used to determine the complex compliance for both the X and the Y directions. The skin was slightly orthotropic, being somewhat stiffer along the Y than the X direction. LC and phase angles also differed slightly between X and Y directions. Because none of these differences was statistically significant, the complex compliances measured along the X and Y directions were averaged together (Fig. 2). The 3 skin phenotypes differed significantly with relation to SC, LC, and phase. C57BL/6 samples had significantly greater LCs and phase angles than Tsk and Pallid samples at almost all frequencies. Phenotypic differences in SC, although significant, were small and were observed only at low frequencies.

MLR analysis was used to determine the strength of association between spikes and all 10 predictors, for memory times between 0 and 50 ms (Fig. 3A). These analyses revealed no differences between the responses to loading along the X and Y directions. Therefore as with the mechanical data, the results from the tests in which the skin was stretched along the X and Y directions were lumped together. Odds ratios for all the neurons studied in each stimulus condition were averaged together for each skin phenotype, yielding an aggregate, mean odds ratio for each memory time for each loading paradigm for each of the 3 mouse phenotypes.

There were similarities in the response of neurons in all loading paradigms in all 3 phenotypes: there was a consistent, strong association between spike responses and dσ/dt, similar to that shown in Fig. 3A. The association with dσ/dt had a peak at memory times ranging from 10 to 16 ms. In addition, there was a single interaction term, σ × dσ/dt, whose odds ratios and
memory time were roughly equal in all groups. Associations with \(dE/dt\), however, appeared to differ between mouse phenotypes.

To compare odds ratios between uniaxial and biaxial stimuli, and across mouse phenotypes, we normalized the magnitudes of odds ratios to the peak odds ratio for \(dH9268/dt\). We chose \(dH9268/dt\) as the norm because it was the most consistent component of response in all groups, and because the odds ratios and memory times for this variable did not differ between skin phenotypes. Figure 3, **B**–**D** shows the normalized odds ratios for mechanical variables of interest, contrasted between mouse phenotypes. Odds ratios in each experimental group were normalized to the maximal value of odds for \(dH9268/dt\) in that group. **B**: \(dE/dt\); **C**: \(dH9268/dt\); **D**: \(H9268\); **E**: \(H9268/H11003\) \(dH9268/dt\).

Differences in odds ratios in the data of Fig. 3, **B**–**E** were analyzed using analyses of variance. Levene’s test revealed inhomogeneity of variance between the 3 mouse types in the data for \(dE/dt\) and \(\sigma\) (Fig. 3, **B** and **D**). In both cases performing a log transformation on the data made the variances homogeneous, and repeated-measures ANOVAs were run on the transformed data. Post hoc comparisons revealed that odds ratios for \(dE/dt\) in Tsk neurons were significantly smaller than in C57 neurons. Pallid neurons had lower odds ratios for \(\sigma\). The odds ratios for \(\sigma \times dH9268/dt\) did not differ between phenotypes.

**DISCUSSION**

The results support the hypothesis that the viscous component of skin’s mechanical response contributes to the dynamic sensitivity of mechanoreceptors. However, of the 3 predictors (\(dH9268/dt\), \(dE/dt\), and \(\sigma \times dH9268/dt\)) that have dynamic components, and that were significantly associated with spikes, only one (\(dE/dt\)) was different between the 3 skin phenotypes. Thus although the data do support the hypothesis, they do so only moderately.
There were significant differences in the dynamic properties of the 3 skin phenotypes. The greater loss compliance and phase angle of C56BL/6 skin can be conceptualized as an increase in the dashpot component in a spring–dashpot (i.e., a Voigt) model of a soft tissue. A tissue with a greater dashpot component will, when stretched, have a greater dynamic (i.e., viscous) component to its mechanical response. C57BL/6 skin had a significantly greater dynamic response than did skin from Tsk or Pallid mice. Nonetheless, the corresponding dynamic components of neuronal response were mixed. The association between spikes and $dE/dt$ and $d^2E/dt^2$ did not vary between phenotypes, and only the association with $dE/dt$ was enhanced in C57 mice. Thus the results can be considered to be only weakly supportive of the hypothesis.

When, as with RA afferents, there are multiple variables that occur at different memory times, and that are positively associated with spikes, there is a question as to how they individually relate to the process of spike initiation. Schafer et al. (1999) deduced that spikes were initiated with zero delay with respect to muscle displacement, which is equivalent to strain. In the current experiments, the variable $dE/dt$ was relatively strongly associated with spikes at 0-ms memory time, similar to the findings of Schafer et al. (1999). However, spike initiation in cutaneous RAs is presumably caused by the summed influences of all the variables that were positively associated with spikes. Indeed Del Prete et al. (2003) found that a prediction model that used the variables and the memory times revealed by logistic regression was able to predict the occurrence of spikes with very high accuracy.

Del Prete et al. (2003) suggested that memory effects in RA afferents might have a basis in the viscoelastic response of skin. That now seems to be an unlikely explanation because of the effects of predictors were observed did not vary between skin phenotypes.

The odds ratios in these experiments were much smaller than those reported in Del Prete et al. (2003). This is likely attributable to the fact that we collected data in very short collection runs with low stimulus levels. This was done because these preparations tended to become poorly responsive with prolonged exposure to stretch stimuli. The precision with which the logistic function is known determines the goodness of likelihood estimation and therefore the magnitude of the odds ratios.

Tissue viscoelasticity might also influence neuron sensitivity in ways other than those we describe: viscoelasticity has been suggested to be a determinant of a slowly adapting component of mechanoreceptor adaptation. After a step change in displacement, both tissue stress and neural response decay with a common time constant. This has been observed in muscle spindles (Boyd et al. 1977; Hunt and Wilkinson 1980), baroreceptors (Xavier-Neto et al. 1996), invertebrate stretch receptors (Rydyvqvist et al. 1990), tendon organs (Houk et al. 1981), and joint afferents (Grigg 1975; Grigg and Greenspan 1977). Similarities of the time constant for stress relaxation and neural adaptation suggest the slow component of adaptation is attributed to viscoelastic relaxation of stress. In such analyses, however, it is necessary that the response clearly be caused by the stimulus. Among the mechanoreceptors mentioned earlier, a specific role for tissue stress in driving a neuron has been shown only for cat joint afferents (Fuller et al. 1991).

As in other experiments addressing the role of tissue viscoelasticity, one should note that this experiment does not preclude the possibility that the relationship between tissue response and neuronal response may not be a causative one; neuronal dynamic responses and skin viscoelasticity may be independent of each other and simply covary.

**Acknowledgments**

We thank S. Baker for advice with statistics.

**Grants**

This work was supported by National Institute of Neurological Disorders and Stroke Grant NS-10783.

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