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Development of cholinergic and inhibitory non-adrenergic non-cholinergic responses in the rat gastric fundus

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1 Cholinergic contractions and inhibitory non-adrenergic non-cholinergic (NANC) relaxations were studied in longitudinal muscle strips of the gastric fundus of 2, 4 and 8 week old rats.

2 Contractions induced by electrical stimulation of the cholinergic neurones and by administration of acetylcholine decreased during development. The potentiating effect of physostigmine was similar in the 3 age groups.

3 Short train stimulation in NANC conditions induced fast relaxations, which were more pronounced in 4 and 8 week than in 2 week old rats. These relaxations were almost completely inhibited by N^G-nitro-L-arginine methyl ester (L-NAME) in the 3 age groups. The nitric oxide-induced relaxations did not change during development.

4 Sustained electrical stimulation in NANC conditions induced an initial relaxation, which was almost totally blocked by L-NAME, followed by an almost complete recovery of tone at lower frequencies of stimulation. At higher frequencies of stimulation, the recovery of tone was incomplete or absent. This sustained relaxation was only partially reduced by L-NAME and almost abolished by L-NAME plus α-chymotrypsin. The initial relaxations increased during development, while the sustained relaxations remained similar during this period. Vasoactive intestinal polypeptide-induced relaxations were also similar in the 3 age groups.

5 These results show that the sensitivity of the gastric fundus to acetylcholine decreases from 2 weeks to 8 weeks postnatally, while the importance of the nitrergic innervation increases during this period.

Keywords: cholinergic responses; nitric oxide; VIP; NANC; rat gastric fundus

Introduction

The rat gastric fundus contains cholinergic excitatory neurones (Lefebvre *et al.*, 1983), important in gastric emptying of liquids and inhibitory non-adrenergic non-cholinergic (NANC) neurones (Lefebvre, 1986), which are responsible for the adaptive and receptive relaxations necessary for food intake without increase of gastric pressure. Nitric oxide (NO) and vasoactive intestinal polypeptide (VIP) are inhibitory NANC co-transmitters in this tissue. NO is involved in shortlasting relaxations and in the initial phase of sustained relaxations, while VIP is mainly involved in sustaining relaxations (for references see Lefebvre, 1993).

As the feeding habit changes from suckling milk to eating solid food in the first period of life in mammals, we were interested in the development of the cholinergic and inhibitory NANC innervation in the rat gastric fundus during this period. The development of the gastrointestinal excitatory and inhibitory innervation has been studied by evaluating changes during the last days of intra-uterine life and the first days of extra-uterine life. In the proximal stomach of the rat for example, cholinergic responses to electrical stimulation can be obtained from approximately embryonic day 16, while inhibitory NANC responses can be obtained only from day 18 (Ito *et al.*, 1988). In the chick oesophagus, the intrinsic cholinergic innervation starts to function at 11 days of incubation (Miyazaki *et al.*, 1989). Gastrointestinal motility responses are also different between the foetal or neonatal state and the adult state. The response to acetylcholine in antral smooth muscle from newborn rabbits is less pronounced than in tissue from adult rabbits (Zitterman & Ryan, 1990). These differences are also seen in gastric fundus smooth muscle strips from foetal and adult guinea-pigs (Paul *et al.*, 1994).

The progression between the neonate and the adult status is infrequently investigated although some studies suggest marked changes of gastrointestinal motility in this period; in the rat duodenum for example the response to the ganglionic stimulant, nicotine and to the purinoreceptor agonist, ATP, changes from contraction to relaxation around the third postnatal week (Furukawa & Nomoto, 1989; Irie *et al.*, 1994). This suggests possible changes in the physiological role of neurotransmitters in gastrointestinal motility.

In this study, we have investigated the responses to electrical stimulation of cholinergic and inhibitory NANC neurones and to addition of the neurotransmitters acetylcholine, VIP and NO in 2, 4 and 8 week old rats. In addition we studied the response to electrical stimulation after treatment with inhibitors of these neurotransmitters.

Methods

General methodology

Male and female Wistar rats (weaning after 21 days) of 14 ± 1 (body mass 38 ± 1 g, $n = 59$), 28 ± 2 (body mass 95 ± 1 g, $n = 29$) and 56 ± 4 (body mass 193 ± 7 g, $n = 31$) days were killed by a blow on the head and decapitation. After laparotomy, the gastric fundus was removed rapidly, placed in ice-cold Krebs solution, and one (for 2 week old rats) or two (for 4 and 8 week old rats) longitudinal muscle strips were prepared according to Vane (1957). The mass and length of the strips were 15 ± 1 g and 1.0 ± 0.1 cm, 29 ± 1 g and 1.2 ± 0.1 cm and 63 ± 3 g and 1.3 ± 0.1 cm for 2, 4 and 8 week old rats, respectively ($n = 54$–59 for the masses and 7–11 for the lengths). The strips were suspended under the mean optimal load (see Results) in 5 ml organ baths containing Krebs solution (with the following composition in mM: NaCl 118.5, KCl 4.8, $CaCl_2$ 1.9, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 10.1) held at 37°C and gassed with 95% O_2/5% CO_2. Changes in auxotonic tension were recorded via a Grass force-displacement transducer FT03 coupled in series with a 1 g cm^{-1} spring on a Kipp en

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Zonen BD112 recorder. Transmural electrical stimulation was performed via two platinum plate electrodes (22 × 5 mm, distance in between 6 mm) by means of a Grass S88 stimulator with a constant voltage unit (40 V, 1 ms duration). At the end of the experiment the tissues were weighed.

Protocols

Transmural electrical stimulation induced contractions. Frequency-response curves were obtained by stimulating the tissues with 10 s trains at increasing frequency (0.25–16 Hz) with 2 min intervals in between the stimulations. The influence of 3×10^{-6} M tetrodotoxin (TTX; incubation time 15 min) and 7.28×10^{-8} M physostigmine (incubation time 30 min) on the electrically induced contractile responses was studied by performing two frequency-response curves before and after addition of these substances or their solvent. The possible influence of the nitrergic system on contractions induced by electrical stimulation was studied in 8 week old rats by performing frequency-response curves before and after addition of 3×10^{-4} M N^G-nitro-L-arginine methyl ester (L-NAME; incubation time 30 min) or its solvent. The acetylcholine-induced concentration-response curve ($10^{-9}-10^{-4}$ M) was obtained in a cumulative way with a stepwise increase of concentration once a stable plateau to the previously added concentration was obtained. The acetylcholine-induced concentration-response curve was also studied before and after addition of 7.28×10^{-8} M physostigmine or its solvent (incubation time 30 min).

To study the relaxant NANC responses, atropine (10^{-6} M) and guanethidine (4×10^{-6} M) were added to the Krebs solution. Tone was raised by addition of 3×10^{-7} M prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and when a stable plateau was reached, relaxant responses were investigated. $PGF_{2\alpha}$ was added at most 3 times in one tissue with a minimum washout period of 45 min between the administrations; the contractile response to the repetitive administrations of $PGF_{2\alpha}$ was consistent. To study the influence of development on the nitrergic apparatus, the relaxant responses to electrical stimulation with isolated trains (10 s trains at increasing frequency with 2 min intervals in between; 1–16 Hz) and to NO ($10^{-7}-10^{-5}$ M; bolus administration of increasing concentrations given at 3 min intervals) were investigated. To study the influence of development on the VIPergic system, the relaxant responses to sustained electrical stimulation (increase of frequency every 2 min; 0.25–16 Hz) and to VIP ($10^{-10}-10^{-7}$ M; administered cumulatively) were investigated. To investigate the neurogenic nature of the responses to both types of electrical stimulation, the responses were studied before and after addition of 3×10^{-6} M TTX (15 min incubation time). To investigate the involvement of NO in the relaxant responses to train stimulation, electrical stimulation with isolated trains was carried out before and after administration of 3×10^{-4} M L-NAME (incubation time 30 min). To investigate the involvement of NO and VIP in the responses to sustained electrical stimulation, these responses were studied before and after administration of 10 u ml^{-1} α-chymotrypsin (30 min incubation), and before and after administration of L-NAME (3×10^{-4} M; incubation time 30 min). In the latter series, the responses were studied a third time in the combined presence of L-NAME (3×10^{-4} M) and α-chymotrypsin (10 u ml^{-1}). α-Chymotrypsin increased tone as previously described in this tissue (De Beurme & Lefebvre, 1987). In order to obtain a similar tone level as before, a smaller concentration of $PGF_{2\alpha}$ was added, depending on the α-chymotrypsin-induced tone.

Data analysis

All contractions are expressed as mN of tension per g mass of tissue. In one series the contractions induced by electrical stimulation and by addition of acetylcholine were also expressed as percentage of the $PGF_{2\alpha}$-induced contraction (3×10^{-7} M) obtained afterwards and as force per cross sectional area (mN cm^{-2}). The cross sectional area was determined as mass × (length × density)$^{-1}$, the latter assumed to be 1.05 g cm^{-3} (Herlihy & Murphy, 1973).

Relaxations are expressed as percentage reduction of the $PGF_{2\alpha}$-induced tone. Train stimulation induced short-lasting relaxations; the maximal amplitude was measured. Upon sustained stimulation, more complex responses were obtained (see Results); in general the amplitude of an initial fast relaxation was measured as well as the amplitude at 2 min stimulation at a given frequency, to assess the nitrergic and VIPergic systems, respectively; the response at 2 min is indicated as sustained relaxation.

The percentage increase of electrically induced contractions by physostigmine and decrease of electrically induced relaxations by L-NAME was calculated as $(R_{after}-R_{before}) \times 100/R_{before}$ and $(R_{before}-R_{after}) \times 100/R_{before}$, respectively, where R_{before} and R_{after} indicate the response before and after addition of the inhibitor, respectively.

Data are given as mean ± s.e.mean and n represents the number of animals used. Analysis of variance (ANOVA) was performed on the frequency- and concentration-response curves with age as factor. If statistical significance was reached ($P<0.05$) a multiple comparison test ($P<0.05$, $P<0.01$) was performed (unpaired t test corrected for multiple comparisons by the Bonferroni procedure) between 2 and 4 week old rats, between 2 and 8 week old rats and between 4 and 8 week old rats (Ludbrook, 1991). For comparison of results before and after addition of an inhibitor in the same tissues a paired t test was used.

Substances used

The following substances were used: acetylcholine chloride (Sigma, St. Louis, U.S.A.), atropine sulphate (Merck, Brussels, Belgium), α-chymotrypsin (Sigma), guanethidine sulphate (Ciba Geigy, Groot Bijgaarden, Belgium), N^G-nitro-L-arginine methyl ester (L-NAME; Sigma), physostigmine salicylate (Federa, Brussels, Belgium), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Sigma), tetrodotoxin (TTX; Janssen Chimica, Beerse, Belgium), VIP (CRB, Northwich, U.K.).

All drugs were dissolved in distilled water. Solutions were prepared on the day of the experiment except for VIP, $PGF_{2\alpha}$ and TTX where stock solutions were stored at −20°C. A saturated NO-solution was prepared from NO gas (Air Liquide, Belgium) as described by Kelm & Schrader (1990).

Results

Preliminary experiments indicated that the mean optimal load, defined as the load where the tissue showed the most pronounced contraction to electrical stimulation at 8 Hz for 10 s, was 0.2, 0.3 and 0.8 g for 2 week, 4 week and 8 week old rats, respectively.

Cholinergic responses

Electrical stimulation with 10 s trains in the absence of atropine and guanethidine induced rapid frequency-dependent contractions, followed by a slower return to baseline after the stimulation train. When stimulating at higher frequencies, tone sometimes did not completely return to baseline before the next stimulation train at higher frequency. The amplitude of the contractions was always measured from the original baseline (Figure 1a). The responses to this type of stimulation decreased with increasing age (Figure 2a). When expressing the contractions as $PGF_{2\alpha}$-induced tone or as mN cm^{-2}, a similar decrease with age was observed (data not shown). TTX abolished the electrically induced contractions ($n=2$ in each age group). Physostigmine increased the basal tone of the tissue by 49 ± 32 ($n=11$), 15 ± 3 ($n=6$) and 15 ± 4 ($n=9$) mN g^{-1} tissue for 2 week, 4 week and 8 week old rats, respectively. It significantly ($P<0.05$) increased the amplitude of the electrically

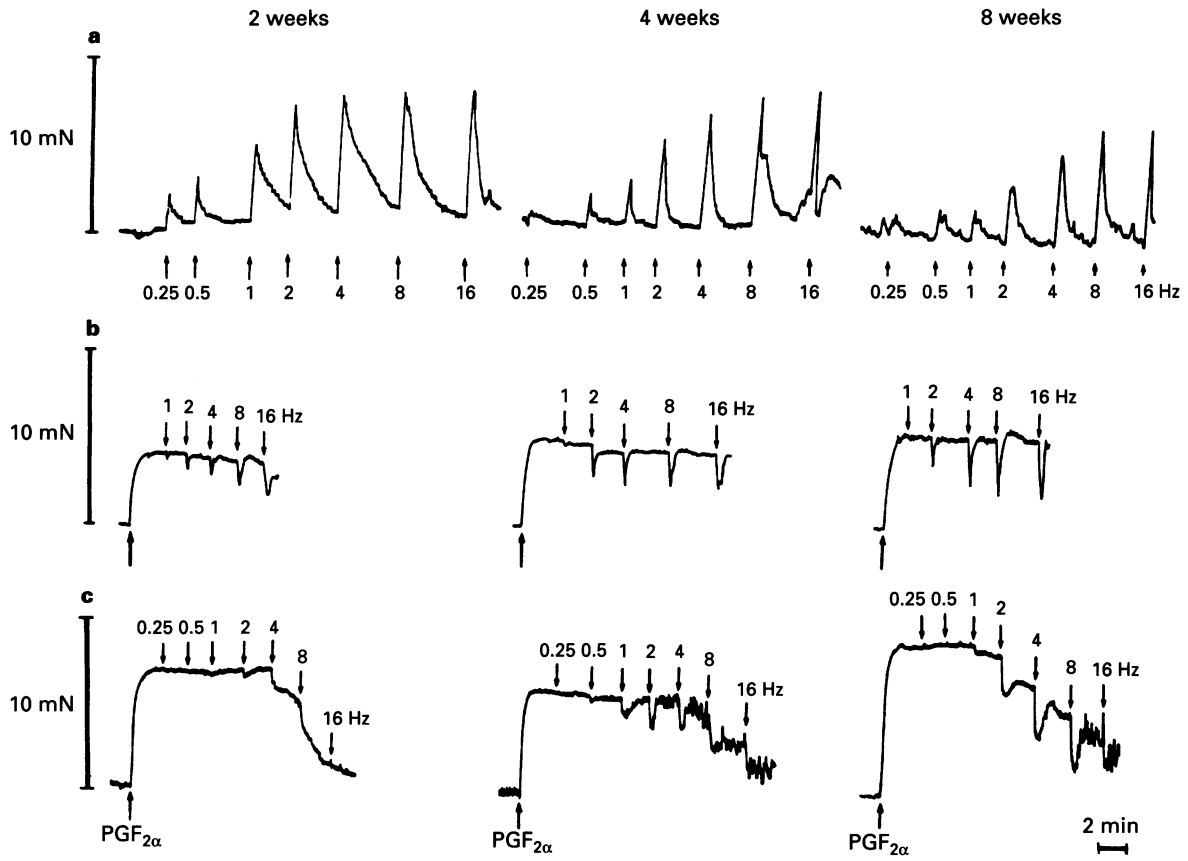

Figure 1 Representative experiments showing the responses to electrical stimulation in gastric fundus strips of 2 (left), 4 (middle) and 8 (right) week old rats (40 V, 1 ms duration) in Krebs solution at basal tone (a, 0.25–16 Hz; 10 s train stimulation) and in Krebs solution containing 10^{-6} M atropine and 4×10^{-6} M guanethidine after increasing tone with 3×10^{-7} M $PGF_{2\alpha}$ (b, 1–16 Hz; 10 s train stimulation and c, 0.25–16 Hz; 2 min sustained stimulation per frequency). In (a) the mass of the tissue was 15.7 mg for the 2 week, 33.4 mg for the 4 week and 52.0 mg for the 8 week old rats.

induced contractions at 0.25 to 1 Hz in 2 week old rats, at 0.25 and 1 Hz in 4 week old rats and at 0.25 to 4 Hz in 8 week old rats. For example, physostigmine increased the electrically induced contractions at 1 Hz from 172 ± 20 to 246 ± 29 mN g^{-1} tissue for 2 week old rats, from 69 ± 6 to 132 ± 21 mN g^{-1} tissue for 4 week old rats, from 24 ± 7 to 60 ± 11 mN g^{-1} tissue for 8 week old rats ($P < 0.05$, in all age groups, $n = 6-11$). However, the % increase by physostigmine was not significantly different in the 3 age groups at any of the frequencies. Physostigmine delayed the return of the tone to baseline after the stimulation. The influence of L-NAME on the electrically induced contractions was tested in 8 week old rats. Incubation with L-NAME potentiated the electrically induced contractions, the potentiation reaching significance at 0.25 and 2 to 8 Hz (Figure 3). However, even in the presence of L-NAME the amplitude of the electrically induced contractions in the 8 week old rats remained clearly smaller than that in the 2 week old rats.

Administration of acetylcholine induced sustained concentration-dependent contractions. In each age group, the amplitude of the maximal acetylcholine-induced contractions was similar to that of the electrically induced contractions; the amplitude of the acetylcholine-induced contractions decreased with increasing age (Figure 2b). When expressing these contractions as percentage of the $PGF_{2\alpha}$-induced tone or as mN cm^{-2} a similar decrease with age was observed (data not shown). The EC_{50} was similar in the three age groups ($2.8 \pm 0.7 \times 10^{-7}$ M for 2 week, $4.6 \pm 1.6 \times 10^{-7}$ M for 4 week, and $3.2 \pm 0.7 \times 10^{-7}$ M for 8 week old rats). Physostigmine had a tendency to increase the contractions to acetylcholine at low concentrations in the three age groups, but this did not reach statistical significance.

NANC relaxant responses

Addition of 3×10^{-7} M $PGF_{2\alpha}$ contracted the tissues in a sustained way (see Figure 1b and c). Preliminary experiments indicated that this concentration of $PGF_{2\alpha}$ contracted the tissues to $91 \pm 2\%$, $88 \pm 2\%$ and $84 \pm 1\%$ of the maximal contraction to $PGF_{2\alpha}$ for 2, 4 and 8 week old rats, respectively ($n = 6-8$ for each age group).

Electrical stimulation with isolated trains in NANC conditions induced TTX-sensitive ($n = 2$ in each age group) short-lasting frequency-dependent relaxations, which were significantly more pronounced in 4 and 8 week old rats than in 2 week old rats (Figure 1b and 4a). L-NAME (3×10^{-4} M) increased the tone in some tissues yielding a mean increase of 19 ± 16, 34 ± 20 and 14 ± 2 mN g^{-1} tissue in 2, 4 and 8 week old rats, respectively ($n = 6-9$ for each age group); it did not reduce the $PGF_{2\alpha}$-induced contractions. L-NAME reduced the relaxations induced by train stimulation in all age groups (Figure 5). The percentage inhibition by L-NAME was similar in the 3 age groups except at 2 and 4 Hz where the percentage inhibition of L-NAME was significantly more pronounced in 2 week old rats than in 8 week old rats. When stimulating the tissue for 2 min, a fast initial relaxation occurred (lasting 15 to 20 s) but tone then slowly recovered during the 2 min stimulation at a given frequency (Figure 1c). From 0.25 to 1 Hz this recovery of tone was almost complete; at 2 and 4 Hz the recovery of tone was incomplete and at 8 and 16 Hz, almost no recovery of tone was observed (Figure 1c). All these responses were abolished by 3×10^{-6} M TTX ($n = 2$ for each age group). For evaluation of these results, the amplitude of the initial fast relaxation and that of the relaxation persisting at 2 min

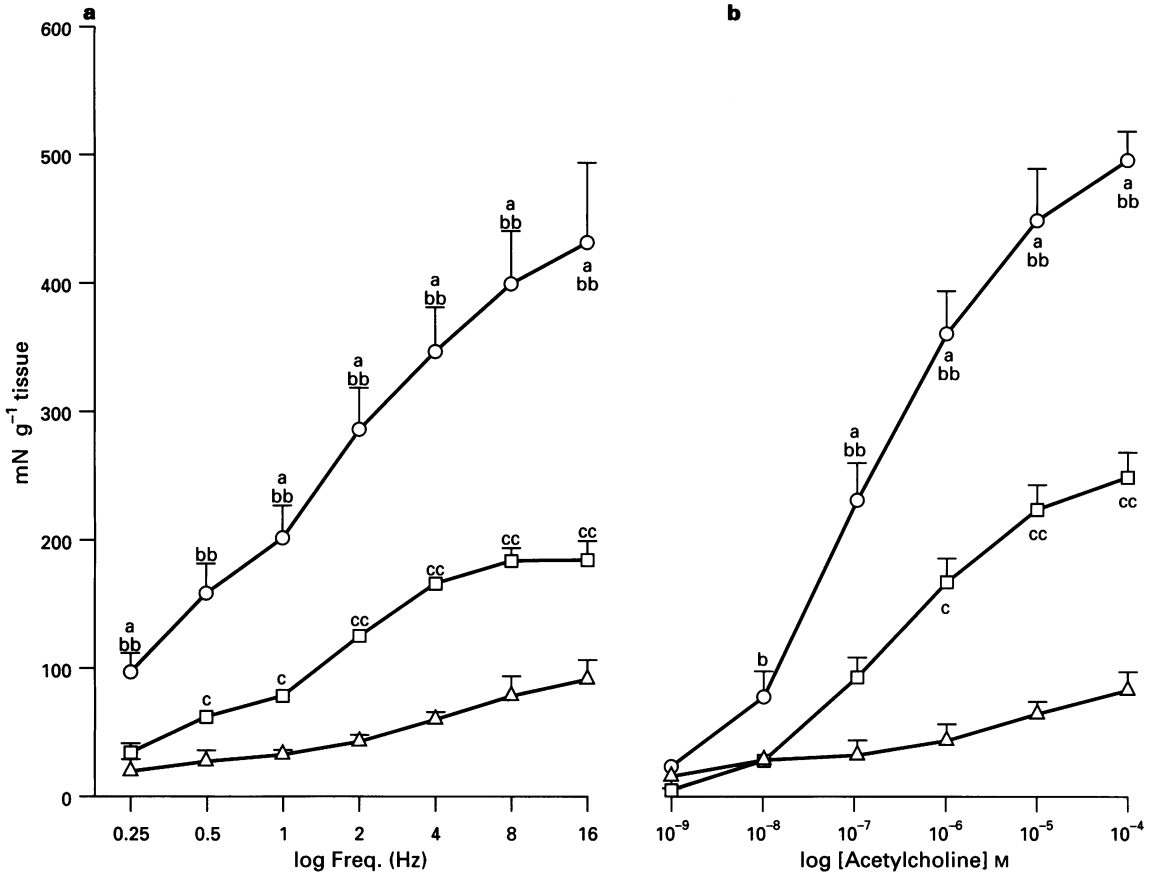

Figure 2 Mean responses for the contractions induced by train stimulation (a, 40 V, 1 ms duration, 10 s trains) and by acetylcholine (b) in Krebs solution at basal tone in gastric fundus strips of 2 (○), 4 (□) and 8 week (Δ) old rats. Mean ± s.e.mean; $n = 6-11$ in each age group. $^{a}P<0.05$, 2 vs 4 week old rats; $^{b}P<0.05$, $^{bb}P<0.01$, 2 vs 8 week old rats; $^{c}P<0.05$, $^{cc}P<0.01$, 4 vs 8 week old rats.

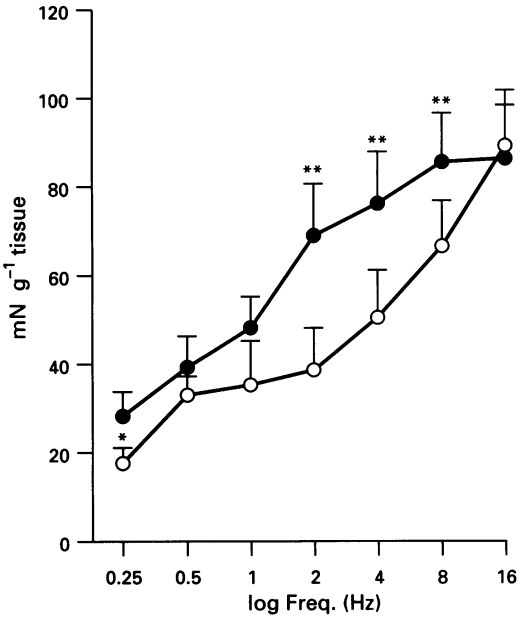

Figure 3 Influence of 3×10^{-4} M L-NAME on the contractions induced by train stimulation (40 V, 1 ms duration, 10 s trains) in Krebs solution at basal tone in gastric fundus strips of 8 week old rats. Responses before (○) and after (●) addition of L-NAME, respectively. Mean ± s.e.mean; $n = 6$; $^{*}P<0.05$, $^{**}P<0.01$, responses after vs those before L-NAME.

of stimulation were measured except at 8 and 16 Hz where the initial fast relaxation was not always discernible. The fast relaxation was increased with age, while the sustained relaxation (after 2 min of stimulation) remained similar with age (Figure 4b and c). The fast relaxation was almost completely abolished by addition of 3×10^{-4} M L-NAME while the sustained relaxation was only moderately decreased by addition of L-NAME (Figures 6 and 7). α-Chymotrypsin (10 u ml^{-1}) increased the tone of the tissues by 166 ± 47, 123 ± 11 and 72 ± 13 mN g^{-1} tissue in 2, 4 and 8 week old rats, respectively ($n = 7-9$ in each age group). In the presence of α-chymotrypsin a lower concentration of $PGF_{2\alpha}$ was added, in order to obtain a similar tone level as with administration of $PGF_{2\alpha}$ before addition of α-chymotrypsin. The average $PGF_{2\alpha}$-concentration in the presence of α-chymotrypsin was $1.9\pm0.6\times10^{-7}$ M, $1.3\pm0.3\times10^{-7}$ M and $1.5\pm0.4\times10^{-7}$ M for 2, 4 and 8 week old rats, respectively. Incubation with 10 u ml^{-1} α-chymotrypsin inhibited the relaxation induced by 10^{-7} M VIP completely ($n = 2$ in each age group), confirming previous results (De Beurme & Lefebvre, 1987). α-Chymotrypsin in combination with L-NAME almost completely inhibited the sustained relaxation (Figure 7). When α-chymotrypsin was incubated alone, it clearly reduced the sustained relaxation from 2 Hz on in all age groups, while the initial relaxation was only moderately reduced at 4 Hz.

The relaxations to NO, which were short-lasting and concentration-dependent, and the relaxations to VIP, which were sustained and concentration-dependent, were similar in the 3 age groups (Figure 8).

Discussion

This study was designed to elucidate possible changes during late postnatal development in the cholinergic and inhibitory NANC innervation of the rat gastric fundus.

Cholinergic contractile responses

Transmural electrical stimulation of the rat gastric fundus in the absence of atripine induces contractions, due to activation of postganglionic intramural cholinergic neurones (Lefebvre *et al.*, 1983). When using short trains of stimulation as in this study, short-lasting contractions are obtained as reported before (Smits & Lefebvre, 1992; Lefebvre *et al.*, 1992). The amplitude of the short-lasting contractions is significantly decreased from 2 to 8 week old rats, the results in the 8 week old rats being comparable to those we reported earlier in 3 month old rats (Smits & Lefebvre, 1992). The way of expres-

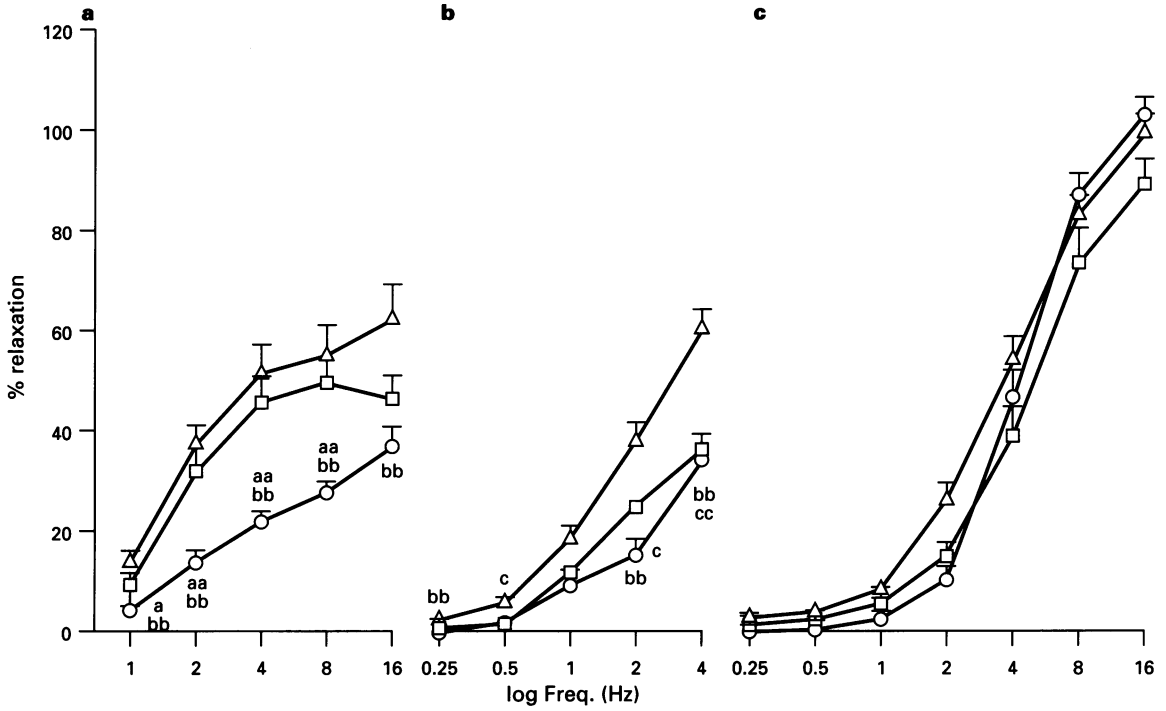

Figure 4 Mean responses for the relaxations induced by train stimulation (a, 40 V, 1 ms duration, 10 s trains) and by sustained stimulation (b and c, 40 V, 1 ms duration, 2 min stimulation per frequency) in NANC conditions of precontracted gastric fundus strips of 2 (○), 4 (□) and 8 week (Δ) old rats; (b) represents the initial fast relaxation, while (c) represents the sustained relaxation after stimulating for 2 min at a given frequency. Mean ± s.e.mean; $n = 6-13$ for (a) and 12–26 for (b) and (c) in each age group. $^{a}P<0.05$, $^{aa}P<0.01$, 2 vs 4 week old rats; $^{bb}P<0.01$, 2 vs 8 week old rats; $^{c}P<0.05$, $^{cc}P<0.01$: 4 vs 8 week old rats.

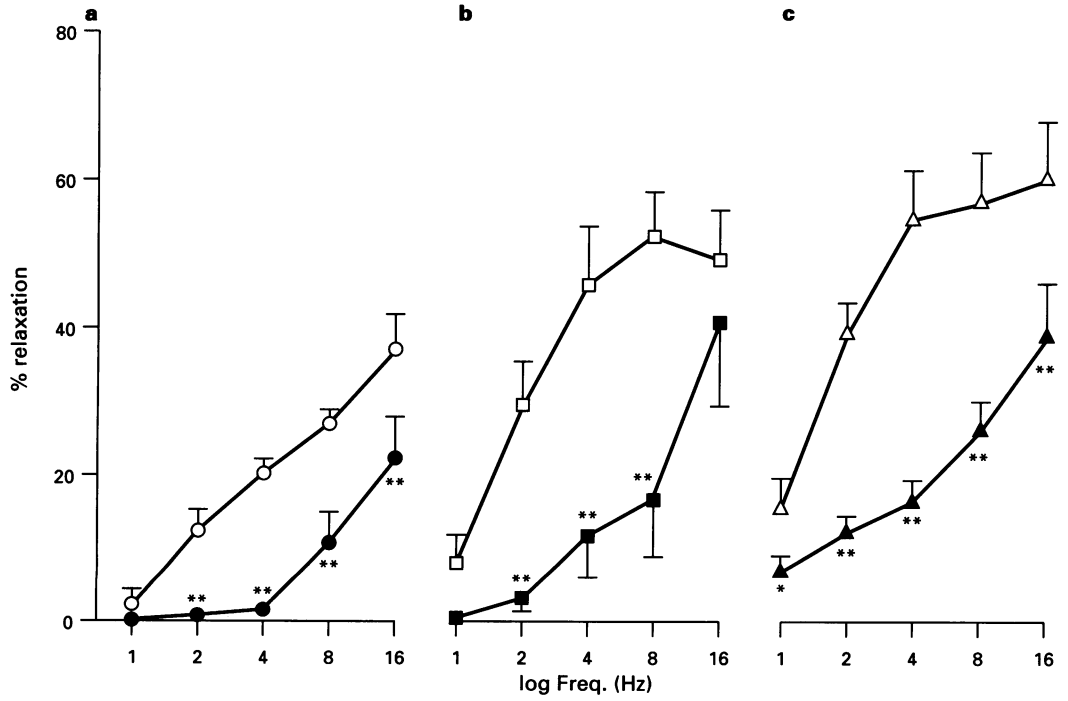

Figure 5 Influence of 3×10^{-4} M L-NAME on the relaxations induced by train stimulation (40 V, 1 ms duration, 10 s trains) in NANC conditions of precontracted gastric fundus strips of 2 (○; a), 4 (□; b) and 8 week (Δ; c) old rats. Open and solid symbols indicate the responses before and after addition of L-NAME, respectively. Mean ± s.e.mean; $n = 6-9$ in each age group. $^{*}P<0.05$, $^{**}P<0.01$, responses after vs those before L-NAME.

sing contractile responses when comparing different groups can be important (Hyland *et al.*, 1987; Mulhern & Docherty, 1989). In the latter study for example, the maximal noradrenaline- and 5-hydroxytryptamine-induced contractions in aortae from diabetic rats did differ from those in control aortae when expressed as force per wet tissue mass, but not when expressed as absolute force or as percentage of the KCl-induced contraction. The significant decrease of the cholinergic contractions with age in this study however was maintained whether the results were expressed as percentage of the contraction induced by 3×10^{-7} M $PGF_{2\alpha}$ or as mN cm^{-2}.

The decrease in the contractions induced by acetylcholine with age was similar to the decline observed with the electrically induced cholinergic contractions. This suggests that the decreased contractility in response to transmural electrical stimulation with age is not due to a decreasing cholinergic innervation but to postsynaptic phenomena. Accepting the EC_{50} of acetylcholine as an estimate of its affinity at the

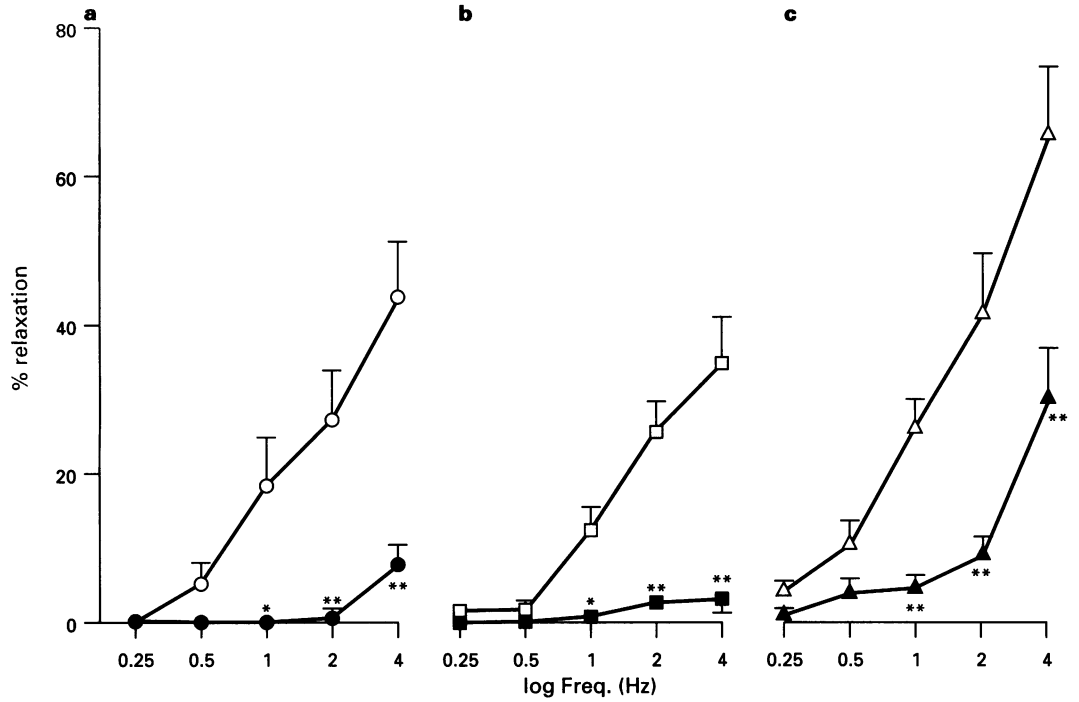

Figure 6 Influence of 3×10^{-4} M L-NAME on the initial, fast relaxation induced by sustained stimulation (40 V, 1 ms duration, 2 min stimulation per frequency) in NANC conditions of precontracted gastric fundus strips of 2 (○; a), 4 (□; b) and 8 week (Δ; c) old rats. Open and solid symbols indicate the responses before and after addition of L-NAME, respectively. Mean ± s.e.mean; $n = 7-9$ in each age group. $^{*}P<0.05$, $^{**}P<0.01$, responses after vs those before L-NAME.

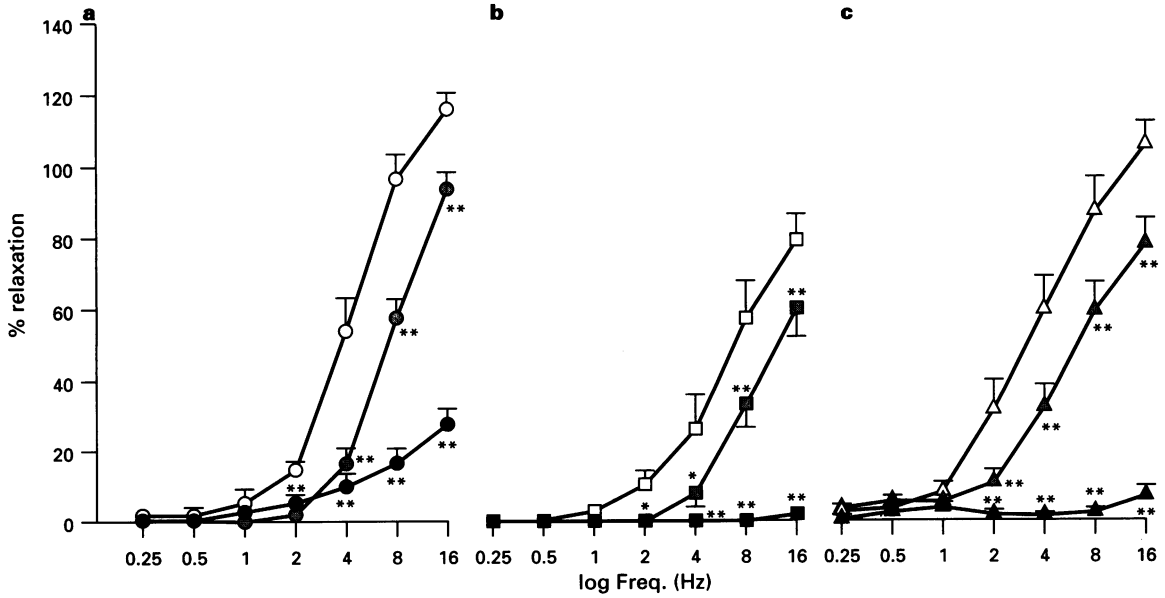

Figure 7 Influence of 3×10^{-4} M L-NAME and 3×10^{-4} M L-NAME plus 10 u ml^{-1} α-chymotrypsin on the relaxation at 2 min induced by sustained stimulation (40 V, 1 ms duration, 2 min stimulation per frequency) in NANC conditions of precontracted gastric fundus strips of 2 (○; a), 4 (□; b) and 8 week (Δ; c) old rats. Open symbols represent the control relaxations; stippled symbols represent the relaxations after addition of L-NAME and solid symbols those after addition of L-NAME and α-chymotrypsin. Mean ± s.e.mean; $n = 7-9$ in each age group. $^{*}P<0.05$, $^{**}P<0.01$, vs control responses.

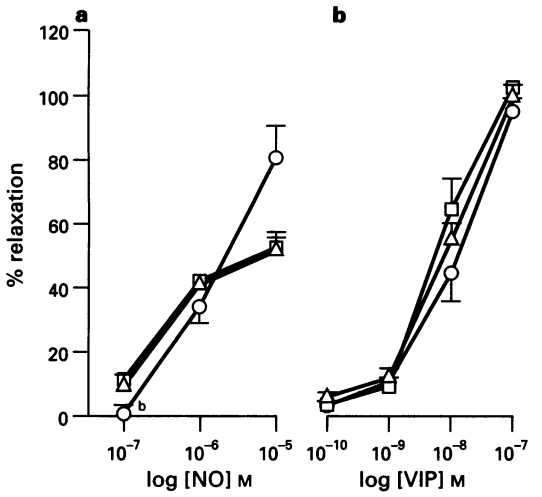

Figure 8 Mean responses for relaxations induced by nitric oxide (a) and vasoactive intestinal polypeptide (b) in precontracted gastric fundus strips of 2 (○), 4 (□) and 8 week (Δ) old rats. Mean ± s.e.mean; $n = 7-11$ in each age group. $^bP < 0.05$, 2 vs 8 week old rats.

muscarinic receptors, an age-dependent decrease in affinity seems unlikely as the EC_{50} of acetylcholine did not differ between the 3 age groups. An age-dependent increase of acetylcholinesterase activity has been reported in the rat jejunum (Kobashi *et al.*, 1985) and could explain our results. However, the potentiating effect of the acetylcholinesterase inhibitor, physostigmine, was similar in the 3 age groups while its effect should increase with age if the acetylcholinesterase activity increases.

When stimulating the tissues with 10 s trains in the absence of atropine, the nitrergic neurones are also stimulated and the released NO can be expected to antagonize the action of acetylcholine at the smooth muscle cell level, the observed contractions thus being the net result of the acetylcholine-induced contraction and the counteracting NO-induced relaxation. This might even hold for contractions induced by exogenous acetylcholine, if it activates nitrergic neurones at the ganglionic level. As the nitrergic inhibition increases with development (see below), this could explain the decreasing electrically and acetylcholine-induced cholinergic contractions. In gastric fundus strips of 8 week old rats, L-NAME indeed potentiated the electrically induced cholinergic contractions, confirming previous results (Lefebvre *et al.*, 1992). But even in the presence of L-NAME, the amplitude of the electrically induced cholinergic contractions remained much smaller in 8 week old rats, than that in the absence of L-NAME in 2 week old rats. The increasing functional antagonism with age of the cholinergic contractions by NO can thus only very partially explain the decreasing amplitude of the cholinergic contractions during the postnatal period.

The decline in cholinergic responses from 2 to 8 weeks might be related to a decrease in the number of the muscarinic receptors or a decreased efficiency of the receptor-effector coupling. Changes in muscarinic receptor number or receptor-effector coupling during the postnatal period have indeed been observed in rat and rabbit tissues (Pulera *et al.*, 1988; Tomomasa *et al.*, 1988; Kojima *et al.*, 1990) but this was not assessed in our study.

NANC relaxant responses

In the rat gastric fundus NO and VIP are inhibitory co-transmitters where NO is mainly involved in short-lasting relaxations and initiating sustained relaxations, while VIP is involved in the second part of sustained relaxations (Li & Rand, 1990; Boeckxstaens *et al.*, 1992; D'Amato *et al.*, 1992a). The nitrergic nature of the short-lasting relaxations induced by train stimulation was confirmed in this study as they were almost completely blocked by L-NAME. The contractile effect of L-NAME *per se* has been reported before in the same tissue with L-NAME and other inhibitors of NO-synthase (Li & Rand, 1990; Boeckxstaens *et al.*, 1991; Lefebvre *et al.*, 1992), and can be attributed to antagonism of basal release of NO or to a specific effect of L-NAME on smooth muscle (Lefebvre *et al.*, 1992).

Sustained electrical stimulation revealed a complex response in our study, consisting of an initial relaxation, followed by a partial or total recovery of tone at lower frequencies of stimulation. We have no explanation why in previous studies in gastric fundus tissue from adult rats, the relaxations induced by prolonged electrical stimulation at different frequencies revealed only a sustained relaxation without recovery of tone, irrespective of the recording method (De Beurme & Lefebvre, 1987; Li & Rand, 1990; Boeckxstaens *et al.*, 1992; D'Amato *et al.*, 1992a; Smits & Lefebvre, 1992; 1995). The initial relaxation during sustained electrical stimulation was clearly nitrergic as it was almost totally blocked by L-NAME, while the sustained relaxation present at 2 min of stimulation was only partially nitrergic, in view of the limited effect of L-NAME. The latter was mainly mediated by a peptide neurotransmitter, most likely to be VIP, as it was almost completely blocked by adding α-chymotrypsin, that abolished the relaxations induced by exogenous VIP, to L-NAME. The release of VIP in the rat gastric fundus has been shown to be more pronounced at higher frequencies of stimulation (D'Amato *et al.*, 1992b), corresponding with the more pronounced sustained relaxation at higher frequencies of stimulation in this study.

The relaxation induced by 10 s train stimulation and the initial relaxation, observed with sustained stimulation, increased with age. As the NO-induced relaxations were similar in the 3 age groups, this increase in electrically induced relaxations with age cannot be related to an increase in smooth muscle sensitivity to NO, suggesting that the nitrergic innervation becomes more important during postnatal development. A relative increase of the nitrergic neurones in the rat myenteric plexus from postnatal day 4 till 6 months, was indeed demonstrated in the proximal colon, but not in the ileum (Belai *et al.*, 1995). The reduction by L-NAME of the relaxations induced by train stimulation and the initial relaxation, observed with sustained stimulation, was similar in the 3 age groups, suggesting that the relative contribution of NO to this type of relaxation does not change with age. The sustained relaxations induced by electrical stimulation and by addition of VIP were similar in the 3 age groups, suggesting that neither the smooth muscle sensitivity to VIP nor the VIPergic innervation changes from 2 to 8 weeks.

Conclusion

The sensitivity of the gastric fundus to acetylcholine decreases from 2 weeks to 8 weeks postnatally, while the importance of the nitrergic innervation increases during the same period. These changes may be part of the physiological changes required during weaning (Henning, 1981).

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