In Vivo Assessment of the Regulatory Mechanism of Cholinergic Neuronal Activity Associated With Motility in Dog Small Intestine

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ABSTRACT—Intestinal motor activity associated with acetylcholine (ACh) release was assessed in the small intestine of anesthetized dogs by simultaneous measurement of motor activity and local ACh concentrations within the intestinal wall with in vivo microdialysis. Basal concentration of ACh measured in the dialysate was 1.12 ± 0.08 pmol/15 min (n = 10), a value that remained constant until 3 h after perfusion. Intraarterial infusion of tetrodotoxin reduced dialysate ACh concentration, while the motor activity accelerated at the early phase after infusion of tetrodotoxin and then decreased, thereby suggesting that the motor activity is regulated by not only excitatory cholinergic neurons, but also inhibitory neurons. Intraarterial infusion of atropine increased dialysate ACh concentration but reduced motor activity, thereby indicating that the cholinergic neurons are tonically active and the muscarinic autoreceptors operate to inhibit the ACh release. Intraarterial infusion of norepinephrine reduced, but yohimbine increased both motor activity and dialysate ACh concentration, thereby indicating that the adrenergic neurons regulate the motor activity due to control of cholinergic neuronal activity. This in vivo microdialysis method demonstrated in the whole body of animals that the activity of cholinergic neurons was physiologically regulated by itself and adrenergic neurons.

Keywords: In vivo microdialysis, Acetylcholine release, Tetrodotoxin, Atropine, Norepinephrine

The mechanism underlying changes in gastrointestinal motility induced by many substances were evaluated by measuring mechanical activity and neurotransmitter release in the isolated preparations. However, findings obtained by in vitro experiments do not always correspond to those in vivo, and thus it cannot be elucidated whether the responses obtained in the isolated preparations are physiologically and/or pathophysiologically important in the whole body. Application of microdialysis to gastrointestinal tissue may offer a potential advantage over traditional methods using isolated preparations because it allows for continuous, long-term sampling of interstitial solute concentration within the region of gastrointestinal tissues in which a dialysis probe has been placed. Prostaglandin E2 in interstitial fluid of dog gastric submucosa (1) and catabolism of neurotensin in interstitial fluid of rat gastric submucosa (2) were measured by the in vivo microdialysis method. These studies did not analyze neuronal regulation of gastrointestinal motility in the whole body, since the concentrations of substances released into the local area related to the motility were not measured. Recently, it has been shown by the in vivo microdialysis method that stimulation of vagal nerve produces release of nitric oxide at concentrations able to cause inhibition of smooth muscle contractions in the stomach and colonic wall of rabbits (3). We previously suggested that the intestinal motility was associated with acetylcholine (ACh) release from enteric nerves in whole body of dogs (4). Thus, we attempted to establish a new method to identify physiological intestinal motor activity associated with mainly the activity of cholinergic neurons in the whole body of animals, by determining simultaneously motor activity and ACh release through a microdialysis fiber implanted around the muscle layers, including the myenteric plexus, of dog small intestine.

MATERIALS AND METHODS

Animal preparation

The study has been carried out in accordance with the Guide for the Care and Use of Laboratory Animals of Nagasaki University as adopted and promulgated by the notification of the Director-General of the Science and
International Affairs Bureau of the Japanese Ministry of Education, Science, Sports and Culture, Japan. The healthy, mature, mongrel dogs of either sex, weighing between 8 and 15 kg, were anesthetized with pentobarbital Na (40 mg/kg, i.v.), and surgical procedures were performed under aseptic conditions. After exposing the abdominal cavity by a low ventral laparotomy, a 3-French Disposable catheter (Atom Corp., Tokyo) connected to a injection-syringe was inserted in the intestinal marginal artery for intraarterial injection of drugs. The area of arterial supply was defined by flushing with 1 ml of Ringer solution (147 mM Na\(^+\), 2.3 mM Ca\(^{2+}\), 155.6 mM Cl\(^-\) and 4 mM K\(^+\)). The animals were intubated and ventilated with a constant volume respirator using room air mixed with oxygen. Anesthetic was supplemented by intravenous injections of pentobarbital Na (30 mg/kg per hour) throughout the experiments.

Procedure for recording of mechanical response
For recording the mechanical response, a strain gauge force transducer was sutured to the serosa of the defined area of the small intestine, and contractility in the circular muscle direction was recorded isometrically.

Procedure for microdialysis
A dialysis probe (O-P-100-10; Eicom, Kyoto) was implanted in the wall of the small intestine. The probe was gently inserted tangentially into the wall of the small intestine; part of the dialysis membrane of the probe was passed through the myenteric plexus, the circular muscle layers and the deep muscular plexus; and then the probe was sutured to the surface of the intestine at approximately the site of the transducer (Fig. 1). The active site of the dialyzer was 10-mm-long, with a 0.2-mm inner diameter and a 0.22-mm outer diameter, and 5-kDa weight cutoff value. At the end of the experiment, the tissue from around the probe was dissected and the correct position of the probe was verified histologically (Fig. 2). Only data from experiments in which the probe was implanted correctly were used for assessments.

![Diagram of positions of strain gauge force transducer and microdialysis probe in dog small intestine.](image1)

![Histological examination of the microdialysis probe placed in the circular muscle layer and myenteric plexus of dog small intestine.](image2)
Measurement of ACh concentration

The dialysis probe was connected to a perfusion pump (EP-60, Eicom) and to the injection valve of the apparatus for high performance liquid chromatography with an electrochemical detector system by means of polyethylene tubing. The motor-driven injection valve of the autoinjector (AS-10, Eicom) was controlled by an adjustable electronic timer. One stainless steel cannula was connected to the perfusion pump by a polyethylene tube, and the outlet of the other cannula was connected to the injection valve. In the present case, the internal standard, ethylhomocholine, delivered by the perfusion pump, was fed into the perfusate tube proximal to the injection valve. The dialysate contained 0.2 mM physostigmine, a cholinesterase inhibitor, to block the degradation of ACh. The intestinal motor activity was not affected by physostigmine. A dialysis probe was exposed to 37°C Ringer solution containing 0.2 mM physostigmine with a constant ACh concentration (10⁻⁷ M), and dialysate samples were collected at various flow rates (1 – 8 μl/min), and then a flow rate of 2 μl/min was chosen for the in vivo experiments. Under the flow rate of 2 μl/min, the in vitro recovery for ACh was almost a steady level of 51.0 ± 1.6% with different ACh concentrations in the testing solution. Thus, the dialysis probe was continually perfused at a flow rate of 2 μl/min with Ringer solution containing 0.2 mM physostigmine. In vitro recovery is higher than in vivo recovery (5), while in the present data, the in vitro value was considered to approximate the in vivo value. The dialysate was collected every 15 min in the sample loop of the automated sample injector, which was set up on line with the high performance liquid chromatography with an electrochemical detector system. Since analysis of choline and ACh was completed within 15 min, the sample loop was set to be held in the load position for 15 min and was automatically switched to the injection position for 60 s, after which the cycle was repeated. The space between the dialysis membrane and detector was measured at the start of each experiment, and the lag time to expression of drug effect was taken into account.

Experimental procedures

Concentration of dialysate ACh collected at 15-min intervals remained almost at a steady state level in each experiment from 60 to 240 min after probe implantation. The samples from the first to 4th fractions after probe implantation were discarded and 4 fractions (15-min dialysates) of the 5th to 8th fractions were determined as a mean basal concentration of dialysate ACh. The intraarterial administrations of saline at the flow rate of less than 0.5 ml/min did not alter the concentration of dialysate ACh; therefore, the saline containing substances were infused at the flow rate of 0.5 ml/min. When effects of the intraarterial administrations of tetrodotoxin (TTX), atropine and norepinephrine (NE) were examined on the motor activity and concentration of dialysate ACh, 4 fractions of 15-min control dialysates were collected; then TTX at 0.1 μM was infused into the marginal artery at a flow rate of 0.5 ml/min for 15 min, atropine at 0.1 μM was infused into the marginal artery at a flow rate of 0.5 ml/min for 2 min or NE at 0.1 μM was infused into the marginal artery at a flow rate of 0.5 ml/min for 30 min. The concentrations of dialysate ACh in the presence of substances were represented as the percentage of the basal concentration of dialysate ACh (before application of substance), in each experiment.

Statistics

The data showing dialysate ACh concentration are represented as the mean ± S.E.M. A statistical analysis was made with Dunnett's test. A probability (P) values of <0.05 was considered statistically significant.

Drugs and chemicals

Substances used were as follows: atropine sulfate, tetrodotoxin, physostigmine (eserine) sulfate, norepinephrine bitartrate, yohimbine hydrochloride and tetramethylammonium chloride (Wako Pure Chemical Industries, Osaka); 1-decanesulfonic acid sodium salt (Tokyo Kasei Organic Chemicals, Tokyo); ethylhomocholine (Eicom); choline chloride (Nacalai Tesque, Kyoto); and acetylcholine perchlorate (Sigma, St. Louis, MO, USA).

RESULTS

Basal concentration of dialysate ACh

Basal concentration of ACh in the dialysate collected at 15-min intervals decreased over 60 min after probe implantation, subsequently reaching an almost steady state level; therefore, the experiment was started 60 min after implantation of the probe. Four samples (dialysates collected at 15-min intervals) of the first to 4th fractions were determined as a mean basal concentration of dialysate ACh. Basal concentration of dialysate ACh was 1.12 ± 0.08 pmol/15 min (n = 10), a value that remained constant until 240 min from 60 min after implantation of the probe and showed little variation among the different animals. Intraarterial infusion of saline did not change either the concentration of dialysate ACh or motor activity (data not shown).

Effects of TTX, atropine, yohimbine and NE infused into marginal artery on motor activity and concentration of dialysate ACh

After 4 fractions of dialysates were collected as a control, TTX, atropine or NE was infused into the marginal artery at a flow rate of 0.5 ml/min. TTX at 0.1 μM for 15 min reduced both the motor activity and concentration of dialysate ACh, although the motor activity accelerated at an early
phase after infusion of TTX (Fig. 3). Concentration of dialysate ACh decreased to approximately 50% of the basal concentration in the first fraction after infusion of TTX.

Atropine at 0.1 μM for 2 min increased dialysate ACh concentration in the first fraction after infusion and to approximately 1.7 times of basal concentration in the second fraction, while the motor activity decreased immediately after infusion (Fig. 4).

NE at 0.1 μM for 30 min reduced both the motor activity and concentration of dialysate acetylcholine (ACh) immediately after infusion of NE (Fig. 5). Concentration of dialysate ACh decreased to approximately 70% of the basal concentration.

Yohimbine at 0.1 μM for 5 min increased both the motor activity and dialysate ACh concentration (Fig. 6). Concentration of dialysate ACh increased in the first fraction after infusion of yohimbine and to approximately 1.4 times of
the basal concentration in the second fraction.

DISCUSSION

The present study demonstrated that the intestinal motor activity was physiologically associated mainly with cholinergic neuronal activity, which was regulated by the cholinergic neuron itself and adrenergic neuron, in the whole body of animals. It is well known that enzymatic activity of extracellular cholinesterase is markedly high; therefore physostigmine, a cholinesterase inhibitor, was added to the perfusion solution to block the degradation of ACh, as in the brain microdialysis method (5). The administration of physostigmine into the microdialysis probe did not affect the motor activity. Physostigmine administered into the microdialysis probe was diffused only to the limited local area, and therefore substances may not affect the motor activity. Basal concentration of ACh measured in the dialysate from the dog small intestine was approximately 74 fmol/min. The value was lower than that in the rat brain (5) and higher than that in the heart of cat (6) and rat (7).

The intraarterial infusion of TTX reduced the concentration of dialysate ACh, thereby indicating that ACh in the dialysate originates from the nerve terminals of enteric cholinergic neurons. On the other hand, the motility was also reduced by infusion of TTX, although accelerated at early phase after infusion of TTX. The motor activity associated with release of ACh has been directly detected in vivo in the previous study, in which intraarterial administration of TTX inhibited the nerve-stimulated contractions and increases in concentrations of dialysate ACh (4). The TTX-induced acceleration of motor activity at the early phase may be attributed to blockade by TTX of the activities of inhibitory neurons such as NO-releasing neuron (8) and VIP-containing neuron (9). Similar results have been shown in the circular muscle of cat jejunum (10) and mouse proximal colon (11). The circular muscles are tonically regulated by inhibitory neurons, and the blockade by TTX of the inhibitory nerve conduction leads to acceleration of motor activity of circular muscle by removal of inhibitory transmission to the smooth muscle (10).

Intraarterial infusion of atropine increased the concentrations of dialysate ACh, but inhibited the motor activity. The increase in concentrations of dialysate ACh is attributed to the blockade by atropine of the muscarinic autoreceptors located on the cholinergic nerve terminals that inhibit ACh release by a negative feedback mechanism, as shown by both in vivo and in vitro studies (12–15). The inhibitory muscarinic autoreceptors were found to be active in the intestine, especially in the presence of a cholinesterase inhibitor in the dialysate, as in the case of brain (16). The release of ACh was increased by blockade of muscarinic autoreceptor with atropine, while the muscarinic receptors located on the smooth muscle cells were blocked by atropine, and thus the motor activity was reduced. The motor activity was not completely abolished by atropine, thereby suggesting that the intestinal smooth muscle receive not only cholinergic neurons but also noncholinergic excitatory neurons. These results indicate that both the cholinergic neurons and the muscarinic autoreceptors are physiologically active in the small intestine of anaesthetized dogs. In vitro experiments, effects of TTX and atropine were detected only on the artificial stimulation-induced release of ACh, but not on the spontaneous release, since the activities of cholinergic neurons were probably very low in the preparations isolated from the body.

The intraarterial infusion of NE reduced both the motor activity and concentration of dialysate ACh. The present study demonstrated in the whole body that NE inhibited the release of ACh from the cholinergic nerve terminals (17–19). The intraarterial infusion of yohimbine increased both the motor activity and concentration of dialysate ACh. The increases in motor activity and dialysate ACh concentrations may be attributed to the blockade by yohimbine of the $\alpha_2$-adrenoceptors located on the cholinergic nerve terminals. Thus, the present study proved in the whole body the concept obtained by in vitro experiments that the adrenergic neurons physiologically regulate the intestinal motor activity due to tonic control of the cholinergic neuronal activity.

In conclusion, the in vivo microdialysis method identified motor activity associated with ACh release. Dialysis fibers were implanted into circular muscle layer including the myenteric plexus of the intestine; therefore, ACh detected in this system may originate from nerve terminals of preganglionic and/or postganglionic cholinergic neurons, although in the dog small intestine, the released ACh, which was associated with motility of circular muscle, was suggested to originate mainly from cholinergic nerve terminals in the deep muscular plexus of the circular muscle layer (8, 15). The present study demonstrated using the whole body of animals that the intestinal motor activity is regulated mainly by the cholinergic neurons and that the local negative feedback system via muscarinic autoreceptors and the adrenergic tonic control of cholinergic activity play physiologically a role in intestinal motor activity. In addition to the cholinergic and adrenergic neurons, the noncholinergic, nonadrenergic excitatory and inhibitory neurons are also tonically involved in the control of intestinal motor activity. With the in vivo microdialysis method, one can analyze mechanisms underlying physiological and pathophysiological motor activity of the gastrointestinal tract, and drugs to treat subjects with gastrointestinal disorders can be developed.
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