Serotonin 5-HT$_3$ receptors on mechanosensitive neurons with cardiac afferents

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Serotonin 5-HT$_3$ receptors on mechanosensitive neurons with cardiac afferents. Am J Physiol Heart Circ Physiol 282: H1828–H1835, 2002; 10.1152/ajpheart.00708.2000.—In rats, the mechanosensitive cardiorenal baroreflex influencing renal excretory function might be impaired by serotonin occurring in coronary arteries, e.g., in hypertension. Because the afferent limb of this reflex could be affected, we investigated the responses of nodose ganglion cells (one neuron of reflex) to osmotic, mechanical stress in presence or absence of the serotonin 5-HT$_3$ receptor agonist phenylbiguanide (PBG). Current-voltage relationships (from −100 to +50 mV) were obtained using cell patch recordings while the cells were exposed to control or hypoosmotic solutions to induce mechanical stress. This protocol was repeated after low doses of PBG (10 μM), angiotensin II (10 nM), or the stretch-activated channel blocker gadolinium (20 μM) were added to the extracellular medium (EM). Hypoosmotic EM induced significant changes in cellular conductance. The full-range current-voltage relationship allowed for the calculation of a mean reversal potential of −13 ± 1.2 mV with respect to this change in cellular conductance (n = 44). This increase in conductance was impaired after addition of either PBG or gadolinium to the EM, which was statistically evaluated at a voltage of −80 mV, where influences of voltage-gated channels are not likely to interfere with the responses recorded. The serotonin 5-HT$_3$ receptor antagonist tropisetron (10 nM) prevented the PBG effect on conductance responses. Angiotensin II had no influence. Hence, serotonin might decrease the mechanical sensitivity of afferent cardiac nerves controlling renal sympathetic nerve activity.

mechanosensitivity; renal innervation

THE SYMPATHETIC NERVOUS SYSTEM influences the circulation not only by its effect on the regulation of peripheral resistance or cardiac performance, but also by controlling volume homeostasis oc occurring in coronary arteries, e.g., in hypertension. Because the afferent limb of this reflex could be affected, we investigated the responses of nodose ganglion cells (one neuron of reflex) to osmotic, mechanical stress in presence or absence of the serotonin 5-HT$_3$ receptor agonist phenylbiguanide (PBG). Current-voltage relationships (from −100 to +50 mV) were obtained using cell patch recordings while the cells were exposed to control or hypoosmotic solutions to induce mechanical stress. This protocol was repeated after low doses of PBG (10 μM), angiotensin II (10 nM), or the stretch-activated channel blocker gadolinium (20 μM) were added to the extracellular medium (EM). Hypoosmotic EM induced significant changes in cellular conductance. The full-range current-voltage relationship allowed for the calculation of a mean reversal potential of −13 ± 1.2 mV with respect to this change in cellular conductance (n = 44). This increase in conductance was impaired after addition of either PBG or gadolinium to the EM, which was statistically evaluated at a voltage of −80 mV, where influences of voltage-gated channels are not likely to interfere with the responses recorded. The serotonin 5-HT$_3$ receptor antagonist tropisetron (10 nM) prevented the PBG effect on conductance responses. Angiotensin II had no influence. Hence, serotonin might decrease the mechanical sensitivity of afferent cardiac nerves controlling renal sympathetic nerve activity.

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have been proposed to help determine whether mechanosensitivity or stretch activation is a physiological property of a distinct class of ion channels (18). It has been suggested that experiments be conducted in tissues that have an established mechanosensitive function by using single channel and whole cell recordings and that alternative methods be used to induce stretch activation, such as hypotonic swelling (21). Previously, this research strategy was successfully applied to demonstrate that mechanosensitive channels are likely to mediate the input of the arterial baroreceptor to the CNS (2).

In the present study, we used hypotonic mechanical stress and the patch-clamp technique to investigate for the first time mechanosensitive currents in putative NGCAs. We tested the hypothesis that the responsiveness of NGCAs is decreased in the presence of even low doses of the serotonin 5-HT3 agonist PBG. In addition, we tested the effect of gadolinium, a trivalent cation that blocks mechanosensitive ion channels (36). In a further group of experiments, NGCAs were exposed to osmotic stress in the presence of ANG II, which putatively influences cardiorenal reflexes at integrating centers in the brain stem rather than peripherally on the level of the first neuron (32).

**METHODS**

For the experiments, male Sprague-Dawley rats (Ivanovas; Kisslegg, Germany) weighing 250–300 g were maintained in cages at 24 ± 2 °C. They were fed a standard rat diet (No. C-1000, Altromin; Lage, Germany) containing 0.2% sodium by weight and were allowed free access to tap water.

**Labeling of Nodose Mechanosensitive Neurons with Cardiac Afferents**

To identify putative mechanosensitive neurons with cardiac afferents, we labeled these cells by applying the dicarbocyanine dye 1,1’-dioleoyl-3,3’,3’-tetramethyl-indocarbocyanine methanesulfonate (DiI) (D9-DiI, 50 mg/ml in DMSO, Molecular Probes; Eugene, OR) intrapericardially to the junction of the great vessels of the heart of Sprague-Dawley rats weighing 250–300 g (Charles River; Sulzfeld, Germany) (2). Initially, rats were anesthetized with a 500-μl bolus injection of methohexital (20 mg/ml) intraperitoneally. After the insertion of a venous line, appropriate anesthesia was achieved with an intravenous maintenance infusion of methohexital (80 μg·100 g −1·min −1) through the venous femoral catheter. Mechanical ventilation was instituted via a tracheal tube, and a high midline thoracotomy was performed. The lobes of the thymus were carefully separated from each other, exposing a small portion of the roof of the pericardial sac adherent to the thymus. The roof of the pericardial sac was slightly opened to insert a glass coverslip. 5-Fluorodeoxy-2-uridine (80 μM) was added to prevent the proliferation of nonneuronal cells. The cells were plated on coverslips for 5–6 days for electrophysiological experiments in a modified L-15 medium. To demonstrate that the labeled cells were neurons, all cells used for experimental procedures were tested for fast sodium currents during repolarization that were characteristic for neuronal cells. Some coverslips were also stained with fluorescein-conjugated tetanus toxin C (Neurotag Green, Böhringer Mannheim), which specifically binds to neurons (2). Stained coverslips were viewed under epifluorescence to permit visualization of the respective neurons before the experiment. Furthermore, a small laser beam (480 nm) powered by a storage battery was mounted to the patch-clamp recording setup. This equipment allowed for the detection of Dil-stained nodose ganglion cells during the experiments using the respective optical filters.

**Patch Clamp**

Patch recordings were obtained from the respective neurons using a recording solution containing (in mM) 104 KCl, 16 KOH, 1 magnesium ATP, and 10 HEPES. The resistances of the electrodes ranged from 3 to 6 MΩ. The seal resistance was between 2 and 10 GΩ, and the series resistance was >100 MΩ. Whole cell voltage-clamp recordings were obtained with the help of a 200 B Axopatch amplifier (Axon instruments; Foster City, CA). Data were sampled at 5 kHz and stored on a computer hard drive using a commercially available software package (pCLAMP, Axon Instruments). Current-voltage relationships were obtained using a voltage-ramp protocol that increased the voltage from −100 to +50 mV over 4 s. The full-range current-voltage relationship allowed for the calculation of the mean reversal potential with respect to changes in cellular conductance after exposure to hypotonic extracellular media. Furthermore, it allowed for the evaluation of the quality of our preparations throughout the experiments (e.g., as a vital parameter of the cells). The increase in conductance was furthermore statistically evaluated at a voltage of −80 mV, where influences of voltage-gated channels were no more likely to interfere with the responses observed.

In general, cells were placed in a one-chamber laminar flow bath and perfused at a rate of 0.5–1 ml/min by gravity feed lines connected to fluid reservoirs. Fluid was removed by a respective carefully applied suction to the bath. The composition of the control bath solution was (in mM) 120 NaCl, 2 CaCl2, 1 Mg Cl2, 1 KCl, 10 HEPES, and 40 mannitol to obtain a solution of 290 mosM. The hypotonic solution had the same ionic content without the mannitol. This solution ex-
hibited a osmolarity of 255 mosM. The osmolality of each solution was controlled for using an osmometer (Micro-Osmometer; Knauer, Germany).

We first exposed the cells to control medium. After we achieved stable current-voltage relationships in this solution (5 min), the extracellular medium was changed, and the cells were exposed to the hypoosmotic medium for 4 min while the voltage-ramp protocol was repeated every minute. The hypoosmotic medium was then again replaced with the original extracellular control solution. We only included neurons in the analysis if their resting membrane potentials was less than −40 mV.

Experimental Protocols

Basic experiments. Respective nodose ganglion cells were exposed twice to hypoosmotic media, and the ramp protocols were performed as described in Patch Clamp. For the final data evaluation, we only used cells that unequivocally stained positive for DiI.

Experiments to inhibit hypoosmotic-induced changes in cellular currents. In two series of experiments, we used the serotonin 5-HT₃ receptor agonist PBG (10 and 100 μM) to impair mechanosensitive currents in putative mechanosensitive neurons with cardiac afferents induced by hypoosmotic mechanical stress. Likewise, in a further series, the putative stretch-activated channel blocker gadolinium (10 μM gadolinium chloride hexahydrate, Aldrich) or the cardiovascular regulator peptide angiotensin II (10 nM) was added to the different media. Again, the respective putative cardiac nodose ganglion cells were exposed twice to the hypoosmotic solution to test for cellular responses with and without the drugs in the media.

Inhibition of the effects of the serotonin 5-HT₃ receptor agonist PBG. We used the serotonin 5-HT₃ receptor antagonist tropisetron (10 nM) to specifically antagonize the impairing effects of the lower dose of PBG (10 μM) on changes in cellular currents due to hypoosmotic stress. As described Experiments to inhibit hypoosmotic-induced changes in cellular currents, the respective putative cardiac nodose ganglion cells were exposed twice to the hypoosmotic solution to test for cellular responses with and without PBG in the media. The serotonin 5-HT₃ receptor antagonist was added constantly to the extracellular control solution and the hypoosmotic medium.

Additionally, we performed time-control experiments by exposing respective nodose ganglion cells to hypoosmotic stress without the addition of PBG.

Data Analyses

The data were statistically analyzed with analyses of variance, analysis of covariance, and Newman-Keuls post hoc test (where appropriate) using a CSS statistical software package (StatSoft; Tulsa, OK). Only a priori fixed comparisons were tested. Statistical significance was defined as P < 0.05. Data are given as means ± SE (35).

RESULTS

Cells in Culture

After several days in culture, nodose ganglion cells could be distinguished in most cases from fibroblasts and other cells by their large rounded soma (20–40 μm). Only in these cells could distinct nucleoli be stained by the tetanus toxin fragment. A fraction of these cells (between 15 and 25%) were brightly labeled with DiI (Fig. 1). The cells clearly labeled were classified as putative NGCAs. No differences in size could be detected between labeled and unlabeled cells.

Neuron Investigation

In all series described below, stable voltage-clamp recordings were obtained in at least six respective neurons from the nodose ganglion. Exposure to hypoosmotic medium without the addition of experimental drugs produced an increase in conductance of these cells, as indicated by a change in the slope of their current-voltage relationship obtained by the voltage-ramp protocol. The increase in conductance was observed 3 min after the change in the solutions and increased to a peak response at ~4–7 min. A steady state could be maintained until the solutions were changed. The current-voltage relationship returned to control levels 5–6 min after the control medium had been added to the cells again. ANOVA and analysis of covariance revealed constant increases of inward currents over the voltage range tested that did not differ between the cells of the
different experimental groups investigated. In the various series of experiments, the increase in conductance was additionally quantitated by measuring the change in holding current at −80 mV. In NGCAs, hypoosmotic medium significantly increased the holding current at −80 mV. In all series of experiments, nonlabeled cells were investigated or the investigator was blinded and could not tell whether they were working with NGCAs. It turned out that all cells that responded to hypoosmotic stress eventually proved to be labeled. We never saw a decrease in conductance when NGCAs were exposed to hypoosmotic media.

**Effects of PBG and ANG II**

NGCAs were tested for the effects of hypoosmotic mechanical stress in the presence of two doses of PBG (10 and 100 μM). Both doses of PBG significantly suppressed the increase in conductance to osmotic stress (Fig. 2, A and B; data for 100 μM PBG not shown; the change in current at −80 mV due to hypoosmotic media under control conditions was 390 ± 40 vs. 230 ± 40 pA in the presence of 100 μM PBG (P > 0.05); current at −80 mV in the presence of control media was 200 ± 53 vs. 230 ± 55 pA, respectively, n = 10 in each group of doses used).

We could never observe an attenuation of the increase in conductance due to hypoosmotic media if ANG II was concomitantly added to the media (change in current at −80 mV due to hypoosmotic media under control conditions was 410 ± 45 vs. 390 ± 65 pA in the presence of ANG II; current at −80 mV in the presence of control media was 190 ± 53 vs. 210 ± 55 pA, respectively, n = 6).

**Experiments with PBG and Concomittant Application of Tropisetron**

NGCAs were furthermore tested for the effects of hypoosmotic mechanical stress in the presence of lower doses of PBG (10 μM) and concomittant application of 10 nM of the serotonin 5-HT3 antagonist tropisetron. Under these circumstances, the serotonin 5-HT3 receptor agonist PBG failed to attenuate the increase in

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**Fig. 2.** A: current-voltage relation to voltage ramps in a putative NGCA in control and hypoosmotic extracellular solution at 5 min; B: comparable curve obtained from cells preexposed to 10 μM of the serotonin 5-HT3 receptor agonist phenylbiguanide (PBG). Note that the curve obtained in hypoosmotic solution with 10 μM PBG is no longer significantly different from that of the control. C: summary of effects of hypoosmotic mechanical stress on the conductance of putative NGCAs at −80 mV. The increase of inward currents was significantly attenuated by adding 10 μM of the serotonin 5-HT3 receptor agonist PBG to the media. Nonshaded crosshatched bars, experimental period without PBG; shaded crosshatched bars, experimental period with PBG.
conductance due to hypoosmotic mechanical stress in NGCAs (Fig. 3, A and B).

Effects of Gadolinium

Eventually, NGCAs were tested for the effects of hypoosmotic mechanical stress in the presence of gadolinium (10 μM). In these experiments, gadolinium suppressed the increase in conductance associated with hypoosmotic mechanical stress (Fig. 4, A and B; P > 0.05).

DISCUSSION

The results of these experiments indicate that hypoosmolar mechanical stress significantly increases the conductance of putative NGCAs. This response could be markedly impaired by concomitant stimulation of serotonin 5-HT₃ receptors with PBG even in quite low doses. This effect of PBG could be prevented if the serotonin 5-HT₃ receptor antagonist tropisetron was constantly added to the media. The putative inhibitor of mechanosensitive channels gadolinium inhibited increases of conductance during mechanical challenge. The peptide angiotensin II had no effect on the NGCAs response to osmotic stretch. It should be noted that our results refer mainly to cell bodies. Comparable studies that used single fiber recordings of baroreceptor neurons and investigations of ganglion nodose cells with baroreceptor afferents did not reveal inconsistencies in the responses of sensory axons and their respective neuronal bodies (7). However, this may not be true under all circumstances.

Our report is one of the first to suggest that pharmacological interventions like stimulation of serotonin 5-HT₃ receptors that are not coupled to G proteins but interfere with cationic conductance (16) can attenuate neurogenic mechanosensitivity of cells like NGCAs.

Fig. 3. A: current-voltage relation to voltage ramps in a putative NGCA in control and hypoosmotic extracellular solution at 5 min either without (A) or with preexposure (B) of cells to 10 μM PBG. In contrast to Fig. 1A, the solutions permanently contained 10 nM of the serotonin 5-HT₃ receptor antagonist tropisetron in these experiments. Note that now the curve obtained in hypoosmotic solution with 10 μM PBG is no longer affected if compared with increases of conductance under control conditions. C: summary of effects of hypoosmotic mechanical stress on the conductance of putative NGCAs at −80 mV. The increase of inward currents was no longer affected, in contrast to Fig. 2C, by the addition of 10 μM of the serotonin 5-HT₃ receptor agonist PBG to the medium if all solutions permanently contained 10 nM of the serotonin 5-HT₃ receptor antagonist tropisetron. Nonshaded crosshatched bars, experimental period without PBG; shaded crosshatched bars, experimental period with PBG.
any case, the mechanosensitivity of cells like NGCAs is prominently dependent on mechanosensitive cationic channels (2), and our results point to a complex pattern of cationic conductance alterations during mechanical stretch and concomittant serotonin 5-HT3 receptor stimulation. A mere unspecific artifact is unlikely:

First, angiotensin II had no effect on conductance increases due to mechanical stretch of NGCAs. This peptide is putatively involved in the neurogenic control of the cardiovascular system on the level of the brain stem or of efferent sympathetic synapses but does not likely modulate sensoric afferent input to the first neuron (32). Second, the effects of serotonin 5-HT3 receptor stimulation on cellular conductance could be specifically blocked with the help of the serotonin 5-HT3 receptor inhibitor tropisetron (14). We cannot decide from our whole cell investigations of ganglion nodosum cells how PBG was able to impair the responses to mechanical stress. One might speculate that subthreshold doses of PBG induced a “leakage” flow of ions that altered the overall conductance of the cell membrane in such a way that increased current through mechanosensitive channels no longer had a detectable effect on the whole cell level.

The reversal potential for the change in conductance of NCGAs produced by hypoosmotic mechanical stress proved to be comparable to the reversal potential predicted for a nonselective cationic conductance. A chloride efflux has been observed with a macroscopic mechanosensitive current in some systems (12). They might contribute to the effect observed here. The change in the current-voltage relationship produced by hypoosmotic mechanical stress was considerably prominent in the linear portion of the curve between -100 to +40 mV but not in the region of outward rectification (from -40 to 0 mV). The linearity of the increase in conductance from -80 to -40 mV implies a lack of voltage dependence in the response to hypoosmotic mechanical stress. However, the obvious absence of an effect on the whole cell current from the reversal potential to 0 mV might represent either voltage dependence or a masking of the current induced by hypoosmotic mechanical stress by voltage-gated K⁺ currents (8, 21, 24). The response pattern of NGCAs proved to be considerably similar to aortic baroreceptor neurons from the nodose ganglion (2).

The increase in whole cell conductance produced by hypoosmotic mechanical stress proved to be specific for putative NGCAs. The DiI-labeled neurons uniformly showed an increase in conductance. Unlabeled neurons that responded to hypoosmotic mechanical stress are likely other types of nodose neurons that are mechano-
Sensitiv...e.g., aortic afferents and perhaps cells with gastric axons.

A really selective antagonist for mechanosensitive ion channels is still not at hand. However, gadolinium (22, 36) has been often used to inhibit mechanosensitive channels. Gadolinium blocked the increase in conductance produced by hypoosmotic mechanical stress in putative NGCAs investigated by us. These data are consistent with earlier reports on mechanosensitive ion channels in other tissue (20, 22, 36) and most notably in aortic baroreceptor neurons from the nodose ganglion (2). Because gadolinium also inhibits other channels besides mechanosensitive channels (1, 6), the mechanosensitivity of aortic baroreceptors neurons was also investigated with lanthanum, another trivalent cation, and a calcium channel blocker, ω-conotoxin (2). Neither of these substances significantly affected the responses of aortic baroreceptor neurons to hypoosmotic mechanical stress, suggesting that the gadolinium effect could be due to its action on mechanosensitive channels and is not mediated by calcium channels. However, one should keep in mind that gadolinium has been reported to block increases in [Ca^{2+}], produced by mechanical stimulation of nodose neurons in vitro (25, 26).

In case one assumes that osmotic stretch (being widely used to induce mechanical stress) is a valid tool to test mechanosensitivity of neurogenic cells in certain respects, our results with mechanosensitive NGCAs can be carefully related to our previous in vivo work in rats. First, they support the in vivo observation that the control of RSNA by mechanosensitive cardiopulmonary reflexes stimulated with a saline volume load was considerably impaired in rats preinfused with subthreshold doses of the serotonin 5-HT\textsubscript{3} receptor agonist PBG (30). In this respect, our findings allow for the hypothesis that serotonin 5-HT\textsubscript{3} receptors on cardiac afferent axons contained within the vagal nerve may directly influence the mechanosensitivity of these neural pathways.

It is likely that in vivo platelets serve as an endogenous source for serotonin, stimulating 5-HT\textsubscript{3} receptors on cardiac nerves. Because the release of serotonin by platelets appears to occur predominantly in coronary arteries with endothelial damage (9, 26), it is possible that these chemoreceptor-mediated reflexes are important in certain forms of coronary heart disease. In some patients with complex coronary artery lesions, the transcardiac serotonin concentration was permanently increased (29). In a recent study in patients with coronary artery disease, increased serotonin levels augmented the likelihood of severe cardiac events (34). Under these circumstances, when, in addition, cardiac filling pressures are often above normal due to developing cardiac insufficiency and subsequently mechanosensitive cardiopulmonary reflexes are chronically stimulated, volume homeostasis could be adversely influenced by serotonin 5-HT\textsubscript{3} receptors on cardiac vagal afferent fibers projecting to their respective NGCAs.

In conclusion, the results of our study on NGCAs extend our knowledge concerning the evidence for mechanosensitive currents in neurons of the nodose ganglion involved in cardiovascular control. So far, this evidence was only reported for aortic baroreceptor neurons, but not for NGCAs. Our results further substantiate the notion that mechanosensitive ion channels may be involved in mechanoelectric transduction not only of aortic baroreceptors, but also of sensoric mechanisms related to the heart.

Eventually, we could demonstrate that stimulation of serotonin 5-HT\textsubscript{3} receptors influencing cationic conductance will impair the mechanosensitive increase in conductance of NGCAs to osmotic stress. Further research on the involvement of specific channels during mechanical stimulation of NGCAs and concomittant serotonin 5-HT\textsubscript{3} receptor activation might be helpful to understand the interaction of chemosensitive and mechanosensitive properties of cardiorenal reflexes in vivo under physiological and pathophysiological conditions.

REFERENCES