Development of Calcium Channels in Gastric Smooth Muscle

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ABSTRACT. We used [3 H]nitrendipine to characterize dihydropyridine sensitive calcium channels on cells isolated from neonatal (1 d) and weanling (11 wk) rabbit gastric fundic and antral smooth muscle. Incubating with and without nifedipine 20 μ M, specific binding was $56 \pm 4\%$ of total binding at 0.1 nM [3 H]nitrendipine. Specific binding was saturable, reversible, achieved equilibrium by 10 min at 4°C, and was linearly related to cell concentration. The affinity constant for [3 H]nitrendipine was higher in weanling fundus ($k_d = 243 \pm 121$ pM) versus antrum ($k_d = 771 \pm 190$ pM), $p < 0.05$. There were no age-related changes in affinity. In the antrum, the number of binding sites (B_{max}) increased from $6\,000 \pm 266$ /cell in neonates to $27\,500 \pm 8\,440$ /cell in weanlings ($p < 0.05$). In the fundus B_{max} was $7\,750 \pm 2\,100$ /cell in neonates, and there was no age-related change. To assess function, we compared isometric stress in full thickness muscle strips oriented to the circular layer. Bethanechol stimulated dose-dependent tonic contractions in the fundus and phasic contractions in the antrum. Maximal stress increased with age from 305 ± 54 mN/cm 2 to 1140 ± 73 mN/cm 2 ($p < 0.05$) in the fundus and from 72 ± 20 mN/cm 2 to 154 ± 30 mN/cm 2 ($p < 0.05$) in the antrum. Preincubation and incubation without calcium resulted in reversible inhibition of contraction at both ages. Nifedipine 10- μ M inhibited 100% of bethanechol-stimulated contraction in the antrum, but only 25% in the fundus at both ages. In summary, in rabbit gastric smooth muscle: 1) [3 H]nitrendipine identified functional calcium channels in neonates and weanlings, 2) there are age-related increases in calcium channels on antral, but not on fundic cells, 3) there are age-related increases in stress in strips from antrum and fundus, 4) the fundus utilizes intracellular calcium stores for bethanechol-stimulated contraction, whereas the antrum requires extracellular calcium. Age-related increases in dihydropyridine sensitive calcium channels are consistent with the requirement of the antrum for extracellular calcium to support contraction. (*Pediatr Res* 25:600-604, 1989)

The mechanisms and controls for smooth muscle contraction are complex, and there are many factors that may contribute to the increasing contraction that is observed during postnatal development. One area of interest is the control of intracellular calcium concentration. An increase in intracellular calcium is required for the initiation of smooth muscle contraction (3). The sources of calcium mediating the increase may be the extracellular space or intracellular organelles. For example, in the cat, muscle from the esophageal body required calcium from the extracellular space, but the lower esophageal sphincter used calcium released from the endoplasmic reticulum (4).

As in the esophagus and lower esophageal sphincter, there are no distinct anatomic features separating the proximal and distal portions of the stomach, but there are striking differences in their motor functions (5). The proximal stomach (fundus and body) stores the gastric contents and exhibits slow, receptive relaxation and tonic contractions. The distal stomach (antrum) grinds the gastric contents during well-coordinated phasic contractions. Preliminary results in the cat indicated that these two portions of the stomach utilized different sources of calcium to support contraction (6).

Calcium influx from the extracellular space is achieved by movement through distinct calcium channels in the plasma membrane. These channels can be quantitated with radioligand binding, because dihydropyridine binding sites copurify with membrane-bound calcium channels (7).

The present studies were designed 1) to quantitate dihydropyridine-sensitive calcium channels in rabbit gastric fundus and antrum, 2) to assess the sources of calcium required for contraction in these tissues, and 3) to determine if there were age-related changes in calcium channels that would help to explain the age-dependent increases in maximal contraction.

MATERIALS AND METHODS

Male New Zealand white rabbits, both neonatal (1 d old) and weanling (11 wk old), were obtained from ABC Rabbitry, Pomona, CA. HEPES was from Research Organics, Cleveland, OH. Glutamine (tissue calcium grade) and BSA were from Sigma Chemical Co., St. Louis, MO. Basal Eagle's medium amino acids (100 times concentrated) were from Grand Island Biological Co., Grand Island, NY. Soybean trypsin inhibitor was from Cooper Biomedical, Inc., Malvern, PA. Collagenase (179 U/mg) was from Boehringer Mannheim, Indianapolis, IN. Tritiated nitrendipine (73.7 Ci/mmol) came from New England Nuclear, Boston, MA. Unlabeled nifedipine was from Pfizer Laboratories, New York, NY, and bethanechol chloride from Merck, Sharp and Dohme, West Point, PA.

Preparation of dispersed cells. The standard buffer contained (in mM): NaCl 115, KCl 5.8, MgCl $_2$ 0.6, CaCl $_2$ 1.1, KH $_2$ PO $_4$ 2.1, HEPES 25.0, glutamine 2.0, glucose 4.15 g/liter, and basal Eagle's medium amino acids 2.6% (vol/vol). The pH of the

In muscle strips from rabbit stomach, maximal contraction was greater in adult compared to newborn animals (1). These data were compatible with studies in humans, showing that the intraluminal pressures achieved in the stomach are greater in adults compared to neonates (2). The age-related changes in gastric smooth muscle physiology that are responsible for increasing contraction have not been examined.

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solution was adjusted to pH 7.4 with NaOH. The osmolarity was 286 mosmol. For high potassium experiments, KCl was exchanged for NaCl to maintain osmolarity. All solutions were gassed with 100% O₂.

Smooth muscle cells were isolated from the gastric antrum and fundus of the rabbit by a technique similar to that introduced by Bitar and Makhoul (8) and modified by Collins (9). After a weanling rabbit was killed, a 4 × 2-cm strip of stomach tissue was removed, washed in cold (4°C) buffer, and pinned to the bottom of a dissecting tray filled with cold buffer with 100% O₂ bubbled through the solution. Using a dissecting microscope the mesentery was trimmed, the segment was turned mucosal side up, and the mucosa was removed by peeling it away from the muscularis with fine forceps. Confirmation of isolation of smooth muscle by this technique was obtained by examination of hematoxylin-eosin stained cross-sections of the muscle tissue preparation.

The tissue was minced with fine scissors and suspended in 15 mL of buffer with BSA 0.1% (wt/vol), collagenase 0.1% (wt/vol), and soybean trypsin inhibitor 0.01% (wt/vol). The tissue was incubated twice at 30°C for 55 min in a Dubnoff metabolic shaking incubator at 60 cycles/min. After the second incubation, the tissue was washed twice with 40 mL of buffer, resuspended in 10 mL of buffer without collagenase and incubated for 20 min. After the third incubation, tissues were passed gently through a siliconized glass pipette with a 3-mm orifice, and the dispersed cells were collected after passage through Nitex filter (500-μm pore size). The filtrate of dispersed cells was gassed with 100% O₂ and allowed to equilibrate to room temperature before use.

Adequate numbers of isolated myocytes from neonatal fundus and antrum were obtained by killing five animals for each experiment. Incubation techniques were similar to that used for weanling preparations.

The concentration of cells in the final filtrate was measured using phase-contrast hemocytometer and ranged between 2–6 × 10⁶ cells/mL. A 4-cm piece of antral or fundic smooth muscle isolated from a single weanling yielded approximately 10⁵ to 10⁶ cells. Cell viability, assessed by the ability of the cells to exclude 0.1% trypan blue, ranged from 80% to 95%. The cells remained viable for at least 2 h, and all experiments were performed within 1 h of completing the preparation of isolated cells.

Binding of [³H]nitrendipine. Aliquots of cell suspension containing 150 000 to 400 000 cells were added to silicon-coated test tubes containing various concentrations of [³H]nitrendipine and either 200-μM unlabeled nifedipine or an equivalent amount of buffer to a final vol of 1.0 mL. After incubation at 4°C for 45 min the contents of the tube were poured on to BSA-washed Whatman GF-A glass filters and the supernatant removed by vacuum extraction using a Millipore model 1225 vacuum filter apparatus. Unbound [³H]nitrendipine was washed through the filter with 10 mL washes with 0.9% NaCl at 4°C. The filters were placed into scintillation vials and 10 mL of budget-solve counting fluid was added to each vial. Measurements of radioactive decay were made with a Beckman LS 7800 Liquid Scintillation Counter (efficiency 40%). Specific binding ranged from 20 to 60% of total binding. At concentrations of 400 pM [³H]nitrendipine or higher, more than 95% of the nonspecific binding was due to counts trapped on the filters.

Isometric tension. Full thickness muscle was obtained from neonates and weanling rabbits as described above. The muscle segments were cut into 10.0- × 2.0- to 2.5-mm strips along the circular axis with fine scissors. The strips were placed in a muscle bath holding 1.0 mL of oxygenated Krebs' solution and studied as previously described (10). One end of the muscle 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 composition (in mM): NaCl 115, KCl 4.6, MgCl₂ 2.1, NaH₂PO₄ 1.2, NaHCO₃ 21.1, CaCl₂ 2.5 and glucose 15.5. The temperature was main-

tained at 37°C and the bath was bubbled continuously with 95% O₂ and 5% CO₂.

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 (L_i). From this initial length, the muscle strip was progressively stretched by 0.5- to 1.0-mm increments. After stretching, the muscle was contracted maximally using 10-μM 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_o) was the muscle length at which peak active tension developed. L_o was determined for each strip. Contraction was expressed as stress per cross-sectional area (10). Bethanechol and nifedipine were diluted in Krebs-Ringer solution and added to the bath to provide the desired concentrations. Agonists and antagonists were added simultaneously to the bath. To increase extracellular potassium concentration, we drained all buffer solution from the bath. We immediately refilled the bath with a high potassium solution (10–120 mM) which was kept isotonic by replacing NaCl with KCl. To study the effect of extracellular Ca²⁺, buffer composition was changed by omitting CaCl₂ and adding 5 mM EGTA.

After each drug administration, the muscle was washed free of the preceding test solution by replacing the chamber buffer twice with warm, well-oxygenated buffer. The chamber was refilled a third time, and the tension allowed to return to its previous resting level.

Data analysis. K_d and the total number of receptors (B_{max}) were determined from the results obtained from each separate experiment by using a nonlinear least-squares computer program (EBDA) (11). Unpaired *t* tests were used to determine differences between experimental groups. *t* tests with probabilities adjusted for multiple comparisons using Bonferroni criteria were used to compare age groups. All results are expressed as mean ± SEM.

RESULTS

Kinetics of [³H]nitrendipine binding. As demonstrated in Figure 1, specific binding was rapid, and achieved equilibrium by 10 min in antral weanling smooth muscle cells. Binding remained constant for at least 60 min at 4°C. The time required for half of the bound radioligand to bind (t_{1/2}) was 2.5 min. After 10 min of preincubation, the reversibility of [³H]nitrendipine was demonstrated by the addition of nifedipine 200 μM (Fig. 1). The time

Fig. 1. Time course of [³H]nitrendipine association and dissociation. Cells from circular smooth muscle of the weanling antrum were incubated with 250 pM [³H]nitrendipine with or without 200 μM unlabeled nifedipine at 4°C (closed circles). Dissociation was measured after adding unlabeled nifedipine to a final concentration of 200 μM after 30 min of incubation with [³H]nitrendipine (open circles). Results are mean ± SEM of five experiments.

required for half of the bound radioligand to dissociate was 1 min. There were no age or tissue-related differences in the time courses for association or disassociation.

Effect of tissue concentration on [^3H]nitrendipine binding. In the antrum of the weanling [^3H]nitrendipine binding was linearly related to cell concentration within the range tested (Fig. 2).

Concentration dependence of [^3H]nitrendipine binding. Specific binding of [^3H]nitrendipine to adult antral myocytes was concentration dependent to 400 pM, but was saturated above this value (Fig. 3). Nonspecific binding was not saturable and in-

Fig. 2. Effect of tissue concentration on [^3H]nitrendipine binding. Myocytes isolated from weanling antrum were incubated with 250 pM [^3H]nitrendipine with and without 200 μM unlabeled nifedipine at 25°C for 30 min. Results are the means of triplicate determinations from a single study, and are representative of four other experiments.

Fig. 3. Concentration dependence of [^3H]nitrendipine binding to antral myocytes. Myocytes from weanling antrum were incubated with various concentrations of [^3H]nitrendipine for 30 min at 25°C with and without 200 μM unlabeled nifedipine. Specific binding (closed circles) is obtained by subtracting nonspecific binding (open circles) from total binding. Results are mean $\pm$ SEM from six separate experiments.

creased with [^3H]nitrendipine concentration. Incubating with and without excess nifedipine (20 μM), specific binding was $57 \pm 4\%$ of the total binding at 0.1 nM [^3H]nitrendipine. Specific binding was saturable in adult fundus and neonatal tissues as well.

Table 1 summarizes the affinity and number of binding sites for nitrendipine on fundic and antral myocytes. The K_d was greater for weanling fundus (243 ± 121 pM) than for weanling antrum (771 ± 190 pM), $p < 0.02$). There were no age-related changes. In the antrum, the number of binding sites (B_{max}) increased from 6000 ± 266 /cell in newborns to $27,500 \pm 8440$ in weanlings, ($p < 0.05$). In the fundus, there were 7700 ± 2100 binding sites/cell in newborns and 8700 ± 1500 in weanlings, ($p > 0.10$). In the weanling rabbit there were more sites on antral compared to fundic myocytes ($p < 0.05$).

Isometric tension studies. Bethanechol-stimulated dose-dependent tonic contraction in the fundus, and phasic contractions in the antrum. As shown in Figure 4, there was an age-dependent 3-fold increase in maximal stress in the fundus, from 305 ± 54 mN/cm 2 in neonates to 1140 ± 73 mN/cm 2 in weanlings, $p < 0.05$. There was a similar age-related 2-fold increase from 72 ± 20 mN/cm 2 to 154 ± 30 mN/cm 2 , ($p < 0.05$) in the antrum.

To assess the effect of receptor-independent membrane depolarization on contraction, we determined the effect of increased extracellular K^+ concentration on stress. Depolarization of the plasma membrane allows entry of calcium from the extracellular space into the cell. In both fundus and antrum, 80-mM K^+ stimulated the maximal response, with further increases in extracellular K^+ concentration resulting in no further increases in stress. In four separate experiments the maximal response in the fundus was 297 ± 67 mN/cm 2 in the neonate and 725 ± 105 mN/cm 2 in the weanling. In the antrum the maximal response was 35 ± 9 mN/cm 2 in the neonate and 81 ± 32 mN/cm 2 in the weanling.

When strips of smooth muscle were pre-incubated for 3 min

Fig. 4. Effect of age on bethanechol-stimulated contraction. Data represent mean $\pm$ SEM of five separate experiments.

Table 1. Age-related changes in [^3H]nitrendipine binding sites (B_{max}) and affinity (k_d); B_{max} and k_d were determined by nonlinear regression analysis for six separate experiments (results are mean $\pm$ SEM)

	Antrum		Fundus	
	Neonate	Weanling	Neonate	Weanling
B_{max} (sites/cell)	$6000 \pm 266^*$	$27500 \pm 8440^\dagger$	7750 ± 2100	8700 ± 1500
k_d (pM)	950 ± 200	$771 \pm 190^\dagger$	530 ± 180	243 ± 121

* Neonate different from weanling $p < 0.05$.

† Antrum different from fundus.

Fig. 5. Effect of nifedipine on bethanechol-stimulated contraction. Data represent the mean $\pm$ SEM of four separate experiments.

Fig. 6. Effect of nifedipine on contraction stimulated by high extracellular potassium concentration. Data represent the mean $\pm$ SEM of four separate experiments.

and then incubated in Ca^{+2} free buffer (0 Ca^{+2} , 5-mM EGTA) there was complete inhibition of both bethanechol- and K^{+} -stimulated contraction at both ages. Contractions in response to these stimulants returned when the calcium free buffer was replaced with buffer containing calcium.

As shown in Figure 5, nifedipine 10 μM inhibited 100% of bethanechol stimulated contraction in the antrum but only 25% in the fundus at both ages. In contrast, nifedipine 10 μM completely inhibited contraction stimulated by increased extracellular K^{+} concentration in both fundus and antrum at both ages (Fig. 6).

DISCUSSION

In isolated smooth muscle cells from the fundus and antrum of rabbit stomach [^3H]nitrendipine binding was rapid, saturable, reversible and linearly proportional to tissue concentration. Nitrendipine inhibited isometric tension generated by bethanechol and potassium in concentration-dependent fashion in antral muscle strips. These results satisfy the generally accepted criteria for specific receptor binding. There were two advantages to using isolated cells rather than tissue homogenates or membranes for binding studies. First, it was possible to measure directly the number of [^3H]nitrendipine binding sites/cell. Second, we avoided [^3H]nitrendipine binding to intracellular receptors exposed by disruption of the cells (12).

Although the ratio of specific to nonspecific binding was relatively low, these results were consistent with earlier studies of colonic myocytes (13), and homogenates of ileal muscle (14). In our laboratory colonic myocytes have double the number of receptors/cell as the adult gastric antral myocytes, and so have nearly double the specific binding; by contrast, nonspecific binding to the filters remained constant (13).

There is a direct relationship between intracellular Ca^{+2} concentration and the force developed by smooth muscle (15). There is also a relationship among the number of calcium channels, the percentage of time that they are open, and the intracellular calcium concentration (16). This relationship is not exactly linear, but all calcium channels do not open until the membrane is

depolarized more positive to +100 mV. The maximum contraction of the muscle occurs at approximately pCa^{+2} 6.5. Therefore, although increases in contraction of adult compared to neonatal tissues may be due to increased myocyte size (17), maturation of contractile proteins or other unknown factors, increasing numbers of calcium channels may be necessary to promote a rapid and equal diffusion of Ca^{+2} throughout the cytoplasm. Unlike many receptors for agonist-mediated events, there is no evidence for "spare" calcium channels. The control of Ca^{+2} influx is due to the activation and inactivation of the channels. Calcium channels inactivate as the intracellular calcium concentration increases (18).

In the present study there was an age-related increase in the number of nitrendipine-sensitive calcium channels in antral but not fundic cells. This divergence in the number of nitrendipine binding sites during maturation of the two gastric muscle tissues is consistent with their different means of increasing intracellular calcium for stimulus-contraction coupling. Previous studies in cats established that fundic smooth muscle utilized calcium stored in intracellular sites during contraction, but antral myocytes were dependent on an extracellular supply of calcium (6).

In the present study there were age-related changes in the maximal stress generated by muscle strips as measured by isometric techniques. In the antrum the combination of age-related increases in maximal stress and in the number of calcium channels was consistent with the association between stress and requirement for extracellular Ca^{+2} influx (15). Age-related increases in the number of calcium channels may increase Ca^{+2} influx and thus support the stress generated by developing antral muscle.

In the antrum the calcium channel blocker nifedipine inhibited both voltage-dependent potassium-stimulated, and receptor-operated bethanechol-stimulated contraction, observations consistent with a requirement for extracellular Ca^{+2} influx for contraction. In contrast, in the fundus receptor-operated bethanechol-stimulated contraction was inhibited only 25% by maximally effective concentrations of the calcium channel blocker nifedipine. These observations were consistent with the absence of a requirement for extracellular Ca^{+2} influx for agonist-stimulated contraction in the fundus.

In summary, in the fundus, which used intracellular sources for the calcium required for contraction, calcium channels did not increase with age. In the antrum, which used extracellular Ca^{+2} for contraction, dihydropyridine-sensitive calcium channels increase in number with postnatal age.

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