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Electrical Field Stimulation to Study Inhibitory Mechanisms in Individual Sensory Neurons in Culture

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CHANGES in the concentration of intracellular free calcium ($[Ca^{2+}]_i$) regulate activation and control of many cellular processes, including excitability, neuronal development, and secretion of neurotransmitters. As such, increased $[Ca^{2+}]_i$ can be considered a surrogate measure for neuronal activation and has been applied to examine the effects of mechanical, thermal, and chemical stimulation of sensory neurons cultured from dorsal root ganglia (DRG).[1,2] Transient depolarization of DRG neurons increases inward Ca^{2+} currents, with a linear relation between magnitude of current and increase in $[Ca^{2+}]_i$ up to approximately 300 nM, beyond which complex buffering mechanisms result in a nonlinear relation.[3]

We describe a method, using fluorometric measurement of $[Ca^{2+}]_i$ by videomicroscopy, to examine inhibitory pharmacology in individual DRG neurons. Previous studies using this method have examined stimuli such as capsaicin exposure,[1] which only activate some nociceptive neurons or can only be applied once because of rapid desensitization. The method we describe can be used to rapidly examine inhibitory pharmacology in populations of individually identified DRG neurons, whose receptor and neurotransmitter phenotype could be subsequently identified using immunocytochemistry.

Materials and Methods

After Animal Care and Use Committee approval (Wake Forest University School of Medicine, Winston-Salem, North Carolina), DRG neurons were prepared from male Sprague-Dawley rats weighing 250 g (Harlan, Indianapolis, IN) as previously described.[4] DRGs from the mid and lower lumbar levels were removed aseptically and collected in Hank's balanced salt solution. After being minced into small pieces, all DRGs were digested in 0.25% collagenase (Sigma-Aldrich, St. Louis, MO) in Ham's F12 medium at 37°C for 45 min with agitation at 15-min intervals. After a 15-min incubation in Hank's balanced salt solution containing 0.25% trypsin at 37°C, the tissues were triturated with a thin flame-polished pipette in Dulbecco's Modified Eagle's Medium containing 1% HEPES buffer solution, and 10% heat-inactivated fetal bovine serum. Cells were next subjected to centrifugation at 1,000*g* for 5 min. The resulting pellet was resuspended with Dulbecco's Modified Eagle's Medium, and the cell suspension was filtered through a cell strainer (70 μm; Becton Dickinson Labware, Franklin Lakes, NJ), yielding a density of 5×10^5 cells/mm^3. Cells were plated in 35-mm culture dishes on 0.02% poly-L-lysine–coated glass coverslips (Sigma-Aldrich). Cells were placed in a 37°C, 5% CO_2 atmosphere and used between 16 and 24 h after preparation. All media were obtained from Gibco/BRL (Carlsbad, CA).

Measurement of $[Ca^{2+}]_i$

Cells were loaded with 5 μM Fura-2 AM (Molecular Probes, Eugene, OR) and 0.1% Pluronic F-127 (Molecular Probes) for 30 min at room temperature and then washed with a buffer solution consisting of 140 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM glucose, and 10 mM HEPES. Cells were left at room temperature in a dark environment for 30 min. Then, coverslips were mounted into a temperature-controlled chamber equipped with a superperfusion pressure valve system and viewed through an inverted microscope, maintained at 25°C. Fura-2 fluorescence was recorded in 6–10 cells/field using a region of interest defined as the outline of each cell in the field using a digital video camera at 510 nm during excitation at 340 and 380 nm at 2 Hz *via* a monochromator (PTI Deltascan; Photon Technology International, South Brunswick, NJ). $[Ca^{2+}]_i$ was calculated from the ratio of emission at 340–380 nm excitation as previously described[5] according to the standard equation:

$$[Ca^{2+}]_i = K_d\,(R - Rmin)/(Rmax - R)\,(Sf2/Sb2)$$

where K_d is the dissociation constant determined *in vitro* ($K_d = 250$), R is the ratio emission with excitation at 340 nm divided by excitation at 380 nm, Rmin is the ratio in the presence of no Ca^{2+}, Rmax is the ratio of saturating $[Ca^{2+}]_i$, and Sf2/Sb2 is the ratio of 380 nm excitation fluorescence at zero and saturating $[Ca^{2+}]_i$. These were determined in solutions alone or in some

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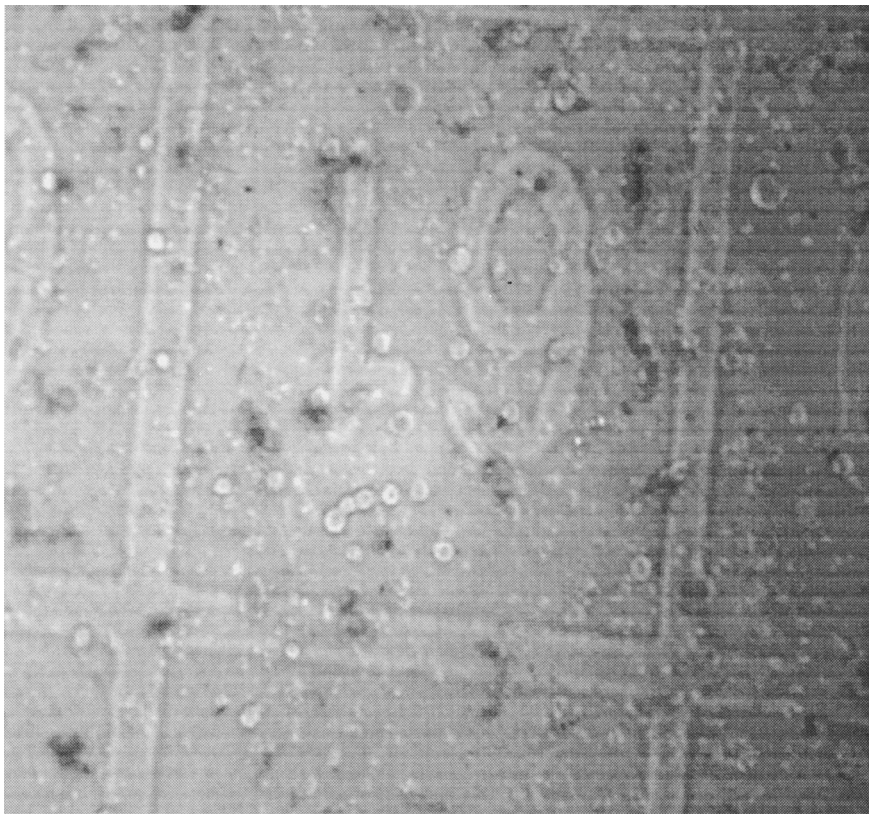

Fig. 1. Phase contrast image of dorsal root ganglion cells used for videomicroscopic examination of intracellular Ca^{2+} concentration. Note the easily visible marker on the surface of the coverslip grid for subsequent identification of location of cells.

cells rendered permeable to Ca^{2+}, although not in each individual cell.

Cell Stimulation

Two wire platinum electrodes separated by 4 mm were positioned onto the coverslip. These wires were nearly 1 cm long, and the cells studied were approximately in the center of the distance between the wires. A constant-voltage generator delivering pulses of 30 V of fixed polarity was used. A stimulator was connected in series to a Med-Lab Stimu-Splitter II (Med-Lab Instruments, Loveland, CO), which allowed measurement of current applied to the coverslip electrodes or shunted. To optimize the stimulation parameters, different numbers of 2-ms-pulse trains at 20 Hz (5, 10, 20, and 40 pulses) and different intensities (60–160 mA) were tested.

Drug Application

All solutions were made daily. All chemicals were purchased from Sigma-Aldrich, Molecular Probes, or Tocris (Ellisville, MO). Solvents used were buffer solution alone or with 0.01% dimethyl sulfoxide (Sigma-Aldrich). After application of the first single-train electrical field stimulus, cells were superperfused with study drugs, vehicle, or buffer for a minimal duration of 8 min after complete return of $[Ca^{2+}]_i$ to baseline.

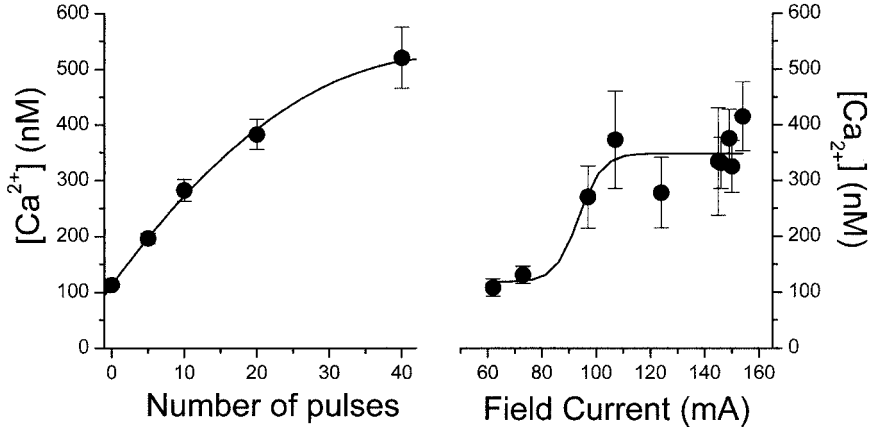

Fig. 2. (*Left*) Relation between number of electrical field pulses (2 ms in duration, 20 Hz, 100 mA, 30 V) and peak intracellular $[Ca^{2+}]$ in dorsal root ganglion neurons. Each *symbol* represents the mean ± SEM of 21–37 cells. Fit to single polynomial. (*Right*) Relation between electrical field current and peak intracellular $[Ca^{2+}]$ in dorsal root ganglion neurons, with stimulus parameters of 10 pulses of 2 ms in duration (20 Hz, 30 V). Each *symbol* represents the mean ± SEM of 4–10 cells. Fit to Boltzmann relation.

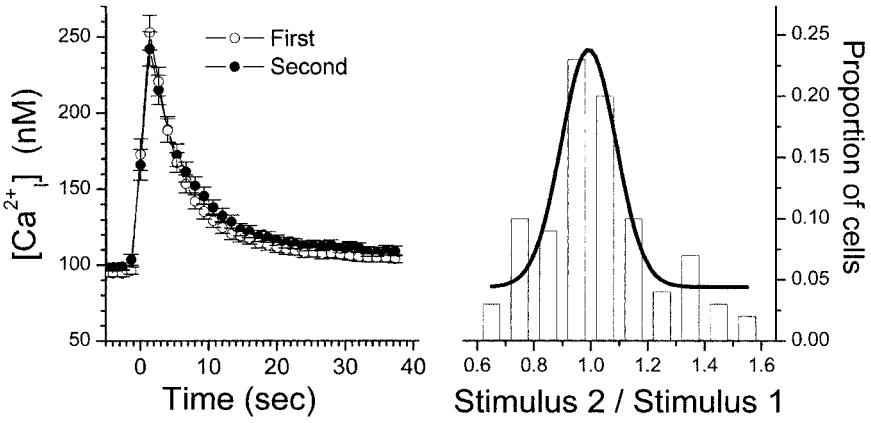

Fig. 3. (*Left*) Response to pulse trains of electrical field stimulation separated by 5 min. Each *symbol* represents the mean ± SEM of 94 cells. (*Right*) Frequency histogram of ratio of peak in intracellular $[Ca^{2+}]$ in dorsal root ganglion neurons to the second train of electrical stimuli compared with the first. Data are from 94 cells. *Solid line* = Gaussian fit of data.

Statistical Analysis

Unless otherwise stated, data are presented as mean ± SE. Only cells with a resting $[Ca^{2+}]_i$ of less than 150 nM were used. The relation between pulse number and $[Ca^{2+}]_i$ was fit to a single polynomial, and the relation between stimulus intensity and $[Ca^{2+}]_i$ was fit using a Boltzmann approach. Subsequent studies used a fixed stimulus of 10 pulses and a current of 100 mA. The ratio of the response to the second train of pulses to the first, as measured by peak minus baseline $[Ca^{2+}]_i$, was calculated, these ratios in the control cells were fit to a Gaussian distribution, and the 3rd and 97th percentiles for this ratio were calculated. For drug studies, two analyses were performed. First, the effect of drug on $[Ca^{2+}]_i$ before and after stimulation was determined by analysis of variance for repeated measures. Second, the proportion of cells meeting criteria for inhibition or excitation ($<$ 3rd or $>$ 97th percentile of control ratios of response to the two trains of stimulation, respectively) were compared by chi-square test with the Yates correction. $P < 0.05$ was considered significant.

Results

Cell Localization

Cells were dissociated onto coverslips with a grid, allowing subsequent identification of cells that had been functionally studied using Ca^{2+} imaging. A representative phase contrast image is provided (fig. 1), indicating the ease of localization of cells on such a grid. Drugs used to study ion channel mediation of Ca^{2+} concentration change or morphine inhibition had no effect on resting Ca^{2+} concentrations (data not shown).

Optimizing Field Stimulation Parameters and Reproducibility

Peak $[Ca^{2+}]_i$ during stimulation using a 100-mA current increased with the number of pulses in a linear fashion up to 10 pulses but less than linear increases with more than 10 pulses (fig. 2). Using a 10-pulse train, there was a threshold to increase $[Ca^{2+}]_i$ at approximately 70 mA and a plateau increase from 90 to 160 mA (fig. 2). Therefore, we used a 10-pulse train at 100 mA for subsequent studies.

Control studies with buffer superfusion showed a slight increase in baseline $[Ca^{2+}]_i$ (95 ± 2.3 nM before the first train, 99 ± 2.4 nM before the second train; $P = 0.03$) but no difference in peak $[Ca^{2+}]_i$ during stimulation (fig. 3). The ratio of peak $[Ca^{2+}]_i$ response after the second compared with the first stimulus was normally distributed and centered around 1.0 (fig. 3), with a 3rd percentile of 0.71 and 97th percentile of 1.68.

Table 1. Ion Channels Involved in $[Ca^{2+}]_i$ Response to Electrical Field Stimulation

Treatment	No.	Ratio of Second Peak of First	% Inhibited	% Excited
Control	94	1.09 ± 0.042	3	3
Cadmium, 100 μM	10	0.15 ± 0.023*	100*	0
Nifedipine, 10 μM	8	0.56 ± 0.100*	63*	0
ω-Conotoxin GVIA, 1 μM	9	0.43 ± 0.042*	100*	0
Na-free (choline, 140 mM)	36	0.47 ± 0.088*	64*	3
Tetrodotoxin, 1 μM	8	0.65 ± 0.145	38*	0
K-free	12	1.26 ± 0.271	0	8
4-Aminopyridine, 200 μM	15	1.26 ± 0.141	7	20*
Charybodotoxin, 100 nM	13	0.98 ± 0.084	8	0

Data are presented as mean ± SE or percentage.

* $P < 0.05$ compared with control.

$[Ca^{2+}]_i$ = concentration of intracellular free calcium.

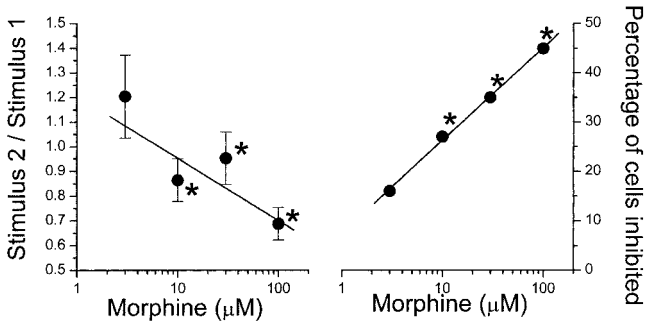

Fig. 4. Relation between morphine concentration and response to electrical stimulation. (*Left*) Mean ± SEM of ratio of the second peak in intracellular [Ca^{2+}] (after morphine exposure) compared with the first (before morphine exposure). (*Right*) Proportion of cells inhibited, as defined by ratio of second to first peak less than the third percentile in control cells. n = 19–34 cells/group. * $P < 0.05$ compared with control. Fit is by linear regression in the log domain.

Ion Channel Dependence of Response

Electrical field stimulation increased $[Ca^{2+}]_i$ by a process involving voltage-gated Ca^{2+} channels, as indicated by inhibition with cadmium, a nonspecific Ca^{2+} channel blocker; nifedipine, an L-type Ca^{2+} channel blocker; and ω-conotoxin GVIA, an N-type Ca^{2+} channel blocker (table 1). Na^+ channels were also involved, as indicated by inhibition with pretreatment with sodium-free buffer and tetrodotoxin, a nonsubtype selective Na^+ channel blocker (table 1). In contrast, experiments with 4-aminopyridine, a nonselective K^+ channel blocker, indicated a minor involvement of K^+ channels, whereas other experiments (K^+-free solution and charybdotoxin, a Ca^{2+}-activated potassium channel blocker) failed to do so (table 1).

Response to Morphine

Morphine (3, 10, 30, and 100 μM) decreased the $[Ca^{2+}]_i$ response to field stimulation in a concentration-dependent manner. This effect was significant by analyzing either the average response in the entire population or the number of cells inhibited, as defined by response to the second stimulus below the third percentile in controls, although the relation was more obvious with the latter analysis (fig. 4).

Discussion

Although in some circumstances the soma of DRG neurons may be important in pain states,[6] most investigators examine the physiology and pharmacology of these cell bodies as models for their peripheral and central terminals. Previous studies to characterize DRG neuronal responses *in vitro* have used either tedious methods to examine a few individual cells (*e.g.*, single-cell electrophysiology) or simpler methods to examine population responses (*e.g.*, neurotransmitter release). Because sensory neurons are heterogeneous, population responses of undefined neurons are difficult to interpret. This is partially addressed by the response measured, such as only studying cells that express vanilloid receptors by using capsaicin stimulation or only studying cells that release a certain neurotransmitter, such as substance P. However, there is no single receptor or neurotransmitter marker for nociceptive neurons in the normal condition, and there is considerable plasticity in receptor and neurotransmitter phenotypes in models of chronic pain.[7] The advantage of our method is that it can easily be applied to populations of DRG neurons, it uses a stimulus and response that involve no assumptions of afferent phenotype, and it could, when coupled with subsequent immunocytochemistry or *in situ* hybridization, provide functional information coupled with phenotypic definition of populations of cells.

As anticipated by previous studies of $[Ca^{2+}]_i$ in DRG neurons,[3] there is a linear association between stimulation strength and increase in $[Ca^{2+}]_i$ up to approximately 300 nM, and this increase reflects activation of voltage-gated Ca^{2+} channels and Na^+ channels. The $[Ca^{2+}]_i$ response to electrical field stimulation has previously been examined with similar results,[8] although that study used cerebellar granule cells and whole population responses in cells in cuvettes rather than videomicroscopy of individual cells.

In summary, DRG neurons respond to an electrical field stimulus with an increase in $[Ca^{2+}]_i$ by activation of voltage-gated Ca^{2+} channels and Na^+ channels in a reproducible manner with repeated stimulation and with concentration-dependent inhibition by the μ-opioid receptor agonist, morphine. This method may be useful to define the physiology and pharmacology in large populations of DRG neurons in normal and chronic pain conditions.

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