Ultrafast optical recording reveals distinct capsaicin-induced ion dynamics along single nociceptive neurite terminals \textit{in vitro}

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Abstract. Pain signals are detected by terminals of nociceptive peripheral fibers situated among the keratinocytes and epithelial cells. Despite being key structures for pain-related stimuli detection and transmission, little is known about the functional organization of terminals. This is mainly due to their minute size, rendering them largely inaccessible by conventional experimental approaches. Here, we report the implementation of an ultrafast optical recording approach for studying cultured neurite terminals, which are readily accessible for assay manipulations. Using this approach, we were able to study capsaicin-induced calcium and sodium dynamics in the nociceptive processes, at a near-action potential time resolution. The approach was sensitive enough to detect differences in latency, time-to-peak, and amplitude of capsaicin-induced ion transients along the terminal neurites. Using this approach, we found that capsaicin evokes distinctive calcium signals along the neurite. At the terminal, the signal was insensitive to voltage-gated sodium channel blockers, and showed slower kinetics and smaller signal amplitudes, compared with signals that were measured further up the neurite. These latter signals were mainly abolished by sodium channel blockers. We propose this ultrafast optical recording approach as a model for studying peripheral terminal signaling, forming a basis for studying pain mechanisms in normal and pathological states. © The Authors. Published by SPIE under a Creative Commons Attribution 3.0 Unported License. Distribution or reproduction of this work in whole or in part requires full attribution of the original publication, including its DOI. [DOI: 10.1117/1.JBO.22.7.076010]

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1 Introduction

The detection of painful stimuli takes place at the nociceptive peripheral sensory nerve terminals in the skin or the viscera. These miniature structures (0.5 to 5 μm in diameter, Fig. 1) are pivotal for the detection of noxious stimuli.\textsuperscript{3} Consequently, the comprehensive understanding of nociception and hence mechanisms of pathological pain can be achieved only by detailed exploration of nociceptive terminal function. However, not much is known about their properties because they are barely accessible by conventional electrophysiological methods. The saphenous skin–nerve preparation and \textit{ex vivo} somatosensory system models developed by Reeh\textsuperscript{6} and Koerber and Woodbury\textsuperscript{8} respectively, as well as other models using extracellular recordings from nerve fibers\textsuperscript{7} monitor axonal activity following activation of the terminals, in different conditions, but not the activity at the terminals themselves. Electrophysiological recordings and ion imaging from terminal axons provided essential information about Na\textsuperscript{+} currents, which underlie action potential generation\textsuperscript{8} and terminal Ca\textsuperscript{2+} signaling\textsuperscript{9}. Nevertheless, spatial constraints of the electrophysiological methods make it unsuitable for studying biophysical properties of signal propagation along the cylindrical terminal and distal axon. Conventional imaging assays overcome these spatial limitations; however, they are lacking in sufficient temporal resolution crucial for exploring ion dynamics underlying action potential generation and propagation. Many molecular and biophysical aspects of nociception have been described using nociceptor cell bodies, situated in dorsal root ganglions (DRG) or trigeminal ganglions\textsuperscript{10}. The functional environment\textsuperscript{11,12} and geometry of terminals differ from that of cell bodies, and likely, their passive membrane properties, density, and specific repertoire of transducer and voltage-gated channels. Hence, detailed characterization of ion signal onset, kinetics, and magnitude, along the terminals and terminal axons, have yet to be achieved, albeit being highly valuable for understanding nociceptive physiology. Consequently, the basic questions in nociceptive physiology, such as where action potentials are generated and how they propagate along nonmyelinated tiny axons in normal and pathological conditions, still remain obscure.

Here, we introduce a new approach for high-temporal resolution optical recordings of ion dynamics from a single nociceptive neurite terminal \textit{in vitro}. This approach allows us to monitor capsaicin-induced calcium (Ca\textsuperscript{2+}) and sodium (Na\textsuperscript{+}) dynamics at the terminal neurites and characterize their propagation along the distal neurites. Using this approach, we have demonstrated that the capsaicin-induced Ca\textsuperscript{2+} and Na\textsuperscript{+} signals change as they propagate from the terminal and along the neurite. We showed that capsaicin-induced calcium signals initiated at the terminals were not dependent on voltage-gated Na\textsuperscript{+} channels, whereas signals measured 15 μm away and onward, toward the cell body, were faster, stronger, and at least partially mediated by voltage-gated Na\textsuperscript{+} channels.

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Goldstein et al.: Ultrafast optical recording reveals distinct capsaicin-induced ion...

2 Materials and Methods

2.1 Animals

All animal procedures were approved by the Ethics Committee of the Hebrew University. Five- to six-week-old male C57BL/6 mice were used.

2.2 Dorsal Root Ganglion Compartmental Cultures

The compartmental chamber dishes were prepared similarly to that described by Campenot et al. Briefly, poly-lysine- and laminin-coated dishes were scraped with a pin-rake (200-μm width of pins; Tyler Research) to create a series of parallel tracks on the laminin coating, limiting neurite growth to the areas left with laminin, in parallel lanes. A droplet of 0.4% methylcellulose (Sigma) in DMEM (Biological Industries) was spread to cover the scratched region where the Teflon compartmentalized chamber is placed. Teflon compartmentalized chambers (Teflon dividers, Camp10 or Camp320, Tyler Research) were attached to the culture dish with silicon grease (Dow Corning), applied with a syringe grease applicator (CAMP-GLSS, Tyler Research). The central compartment was used for plating the cell soma, flanked by peripheral compartments, containing lanes in which neurites can extend [Fig. 1(b)].

DRG culture preparation: Adult mice were deeply anesthetized (3% isoflurane), and DRG were isolated and dissociated in a manner similar to that described previously with the following modifications: dissected ganglia were placed in ice-cold DMEM (Biological Industries), pelleted, and reconstituted for enzymatic digestion in solution containing 5 mg/ml collagenase and 1 mg/ml dispase II (Roche) for 45 min. Cells were triturated in the presence of 50 U DNase 1 (Sigma) and centrifuged through 10% bovine serum albumin (Sigma). The cell pellet was resuspended in 1 ml of Neurobasal (Gibco) medium containing B27 supplement (Invitrogen), penicillin and streptomycin (Biological Industries), 100 ng/ml 2.5S NGF (Promega), and 2 ng/ml GDNF (Sigma). Cells were plated on to polylsine- (500 μg/ml) and laminin- (5 mg/ml) coated 35-mm glass bottom tissue culture dishes [World Precision Instruments (WPI)] in the middle compartment of the compartmented chambers [Fig. 1(a), see also Ref. 20].

On the day of seeding the cells (day 0), both chambers contained the above-mentioned Neurobasal media with 100 ng/ml 2.5S NGF; thereafter, the concentrations of NGF were altered to promote the sprouting neurites to grow toward the distal chambers as follows (cell bodies and peripheral compartments, respectively): day 1: 10 and 100 ng/ml; day 2: 1 and 10 ng/ml; and days 5, 8, and day 11: 0 and 1 ng/ml.

 Cultures were maintained at 37°C in a humidified incubator containing 5% CO2. The cultured DRG cell somata underwent viral coinfection on either of 8- to 10-days postseeding. Imaging experiments of neurite terminals were carried out on days 11 to 14 from seeding. Teflon dividers were removed prior to the experiment.

2.3 Viral Vectors

To measure the changes in intraterminal and intraneuritic Ca2+ concentrations ([Ca2+]i), the adenov-associated virus serotypes 1 or 6, carrying an expression cassette for the genetically encoded Ca2+ indicator (GCaMP6s) or the mRuby fluorescent protein indicator (under a CMV promoter; ELSC Virus Core Facility), were used. Cells were infected by the two viruses simultaneously.

2.4 Imaging

Image data were processed by Neuroplex software (RedShirt Imaging) and analyzed using pCLAMP 10.2 (Molecular Devices) and OriginPro 9.0.0 (OriginLab Corporation).

Number of repeats in this study (n’s) refers to the number of terminals examined in each experiment. In the Ca2+ imaging assay, each analyzed terminal was from a culture made from a different animal. For the Na+ imaging assay, n reflects different terminals in different dishes, however, not all from different animals.

All analyses were performed on the processes in the same field of view.
We used a short imaging protocol of up to 5 s to avoid photo damage, which occurred during longer exposure, probably due to the recurrent excitation with high-intensity ultraviolet and blue excitation wavelengths used here.

2.4.1 \( Ca^{2+} \) imaging

Fluorescent excitation was performed with a 75 W Xenon arc lamp (Lambda DG4; Shutter Instruments) and a GFP filter set (Ex 480, Em 535, dichroic Lp 510; Chroma). An inverted microscope (Eclipse Ti; Nikon) equipped with a x60 oil 1.4NA objective, Epi-Fl attachment, and perfect focus system was used. For bright field imaging and fluorescent still image acquisition, an EXI Aqua monochromator camera (QImaging) and Nikon elements AR software (Nikon) were used. Changes in intraterminal and intraneuritic fluorescence were acquired using a back-illuminated 80 × 80 pixel cooled CCD camera (NeuroCCD-SMQ; RedShirt Imaging), controlled by Neuroplex software (RedShirtImaging). In these conditions, pixel size was 1 × 1 \( \mu \)m. This spatial resolution was sufficient for analyzing the signal propagation along terminals and distal neurites, even though in some locations the diameter of the structure (0.5 to 5 \( \mu \)m) could have been less than the camera resolution.

Images were acquired at 1000 frames/second (fps).

2.4.2 \( Na^+ \) imaging

To measure changes in intraterminal and intraneuritic \( Na^+ \) concentrations \([Na^+]_{in}\) \textit{in vitro}, the cell-permeable \( Na^+ \) indicator SBFI-AM (Molecular Probes) was used. Five-millimolar stock was prepared in dimethyl sulfoxide with 10% Pluronic F-127. Cells were incubated for 1 h with a 5 \( \mu \)M final concentration of SBFI-AM in the culture media, followed by a 30-min rinse period. Images were acquired at 125 fps. Florescent excitation was performed using a CoolLed fluorescence excitation system, using a 380-nm LED and a Fura2 filter set (Ex: 380 nm, Em: 510 nm, dichroic Lp: 400 nm; Chroma). To compensate for time-dependent decrease in fluorescence, due to fluorophore bleaching, fitted linear curves derived from the first 500 ms of the acquired data were subtracted from the recorded data.

2.5 Solutions

All imaging experiments were performed in standard external solution (SES) composed of 145 mM NaCl, 5 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM glucose, and 10 mM HEPES (pH \( \approx 7.4 \)).

Capsaicin (300 nM) or vehicle (SES with 0.3 \( \mu l/\text{ml} \) ethanol) was focally puff applied (500 ms puff, 2 psi, PV820 Pneumatic PicoPump; WPI) by a pipette with 4 to 6 \( \Omega \)M2 resistance, when filled with SES. The application regime was commanded by a Digidata 1440 A/D interface (Molecular Devices).

In some experiments, the \( Na^+ \) channel blocker, tetrodotoxin (TTX, 10 \( \mu M \); Alomone Labs) together with a Na(+) 1.8 channel blocker, A803467 (10 \( \mu M \); Tocris), were applied to bath for 10 min before the recordings.

2.6 Puff Calibration

We calibrated the dispersion profile of puffed substances by measuring changes in the sulforhodamine (2 \( \mu M \)) fluorescent intensity profile with distance from the pipette tip [Fig. 1B(15)] according to

\[
\frac{F_{x_1,y_1}}{F_{x_0,y_0}} \times C_{x_0,y_0} = C_{x_1,y_1},
\]

where \( F \) is the fluorescence intensity at \( x, y \) coordinates and \( C \) is the concentration.

2.7 Data Analysis

To measure changes in \([Ca^{2+}]_{in}\) \textit{within} a terminal at the highest possible resolution our conditions permit, the data were collected from 1 × 1 \( \mu \)m sized regions of interest (ROIs) [1 pixel; Fig. 1C(1)]. To examine changes in \( Ca^{2+} \) concentration propagating along the neurite and to improve the signal-to-noise ratio, the data were collected using Kernel smoother from 2 × 2 \( \mu \)m sized ROIs (2 × 2 pixels; Fig. 1C(3)), which were distributed every 5 \( \mu \)m along the neurite’s terminal and processes, dividing the terminals into \( m \) segments. All the data were analyzed from the ROIs delimiting the examined segments, and the size of the ROI was neglected when the actual distance was calculated. Therefore, the distance is represented as an estimated location rather than an absolute distance.

The time of onset of the signal was measured as the time at the onset of a clear sharp deviation from fluorescent base level measured locally, while analyzing the trace from its positive peak toward time “0.” All the values were subtracted from the value at the terminal tip (\( x = 0 \)) of the corresponding terminal.

Difference in time of onset between two adjacent segments, i.e., the delay in onset at one segment compared with the time of onset at the previous segment, were calculated according to

\[
t_m - t_{m-1},
\]

where \( m \) is the \( m \)’th segment.

The fluorescence propagation velocity within 5 \( \mu \)m segments was calculated according to

\[
v = \frac{5}{t_m - t_{m-1}},
\]

where \( v \) is the fluorescence propagation velocity and 5 is the distance between each ROI in micrometers (\( \mu \)m).

Maximum rate of rise of \( Ca^{2+} \) transients was calculated as \( \max(dF/dt) \) at different locations along the neurite terminal. The values were normalized to the value of the terminal tip of the corresponding terminal and plotted as a function of distance from the terminal tip.

All averaged data are presented as the mean ± SEM. Assessment of statistical significance of differences between means was performed with repeated-measures of analysis of variance (ANOVA), with posthoc Bonferroni, as found appropriate.

3 Results and Discussion

To study processing of nociceptive information directly at its most relevant location, with sufficient time and space resolution, we introduced an approach for ultrafast optical recording of cultured nociceptive processes. To that end, we used compartmented chambers [Fig. 1E(16)] see Sec. 3 that have been previously used to study neurites of DRG neurons [17][18]. In these chambers, DRG cell bodies are prompt to grow neurites, and terminal processes can be easily identified and accessed [Figs. 1D(19) and 1C(16)].

We selectively stimulated these neurite terminals with a calibrated focal puff application [Fig. 1D(19)] of capsaicin, an agonist of the noxious heat-sensitive transient receptor potential cation...
Fig. 2 Propagation of capsaicin-induced Ca\(^{2+}\) transients along the neurite terminal. (a) Fluorescence image of representative nociceptive terminals expressing GCaMP6s with illustration of pipette location and with the arrows indicating the locations of ROIs from which the measurements shown in (b) and (c) were performed. (b, c) Representative traces of capsaicin-induced changes in \(\frac{\Delta F}{F_0}\) measured every 5 \(\mu\)m at distances shown in left, from the terminal tip before (b) and 10 min after (c) application of 10-\(\mu\)M TTX together with 10-\(\mu\)M A803467. Changes in fluorescent were measured at 1000 fps. Note the substantial increase in signal amplitude after 15 \(\mu\)m, which was abolished by applying TTX and A803467. Insets: Expanded timescale of the traces presented in (b) and (c) emphasizing the signal onset. Arrows indicate calculated time of signal onset (see Sec. 2). Note that the timescale of the inset for (b) is further expanded to better illustrate the differences in time of signal onset along the neurite in the control conditions. (d) Time of onset of capsaicin-induced Ca\(^{2+}\) signals measured from terminal tip (point “0”) in control conditions (SES, black squares) and after bath application of TTX together with A803467 (red circles), plotted versus distance from the terminal tip. Note much smaller changes in time of onset after 15 \(\mu\)m in control conditions. Application of TTX together with A803467 significantly increased the difference in time of onset after 15 \(\mu\)m. ns, not significant; * \(p<0.05\), ** \(p<0.01\), *** \(p<0.001\); black letters and symbols, comparison between values measured at each location in control condition, \(n=4\), one-way ANOVA with posthoc Bonferroni; orange letters and symbols, comparison between “SES” and “TTX + A803467,” \(n=2\); two-way ANOVA, with posthoc Bonferroni. (e) Peak amplitudes of capsaicin-induced changes in fluorescence, measured every 5 \(\mu\)m and plotted as a function of distance from terminal tip, in control conditions (SES, black) and after (red) application of TTX together with A803467. Note that in control conditions, signal amplitude significantly increased after 15 \(\mu\)m. TTX together with A803467 did not affect the signal measured at the first 15 \(\mu\)m, but significantly reduced the signal amplitude thereafter. Symbols and statistics as in (d). (f) Maximum rate of rise of Ca\(^{2+}\) transients, \(\max \left(\frac{\Delta F}{\Delta t}\right)\), at the different locations along the neurite terminal in control condition (SES, black), and TTX together with A803467 (red) plotted as a function of distance from the terminal tip. Note that in control conditions, the rise rate significantly increased at \(\sim15\) \(\mu\)m and plateaued afterwards. Application of TTX together with A803467 prevented the increase in rise rate. Statistics as in (d).
channel subfamily V member 1 (TRPV1) channel, expressed by nociceptive neurons. We placed the pipette, containing 300 nM capsaicin, about 10 μm from the terminal tip [Figs. 1(a) and (b)] because at this distance, the application of vehicle did not induce changes in GCaMP6s fluorescence [Fig. 1(c)]. We further supported the latter notion by directly measuring that the increase in [Ca^{2+}]_{in} is partially activated along the neurite; Figs. 2(e) and 2(f)]. These data suggest that the increase in [Ca^{2+}]_{in} beyond the ~15 μm is partially dependent on activation of voltage-gated Na^+ channels.

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utilizing fast and sensitive optical recordings of ion dynamics from easily accessible cultured neurite terminals. Altogether, the data shown here demonstrate that our methodology gains in sensitivity and temporal acquisition capabilities, compared with standard imaging techniques, allowing fast optical recordings of weak signals from minute nociceptive terminal processes. Moreover, our approach gains in functional spatial resolution, compared with single electrode electrophysiological approaches, permitting characterization of signal propagations along the terminal and terminal neurites by sampling the signals in action potential relevant time resolution. Thus, the ultrafast and high-resolution optical recording technique described here provides the only tool for a detailed study of a nociceptive terminal functional molecular network, which underlies noxious stimuli detection and transmission in normal and pathological conditions. This could advance the very much needed knowledge for understanding pain physiology and pathophysiology. Moreover, this platform can be utilized to enable studying the terminals of other neuronal types, such as other primary sensory neuron terminals.

Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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Goldstein et al.: Ultrafast optical recording reveals distinct capsaicin-induced ion...