Anesthesiology 2003; 99:232–5

Functional Evidence for P2X Receptors in Isolated Human Vagus Nerve

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IONOTROPIC adenosine 5'-triphosphate (ATP) (P2X) receptors are expressed in some primary afferent neurons, and activation of these receptors results in membrane depolarisation.[1] Many studies indicate that one subtype, the $P2X_3$ receptor, may contribute to the transduction of nociceptive stimuli in somatosensory[2] and visceral afferents.[3,4] However, the function of this receptor in the peripheral human nervous system is poorly understood. Using sensory testing, pain was experienced on local application of ATP on a human blister base preparation[5] or by iontophoresis of ATP to human skin.[6] However, it was not possible to identify the subtype of purinergic receptor underlying this effect. In the current study, we have used a new human nerve preparation. We tested the sensitivity of isolated fascicles of human vagus nerve to agonists at $P2X_3$ receptors. It is known that persistent changes in membrane potential and axonal excitability occur during application of the $P2X_3$ receptor agonist α,β-methylene ATP (α,β-meATP) to rat nodose ganglion cells,[1] to isolated rat vagus nerve[7,8] and in single vagal afferents in the mouse.[9] Our data indicate that isolated fascicles of human vagus nerve can be used to study the pharmacology of $P2X_3$ receptors in the peripheral human autonomic nervous system.

Materials and Methods

Preparations

The experiments on human vagus nerves were conducted using 12 isolated fascicles from four patients. Approval for this procedure was obtained from the Ethics Committee of the University of Munich, and the patients gave written informed consent. The patients (male, n = 4) were aged 49, 55, 66, and 69 yr when they underwent the procedure. All of them underwent complete surgical resection of the stomach because of cancer. A 2- to 3-cm part of the anterior vagal trunk at the distal abdominal esophagus (subdiaphragmatic) was removed from the area of gastrectomy.

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Received from the Departments of Anesthesiology, Physiology, and Surgery, University of Munich, Munich, Germany. Submitted for publication November 15, 2002. Accepted for publication February 27, 2003. Supported by Deutsche Forschungsgemeinschaft (SFB 391/A1), Bonn, Germany.

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Experimental Setup

Experimental procedures were done as described previously.[10] Briefly, isolated human nerve fascicles nerves were held at each end by suction electrodes in an organ bath. One suction electrode was used to elicit action potentials; the other was used as a recording electrode. The distance between stimulating and recording electrodes was approximately 3 mm. The organ bath (volume, 2 ml) was continuously perfused with solution at a flow rate of 6–8 ml/min (32°C). The perfusion solution contained (in mM) NaCl 118, KCl 3.0, $CaCl_2$ 1.5, $MgCl_2$ 1.0, D-glucose 5.0, $NaHCO_3$ 25, and NaH_2PO_4 1.2, and it was bubbled with 95% O_2/5% CO_2 (pH 7.4).

Electrophysiologic Recordings

Isolated fascicles of human vagus nerve were stimulated with a linear stimulus isolator (A395, WPI, Sarasota, FL) with 1.0-ms current pulses (100–500 μA) at a frequency of 1 Hz. The stimulator was controlled by a computer *via* a data acquisition board (Data Translation DT2812, Marlboro MA). Compound action potentials were elicited and recorded, making use of the QTRAC program (copyright, Hugh Bostock, Ph.D., Professor, Institute of Neurology, London, United Kingdom). The peak amplitude of the C-fiber component was measured continuously from the negative to the positive maximum.

Intracellular Calcium Concentration

Cells in the nerve fascicles were loaded with membrane-permeant esters of the fluorescent dyes Calcium Green-1 and Fura Red. The organ bath containing the nerve fascicles was mounted on an inverted fluorescence microscope (Zeiss Axiovert 35, Jena, Germany) with a custom-made photometric attachment and was illuminated at 0.33Hz with 10-ms light pulses at 485 nm. Intensity of the emitted fluorescent light was measured after filtering by two photodiodes at 530 nm (Calcium Green) and 660 nm (Fura Red). Emission intensities were recorded using the QTRAC program. The ratio of the two emission intensities was calculated off-line to give a measure of intracellular calcium concentration.

Chemicals

α,β-meATP (α,β-methylene-adenosine 5'-triphosphate), Ap5A (diadenosine pentaphosphate), ATP (adenosine 5'-triphosphate), and ATPγS (adenosine 5' [γ-thio] triphosphate) were purchased from Sigma (St. Louis, MO). Trinitrophenyl (TNP)-ATP (2' (or 3')-*O*-adenosine 5'-

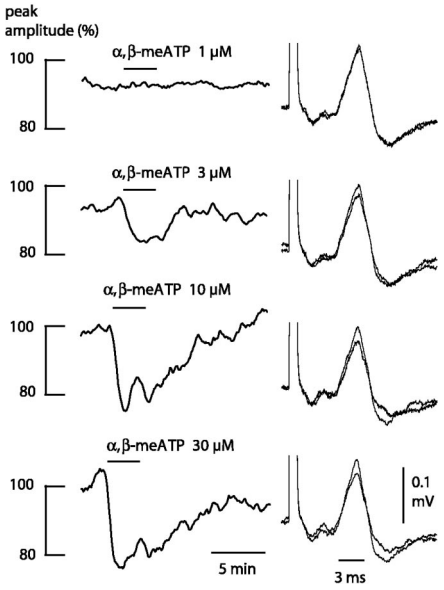

Fig. 1. Effects of α,β-methylene-adenosine 5'-triphosphate (*α,β-meATP*) on the peak amplitude of the C-fiber compound action potential in isolated human vagus nerve. Continuous recordings of the peak amplitude are shown on the *left*, and individual compound action potentials taken before and after application of α,β-meATP to the bathing solution are shown on the *right*. α,β-meATP was tested in ascending concentrations on a single fascicle.

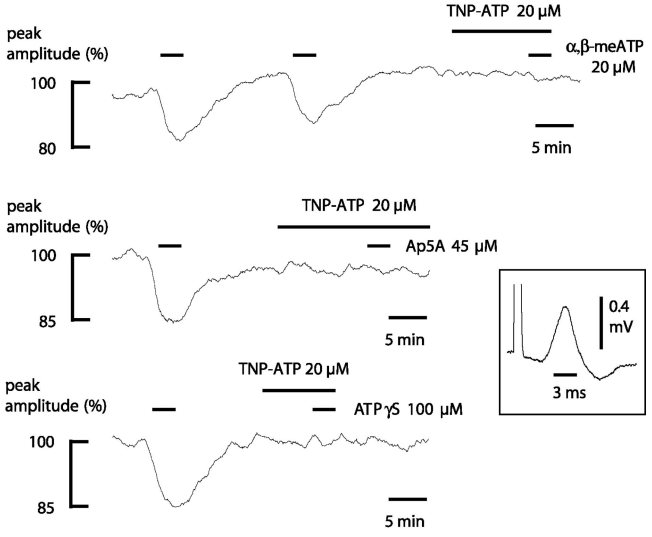

Fig. 2. Pharmacologic profile of ionotropic adenosine 5'-triphosphate (P2X) receptors of unmyelinated axons in isolated human vagus nerve. Changes in the peak amplitude of the C fiber compound action potential are shown. The agonists (α,β-meATP, Ap5A, ATP-γ-S) and one antagonist (TNP-ATP) at P2X receptors were applied *via* the bathing solution. The *inset* shows a representative example of the C fiber compound action potential following the stimulus artifact (stimulus duration: 1 ms).

triphosphate), Calcium Green-1, and Fura Red were purchased from Molecular Probes (Eugene, OR). MRS 2179 (2'-deoxy-N^6-methyladenosine 3',5'-bisphosphate) was purchased from Tocris (Bristol, UK). Agonists at purinergic receptors were administered for 2–4 min with application-free intervals of at least 10 min after recovery to baseline. TNP-ATP and MRS 2179 were usually given 10 min before application of the agonists.

Statistical Analysis

Data are given as deviation from the resting "peak amplitude" and the intracellular Ca^{2+} baseline, both set as 100% (mean ± SD).

Results

Compound action potentials of unmyelinated axons (C-fiber component) were recorded from isolated fascicles of human vagus nerve during repetitive electrical stimulation at 1 Hz. Agonists and one antagonist at P2X receptors were applied *via* the bathing solution and tested on the peak amplitude of the C-fiber compound action potentials. A representative experiment is illustrated in figure 1. α,β-meATP was tested in concentrations from 1 to 30 μM, and a reduction in compound action potential amplitude was observed. Quantitatively (mean ± SD), α,β-meATP in a concentration of 1 μM did not change the peak amplitude, whereas a reduction in peak amplitude was observed in concentrations of 3 μM (−9.6 ± 4.8%, n = 3), 10 μM (−18.6 ± 10.3%, n = 3), 20 μM (−12.3 ± 3.3%, n = 9), and 30 μM (−20.1 ± 9.7%, n = 3).

In further experiments, other agonists at P2X receptors were tested. Reduction in peak amplitude was also observed by using Ap5A (45 μM) and ATPγS (100 μM). These compounds reduced the amplitude of the C-fiber compound action potential by 8.4 ± 2.9% (n = 3) and 14.1% (n = 2), respectively. The effects of α,β-meATP (n = 4), Ap5A (n = 2), and ATPγS (n = 1) were completely blocked in the presence of 20 μM TNP-ATP (fig. 2).

In additional experiments, application of ATP (100 μM) to the bathing solution induced a rapid and transient rise in intracellular Ca^{2+}. Quantitatively, ATP (100 μM) increased the emission ratio of Calcium Green/Fura Red by 19.4 ± 10.8% (n = 7). MRS 2179 (20 μM), an antagonist at $P2Y_1$ receptors, blocked the ATP-induced intracellular Ca^{2+} transient (fig. 3).

Discussion

The data indicate that recordings from isolated fascicles of human vagus nerve can be used for functional studies of P2Y and P2X receptors in the peripheral human nervous system. To our knowledge, this is the first optical and electrophysiologic study on an isolated human vagus nerve preparation. The presence of P2Y receptors in human peripheral nerve has already been demonstrated using ATP-induced Ca^{2+} transients in isolated sural nerve.[10,11] Schwann cells are most likely the

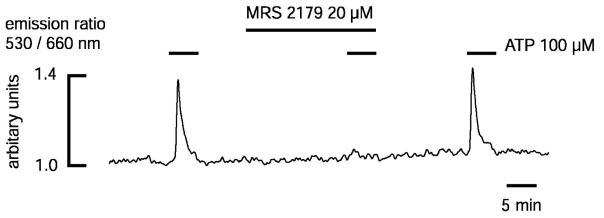

Fig. 3. Changes in the free intracellular Ca^{2+} concentration in a fascicle of human vagus nerve. The nerve fascicle was loaded with the Ca^{2+}-sensitive fluorescent dyes Calcium Green and Fura Red. An increase in the emission ratio e(530 nm)/ e(660 nm) indicates an increase in $[Ca^{2+}]_i$. ATP produces an increase in $[Ca^{2+}]_i$. MRS 2179 antagonizes the intracellular Ca^{2+} transient.

cellular elements causing these intracellular Ca^{2+} transients.[12] In the current study, ATP-induced Ca^{2+} transients were also found in isolated human vagus nerve (fig. 3). These Ca^{2+} transients were blocked by MRS 2179, a potent antagonist at $P2Y_1$ receptors.[13] This indicates that P2Y receptors are functional in both somatosensory and visceral human nerves.

To our knowledge, electrophysiologic evidence for the activity of P2X receptors in the peripheral human nervous system has not been described, except for the observation that α,β-meATP induces contraction of muscle specimen from human bladder.[14] In the current study, clear effects of agonists at P2X receptors were found on action potentials of unmyelinated axons in segments of human vagus nerve. The pharmacologic profile indicates the presence of $P2X_3$ and/or heteromeric $P2X_{2/3}$ receptors, because α,β-meATP is an agonist and TNP-ATP is an antagonist at these subtypes of P2X receptor.[15–17] P2X receptors in human vagus nerve were also activated by the less specific P2X receptor agonists Ap5A[18] and ATPγS. Recently, desensitization of $P2X_3$ receptors by low concentrations of Ap5A has been described.[19] This effect was not tested in the current study. It is known that P2X receptors depolarize neurons. We therefore interpret the changes in compound action potentials as a consequence of axonal depolarization in the human vagus nerve (*e.g.*, due to inactivation of sodium channels).

In previous studies, effects of ATP on afferent human nerve fibers have been observed using microneurography[20] and sensory testing. In the latter case, pain was experienced on local application of ATP on a human blister base preparation[5] or by iontophoresis of ATP to human skin.[6] However, in intact nervous tissue, there is rapid enzymatic degradation of ATP to adenosine,[21] and the effects of ATP seen in these studies might have been caused by activation of adenosine/P1 receptors. In fact, the effects of ATP on the excitability of unmyelinated axons in isolated segments of human sural nerve were blocked by adenosine receptor antagonists; evidence for activation of P2X receptors (using α,β-meATP) was not found.[10,22] The effectiveness of α,β-meATP on human vagus nerve (current study) and its ineffectiveness on human sural nerve[10] might be attributable to the expression of heteromultimeric $P2X_{2/3}$ receptors in human vagus nerve and homomeric $P2X_3$ receptors in human sural nerve. In this case, bath application of α,β-meATP is sufficient for activation of slowly desensitizing $P2X_{2/3}$ receptors[16] and is probably too slow for the activation of rapidly desensitizing $P2X_3$ receptors.

The function of P2X receptors on unmyelinated afferent nerve fibers in the vagus nerve is not well understood. There is evidence that 80–85% of the vagal nerve fibers are afferent.[23] Mice lacking $P2X_3$ receptors have marked urinary bladder hyporeflexia,[24,25] and it is plausible that a similar mechanosensory function may be found for P2X receptors in reflex control of distension of the esophagus and/or stomach.[4,9] Models of visceral pain were not investigated in $P2X_3$ knock-out mice. However, TNP-ATP blocks acetic acid-induced abdominal constriction in mice, which indicates a contribution of $P2X_3$ and heteromeric $P2X_{2/3}$ receptors to inflammatory visceral pain.[3] A possible function of P2X receptors in vomiting, emesis, and digestion should be explored. More selective, stable, and bioavailable P2X receptor antagonists might be helpful in such studies and in the treatment of visceral pain.[26] Studies on isolated segments of human vagus nerve could be useful in such investigations.

The authors thank Klaus Hallfeldt, M.D., Ph.D., and Thomas Mussack, M.D. (Staff Surgeons, Department of Surgery, University of Munich, Germany), for preparation of the vagus nerve; Christina Müller (Laboratory Technician, Department of Physiology, University of Munich) for technical assistance; and David J. Tracey, Ph.D. (Professor, Department of Anatomy, University of New South Wales, Sydney, Australia), for helpful discussions on the manuscript.

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