Developmental Changes in Agonist-Mediated Gastric Smooth Muscle Contraction in the Rabbit

TAKESHI TOMOMASA, HIDEKI YAGI, SABURO KIMURA, WILLIAM J. SNAPE, JR. AND
PAUL E. HYMAN

Department of Pediatrics and Medicine and the Inflammatory Bowel Disease Center, Harbor-UCLA Medical Center, Torrance, California 90509

ABSTRACT. We studied smooth muscle strips from rabbit proximal stomach to explore the age-related changes in agonist-mediated contraction. Strips from neonatal (1 d) and weanling (11 wk) rabbits were oriented to measure isometric tension in circular muscle. Bethanechol stimulated maximal tension in both age groups. Although the potencies for bethanechol were similar ($ED_{50} \sim 5 \mu M$), the maximal response was nearly 4-fold greater in weanling ($1140 \pm 73 \text{ mN/cm}^2$) versus neonate ($305 \pm 54 \text{ mN/cm}^2$), $p < 0.001$. Maximum stress increased with age for bethanechol, high extracellular K^+ , and substance P, but not for serotonin, cholecystokinin octapeptide, neurotensin, or bombesin. Only bombesin stimulated larger contraction in neonates ($152 \pm 37 \text{ mN/cm}^2$) versus weanlings ($86 \pm 20 \text{ mN/cm}^2$), $p < 0.05$. Potencies did not change with age, except for substance P and serotonin. Substance P and serotonin induced early phasic and prolonged tonic contractions, which were unaffected by tetrodotoxin or atropine. ED_{50} for the phasic and tonic components of substance P-stimulated contraction in neonates were 1.8 and 7.7 nM. Substance P was 60–70 times more potent in neonates versus weanlings ($p < 0.001$). ED_{50} for serotonin-stimulated contraction in neonates (33 and 22 nM, respectively) were 20–30 times more potent than in weanlings ($p < 0.05$). Motilin, morphine, epidermal growth factor, and gastrin did not stimulate contraction at either age. We conclude that age-dependent changes in agonist potency and efficacy may be one factor to explain in part the changes that occur in gastric motility during postnatal development. (*Pediatr Res* 26:458–461, 1989)

Abbreviations

CCK-OP, cholecystokinin octapeptide
 ED_{50} , half of maximal response

The control of gastric motility involves interactions among nerves, hormones, paracrine substances, and smooth muscle. The coordinated contraction and relaxation of gastric smooth muscle is the measurable expression of these complex interactions. It is not surprising that the patterns of *in vivo* gastric motility change during development in both animals (for review see Reference 1) and man (2, 3), as morphology, physiology, and biochemistry change during development.

A variety of techniques has been used to examine age-depend-

ent changes in morphology and neurohormonal interactions related to gastric intestinal motor function. For example, morphologic studies (4) have shown that smooth muscle cells grow larger during maturation, gaining in the ratio of cytoplasm to nucleus. Radioligand binding studies were used to demonstrate changes in the number of adrenergic (5) and cholecystokinin (6) receptors in maturing tissues. RIA (7) and histochemistry (8) were used to document age-related changes in tissue quantity of substance P and serotonin, respectively. Surgically placed serosal electrodes recorded myoelectrical activity of animal intestine during the perinatal period (9, 10). All of these methods have yielded evidence of age-related changes that may affect gut motility.

One area that has not yet been examined systematically for developmental changes is that of agonist-mediated stimulus-contraction coupling in muscle strips. Modulation in receptor-mediated contraction stimulated by neurotransmitters and hormones may be an important factor in the maturation of gastric motility. Our study was designed to examine the effect of postnatal age on gastric smooth muscle contraction in response to a number of putative agonists. The purpose of our study was to identify agonists whose effects on gastric contraction change with postnatal age.

MATERIALS AND METHODS

Tissue preparation. Newborn (1 d old) and weanling (2.0–2.5 kg, approximately 11 wk old) New Zealand white rabbits were used. Small segments were cut from the greater curvature approximately 20–25% of the distance from the lower esophageal sphincter to the pylorus. With the aid of a dissecting microscope, the mucosa and the serosa were removed. Care was taken to avoid stretching or damaging the underlying muscle. The full-thickness muscle segments were cut into 10.0×2.0 – 2.5 mm strips along the circular axis.

The strips were placed in a 1.0-mL well filled with oxygenated Krebs' solution and studied as previously described (11). One end of the muscle was attached to a stationary clamp. The other end was attached via an inelastic wire to an isometric force transducer (Grass model FT-03C, Grass Instrument Co., Quincy, MA). The muscle was bathed in Krebs-Ringer solution with the following composition (in mM): NaCl 115, KCl 4.6, $MgCl_2$ 2.1, NaH_2PO_4 1.2, $NaHCO_3$ 21.2, $CaCl_2$ 2.5, glucose 15.5. Temperature maintained at $37^\circ C$, and the bath was bubbled continuously with 95% O_2 and 5% CO_2 . When a peptide was studied, BSA (0.03% w/v) was added to the Krebs-Ringer solution.

The muscle tissue was allowed to stabilize in the bath for 1 h and then was stretched by increments of 1 mm until muscle tension was ready to increase with any further increase in muscle length. The length was noted as the L_i . From this initial length, the muscle strip was progressively stretched by 0.5–1.0 mm increments. After stretching, the muscle was allowed to reach a

Received February 20, 1989; accepted June 22, 1989. Correspondence Paul E. Hyman, M.D., Harbor-UCLA Medical Center, 1124 West Carson Street, Torrance, CA 90502.

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level of tension that was stable. At each increment of stretch, the muscle was contracted maximally using 10 μ M bethanechol. The bethanechol-stimulated active muscle tension was measured from the stable level of resting tension to the peak tension recorded after bethanechol administration. The optimum length of the muscle L_0 was the muscle length at which peak active tension developed. L_0 was determined for each strip. Experiments were performed at 95–100% L_0 .

Drugs tested in these studies were diluted in Krebs-Ringer solution and were added to the bath to provide the desired final concentration. After each administration, the muscle was washed free of the test agent and the tension allowed to return to its previous resting level. Contraction was expressed as stress per cross-sectional area (A), calculated from the equation $A = M/pl$, where M was mass (g), p was density (g/mL) and numerically equal to the specific gravity and l was length of the muscle (cm). The muscle wt was measured after experiments were completed, and after blotting to eliminate surface water.

A single agonist was tested on each muscle strip. Dose-response curves were cumulative, beginning with the lowest dose and progressing to the maximal dose. After completion of the dose-response curve, the strip was tested with 10 μ M of bethanechol to assure that the strip had maintained its ability to contract.

To increase extracellular potassium concentration, we drained all buffer solution from the bath. We immediately refilled the bath with a high potassium buffer solution (10–120 mM) made by replacing NaCl with KCl. To evaluate the effect of tetrodotoxin or atropine, we first studied the response to each agonist alone. Tissues were washed, and 3 μ M tetrodotoxin or 3 μ M atropine were added. After 20 min preincubation with tetrodotoxin or atropine, the strip was incubated with the agonist. Response of the strip after the second incubation with the agonist was compared to that after the first incubation and expressed as percent of the first response.

The drugs used in these studies were bethanechol chloride (Merck, Sharp and Dohme, West Point, PA), atropine sulfate (Elkins-Sinn, Cherry Hill, NJ), tetrodotoxin, CCK-OP, 5-hydroxytryptamine (Sigma Chemical Co., St. Louis, MO), substance P, neurotensin, bombesin, motilin (Peninsula Laboratories, Belmont, CA), pentagastrin (Ayerst Lab., Inc., New York, NY), epidermal growth factor and morphine (Wyeth Laboratories, Philadelphia, PA).

Data analysis. The agonist concentration causing ED_{50} and the maximal stress were calculated from dose-response data for

each agonist tested using a nonlinear least squares computer program (12). Unpaired *t* tests were conducted to compare ED_{50} or maximal stress between the age groups. All results were expressed as mean $\pm$ SEM.

RESULTS

As shown in Figure 1, there were qualitative differences in the agonist-induced contractions. Bethanechol stimulated sustained tonic contractions, whereas bombesin and neurotensin stimulated single phasic contractions. CCK-OP stimulated single phasic contractions in two of five strips from weanlings and in all strips from neonates. CCK-OP stimulated a sustained tonic increase in tension in three of five strips from the weanlings. Substance P stimulated biphasic contractions in eight of 10 strips in neonates and in six of 10 strips from weanlings. These biphasic responses were characterized by an initial phasic response of short duration followed by a prolonged tonic response. In studies from two neonates substance P stimulated only phasic contractions. In studies from four weanlings, only the tonic component was observed. Serotonin stimulated biphasic contraction in all five strips from neonates, and in three of five strips from weanlings. In studies from two weanlings, serotonin stimulated only tonic contractions. Pentagastrin 1 μ M, morphine 100 μ M, epidermal growth factor 100 nM, and motilin 1 μ M had no effect in either age group.

Table 1 shows quantitative differences in agonist-induced contractions. Bethanechol stimulated the strongest responses in both neonates (305 ± 54 mN/cm²) and weanlings (1140 ± 73 mN/cm²). All other ligands stimulated less than maximal force development.

The maximal force development in response to bethanechol, potassium, and substance P increased in weanlings compared to neonates, $p < 0.05$. Figure 2 shows the relationship between concentration and response for extracellular potassium (for the dose-response curves for bethanechol, see Reference 11). The strength of potassium-stimulated contraction increased with age. The maximal force development in response to serotonin, CCK-OP, and neurotensin did not differ significantly in the two age groups. In contrast, only bombesin stimulated greater force development in newborns compared to weanlings (152 ± 37 versus 86 ± 23 mN/cm², $p < 0.05$). Figure 3 shows the dose-response curve for bombesin, demonstrating decreased maximal response in weanlings compared to neonates.

Table 2 shows an absence of age-related changes in potency for bethanechol, potassium, CCK-OP, neurotensin, or bombesin. In contrast, substance P and serotonin were 30 to 70 times more potent in newborn rabbits than in weanlings ($p < 0.05$). An

Fig. 1. Qualitative differences in contractions. Muscle strips from the rabbit proximal stomach were incubated with each agonist and isometric tension was measured and recorded as described in "Materials and Methods." The three types of contractions are shown.

Table 1. Effect of age on efficacy of agonist-stimulated gastric muscle contraction*

Agonist	Maximal contraction (mN/cm ²)	
	Newborn	Weanling
Bethanechol	305 $\pm$ 54	1140 $\pm$ 73
Increased extracellular K ⁺	310 $\pm$ 17	823 $\pm$ 137
Substance P		
Phasic component	244 $\pm$ 48	576 $\pm$ 46
Tonic component	221 $\pm$ 85	797 $\pm$ 113
Serotonin		
Phasic component	247 $\pm$ 18	215 $\pm$ 85
Tonic component	226 $\pm$ 30	306 $\pm$ 85
CCK-OP	153 $\pm$ 65	196 $\pm$ 61
Bombesin	152 $\pm$ 37	86 $\pm$ 20
Neurotensin	71 $\pm$ 26	79 $\pm$ 15

* For each agonist, maximal contraction was calculated from individual dose-response studies. Each value is the mean $\pm$ SE from five or more experiments.

Fig. 2. Effect of age on contraction stimulated by increased extracellular potassium concentration. Muscle strips from the proximal stomach of newborn rabbits (closed circles) and weanling rabbits (open circles) were incubated in buffer with various concentrations of potassium as described in "Materials and Methods." Data represent the mean $\pm$ SEM of five separate experiments.

Fig. 3. Effect of age on bombesin-stimulated contraction. Muscle strips from the stomach of newborn rabbits (closed circles) and weanling rabbits (open circles) were incubated with various concentrations of bombesin. Data represent the mean $\pm$ SEM of six separate experiments.

example of age-related changes in agonist potency is shown in Figure 4, the dose-response curve for the tonic, second component of substance P-stimulated contraction.

To determine if the intrinsic nerves within the muscle strips were involved in agonist-stimulated contractions, we studied the effect of the tetrodotoxin on the contractions induced by submaximal concentrations of each agonist. Preincubation with 3 μ M tetrodotoxin for 15 min had no effect on any agonist at either age. In addition, we studied the effect of atropine on the contractions induced by 1.0 μ M substance P and 1.0 μ M serotonin. A 20-min preincubation and incubation with 3 μ M atropine caused no significant changes in either phase of contraction by each agonist at either age.

DISCUSSION

In our study the M_2 -muscarinic agonist bethanechol, CCK-OP, neurotensin, bombesin, substance P, and serotonin stimulated gastric smooth muscle contraction in both newborn and weanling rabbits. The study was designed to identify agonists

Table 2. Effect of age on potency of agonists*

Agonist	ED ₅₀	
	Newborn	Weanling
Bethanechol (μ M)	5.6 (4.8–6.6)	5.5 (4.5–6.8)
K ⁺ (mM)	29 (25–33)	23 (21–27)
Substance P (nM)		
Phasic component	1.8 (0.7–3.0)	134 (83–219)†
Tonic component	7.7 (3.8–15.6)	443 (259–759)†
Serotonin (nM)		
Phasic component	34 (17–68)	980 (540–1780)†
Tonic component	22 (12–44)	540 (280–1050)†
CCK-OP (nM)	10.2 (7.2–14.5)	4.4 (3.2–5.9)
Bombesin (nM)	1.4 (0.8–2.3)	1.1 (0.9–1.3)
Neurotensin (nM)	2.8 (1.3–5.8)	18.2 (12.6–26.3)

* Values were calculated from five or more experiments. ED₅₀ were calculated in logarithmic units, so that estimates of central tendency are given as 95% confidence ranges rather than SEM.

† $p < 0.05$ compared to newborn.

Fig. 4. Effect of age on the second phase of substance P stimulated contraction. Muscle strips from the stomach of newborn rabbits (closed circles) and weanling rabbits (open circles) were incubated with various concentrations of substance P. The second phase or phasic component was noted in eight of 10 strips from newborns and all of 10 strips from weanlings. Data represent the mean $\pm$ SEM of 10 separate experiments.

that might have some special role during postnatal development. In this regard we first identified general trends in muscle function, and then noted divergence from the usual patterns with several agonists.

The general trend was that after correcting for surface area of the muscle strips, tissue from weanlings generated greater maximal force than tissue from neonates. Several pieces of evidence suggested that this trend was due to muscle growth rather than increasing responses to specific agonists. Depolarization of the cell membrane by increasing extracellular potassium stimulated stronger contractions in weanlings compared to neonates. Because the mechanism of potassium-induced contraction is membrane depolarization, unrelated to any membrane receptor, the stimulus serves as proof that there are receptor-independent mechanisms responsible for the increased stress in the weanlings. Further indirect evidence for receptor-independent mechanisms for increased stress with age was provided in previous studies of muscarinic receptors. There are large numbers of M_2 -muscarinic receptors in neonatal rabbit gastric smooth muscle (11). Therefore, the age-dependent increase in bethanechol-stimulated con-

traction was not due to a change from suboptimal to an optimal receptor number. Age-related increases in gastrointestinal myocyte size and content of myofilaments (4) may help to explain receptor-independent increases in the magnitude of contraction during postnatal development. There was a nearly 2-fold increase in isolated gastric myocyte length in weanlings (mean 150 μ) compared to neonates (80 μ). Planimetry of myocytes in electron micrographs demonstrated a 4-fold increase in surface area over the same period, with a corresponding decrease in the amount of extracellular matrix.

Bombesin is a tetradecapeptide isolated from frog skin. Bombesin is chemically and biologically homologous to mammalian gastrin-releasing peptide. Results from our study suggest that bombesin-like peptides may be most important for motility during the neonatal period, and have a declining role as development proceeds. In contrast to results with potassium, bethanechol, and substance P, bombesin stimulated greater contraction in the neonate than in the weanling. Maximally effective concentrations of bombesin stimulated 45% of the maximal response to bethanechol in the neonate, and only 9% of the maximal response in the weanling. Bombesin-like immunoreactivity was present in human and rabbit alimentary tract (13) and milk (14). Circumstantial evidence linking bombesin to early development was found in studies of human lung, where concentrations of bombesin-like immunoreactivity were highest during childhood, and decreased in the adult to one-tenth of values found during adolescence (15). In a preliminary report oral bombesin inhibited gastric emptying in neonatal rats but not in weanlings (16). This result was consistent with the increased importance of bombesin in early life, although the effects of bombesin were unexpected, given the stimulatory effect of this peptide on smooth muscle.

The responses to CCK-OP, neurotensin, and serotonin did not change with age, so that their relative efficacy, like that of bombesin, declined with age, suggesting that these peptides may have a greater role in gastric muscle contraction early in postnatal development.

Gastrin (17), and motilin (18) and morphine (19) were effective gastrointestinal smooth muscle agonists in studies with different methods or in other species. The lack of response to these agents in our study indicated that they do not stimulate contraction in strips of rabbit circular muscle from the proximal stomach. These agonists may have a role in other gastrointestinal muscle tissues, or in the regulation of fasting cyclic motor activity, a feature not evaluable during *in vitro* studies.

In addition to quantitative changes in maximal response and potency, we noted age-related qualitative changes in contraction. CCK-OP stimulated phasic contraction in muscle strips from neonates, but tonic contraction in the majority of muscle strips from weanlings. Although the majority of muscle strips treated with substance P yielded biphasic response at both ages, the trends in strips with single responses suggested a transition from phasic responses in the immature tissues to tonic responses in the more mature tissues.

Substance P and serotonin were more potent in newborns than in weanlings. There were 30- to 70-fold differences in the ED₅₀ for these agonists. One possible explanation for age-related decreased potency of substance P and serotonin is the loss of a high affinity receptor. We recently presented preliminary results demonstrating with radionuclide binding studies the age-dependent loss of a high affinity substance P receptor from gastric muscle homogenates (20).

The biphasic responses to substance P and serotonin were not explained by the presence of both neurogenic and myogenic components, as was the case in other gastrointestinal tissues. For example, in classic studies from longitudinal muscle of guinea pig ileum, substance P produced a brief phasic response that faded to a weaker but sustained plateau. Atropine did not affect the initial peak contraction but substantially reduced the tonic phase of contraction (21). Also in guinea pig ileum, serotonin-stimulated contraction was mediated both by a direct myogenic

effect and by the release of acetylcholine or substance P from nerves of the myenteric plexus (22). In contrast, in our study, tetrodotoxin and atropine did not alter either the phasic or tonic component of substance P or serotonin-stimulated contractions, suggesting that in rabbit proximal gastric smooth muscle both the tonic and phasic components of substance P and serotonin-stimulated contraction are myogenic.

In summary, this survey of putative agonists was used to identify ligands that elicit substantially different responses in neonatal compared to weanling gastric tissues. We found four kinds of age-related changes: 1) increasing maximal response of the tissue; 2) qualitative differences in the type of contraction, with trends from phasic in the neonate to tonic in the weanling; 3) decreasing potency for substance P and serotonin; and 4) decreasing efficacy for bombesin. Continuing studies of the role of bombesin, substance P and other neurokinins, and serotonin may provide insight into one aspect of maturation and the control of gastric motility.

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