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TARGETED ELECTROPHYSIOLOGICAL ANALYSIS OF VISCEROFUGAL NEURONS IN THE MYENTERIC PLEXUS OF GUINEA-PIG COLON

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Abstract—Enteric viscerofugal neurons are mechanosensory interneurons that form the afferent limb of intestinointestinal reflexes involving prevertebral sympathetic neurons. Fast synaptic inputs to viscerofugal neurons arise from other enteric neurons, but their sources are unknown. We aimed to describe the origins of synaptic inputs to viscerofugal neurons by mapping the locations of their cell bodies within the myenteric plexus. Viscerofugal neuron somata were retrogradely traced with 1,1′-didodecyl-3,3,3′,3′-tetramethyl indocarbocyanine perchlorate (DiI) from colonic nerve trunks and impaled with microelectrodes, in longitudinal muscle/myenteric plexus preparations of the guinea-pig distal colon (39 impalements, $n = 14$). Thirty-eight viscerofugal neurons were uni-axonal and had the electrophysiological characteristics of myenteric S-neurons; one neuron was multipolar with AH-neuron electrophysiological characteristics. Depolarizing current pulses evoked either single- or multiple-action potentials in viscerofugal neurons (range 1–25 spikes, 500 ms, 100–900 pA, 21 cells). Electrical stimulation of internodal strands circumferential to viscerofugal neurons evoked fast excitatory postsynaptic potentials (EPSPs) in 19/24 cells. Focal pressure-ejection of the nicotinic agonist 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP, 10 μm) directly onto viscerofugal nerve cell bodies evoked large depolarizations and action potentials (23 ± 10 mV, latency 350 ± 230 ms, 21/22 cells). DMPP was then focally applied to multiple sites, up to 3 mm from the recorded viscerofugal neuron, to activate other myenteric S-neurons. In a few sites in myenteric ganglia, DMPP evoked repeatable fast EPSPs in viscerofugal neurons (latency 300 ± 316 ms, 38/394 sites, 10 cells). The cellular sources of synaptic inputs to viscerofugal neurons were located both orally and aborally (19 oral, 19 aboral), but the amplitude of oral inputs was consistently greater than aboral inputs (13.1 ± 4.3 mV vs. 10.1 ± 4.8 mV, respectively, $p < 0.05$, paired *t*-test, $n = 6$). Most impaled viscerofugal neurons were nitric oxide synthase (NOS) immunoreactive (20/27 cells tested). Thus, the synaptic connections onto viscerofugal neurons within the myenteric plexus suggest that multiple enteric neural pathways feed into intestino-intestinal reflexes, involving sympathetic prevertebral ganglia. © **2014 Published by Elsevier Ltd. on behalf of IBRO.**

Key words: enteric nervous system, sympathetic nervous system, sensory neurons, cholinergic, synaptic potentials.

INTRODUCTION

Enteric viscerofugal neurons form the afferent pathway of reflex circuits between the gut and sympathetic prevertebral ganglia which regulate motility (Weems and Szurszewski, 1977) and secretion (Quinson and Furness, 2002). Mechanical distension of the gut activates these reflexes, and directly evokes viscerofugal neuron firing (Kuntz, 1940; Kuntz and Saccomanno, 1944; Hibberd et al., 2012a). However, viscerofugal neurons also receive prominent synaptic inputs via nicotinic receptors. This was shown by intracellular recordings from sympathetic prevertebral neurons, which receive synaptic inputs from viscerofugal neurons. During nicotinic blockade in the gut wall, decreases in both ongoing and distension-evoked synaptic activity were observed (Crowcroft et al., 1971; Bywater, 1993). Subsequent intracellular recordings of viscerofugal neurons confirmed directly that they receive fast excitatory nicotinic inputs (Sharkey et al., 1998). Thus, viscerofugal neurons function in part as interneurons, and may comprise a pathway for transmitting output from enteric circuits to the sympathetic nervous system. However, the enteric pathways that converge upon viscerofugal neurons have not been functionally identified. Immunohistochemical evidence shows that cholinergic varicosities surrounding viscerofugal neurons appear to arise mainly from descending interneurons, with decreases in synaptic density occurring around viscerofugal neurons immediately aboral, but not oral, to severed myenteric plexus (Lomax et al., 2000). The major aim of the current study was to characterize the functional synaptic inputs to viscerofugal neurons in the myenteric plexus of the isolated guinea-pig distal colon. This was performed by focal pharmacological activation of parts of myenteric ganglia, combined with

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Abbreviations: ChAT, choline acetyltransferase; DiI, 1,1′-didodecyl-3,3,3′,3′-tetramethyl indocarbocyanine perchlorate; DMPP, 1,1-dimethyl-4-phenylpiperazinium iodide; EPSP, excitatory postsynaptic potential; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; NOS, nitric oxide synthase.

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intracellular recording from retrogradely labeled viscerofugal neurons.

EXPERIMENTAL PROCEDURES

Dissection and DiI-labeling

Adult guinea pigs, weighing 200–350 g, were euthanized by stunning and exsanguination as approved by the Animal Welfare Committee of Flinders University. Segments of distal colon (10–20 mm long, > 20 mm from the anus) and attached mesentery were removed and immediately placed into a Sylgard-lined Petri dish (Dow Corning, Midland, MI, USA) filled with filtered (polyethersulfone membrane, 0.22-μm pores, Merck Millipore, Cork, Ireland) and oxygenated Krebs solution at room temperature. The Krebs solution contained (in mM concentrations): NaCl 118; KCl 4.7, $NaH_2PO_4.2H_2O$ 1; $NaHCO_3$ 25; $MgCl_2.6H2O$ 1.2; D-glucose 11; $CaCl_2.2H_2O$ 2.5; bubbled with 95% O_2 and 5% CO_2. Segments were cut open along the mesenteric border, pinned flat with the mucosa uppermost. Mucosa and submucosa were removed by sharp dissection. Extrinsic nerve trunks (5–10 trunks per preparation, 3–10 mm long) were dissected free from surrounding mesentery. The fluorescent dye, 1,1′-didodecyl-3,3,3′,3′-tetramethyl indocarbocyanine perchlorate (DiI; D383, Molecular Probes, Eugene, OR, USA) was used for retrogradely labeling viscerofugal neurons. DiI was evaporated from ethanol solution onto small glass beads (212–300 μm diameter; G-1277, Sigma, St. Louis, MO, USA). A single glass bead was gently placed onto each dissected extrinsic nerve trunk and was confirmed to have remained on the nerve during and after organ culture.

Organotypic culture

Following repeated washes with filtered Krebs solution, preparations were re-pinned into sterilized Petri dishes containing sterile culture medium (Dulbecco's modified Eagle's [DME]/Han's F12, Sigma [1:1 ratio mix, supplemented with L-glutamine and 15 mM HEPES]; including 10% fetal bovine serum (Gibco, Life Technologies Corporation, USA), 100 IU/ml penicillin (Pen Strep, Gibco), 100 μg/ml streptomycin D (Pen Strep, Gibco), 10 μg/ml gentamycin (Gibco), 2.5 μg/ml amphotericin B (Sigma), and 1.8 mM $CaCl_2$. The preparations were incubated for 2–4 days in a humidified incubator (36 °C, 5% CO_2 in air), and agitated on a rocking tray. Sterile culture medium was changed every 24 h.

Intracellular recording

Preparations were pinned, using 50-μm tungsten pins, circular muscle uppermost, into a Sylgard-lined recording chamber of 1-ml volume. Circular muscle was removed by sharp dissection, leaving a preparation of longitudinal muscle and myenteric plexus. The recording chamber was fixed onto the stage of an inverted microscope (IX71, Olympus Corporation, Japan) fitted with fluorescent optics. Krebs solution at 35 °C was constantly superfused at a rate of 3 ml/min. Neurons were impaled using borosilicate glass capillary electrodes (1 mm OD, 0.58 mm ID; Harvard Apparatus) filled with 5% 5,6-carboxyfluorescein (Sigma; 21877) in 20 mM Tris buffer (pH 7.0) in 1 M KCl solution. Electrode resistances ranged from 110 to 175 MΩ. Membrane potential of viscerofugal neurons was recorded using an Axoclamp 2A amplifier (Axon Instruments), viewed on an oscilloscope (model VP-5220A, Matsushita), digitized at 10 kHz, and stored via an analog-to-digital interface (MacLab 8SP, ADInstruments, Sydney, Australia) using Chart 7 software (ADInstruments). Single-pulse electrical stimuli (0.4 ms, 10–15 V) were delivered focally to local internodal strands or ganglia via a pair of insulated Pt–Ir wire stimulating electrodes placed 0.5 mm circumferential to the recording electrode. A Grass S48 stimulator and a Grass S1U5 stimulator isolation unit were used to generate the stimulus. The nicotinic receptor agonist, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP; 10 μM in Krebs solution, mixed 1:10 with blue food dye, Rainbow Food Colours, Australia), was applied focally to the tissue through a glass micropipette (10–20 μm tip). Agonists were delivered using nitrogen gas under 70–80-kPa pressure via a solenoid-operated valve driven by short electrical pulses 20–50 ms in duration. Visualization of the spatial field and trajectory of drug ejection was enabled by the dye; ejection of dye solution without DMPP was used as a control. Locations of the recording and stimulating electrodes, and all sites tested with DMPP were recorded on photomicrographs (Camedia, C3040ZOOM, Olympus Corporation, Japan).

Targeted impalements

DiI-filled neurons were visualized using narrow-band light-emitting diodes of the appropriate excitation wavelength and emission filters (Cool Light-Emitting diode (LED) *p*E excitation system, Andover, UK). To avoid damaging DiI-filled neurons, exposure to green excitation wavelength photons was limited by using low-power objectives (20×), reducing power supply to LEDs to a minimum required for visualization, and by using short exposure time (< 1 s). Microelectrode tips were vertically aligned to the center of the cell body and advanced using the vertical drive of a micromanipulator.

Immunohistochemistry

Preparations were pinned tightly and fixed in modified Zamboni's fixative (2% formaldehyde, 0.2% saturated picric acid in 0.1 M phosphate buffer, pH 7.0) after recordings. Tissue was then cleared for 1 h in bicarbonate-buffered glycerol (0.5 M sodium carbonate in 50%, 70% and 100% glycerol solutions, pH 8.6, 20 min each, in series). Cleared preparations were incubated with antisera to choline acetyltransferase (ChAT, 1:1,000) and nitric oxide synthase (NOS, 1:1,000) at room temperature for two days. Preparations were rinsed three times in PBS and incubated with secondary antisera for 4 h at room temperature. The primary antibodies were as follows: polyclonal rabbit anti-ChAT (cat. No. P3YEB generously provided by Dr.

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