Inhibitory motor neurons of the esophageal myenteric plexus are mechanosensitive

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Dong H, Jiang Y, Dong J, Mittal RK. Inhibitory motor neurons of the esophageal myenteric plexus are mechanosensitive. Am J Physiol Cell Physiol 308: C405–C413, 2015. First published December 24, 2014; doi:10.1152/ajpcell.00159.2014.—Mechanosensitivity of enteric neurons has been reported in the small intestine and colon, but not in the esophagus. Our earlier in vivo studies show that mechanical stretch of the esophagus in the axial direction induces neurally mediated relaxation of the lower esophageal sphincter, possibly through mechanosensitive motor neurons. However, this novel notion that the motor neurons are mechanosensitive has not been examined in isolated esophageal myenteric motor neurons. The goal of our present study was to examine the mechanosensitivity of esophageal motor neurons in primary culture and elucidate the underlying molecular mechanisms. Immunocytochemical analysis revealed that >95% cells were positive for the neuronal marker protein gene product 9.5 and that 66% of these cells contained with protein gene product 9.5 and neuronal nitric oxide (NO) synthase. Hypotonic solution induced an increase in the cytoplasm volume in all cells that was independent of extracellular Ca2+. Hypotonic solution and mechanical stretch induced cytoplasmic free Ca2+ signaling in ~65% of neurons in the presence, but not absence, of extracellular Ca2+. Neurons grown on the elastic membrane responded to mechanical stretch by an increase in neuronal size and Ca2+ signaling simultaneously. Hypotonic stretch-induced cytoplasmic free Ca2+ signaling was not affected by extracellular Mg2+, 5-nitro-2-(3-phenylpropylamino)benzoic acid, and nifedipine but was attenuated by 2-aminoethoxydiphenyl borate, Gd3+, and Grammostola mechanotoxin 4, blockers of the stretch-activated ion channels. In ~57% of the neurons, hypotonic stretch also induced Ca2+ dependent cytoplasmic NO production, which was abolished by Grammostola mechanotoxin 4. These results prove that the esophageal inhibitory motor neurons possess a mechanosensitive property and also provide novel insights into the stretch-activated ion channel-Ca2+-NO signaling pathway in these neurons.

neuronal nitric oxide synthase; nitric oxide production; cytoplasmic free calcium; stretch-activated cation channels

THE ENTERIC NERVOUS SYSTEM regulates gastrointestinal (GI) functions, such as digestion, absorption, secretion, motility, and sensation (1, 14, 37). The enteric nervous system contains three types of neurons: 1) sensory neurons [intrinsic primary afferent neurons (IPANs)], 2) interneurons, and 3) motor neurons (1, 14). The latter are further subdivided into excitatory and inhibitory motor neurons on the basis of their regulatory functions (1, 15): excitatory motor neurons contain neurotransmitters, such as acetylcholine, substance P, and glutamate (14), and inhibitory motor neurons contain nitric oxide (NO), ATP, and vasoactive intestinal polypeptide (14, 37).

Similar to neurons in the rest of the GI tract, neurons in the wall of esophagus also play an important role in the sensory and motor functions of the esophagus (14, 15, 47). The esophageal motor neurons have their cell bodies in the myenteric ganglia, and their axons innervate the longitudinal and circular smooth muscle layers of the esophagus and lower esophageal sphincter (LES) (11, 47). In whole animal studies, we found that mechanical stretch of the esophagus in the axial and transverse directions induces neurally mediated LES relaxation and LES contraction, respectively (22, 23). Pharmacological analysis of in vivo studies suggests that esophageal motor neurons might be mechanosensitive (22); however, this novel notion needs direct evidence. Moreover, although a growing body of literature suggests that enteric neurons respond directly to mechanical stretch in the small intestine and colon (6, 28, 36, 41), whether isolated enteric neurons are mechanosensitive has not been studied. Recently, we successfully cultured myenteric neurons from the rat esophagus (9). We characterized morphology and chemical coding of these neurons and identified functional expression of G protein-coupled receptors and ion channels (9). Because of the integrity and high quality of these isolated neurons in primary culture, they represent a useful cell model for further studies of myenteric neurons.

Cytoplasmic free Ca2+ ([Ca2+]cyt) plays a central role in a wide variety of neuronal functions, including synthesis and release of neurotransmitters and synaptic transmission (4, 8, 19, 42). Similar to many other types of mammalian cells, different Ca2+ influx pathways, voltage-gated Ca2+ channels (VGCCs) (3, 40), receptor-operated Ca2+ channels, and store-operated Ca2+ channels are observed in various types of neurons. A Ca2+ influx pathway, Ca2+-permeable channels activated by mechanical stretch, so-called stretch-activated cation channels (SACs), has been found in various cell types, including neurons (32, 35). Recent evidence indicates that some SACs are encoded by mammalian homologs of Drosophila transient receptor potential (TRP) genes, such as the TRP canonical (TRPC) subfamily (16, 17). The involvement of Ca2+ influx pathways, including SACs and TRPC channels, in the mechanosensitive process is poorly understood in the enteric neurons. The present study was undertaken to investigate 1) whether the inhibitory motor neurons of the esophageal myenteric plexus are mechanosensitive and 2) the underlying mechanisms of their mechanosensitivity.

MATERIALS AND METHODS

Isolation and primary culture of esophageal enteric neurons. The Institutional Animal Care and Use Committee at the Veterans Affairs San Diego Healthcare Systems and University of California, San
Diego approved the study protocol, and all experiments were conducted in accordance with the National Institutes of Health “Guide for the Care and Use of Laboratory Animals.” Isolation and primary culture of enteric neurons from the rat esophagus were conducted as described previously (9). To isolate fresh enteric neurons from the rat esophagus, we used magnetic bead immunoselection of cells expressing p75 neurotrophin receptor (a neural marker). Briefly, the whole esophagus was isolated from postnatal day 5 rats and placed in L-15 medium (Mediatech Cellgro). After removal of the mucosa, the L-15 medium was removed and 1 ml of collagenase (2 mg/ml)-disperse (0.2 mg/ml) was added to prepare the single-cell suspension, which was then incubated for 30 min at 37°C. The tissues were triturated in N-2 medium with a sterile-polished glass pipette, and dissociated cells were incubated with monoclonal antibody to low-affinity nerve growth factor receptor (p75NTR, Millipore, Temecula, CA) at 4°C on a shaker for 1 h. Washing buffer was added, and the solution was spun at 1,300 rpm (300 g) for 10 min at 4°C. The supernatant was aspirated, the cells were resuspended in washing buffer, and magnetic beads labeled with goat anti-mouse IgG were added for 15 min at 4°C. Then all of the solution was added to allow passage of all the cells through a column mounted on the MACS MultiStand, taken off the column, and placed into a 15-ml tube. One milliliter washing buffer was added to the column, and a plunger was placed into the column and used to obtain the positive fraction. Ten microliters of the cell aliquot (positive fraction) were mixed with 10 μl of Trypan blue (1:2 dilution), and the cells were counted in the hemocytometer under a microscope. The number of cells from each esophagus equals the average number of cells counted from four squares of the hemocytometer and was adjusted for the dilution factor and then divided by the total number of esophagi. The remainder of the cell aliquot was spun and resuspended in N-2 medium with penicillin G (50 U/ml) and streptomycin (100 μg/ml). The cells were cultured for 2 wk in a humidified tissue culture incubator.

**Immunocytochemical staining of esophageal myenteric neurons.**

Neurons were immunostained with anti-protein gene product (PGP) 9.5 and anti-neuronal NO synthase (nNOS) antibodies as reported previously (9). Primary culture of the neurons was fixed by Formal-Fixx (Thermo Scientific, Kalamazoo, MI) for 30 min, washed with PBS for 5 min, treated twice with PBS + 0.05% Triton X-100 + 1% bovine serum albumin (BSA) for 5 min, and then rinsed in PBS for 5 min and incubated in PBS for 30 min at 37°C. The tissues were triturated in N-2 medium with a sterile-polished glass pipette, and dissociated cells were incubated with monoclonal antibody to low-affinity nerve growth factor receptor (p75NTR, Millipore, Temecula, CA) at 4°C on a shaker for 1 h. Washing buffer was added, and the solution was spun at 1,300 rpm (300 g) for 10 min at 4°C. The supernatant was aspirated, the cells were resuspended in washing buffer, and magnetic beads labeled with goat anti-mouse IgG were added for 15 min at 4°C. Then all of the solution was added to allow passage of all the cells through a column mounted on the MACS MultiStand, taken off the column, and placed into a 15-ml tube. One milliliter washing buffer was added to the column, and a plunger was placed into the column and used to obtain the positive fraction. Ten microliters of the cell aliquot (positive fraction) were mixed with 10 μl of Trypan blue (1:2 dilution), and the cells were counted in the hemocytometer under a microscope. The number of cells from each esophagus equals the average number of cells counted from four squares of the hemocytometer and was adjusted for the dilution factor and then divided by the total number of esophagi. The remainder of the cell aliquot was spun and resuspended in N-2 medium with penicillin G (50 U/ml) and streptomycin (50 μg/ml). The cells were cultured for 2 wk in a humidified tissue culture incubator.

**Determination of cell volume and [Ca^{2+}]_{cyt} in individual esophageal myenteric neurons.**

Neurons were cultured on 10-nm glass coverslips as well as on elastic silicone membranes for 2 wk. They were then loaded with 5 μM fura 2-AM in physiological standard solution (PSS; see below) at room temperature (22°C) for 50 min and washed in PSS for ≥20 min (2). The coverslips with neurons were mounted in a perfusion chamber on the Nikon microscope stage. To monitor cell volume, the neurons were excited at 360 nm, which is the isosbestic point for fura 2 fluorescence (16, 25). The majority of the loaded fura 2 is contained in the cytoplasm at room temperature, and at 360-nm excitation (F360) and 510-nm emission, fura 2 fluorescence in cells is unaffected by [Ca^{2+}]_{cyt}. Therefore, F360 can be used to measure cytoplasmic concentrations of fura 2, as well as cell volume (10). The change in F360 is an indicator of cell stretch induced by changes in the osmolality of extracellular solution, [Ca^{2+}]_{cyt} in the neurons was measured using fura 2-AM at 340- and 380-nm excitation and 510-nm emission, as described previously (2, 9).

PSS contained (in mM) 140 Na+, 5 K+, 2 Ca2+, 147 Cl−, 10 HEPES, and 10 glucose at pH 7.4 (~300 mosM). In the high-K+ solution (100 mM), 100 mM NaCl was replaced with KCl to maintain isotonic osmolality. Hypotonic solution contained (in mM) 70 Na+, 5 K+, 2 Ca2+, 77 Cl−, 10 HEPES, and 10 glucose (~170 mosM). Hypertonic solution contained (in mM) 200 Na+, 5 K+, 2 Ca2+, 207 Cl−, 10 HEPES, and 10 mM glucose. Osmolality of the hypertonic solution was ~430 mosM. For the Ca2+-free solution, 0.5 mM EGTA was substituted for Ca2+.

**Determination of cytoplasmic NO in neurons.**

Cytoplasmic NO ([NO]_{cyt}) production in the neurons was measured as described previously (5, 38). Briefly, after neurons were cultured on 10-mm glass coverslips for 2 wk, they were loaded with 1 μM 4-aminophenylmethylene-2',7'-difluorofluorescein (DAF-FM) diacetate in PSS at room temperature (22°C) for 30 min and washed in PSS for ≥20 min. The coverslips with neurons were mounted in a perfusion chamber on a Nikon microscope stage. Fluorescence intensity [540-nm light emission excited by 484-nm illuminations (F484)], as well as background fluorescence, was collected using a ×20 Nikon UV-Fluor objective and an intensified CCD camera (ICCDD200). PSS used in digital [NO]_{cyt} measurement contained (in mM) 140 Na+, 5 K+, 2 Ca2+, 147 Cl−, 10 HEPES, 10 glucose, and 1 L-arginine.

**Mechanical stretch of neurons grown on an elastic silicone membrane.**

To test the response of neurons to mechanical stretch, we used a custom-built device. The device is equipped with two metal bars that are close to the edge of the silicone membrane. When the bars are depressed, they stretch the elastic membrane and, thus, the cells on the membrane (see Fig. 4A). After neurons were grown on the membrane for 2 wk, they were loaded with 5 μM fura 2-AM for 50 min and washed in PSS for 20 min. The membrane was mounted on the stretch device, which was placed on the microscope stage. The neurons on the membrane were continuously superfused with PSS at room temperature. Mechanical stretch-induced Ca2+ imaging in the neurons was recorded as described above (see Determination of cell volume and [Ca^{2+}]_{cyt} in individual esophageal myenteric neurons).

**Chemicals.**

ATP, fluorogenic acid, sodium nitroprusside (SNP), nifedipine, Gd^{3+}, and 5-nitro-2-(3-phenylpropionamido)benzoic acid (NPPB) were purchased from Sigma (St. Louis, MO); Grammestola mecanotoxin 4 (GsMTx4) and 2-aminoethoxydiphenyl borate from Toesri Bioscience (Ellisville, MO); 1-octanol-2-acetyl-sn-glycerol from Calbiochem (Billerica, MA); and fura 2-AM and DAF-FM diacetate from Molecular Probes (Eugene, OR). The other chemicals were obtained from Fisher Scientific (Santa Clara, CA).

**Statistical analysis.**

Values are means ± SE. Differences between means were considered to be statistically significant when P < 0.05 by Student’s t-test or one-way ANOVA followed by Newman-Keuls post hoc test when appropriate.
RESULTS

Immunocytochemical analysis of neurons. We performed immunocytochemical staining to determine if cells in our primary culture were indeed neurons and, if so, whether they contained the coding of the inhibitory neurotransmitter nNOS. Almost all cells stained positive for the neuronal marker PGP 9.5 (Fig. 1A), and some of these neurons costained with nNOS (Fig. 1A). Positive staining for PGP 9.5 and nNOS was specific, because all cells stained negative in the absence of primary antibodies (Fig. 1B). PGP 9.5-positive and nNOS-positive neurons were counted in six high-power fields to determine the percentage of nNOS-positive neurons. We found that 66 ± 13% of the PGP 9.5-positive neurons colabeled with nNOS (n = 6).

Osmotic change-induced cell stretch and Ca\(^{2+}\) signaling in neurons. We used fura 2-AM to measure neuronal cell volume and neuronal [Ca\(^{2+}\)]\(_{\text{cyt.}}\). F\(_{360}\) was used to monitor cell volume, since [Ca\(^{2+}\)]\(_{\text{cyt.}}\) is unaffected at this excitation (25). Figure 2A shows that when F\(_{360}\) baseline was stable in PSS (300 mosM), switching extracellular solutions to hypotonic solution (170 mosM) produced a marked decrease in F\(_{360}\), indicating cell swelling or cell volume increase. When extracellular solution was switched back from hypotonic solution to PSS, F\(_{360}\) returned to baseline, indicating the ability of cells to recover their volume. All neurons were sensitive to hypotonic solution-induced cell swelling. The effect of hypotonic solution on cell volume was reproducible and independent of extracellular Ca\(^{2+}\). Furthermore, a switch in the extracellular solution from PSS to hypertonic solution (430 mosM) induced an increase in F\(_{360}\), indicating cell shrinkage. Figure 2B shows summary data of cell swelling and shrinkage assessed by changes in F\(_{360}\). Figure 2C shows changes in F\(_{360}\) induced by hypotonic and hypertonic solutions with or without extracellular Ca\(^{2+}\). Hypotonic solution markedly reduced F\(_{360}\) in neurons (P < 0.001, n = 37 cells). Repeated measurements with hypotonic solutions with or without Ca\(^{2+}\) induced reproducible changes in F\(_{360}\) (P > 0.05, n = 37). Cell size was not affected by the presence or absence of extracellular Ca\(^{2+}\) (Fig. 2C). On the
other hand, hypertonic solution induced an increase in F360 (P < 0.05, n = 37 cells); however, it was not as reproducible as the hypotonic solution-induced decrease in F360 (Fig. 2, A and B). For the above-described reasons and because the hypotonic solution-induced cell swelling has been commonly applied in the study of mechanosensitivity of many different types of cells (16, 32, 43, 45), we chose hypotonic solution-induced cell swelling to produce neuronal membrane stretch in our studies.

Next, we measured the fluorescence ratio at excitations of 340 and 380 nm (F340/380) to specifically assess [Ca2+]cyt, a cell signaling molecule essential for production and release of neurotransmitters (18, 19, 42). Figure 3A shows that after a stable F340/380 baseline was achieved in PSS (300 mosM), a switch of extracellular solution to hypotonic solution (170 mosM) with Ca2+ produced a marked increase in F340/380 in 64 ± 5% of neurons (n = 221 cells in 6 separate experiments). Ca2+ responses to hypotonic stretch could be classified into three different categories: 1) an initial transient rise of large amplitude followed by a second rise of small amplitude (top trace in Fig. 3B), 2) a slow and sustained rise of small amplitude (Fig. 3C), and 3) no response (Fig. 3D). After recovery of Ca2+ responses to hypotonic stretch, addition of ATP (10 μM) or high K+ (100 mM) immediately activated Ca2+ signaling in almost all neurons (Fig. 3, A–D), indicating that these cells were functional and sensitive to G protein-coupled receptor activation and membrane depolarization. Figure 3, E and H, shows summary data indicating Ca2+ responses to hypotonic solution, ATP, and high K+ (n = 52 cells) in the presence of extracellular Ca2+.

Since the neurons were in culture for 2 wk, it is possible that synaptic contact may have been established among some of these neurons. However, direct observation under a microscope did not reveal obvious physical contacts between individual neurons (Fig. 4B). To functionally test if stretch-activated responses in our preparation were due to synaptic transmission between neurons, Ca2+ signaling was compared in the presence and absence of extracellular Mg2+, a blocker of N-methyl-D-aspartate receptors that plays a critical role in synaptic transmission (24). Figure 3E shows summary data of Ca2+ responses to hypotonic solution with Ca2+ and to ATP in the presence of extracellular Mg2+ (1 mM). Figure 3F shows summary data of neuronal responses to hypotonic solution with Ca2+ in the presence or absence of Mg2+. Hypotonic solution induced a 1.38-fold increase (compared with baseline) in F340/380 without Mg2+ vs. a 1.37-fold increase with Mg2+ (P > 0.05, n = 52 cells); the difference was not significant. Hypotonic solutions with or without Mg2+ also induced a comparable Ca2+ response in the esophageal neurons (P > 0.05, n = 52 cells).

Mechanical stretch-induced Ca2+ signaling in esophageal neurons. Neurons were grown on the stretchable silicone membrane and loaded with fura 2, and the membrane was mounted on our custom-built device (Fig. 4A). The latter was placed under the microscope and superfused with PSS. As shown in Fig. 4B, left, most individual neurons did not have

![Figure 3](http://ajpcell.physiology.org/)
obvious physical contacts with each other. After cells were superfused with PSS for 5 min, mechanical stretch significantly increased the size of most neurons (compare Fig. 4B, middle with left and right), indicating an effective cell stretch. Mechanical stretch induced a marked increase in \( \text{Ca}^{2+} \) signaling (shown as yellow or red color) in 65 ± 1% of neurons (\( n = 336 \) cells in 5 separate experiments). Moreover, as shown in Fig. 4B, middle, when the silicone membrane was stretched, the activated neurons (shown as yellow or red color) were moved from their original place (shown as green or purple color) on the membrane. The neurons that were not activated (shown as green or purple color) also moved from their original place with mechanical stretch. The mechanical stretch-activated \( \text{Ca}^{2+} \) signaling in the neurons appeared and disappeared with the onset and cessation of stretch, respectively (compare Fig. 4B, middle with left and right). Taken together, these data suggest that \( \text{Ca}^{2+} \) signaling was activated by mechanical stretch in some, but not all, neurons.

Fig. 4. Mechanical stretch-induced changes in cell size and \( \text{Ca}^{2+} \) signaling in myenteric neurons on an elastic silicone membrane. A: design of custom-built device for mechanical stretch of neurons grown on a membrane. B: neurons grown on a membrane were loaded with fura 2-AM and mounted on the device, which was then placed on a microscope. Mechanical stretch-induced cell size and \( \text{Ca}^{2+} \) imaging in neurons were monitored before (left), during (middle), and after (right) stretch. Cell size and \( \text{Ca}^{2+} \) signaling were markedly increased in some, but not all, enteric neurons during a stretch (compare left and middle), and these changes recovered with cessation of stretch (compare middle and right).

Fig. 5. Effects of removal of extracellular \( \text{Ca}^{2+} \) and 2-aminoethoxydiphenyl borate (2-APB) on hypotonic stretch-induced \([\text{Ca}^{2+}]_\text{i}\) in esophageal myenteric neurons. A: summary time course of neuronal response to hypotonic solution with \( \text{Ca}^{2+} \). After stable \( F_{340/380} \) baseline was achieved in PSS, extracellular solution was switched to hypotonic solution with \( \text{Ca}^{2+} \). B: summary time course of neuronal response to hypotonic solution without \( \text{Ca}^{2+} \). C: summary time course of neuronal response to hypotonic solution with \( \text{Ca}^{2+} \) in the presence of 2-APB (100 \( \mu \text{M} \)). D: summary data of neuronal response to different hypotonic solutions with or without 2-APB and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB, 10 \( \mu \text{M} \)). Values are means ± SE; \( n = 39–55 \) cells from 6–7 rats. **\( P < 0.01 \) vs. basal.
Mechanisms underlying stretch-induced Ca\textsuperscript{2+} signaling in neurons. First, we tested stretch-induced Ca\textsuperscript{2+} signaling in the presence and absence of extracellular Ca\textsuperscript{2+}. Stretch-induced increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} was observed mainly in 2 mM Ca\textsuperscript{2+} solutions but marginally in 0 mM Ca\textsuperscript{2+} solutions (Fig. 5, A and B). Hypotonic solution with Ca\textsuperscript{2+} induced a 1.40-fold increase in F\textsubscript{340/380} in the neurons (P < 0.01, n = 49 cells; Fig. 5, A and D), but hypotonic solution without Ca\textsuperscript{2+} did not induce significant Ca\textsuperscript{2+} signaling (1.07-fold increase vs. baseline, P > 0.05, n = 39 cells; Fig. 5, C and D), suggesting that stretch induces extracellular Ca\textsuperscript{2+} to move inside the cells. Second, 2-aminoethoxydiphenyl borate (100 \mu M), a blocker of nonselective cation channels, attenuated stretch-induced Ca\textsuperscript{2+} signaling (1.08-fold increase vs. baseline, P > 0.05, n = 39 cells; Fig. 5, C and D); however, NPPB (10 \mu M), an efficient blocker of volume-sensitive Cl\textsuperscript{-} channels, did not prevent stretch-induced Ca\textsuperscript{2+} signaling (1.38-fold increase vs. baseline, P < 0.05, n = 50; Fig. 5D). Third, Gd\textsuperscript{3+} (50 \mu M), a nonselective blocker of SACs, abolished stretch-induced Ca\textsuperscript{2+} signaling (1.06-fold increase vs. baseline, P > 0.05, n = 45; Fig. 6, B and D). Fourth, nifedipine (10 \mu M), a selective blocker of VGCCs (44), did not affect stretch-induced Ca\textsuperscript{2+} signaling (1.36-fold increase vs. baseline, P < 0.05, n = 55), but GsMTx4 (10 \mu M), a selective SAC blocker, completely abolished the stretch-induced Ca\textsuperscript{2+} signaling in all neurons (1.03-fold increase vs. baseline, P > 0.05, n = 55; Fig. 6, C and D).

Since GsMTx4 may also block TRPC channels, which are molecular candidates for the SAC in some cell types, we tested functional expression of the TRPC channel on the esophageal neurons. 1-Oleoyl-2-acetyl-sn-glycerol (300 \mu M), a cell-permeant analog of diacylglycerol that selectively activates TRPC-encoded receptor-operated Ca\textsuperscript{2+} channels (34), did not induce Ca\textsuperscript{2+} signaling in any neurons (1.01-fold increase, P > 0.05, n = 50 cells; Fig. 6, E and F). Moreover, flufenamic acid (300 \mu M), a putative activator of TRPC channels (2, 20), induced only a minor Ca\textsuperscript{2+} signaling response in a few neurons (1.03-fold increase, P > 0.05, n = 50 cells; Fig. 6, E and F). On the other hand, ATP (10 \mu M) induced significant Ca\textsuperscript{2+} signaling in all these neurons (1.90-fold increase, P < 0.01, n = 50 cells; Fig. 6, E and F).

Hypotonic stretch-induced [NO\textsubscript{cys}] in esophageal neurons. We used DAF-FM to monitor hypotonic stretch-induced Ca\textsuperscript{2+}-dependent NO production in neurons. Figure 7A shows that hypotonic solution without Ca\textsuperscript{2+} did not induce NO signaling in neurons (1.01-fold increase in F\textsubscript{340/380} vs. baseline, P > 0.05, n = 49 cells). On the other hand, hypotonic solution with Ca\textsuperscript{2+} induced NO signaling in 57 ± 5% of neurons (n = 184 cells in 3 separate experiments), and NO signaling was increased...
1.19-fold vs. baseline (*P < 0.05, n = 49 cells). A significant population of neurons did not show a NO response to hypotonic solution with or without Ca\(^{2+}\) (Fig. 7B). Addition of SNP (10 \(\mu\)M), a NO donor, induced strong NO signaling in almost all cells (1.59-fold increase in F\(_{484}\) vs. baseline, *P < 0.01, n = 49 cells), proving that our detection technique was sensitive to the presence of NO in the cells (Fig. 7, A and B). Figure 7C shows a summary of the time course of the NO response to hypotonic solutions with or without Ca\(^{2+}\) and SNP. Figure 7D shows a summary of the neuronal responses to hypotonic solutions with or without Ca\(^{2+}\). Some neurons had no NO signaling response to hypotonic solution with or without Ca\(^{2+}\) (A). Some neurons had NO signaling response to hypotonic solution with or without Ca\(^{2+}\) (B). However, almost all neurons responded to SNP. C: summary time course of neuronal response to hypotonic solution and SNP. D: summary neuronal response to hypotonic solution, GsMTx4 (GsMT, 10 \(\mu\)M), and SNP. Values are means ± SE; n = 40–49 cells from 3 rats. *P < 0.05, **P < 0.01 vs. 2 Ca only.

**DISCUSSION**

Our study, for the first time, provides clear evidence for the mechanosensitivity of inhibitory esophageal motor neurons. This mechanosensitivity is due to extracellular Ca\(^{2+}\) influx through the SAC, because it was prevented by omission of extracellular Ca\(^{2+}\) and SAC blockers. Besides activation, we found that, in response to mechanical stretch, these neurons release NO, the major inhibitory neurotransmitter of motor neurons. Our finding suggests a pivotal role of the SAC in the stretch-induced, Ca\(^{2+}\)-dependent neurotransmitter release from inhibitory motor neurons of the esophagus.

Our earlier study revealed that the cells in our primary culture are indeed neurons. Furthermore, they contain choline acetyltransferase (ChAT) and nNOS, potential markers of excitatory and inhibitory motor neurons, respectively (9). ChAT exists in the sensory neurons and motor neurons, as well as interneurons. On the other hand, nNOS is predominantly found in the inhibitory neurons (41). Sixty-six percent of neurons in our culture stained positive for nNOS, which is similar to the in vivo rat and human studies of esophageal myenteric neurons (39, 46). Our function studies demonstrate that hypotonic solution, as well as mechanical stretch, induced Ca\(^{2+}\) signaling in ~65% of esophageal neurons. Moreover, we found stretch-induced Ca\(^{2+}\)-dependent NO production in ~60% of neurons, which is consistent with the percentage of neurons that stained positive for nNOS and showed a [Ca\(^{2+}\)]\(_{\text{cyt}}\) response to hypotonic and mechanical stretch. On the basis of our findings, one may wonder if only nNOS-containing neurons are mechanosensitive. We believe that this is unlikely, because a subpopulation of neurons in our culture contains ChAT, as well as nNOS (9), and we only monitored NO release. A study in which one could identify nNOS and ChAT labeling of all activated and nonactivated neurons is needed to answer this question.

Hypotonic swelling, along with ratiometric measurement of [Ca\(^{2+}\)]\(_{\text{cyt}}\), is widely used in the study of neurons (16, 32, 43, 45). Hypotonic swelling-induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) elevation was found to be associated with neuronal cell swelling, and it was reversible (16, 32, 45). Hypotonic solutions in the presence, but not absence, of extracellular Ca\(^{2+}\) induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) signaling in a significant number of neurons, indicating Ca\(^{2+}\)\(_{\text{cyt}}\) entry through the plasma membrane. Our finding that hypotonic solution-induced cell swelling caused [Ca\(^{2+}\)]\(_{\text{cyt}}\) signaling in some, but not all, esophageal neurons is consistent with findings from a previous study in which trigeminal neurons were grown in primary culture and tested for mechanosensitivity (45). Although hypotonic solution-induced cell swelling is commonly used for the study of cell mechanosensitivity, it might activate volume-sensitive Cl\(^{-}\) channels to induce membrane depolarization and then open VGCCs to induce Ca\(^{2+}\) entry. We therefore used NPPB and nifedipine at concentra-
Mechanosensitive ion channels are widely expressed in mammalian cell types, including the central and enteric nervous systems (26, 27, 32). Even though some neurons in the brain are clearly mechanosensitive and express the SAC in relatively high density on the plasma membranes, their primary function is not sensory (26, 27, 32). Molecular candidates for the SAC in various types of mammalian cells are unclear. The SAC may be a different molecule in different cell types. Some SACs are encoded by mammalian homologs of Drosophila TRP genes, such as the TRPC subfamily (16, 17). The SAC may also be a VGCC in intestinal smooth muscle cells and some enteric neurons (12), but not in airway smooth muscle cells and other types of neurons (21). Our study is the first to show the involvement of Ca\(^{2+}\) entry pathways, including the SAC, in the mechanosensitive response of esophageal neurons. Our findings do not support the involvement of volume-sensitive Cl\(^{-}\) channels and VGCCs in the mechanosensitivity of esophageal motor neurons. We also did not identify functional expression of TRPC channels on the esophageal neurons, arguing against the possibility that TRPC channels are molecular candidates for the SAC on these neurons.

Conventional thinking is that the myenteric plexus contains sensory neurons, interneurons, and motor neurons. Sensory neurons are also called IPANs, which are defined on the basis of morphology (Dogiel type 2), electrophysiological behavior (afterhyperpolarization), and chemical coding (calbindin, neurofilament 145kD, and calretinin positivity) in guinea pig and mouse small intestine and colon. However, there is considerable debate about whether IPANs are truly sensory neurons, because mechanosensitivity has been found in a large number of enteric neurons of the mouse/guinea pig small and large intestine (colon), even when these cells are not consistent with the morphological description, electrophysiological behavior, and chemical coding of IPANs (28, 29, 41). Furthermore, stretch-activated reflexes exist in the stomach, where there are no IPANs (41). Our earlier in vivo studies (7, 22, 23) and the present in vitro study clearly prove that the inhibitory motor neurons are indeed mechanosensitive. Our findings are in agreement with the concept of multifunctionality of myenteric neurons proposed by Schemann and colleagues (28, 29), who demonstrated that a large number of myenteric neurons possess sensory, integrative, and motor functions in the same cell. Mechanosensitivity in a small number of NO synthase-containing interneurons of the myenteric plexus of the small intestine and colon in guinea pigs has also been reported (41).

Our study has a few limitations. 1) We isolated neurons from the rat esophagus, which is mostly composed of striated muscle, whereas the distal part of human esophagus is composed of smooth muscle. The role of myenteric neurons in the rat esophagus is not clear, but it is possible that these neurons provide inhibitory innervation to the LES, a smooth muscle structure. 2) We do not know if the mechanical stretch applied in the present study mimics physiological deformation of the neurons during muscle contraction. 3) In addition to inhibitory motor neurons, our cell culture also contains excitatory motor neurons, but it is not clear if they are activated by stretch, because technical limitations prevented identification of the chemical coding of all neurons activated by stretch. Despite these limitations, our findings have important physiological and clinical relevance. Myenteric neurons are sandwiched between the longitudinal and circular muscle layers of the muscularis propria and, therefore, are subjected to mechanical forces when the two layers move in relationship to each other. Using ultrasound imaging to monitor circular and longitudinal muscle layers of the distal esophagus (31), we found that the two muscle layers slide against each other in different directions during different motor patterns. During peristalsis, axial shortening of the circular muscle layer is greater than that of the longitudinal muscle. On the other hand, the reverse is the case during transient LES relaxation (31), a key motor event that allows oral movement of gastric contents into the esophagus. The relative movement between two layers will result in strain patterns that may activate different myenteric neurons to induce different motor patterns. With regard to clinical significance, we propose that SACs are potential targets to treat esophageal motility disorders. For example, transient LES relaxation, the major mechanism of gastroesophageal reflux, may be inhibited by SAC blockers (30). On the other hand, SAC activators may be used to treat conditions such as achalasia, which is characterized by a lack or reduction of inhibitory motor neuron activity (33).

**REFERENCES**


**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.
MECHANOSENSITIVITY OF ENTERIC MOTOR NEURONS