Can Receptor Potentials Be Detected With Threshold Tracking in Rat Cutaneous Nociceptive Terminals?


Department of Physiology and Pathophysiology, University of Erlangen/Nürnberg, Erlangen, Germany

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Sauer, S. K., C. Weidner, R. W. Carr, B. Averbeck, U. Nesnidal, P. W. Reeh, and H. O. Handwerker. Can receptor potentials be detected with threshold tracking in rat cutaneous nociceptive terminals? J Neurophysiol 94: 219–225, 2005. First published March 16, 2005; doi:10.1152/jn.00655.2004. Threshold tracking of individual polymodal C- and Aδ-fiber terminals was used to assess membrane potential changes induced by de- or hyperpolarizing stimuli in the isolated rat skin–nerve preparation. Constant current pulses were delivered (1 Hz) through a tungsten microelectrode inserted in the receptive field, and the current amplitude was controlled by feedback with a laboratory computer programmed to serially determine the electrical threshold using the method of limits. During threshold tracking, the receptive fields of the fibers were heated (32–46°C in 210 s) or superfused with modified synthetic interstitial fluid containing either 0, 20, 40, 50, or 60 mM [K+]e. Bradykinin (BK, 10−8−10−5 M) and low pH also induced ongoing discharge in 60% of units. Bradykinin in a pure CO2 atmosphere. The skin of the lower hind paw was blunt glass rod.

INTRODUCTION

The transduction site in the nociceptive terminal of C-fibers has long been a target of physiological interest, but no methods for direct experimental access are known. Electron microscopy was used to reconstruct “free” nerve endings (Heppelmann et al. 1990), and these pictures suggest putative transduction sites that are not coated by Schwann cells. However, nothing is known about the receptor or generator potential and the generation of action potentials. Usually, extrapolations for transduction mechanisms are derived from patch-clamp recordings on dorsal root ganglion (DRG) cells; the distribution and expression of membrane proteins responsible for transduction may, however, differ considerably between nerve terminals and the DRG soma. Evidence for crucial differences has accumulated over the past few years (Brock et al. 1998; Caterina et al. 2000). The only technique that allows extracellular recording from terminal branches stimulated at their axons was developed by Brock et al. (1998) in the cornea of the guinea pig. An indirect method using calcium imaging to visualize neuronal activity in sensory nerve terminals was introduced recently (Gover et al. 2003). Brock et al. (1998) found that TTX-resistant sodium channels can give rise to spike generation in the nerve terminal. A heterogeneity of axon and terminal has been suggested for the conductive properties of human C-fibers. The distal parts of human C-fibers are thought to conduct action potentials slower and show more pronounced activity-dependent slowing than the more proximal axon (Weidner et al. 2000). The small size of nociceptive terminals and their embedding in the tissue prevent direct experimental access to the terminals. The “threshold tracking” method, i.e., measuring the electrical excitability, may offer an indirect means to assess the receptor potential, if the current threshold is really determinable at a site where the subthreshold receptor potential is present (Bostock et al. 1991). This hypothesis is tested in this study, parts of which have been published in abstract form (Sauer et al. 1999).

METHODS

Preparation

An in vitro preparation of a flap of rat hairy skin together with the innervating nerve was used for extracellular single fiber recordings of nociceptive primary afferents. The preparation has been previously described in detail and validated (Kress et al. 1992; Reeh 1986). In brief, male Wistar rats (120–420 g body weight, n = 30) were killed in a pure CO2 atmosphere. The skin of the lower hind paw was dissected subcutaneously together with the attached saphenous nerve, which was cut next to the inguinal ligament. The skin flap was pinned out with the corium side up in a chamber and superfused at a rate of 16 ml/min with synthetic interstitial fluid (SIF; Bretag 1969), containing (in mM) 107 NaCl, 3.48 KCl, 26.2 NaHCO3, 1.67 NaH2PO4, 0.69 MgSO4, 9.64 sodium gluconate, 5.5 glucose, 7.6 sucrose, and 1.53 CaCl2. The temperature was set to 32°C, and the pH adjusted to 7.4 by gassing with carbogen (5% CO2–95% O2). The saphenous nerve was pulled through a hole in the tissue chamber wall into a paraffin oil–filled recording chamber and placed on a small mirror. Small filaments were teased out of the whole nerve and subdivided under microscopic control. Afferent nerve fiber activity was recorded via a gold wire electrode. When single nerve fiber activity could be recorded the receptive field was localized by probing the skin with a blunt glass rod.

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* S. K. Sauer and C. Weidner contributed equally to this work.

Address for reprint requests and other correspondence: S. K. Sauer, Inst. für Physiologie and Pathophysiology, Univ. Erlangen/Nürnberg, Universitätsstr. 17, D-91054 Erlangen, Germany (E-mail: sauer@physiologie.uni-erlangen.de).
Recording and stimulation

Single units were characterized as follows: conduction latency in milliseconds was determined by electrical stimulation inside the receptive field with a fine steel electrode, and the conduction distance was assessed to calculate conduction velocity. Mechanical thresholds were determined using a series of calibrated von Frey hairs that exerted forces geometrically scaled from 1 to 256 mN. For thermal, chemical, and electrical stimulation, small hollow steel cylinders (~8 mm) were placed on the receptive field, which was thus isolated from the main organ bath. The chamber formed by this cylinder could be perfused separately (Reeh 1986). For heat stimulation, the radiation of a halogen lamp was focused on the receptive field (within the evacuated chamber) through the translucent bottom of the organ bath. The temperature was controlled by feedback with a thermocouple placed on the corium side of the receptive field. Standardized ramp-shaped heat stimuli were applied rising from 32 to 45°C within 20 s. For cold stimulation, ice-cooled SIF was applied to the receptive field. For chemical stimulation, the small chamber formed by the cylinder around the receptive field was perfused at 2.5 ml/min, allowing the chamber contents to be completely exchanged within 30–40 s.

Threshold tracking

For electrical stimulation inside the mechanoreceptive field of an identified primary afferent unit tungsten microelectrodes (WPI), with a impedance varying from 0.9 to 1.3 M, were inserted into the corium. The position and depth of the electrode tip were adjusted with a three-dimensional micromanipulator under binocular control to find the position at which propagated action potentials were evoked with minimum current. A battery-powered, constant current linear stimulus isolator (WPI A395) that was controlled by a laboratory computer via a DAP interface card (Microstar, Richmond, WA) was used for stimulation. Stimuli of 500 μs duration were delivered at 1 Hz. Response latency and current threshold were tracked automatically using the “method of limits” assessed on-line with custom written software. A data point was only generated if the electrical stimulus induced an action potential, i.e., the stimulus was suprathreshold. Stimulus magnitude was increased stepwise by 0.2 mA until a spike response occurred within a fixed time window or decreased until it vanished. Therefore the variable “tracked threshold” reflects the real threshold with two restrictions: first, the tracked threshold will oscillate around a stable real threshold, and second, the technique could not track threshold changes faster than 0.2 mA/s without delay (Fig. 1, A and B).

Chemical stimulation

The following chemical stimuli were used: [K⁺]e at 5 concentrations (0, 20, 40, 50, and 60 mM), to keep the osmolarity of the modified SIF constant, [Na⁺]e was adjusted accordingly; [H⁺]e, the pH of the SIF solution was altered by exchanging bicarbonate for phosphate buffer (pH 5.2: NaH₂PO₄ 26.54 mM and Na₂HPO₄ 0.66 mM; pH 6.1: NaH₂PO₄ 19.92 mM and Na₂HPO₄ 3.98 mM) and titrated to pH 5.2 and 6.1 with drops of HCl or NaOH; and bradykinin (BK, Sigma), applied to the receptive fields at five decadic concentrations: 10⁻⁸–10⁻⁵ M. Different concentrations were used successively in ascending order until the fiber responded or the highest concentration was reached. Individual concentrations were applied for ≥120 s. Bradykinin was diluted in SIF from 10⁻³ M stock solutions stored at −28°C before each experiment.

Between different chemical stimuli, the solution was replaced by regular SIF, and a washout period followed that lasted for ≥5 min. When units were excited by the chemical stimulus, the superfusion was immediately switched back to SIF to stop the discharge, because electrical threshold detection became impossible because of refractoriness and an activity-dependent threshold increase.

Response latency (ms) and current threshold (mA) were stored in ASCII format and further computed with EXCEL (Microsoft). Sequential threshold and latency data were smoothed using a five-point running average algorithm. A period of ≥30 s before starting the heat/chemical stimulation was recorded as baseline. The SD of the threshold in this period was calculated, and the mean was taken to normalize all subsequent data in summary figures.

Electrical threshold and response latency were assumed to be changed during chemical or heat stimulation when the values deviated from baseline by >2 SD. This time-point was taken as the beginning of a response, and all further times were expressed relative to it. Thereafter, response periods of 30 s were averaged; the observation period was maximally 150 s. All fibers were grouped by stimulus type (i.e., low pH, heat, and bradykinin) and by their responsiveness to these stimuli.

For statistical evaluation, an ANOVA was used (STATISTICA, Statsoft). Single stimulation periods were regarded as independent cases. For each stimulus type, the dependent variable tracked threshold (successive 30 s bins as repeated measures) was tested for the influence of the independent variable responsiveness. P < 0.05 was considered to be significant.
RESULTS

In 12 units of different categories (C and Aδ fibers), the electrical thresholds were followed over 150 s before any conditioning treatment. During this period, the electrical threshold increased slightly by about 2% (from 97.7 ± 1.3 to 100.1 ± 0.08%, n = 12, data not shown).

Potassium stimulation

Electrical thresholds of 28 mechano-heat sensitive C-fibers (CMH; polymodal nociceptors; cv = 0.51 ± 0.1 m/s; median von Frey threshold 32 mN) and two mechano-heat responsive Aδ fibers (cv 12.3 and 7.4 m/s) were tracked during superfusion with five different potassium concentrations (0, 20, 40, 50, and 60 mM; Fig. 2B). Figure 2A shows results from a polymodal C-nociceptor that was exposed to increasing K\(^+\) concentrations. Superfusion with 20 mM [K\(^+\)]\(_e\) led to a distinct reduction in the electrical threshold that became more pronounced during 40 and 50 mM [K\(^+\)]\(_e\) superfusion, consistent with the expected depolarizing effect.

During superfusion of the receptive field with K\(^+\)-free SIF, the mean threshold increased significantly from basal 100.04% to 105.67% (P = 0.02, n = 10, Wilcoxon test, Fig. 2B), consistent with the predicted hyperpolarization. Additionally, after a variable delay, ongoing activity was induced in 6/10 units in K\(^+\)-free SIF.

All SIF variations with higher than normal K\(^+\) concentration (i.e., >3.48 mM) led to a decrease in the electrical threshold but did not induce ongoing activity. However, single spike discharges were observed. The decline of the threshold after switching to 20 mM [K\(^+\)]\(_e\) became significant with respect to baseline 30 s after the operationally defined (see METHODS) onset of the response (P = 0.04 Wilcoxon test, Fig. 2B). At higher concentrations of K\(^+\), 40 (n = 12), 50 (n = 13), and 60 mM (n = 11), threshold decreases that were significantly different from the basal thresholds were observed (40 mM: P < 0.002, 50 mM: P < 0.001, 60 mM: P < 0.007; Wilcoxon tests). However, the threshold changes induced by the K\(^+\) concentrations of 40–60 mM were not significantly different from each other. At 120 s response time, 40 mM [K\(^+\)]\(_e\) led to a final threshold value about 12% lower than baseline, 14% lower at 50 mM [K\(^+\)], and 12.5% lower at 60 mM [K\(^+\)] (P = 0.04, P = 0.02, and P = 0.04, respectively; Wilcoxon tests).

Acidic pH

For superfusion with low pH we used SIF at pH 6.1 and 5.2. The polymodal nociceptor (CMH, cv: 0.5 m/s) shown in Fig. 3A was consecutively exposed to SIF at pH 6.1 and 5.2, and it responded first with a threshold increase and then began to discharge. Overall, we tested solutions with high [H\(^+\)] concentration on 10 CMH fibers (cv: 0.59 ± 0.14 m/s), 3 of which were excited by low pH stimulation. In the pretest period of 150 s, there was no significant change in threshold (from 98.6 ± 1.9 to 99.7 ± 0.8%, data not shown). During low pH stimulation, not only pH-sensitive but also those fibers that were not excited by the acidic stimulus, exhibited a significant increase in their threshold (Fig. 3, B and C; ANOVA, P < 0.01 and P < 0.02, respectively). A lowering of the threshold before excitation was never observed.

BK

BK in concentrations of 10\(^{-8}\) (n = 13), 10\(^{-7}\) (n = 9), 10\(^{-6}\) (n = 11), and 10\(^{-5}\) M (n = 9) was applied consecutively to the receptive fields of 13 primary afferent fibers (12 CMH fibers: cv = 0.58 ± 0.2 m/s, 1 low threshold A-fiber mechanoreceptor: cv = 12.3 m/s). Figure 4 summarizes the results.

The fibers’ electrical threshold remained unchanged when BK did not evoke spike discharge. In contrast, thresholds decreased before activation in those fibers in which BK exerted an excitatory action, and the difference between trials with and without subsequent discharge was significant (ANOVA, all BK concentrations, P < 0.04; n = 14 and 10, respectively). The maximal threshold decrease was 5% with a BK concentration of 10\(^{-5}\) M (LSD posthoc test for 10\(^{-5}\) M, P < 0.01).

Heat stimulation

The effects of slow heating (0.06°C/s, 32–46°C within 210 s) on fiber excitability were studied in nine C-fibers (cv: 0.5 ± 0.01 m/s, 7 of them CMH fibers responding to heat stimulation) and one Aδ low threshold mechanosensitive (LTM) unit (cv: 4 m/s, von Frey 1 mN). An example is shown in Fig. 5A. For six of these fibers, the thresholds could be tracked for 120 s (32–40°C) after the onset of the heat stimulus. Figure 5B shows that initially both heat responsive and heat insensitive fibers exhibited a distinct threshold in-
crease (103.5 ± 7.7% within 60 s). Before excitation, the threshold of the heat responsive fibers decreased to 93.1 ± 11.0% (at 120 s), whereas the threshold of heat insensitive fibers further increased to 114.2 ± 8.9%. This difference was statistically significant (ANOVA, repeated measures, P < 0.01, n = 6).

DISCUSSION

Terminals of nociceptive nerve fibers in the skin are thought to be equipped with membrane receptors and ion channels through which current in response to mechanical, thermal, or chemical stimuli generates a local membrane depolarization, the so-called receptor potentials. From electron microscopic serial sections, it seems that transduction sites for the generation of receptor potentials may be correlated to patches of axon membrane that remain uncovered by membranes of the Schwann cell coating (Heppelmann et al. 1990). From those “windows,” the receptor potentials spread electrotonically according to the length constant potentially influencing voltage-gated sodium channels. A receptor potential that discharges the membrane capacitance rapidly enough to generate a propagated action potential is termed the generator potential. In unmyelinated axons, the site where the receptor potential acts as an action potential generator (transformation site) is unknown and may not be a spatially specialized (Carr et al. 2003).

A stimulus that ultimately activates a nerve fiber must have caused depolarizing currents leading to membrane depolarization at both the transduction site (i.e., the region of membrane equipped with the specific receptor) and that portion of the membrane that is electrotonically influenced. As long as the threshold for voltage-gated sodium channel activation is not reached at any site within this depolarized area, an increase in excitability (i.e., a lower threshold for activation by external electrical stimuli) should be detectable by the threshold tracking technique (Bostock and Baker 1988). However, it may be noted that threshold tracking probably measures the net effect of spatially and temporally dispersed receptor potentials and not the ultimate generator potential that somewhere in the nerve ending exceeds the threshold for sodium channel activation and elicits an action potential.

In this in vitro study, we showed that threshold tracking is a viable method for detecting excitability changes in terminal branches of primary nociceptive skin afferents, and these are likely to reflect changes in membrane potential. Changes of [K+]o known to alter the resting membrane potential induced concentration-dependent changes of the excitability, both increasing excitability with depolarization and decreasing excitability with hyperpolarization. During heat and BK stimulation, both of which activate nociceptive terminals physiologi-

FIG. 3. Influence of low pH on excitability. A: unit (cv = 0.5 m/s, von Frey 22.6 mN) superfused with acidic synthetic interstitial fluid (SIF), which caused both an increase of the electrical threshold and a short burst-like activity (gap and arrow). B: unspecific threshold increase (30 s bins) induced by pH 6.1 both in responsive (○, n = 2) and unresponsive (■, n = 6) fibers. C: even more pronounced threshold increase at pH 5.2 (○, n = 2; ■, n = 3).

FIG. 4. Influence of bradykinin on excitability. A: unit (cv = 0.86 m/s, von Frey 64 mN) superfused with bradykinin in increasing concentrations that caused a decrease of the electrical threshold only at 10⁻⁸ M, i.e., before excitation indicated by the arrow. B: differential effect of bradykinin stimulation leading to excitation or not. If bradykinin superfusion did not induce fiber discharge (■, n = 14), the average electrical threshold increased slightly. In contrast, before onset of excitation, significantly lower thresholds were observed in response to bradykinin (○, n = 10; ANOVA, P < 0.04).

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followed, after a variably long delay, by ongoing discharge in zero $[K^+]_e$ (K 1983; Raymond 1979; Raymond and Lettvin 1978). Although (ANOVA, olds, indicative of a subthreshold depolarization, before the onset of excitation lowering of the electrical activation threshold; likewise, zero $[K^+]_e$ inward currents (Baker et al. 1987). However, the delayed for activation and/or from hyperpolarization-activated cationic ions, changes in the proportion of sodium channels available should decrease the electrical threshold of the respective noci-

Potassium

According to the constant field equation, changes of $[K^+]_e$ should alter excitability by shifting the membrane potential toward or away from the activation threshold of the voltage-dependent sodium channels (Hodgkin and Huxley 1952). Indeed an increase in $[K^+]_e$ led to a concentration-dependent lowering of the electrical activation threshold; likewise, zero $[K^+]_e$, raised the threshold (Kocsis et al. 1983; Malenka et al. 1983; Raymond 1979; Raymond and Lettvin 1978). Although zero $[K^+]_e$, obviously induced a hyperpolarization, this was followed, after a variably long delay, by ongoing discharge in 6/10 fibers. These delayed depolarizing effects of zero $[K^+]_e$ could derive from a block of the Na-K pump (Rang and Ritchie 1968), which ceases to work in the absence of external $K^+$ ions, changes in the proportion of sodium channels available for activation and/or from hyperpolarization-activated cationic inward currents (Baker et al. 1987). However, the delayed depolarization induced by zero $[K^+]_e$ was not preceded by a detectable increase of the excitability as measured by threshold tracking. This means that the site of action potential generation—probably in the terminal region with low intracellular volumes where the blockade of the pump is most effective—must have been different from where the electrical test stimuli were effective to excite the nerve ending (probably in the thicker preterminal or branching point regions).

On the other hand, the presumably slow depolarization during wash-in of high $[K^+]_e$ increased the electrical excitability of the nerve endings and induced impulse activity the frequency of which was irregular. Regular ongoing activity or burst discharge was probably prevented by accommodation. Also, the saturation of the potassium effect at concentrations of 40 mM and higher may have resulted from progressive inactivation of sodium channels that counteracted any further increase in excitability. Inactivation of terminal Na channels due to depolarization seems to be a physiologically relevant factor determining excitability, as shown for corneal cold receptors (Carr et al. 2002).

From these results, we conclude that the threshold changes, as assessed here, indeed reflect changes in membrane potential in the terminal. Hence, we applied the threshold tracking method to assess membrane potential changes evoked by stimuli that activate nociceptive terminals physiologically, i.e., BK, low pH, and noxious heat.

Low pH

Low pH leads to excitation of a subpopulation of nociceptors. Acid-sensing ionic channels (ASICs) and capsaicin receptor channels (TRPV1) are likely candidates for transducing low pH stimuli by inducing depolarizing inward currents (Tominaga et al. 1998; Waldmann et al. 1999). In previous experiments using the skin–nerve preparation, it was shown that 38% of the CMH units in the rat were excited by superfusion with solutions of pH 6.1 (Steen et al. 1992).

Accordingly, in these experiments, we also observed activation of 33% of the C units induced by lowering the pH to 6.1 or 5.2. However, before the onset of firing, we did not observe a gradual threshold decrease that would reflect a depolarizing receptor potential; instead, the thresholds rose, indicating either a membrane hyperpolarization or an impaired sodium channel function. If at all, low pH is known to shift the resting membrane potential (of muscle cells) in a depolarizing direction (Hutter and Warner 1967), whereas the conductance and gating of voltage-gated sodium channels are generally impeded by protons (Benitah et al. 1997; Hille 1968, 1992). Therefore the observed threshold increase is most likely an effect not related to the pH-transduction mechanism, but due instead to a proton blockade of sodium channels rather than to hyperpolarization. This pronounced loss in excitability (threshold increase $\approx 25\%$) obviously decreased the sensitivity of our technique to detect the expected proton-induced subthreshold depolarization.

Noxious heat

The depolarizing receptor potential presumably induced by heat-activated cation currents (Cesare and McNaughton 1996) should decrease the electrical threshold of the respective noci-

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**FIG. 5.** Influence of temperature increase on excitability. A: pronounced threshold and latency decrease induced by a slow temperature increase (stimulus trace in the subpanel) in a mechano-heat sensitive C-fiber (CMH) nociceptor (cv = 0.46 m/s) that then started firing at a temperature close to 40°C (at which time the recording was shortly interrupted and heat stimulation discontinued). B: nonspecific threshold increase with temperature (32–40°C in 120 s) in fibers that were not excited by heat stimulation (n, n = 3). In contrast, heat-sensitive fibers (c, n = 3) developed significantly lower electrical thresholds, indicative of a subthreshold depolarization, before the onset of excitation (ANOVA, P < 0.01).
cceptor before its activation. Indeed, we could observe a small average threshold decrease of all the fibers that were activated by heating. Although this decrease was not statistically significant versus baseline, the threshold significantly differed from the increased thresholds of the fibers not excited by heat, an effect that developed over a similar time course (Fig. 5C). The significant increase in the threshold of units not activated by heat is probably due to a mechanism not related to heat transduction, i.e., it is considered nonspecific. The nonspecific threshold increase was observed in parallel with an increase of the conduction velocity. Temperature primarily affects several membrane properties that could account for changes in electrical threshold and cv changes, including resting membrane potential, membrane resistance (input impedance), and the gating kinetics of sodium channels. The resting membrane potential will be shifted in a hyperpolarizing direction simply due to thermodynamic principles that is the Boltzmann term in the Goldman equation increases by $1 + \frac{\Delta T_{\text{heating}}}{293K}$. In addition, the Na/K pump should gain a higher rate (Glitsch and Pusch 1984) and a more pronounced activity dependence contributing to further hyperpolarization. However, hyperpolarization can only explain the nonspecific threshold increase but not the acceleration of conduction (Raymond and Lettvin 1978). Activation time constants of voltage-gated Na channels decrease with rising temperature as shown for myelinated axons (Jonas 1989), which explains the acceleration of action potential propagation (Schmidtmaier 1989). The nonspecific threshold increase should occur in heat sensitive as well as insensitive fibers. Therefore the threshold difference between activated and nonactivated fibers probably corresponds to the true and specific heat-induced subthreshold depolarization that builds up before the (increased) threshold for action potential generation is reached.

**BK**

BK can induce depolarizing cationic currents in a subpopulation of cultured sensory neurons, and this seems to result from a drastic B2 receptor-mediated sensitization of the capsaicin receptor, TRPV1, a heat-activated ion channel that opens at room temperature when sensitized (Burgess et al. 1989; Chuang et al. 2001). Thus BK could be expected to elicit a detectable receptor potential—without the confounding influences exerted by noxious heat or protons—at least in the subpopulation of polymodal nociceptors in the isolated skin that is overtly excited by BK (Lang et al. 1990). Indeed, before firing, those units responding to BK showed a significant lowering of their electrical threshold before firing, whereas the excitability of fibers not activated by BK remained unchanged. However, the B2 receptor is functionally expressed in 85% of cold-sensitive receptors in the guinea-pig cornea (Liang et al. 2001). This frequent sensitizing effect is obviously not reflected in our threshold tracking data that indicate a pre-excitatory depolarization in only 10/24 cases. This suggests that sensitization to heat by BK indeed affects sensory transduction—through heat-activated ion channels—rather than the action potential generation through voltage-gated sodium channels. The latter could be taken as an argument against a major role of TTX-resistant sodium channels, at least in nociceptor sensitization. BK—through stimulation of prostaglandin E (PGE$_2$) synthesis—lowers their voltage threshold in cultured sensory neurons (Gold et al. 1998), which should increase the excitability as measured here by the threshold tracking technique.

In conclusion, this study verifies the viability of threshold tracking as a means to detect peripheral membrane potential changes in nerve terminals induced by varying [K$^+$]e. Excitatory physiological stimuli evoked detectable subthreshold depolarizations before excitation in those cases where nonspecific effects (e.g., proton block) did not prevent their detection.

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