ELSEVIER

European Journal of Pharmacology 303 (1996) 79–86

ejp

Influence of age on cholinergic and inhibitory nonadrenergic noncholinergic responses in the rat ileum

Geert J.M. Smits, Romain A. Lefebvre *

Heymans Institute of Pharmacology, University of Gent, De Pintelaan 185, 9000 Gent, Belgium

Received 25 September 1995; revised 25 January 1996; accepted 29 January 1996

Abstract

In view of the previously reported marked reduction in the number of neurons in the rat small intestine, the influence of age on the responses to electrical stimulation of the longitudinal muscle-myenteric plexus preparation of the ileum of young (3–4 months), adult (12–13 months) and old (24–25 months) rats was investigated. The differences in responses between the three age groups were varied. At the basal tone level, electrical train stimulation induced complex responses consisting of a primary contraction, which was partly cholinergic, a secondary contraction, which was completely cholinergic and a rebound contraction, which was partly cholinergic. These responses did not differ between young, adult and old rats. The inhibiting effects of atropine and potentiating effects of physostigmine on these responses did not change with age. The acetylcholine-induced concentration-response curve did not differ between the three age groups. Electrical train stimulation of the precontracted longitudinal muscle-myenteric plexus preparation under nonadrenergic noncholinergic (NANC) conditions induced multiphasic responses consisting of a primary contraction, a primary relaxation, a postrelaxation and a rebound contraction. The primary contraction and the postrelaxation, which were nitrergic in nature, were not influenced by age. The primary relaxation was slightly more pronounced in young rats. In young rats, this relaxation was partly inhibited by N^G-nitro-L-arginine methyl ester (L-NAME) and ATP desensitization, while in adult and old rats these primary relaxations were only influenced by ATP desensitization, but not by L-NAME. Nitric oxide-induced relaxations were similar in the three age groups. The cAMP content was not increased by electrical stimulation, while the cGMP content increased with electrical stimulation, but no differences due to age were observed. These results suggest that cholinergic responses in the rat small intestine are well-maintained with age while the nitrergic contribution to NANC relaxation decreases with age.

Keywords: Aging; Nitric oxide (NO); ATP; Non-adrenergic non-cholinergic (NANC); Ileum, rat

1. Introduction

Aging is the process of functional and structural changes that occurs in an organism characterized by an increasing vulnerability to environmental challenges and thereby a decreased ability to survive (Masoro, 1991). Although not studied as intensively as, for instance, the cardiovascular system, the gastrointestinal tract changes structurally and functionally with age in animals and humans, leading to symptoms, such as constipation in old people. Previously, we obtained evidence of age-related functional changes in the rat gastric fundus (Smits and Lefebvre, 1992, 1995). We now investigated the effect of age on the rat small intestine.

In the small intestine of man, guinea-pig and rat, a substantial reduction in the number of myenteric neurons with age has been observed (Santer and Baker, 1988; Gabella, 1989; Desouza et al., 1993). Functionally age-related changes in the rat and guinea-pig small intestine have also been observed (Kobashi et al., 1985; Nowak et al., 1990). In contrast, small intestine transit time in rats did not change with age (Varga, 1976; Smits and Lefebvre, 1996b) or humans (Kupfer et al., 1985), suggesting a great reserve capacity of the intrinsic nervous system or the appearance of compensatory mechanisms in the small intestine with age.

Excitatory cholinergic and inhibitory nonadrenergic noncholinergic (NANC) neurons, involved in ascending excitation and descending inhibition, respectively, play an important role in the motility of the gastrointestinal tract. Previously, we obtained evidence that nitric oxide (NO) and ATP are inhibitory NANC neurotransmitters in the rat

* Corresponding author. Tel.: (32) (9) 240 33 73; fax: (32) (9) 240 49 88; e-mail: romain.lefebvre@rug.ac.be

PII S0014-2999(96)00089-1

ileum (Smits and Lefebvre, 1996a). In a search for possible age-related changes in the rat ileum, we investigated the responses to electrical stimulation of the cholinergic and NANC neurons in the longitudinal muscle-myenteric plexus preparation of the ileum of young (3–4 months), adult (12–13 months) and old (24–25 months) rats. We also studied the effect of the neurotransmitters involved: acetylcholine, NO and ATP as well as the influence of NO synthesis inhibition and ATP desensitization on the electrically induced NANC relaxation. Finally, we studied the effect of electrical stimulation on the cAMP and cGMP content of the tissue. A preliminary account of these results has been presented (Smits et al., 1995).

2. Materials and methods

2.1. General

Male Wistar rats aged 3–4, 12–13 and 24–25 months (young, adult and old rats) were obtained from the Centre For Experimental Animals (University of Louvain, Louvain, Belgium). They were killed by a blow on the head and bleeding. After laparotomy, a segment of 20 cm of the distal part of the ileum (terminal 10 cm not included) was removed rapidly. A maximum of four longitudinal muscle-myenteric plexus preparations of 1.5 cm were prepared as described by Paton and Vizi (1969) for the guinea-pig ileum. Briefly, after rinsing of the lumen, the ileum was mounted on a glass rod and the longitudinal muscle with the myenteric plexus was gently wiped off with cotton-wool soaked in Krebs solution. The strips were suspended under a load of 0.6 g in 7.5-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 isometric tension were recorded via a Grass force-displacement transducer, FT03, on a Graphtec linearcorder F WR3701. Transmural electrical stimulation was performed via two platinum plate electrodes (22 × 7 mm, 6 mm apart) by means of a Grass S88 stimulator with a constant voltage unit (supramaximal voltage, 0.3 ms duration, 10-s trains). After an equilibration period of 60 min (rinsing every 15 min), the optimal load for each tissue was determined; the contraction in response to 3×10^{-7} M methacholine was recorded for different loads ranging from 0.6 to 1.6 g, with the load increased stepwise by 0.2 g. For the rest of the experiments, the tissues were held at their optimal load, where they had shown the most pronounced contraction with methacholine. At the end of the experiment, the tissues were weighed.

2.2. Responses to electrical stimulation and to acetylcholine

Frequency-response curves were obtained by stimulating the tissues with 10-s trains at increasing frequency (0.25–8 Hz) with 3-min intervals between stimulations. The influence of 3×10^{-6} M tetrodotoxin (incubation time 15 min), 10^{-6} M atropine (incubation time 15 min), 1.82×10^{-8} M physostigmine (incubation time 30 min) and 10^{-6} M atropine + 3×10^{-4} M N^G-nitro-L-arginine methyl ester (L-NAME; incubation time 30 min) on these electrically induced contractile responses was studied by making two frequency-response curves before and after addition of these substances or their solvent. The acetylcholine-induced concentration-response curve (10^{-9}–10^{-4} M) was obtained cumulatively with a stepwise increase in concentration once a stable plateau was reached with the previously added concentration. The acetylcholine-induced concentration-response curve was studied before and after addition of 1.82×10^{-8} M physostigmine or its solvent (incubation time 30 min).

2.3. Responses to electrical stimulation and to NO and ATP under NANC conditions

The Krebs solution now contained atropine (10^{-6} M) and guanethidine (4×10^{-6} M) in order to obtain NANC conditions. To study relaxant responses, the tone was raised by addition of 3×10^{-7} M prostaglandin $F_{2\alpha}$ and relaxant stimuli were tested when a stable plateau was reached. A maximum of four prostaglandin $F_{2\alpha}$ additions was done in one tissue with a minimum washout period of 60 min in between. The contractile response to repeated administration of prostaglandin $F_{2\alpha}$ was consistent.

The response to electrical stimulation (10-s trains at 1, 2, 4 and 8 Hz; minimum interval dependent on the recovery of tone after a stimulation train) and addition of putative transmitters (NO: 10^{-7}, 10^{-6} and 10^{-5} M, 2-min interval; ATP: 10^{-5} and 10^{-4} M, only one concentration per prostaglandin $F_{2\alpha}$ plateau being tested) was investigated. NO and ATP were tested on different prostaglandin $F_{2\alpha}$ plateaus; ATP was also studied at the basal tone level. The neurogenic nature of the response to electrical stimulation was investigated using electrical stimulation in the absence and presence of 3×10^{-6} M tetrodotoxin.

To investigate the degree of nitrergic involvement in the responses, electrical stimulation (see above) was studied first in the absence and then in the presence of L-NAME (3×10^{-4} M; incubation time 30 min) or its solvent (control). To study the purinergic contribution, electrical stimulation was also performed before and after ATP desensitization. Muscular desensitization to ATP was established by incubating 10^{-4} M ATP for 30 min before increasing tone with prostaglandin $F_{2\alpha}$ and was tested by investigating the responses to 10^{-4} M ATP and 10^{-5} M NO. The control tissue was not desensitized to ATP. The responses of control tissues were reproducible unless otherwise indicated.

2.4. Assay of cyclic nucleotides

Strips were now mounted in an isotonic setup that allowed quick clamping of the tissues (see below). Changes

Table 1
The mass of the rats and the mass, the optimal load and the spontaneous activity of the tissues in young, adult and old rats

	Young	Adult	Old
Rat mass (g) [aa,bb,cc]	357 ± 7	473 ± 7	523 ± 14
Tissue mass (mg)	16.4 ± 0.6	16.8 ± 0.7	15.6 ± 0.6
Optimal load (g)	0.75 ± 0.06	0.73 ± 0.07	0.75 ± 0.07
Spontaneous activity (mN/mg)			
A	0.070 ± 0.009	0.075 ± 0.011	0.093 ± 0.008
B	0.074 ± 0.009	0.074 ± 0.008	0.095 ± 0.008
C	0.108 ± 0.024	0.091 ± 0.013	0.115 ± 0.025

Spontaneous activity was measured in Krebs solution (A) and in Krebs solution containing atropine and guanethidine at basal tone level (B) and when the tissue was contracted with prostaglandin $F_{2\alpha}$ (C). Mean ± S.E.M.; $n = 14–16$ for spontaneous activity and $n = 29–34$ for mass of the rats, tissue mass and optimal load, in each age group. [aa,bb,cc] $P < 0.01$. young vs. adult, young vs. old and adult vs. old, respectively.

in length were recorded via a HSE lever transducer B type 368 on a Graphtec linearcorder, WR3500. The load used was 0.75 g, as the experiments performed under isometric conditions showed that this was the mean optimal load for the three age groups (Table 1). After 1-h equilibration, the tone was raised with prostaglandin $F_{2\alpha}$ and the tissue was stimulated electrically (2 Hz). The tissue was quickly clamped between two liquid nitrogen-cooled plates after 7 s of stimulation or 1 or 10 s after the end of a 10-s stimulation train. Some tissues were clamped after induction of tone with prostaglandin $F_{2\alpha}$, without application of a relaxant stimulus (control). The tissue was homogenized, first with a membrane dismembrator (B. Braun Melsungen, 100%) for 45 s, and, second, with an ultrasonic probe (B. Braun Melsungen) for 4 × 5 s in 6% trichloroacetic acid on ice. The homogenate was centrifuged for 20 min at 2600 × *g* and the trichloroacetic acid was extracted from the supernatant 4 × with 5 vols. of water-saturated ether. The cAMP content was measured with a binding assay based on the method of Tovey et al. (1974) and the cGMP content was determined in a radioimmunoassay. The protein content on the pellet was determined with the method of Lowry et al. (1951) with bovine serum albumin as standard.

2.5. Statistical analysis

Contractions induced by electrical stimulation at basal tone, by acetylcholine and by 3×10^{-7} M prostaglandin $F_{2\alpha}$ as well as spontaneous activity were expressed as mN × (mg mass of the tissue)$^{-1}$. Spontaneous activity was assessed as the mean amplitude of the spontaneous contractions measured for 1 min. Relaxations and contractions induced by electrical stimulation and by addition of ATP and NO under NANC conditions were expressed as percentage of the prostaglandin $F_{2\alpha}$-induced tone. The cyclic nucleotide contents were expressed as pmol cyclic nucleotide × (mg protein)$^{-1}$. The data are given as mean ± S.E.M. values, *n* referring to tissues obtained from different animals unless otherwise indicated.

To evaluate the effect of age on the responses, a one-way ANOVA was performed with age as factor. When statistical significance ($P < 0.05$) was found, comparisons between young and adult, young and old and adult and old were performed with a Bonferroni-corrected unpaired *t*-test (Ludbrook, 1991). Comparisons between the responses in the absence and in the presence of inhibitors in the same tissues were done with a paired *t*-test. When comparison between results obtained in parallel tissues from the same age group was required, an unpaired *t*-test was used. In all statistical procedures, a *P* value ≤ 0.05 was considered to be statistically significant.

2.6. Substances used

Adenosine-5′-triphosphate (ATP; Boehringer, Mannheim, Germany), acetylcholine chloride (Sigma, St. Louis, MO, USA), atropine sulphate (Sigma), bovine serum albumin (Sigma), guanethidine sulphate (Sigma), methacholine chloride (Schuchardt, Munchen, Germany), N^G-nitro-L-arginine methyl ester (L-NAME; Sigma), physostigmine salicylate (Federa, Brussels, Belgium), prostaglandin $F_{2\alpha}$ (Sigma), tetrodotoxin (Janssen Chimica, Beerse, Belgium). The cAMP [^{3}H] assay system and the cGMP [^{125}I] RIA-kit were bought from Amersham (Buckinghamshire, UK) and DuPont Canada (Ontario, Canada), respectively. All drug solutions were prepared on the day of the experiment except for prostaglandin $F_{2\alpha}$ and tetrodotoxin for which stock solutions were made and kept at −20°C. Saturated NO solutions were prepared from the gas (Air Liquide, Belgium) as described by Kelm and Schrader (1990).

3. Results

3.1. General

The mass of the rats, the tissue mass, the optimal load and the mean amplitude of the spontaneous contractions are given in Table 1. The mean amplitude of the spontaneous contractions did not change significantly after the addition of atropine and guanethidine, nor did it change significantly after contraction with prostaglandin $F_{2\alpha}$. No difference between age groups was observed for spontaneous contractility, optimal load and tissue mass.

3.2. Responses to electrical stimulation and to acetylcholine

A representative trace of the responses to electrical stimulation at basal tone is shown in Fig. 1A. At 0.25 and 0.5 Hz, electrical stimulation induced phasic contractions (the first of these contractions will be termed the primary

contraction) corresponding to the pulses of electrical stimulation (Fig. 1A, three and five pulses for 0.25 and 0.5 Hz, respectively). Sometimes, at 0.5 Hz, a tonic contraction was observed after the first phasic contraction. At 1 and 2 Hz, the primary contraction was still observed, but a more tonic contraction then developed (secondary contraction), further, superimposed phasic contractions were observed which did not correspond to the number of pulses of electrical stimulation (Fig. 1A). At 4 and 8 Hz, the primary contraction was immediately followed by the secondary contraction; at 8 Hz, the primary contraction was sometimes difficult to distinguish from the more pronounced secondary contraction (Fig. 1A). A sustained rebound contraction was observed after stopping of the stimulation.

The amplitude of the primary contraction was similar between 0.25 and 1 Hz, while it increased slightly from 2 Hz on (Fig. 2A). The amplitude of the secondary contraction and of the rebound contraction increased with increasing frequency (Fig. 2B,C). No age-related differences in amplitude of the three contractions described were observed (Fig. 2A–C). All these responses were abolished by 3×10^{-6} M tetrodotoxin ($n = 2$–3 in each age group).

Atropine (10^{-6} M) partially inhibited the primary contractions at 0.25–2 Hz. At 0.25 and 0.5 Hz, the phasic contractions occurring after the primary contraction were inhibited in a similar manner. The primary contractions at 0.25–2 Hz were totally inhibited by 10^{-6} M atropine + 3×10^{-4} M L-NAME (only tested in young rats, $n = 3$). At 4 and 8 Hz, the primary contraction was abolished by atropine (Fig. 3A). The secondary contractions were completely inhibited by atropine (Fig. 3B) and sometimes even a small relaxation was observed. The rebound contraction was not consistently influenced by atropine. The influence of atropine on the electrically induced contractions did not differ significantly between the three age groups.

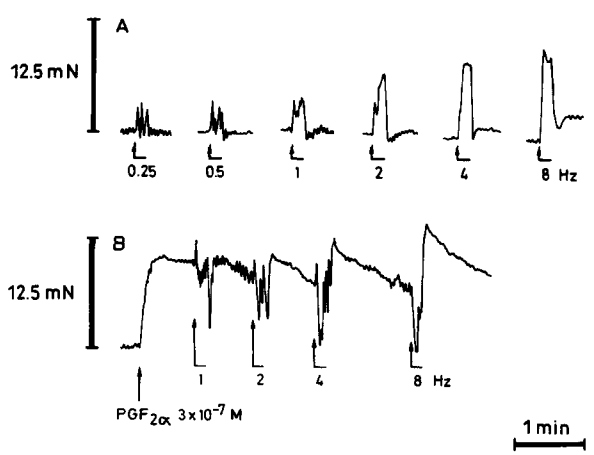

Fig. 1. Representative traces showing the effects of electrical stimulation (supramaximal voltage, 0.3 ms duration, 10-s trains) on the longitudinal muscle-myenteric plexus preparation of the ileum of a 1-year-old rat: (A) in Krebs solution at basal tone level; (B) in Krebs solution containing atropine and guanethidine when the preparation was precontracted with prostaglandin $F_{2\alpha}$.

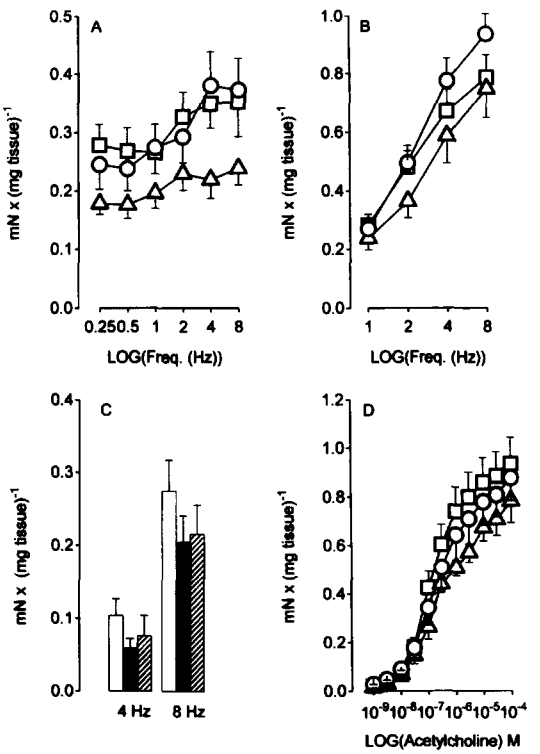

Fig. 2. Mean responses for the primary contraction (A), secondary contraction (B) and rebound contraction (C) induced by electrical stimulation (supramaximal voltage, 0.3 ms duration, 10-s trains) and for contractions induced by acetylcholine (D) of the longitudinal muscle-myenteric plexus preparation of the ileum of young (□, open bars), adult (△, black bars) and old (○, hatched bars) rats. Contractions are expressed as mN × (mg mass of the tissue)$^{-1}$. Mean ± S.E.M.; $n = 12$–14 in each age group for the electrically induced responses and $n = 6$–8 in each age group for the responses to acetylcholine.

The addition of 1.82×10^{-8} M physostigmine did not increase the tone of the tissues. Physostigmine increased the primary contraction at 0.25 and 0.5 Hz. At 1–8 Hz, an increase in amplitude was also seen but it was variable and did not reach significance (Fig. 3C). The secondary contraction was augmented by physostigmine at all frequencies (Fig. 3D). The rebound contraction was also increased by physostigmine. The potentiation of the contractions by physostigmine was not influenced by age.

Acetylcholine (10^{-9}–10^{-4} M) induced concentration-dependent contractions which were increased by physostigmine ($n = 6$–8, experiments not shown). Acetylcholine-induced contraction and the influence of physostigmine on it was not altered with age (Fig. 2D).

3.3. Responses to electrical stimulation and to NO and ATP under NANC conditions

Preliminary experiments had indicated that 3×10^{-7} M prostaglandin $F_{2\alpha}$ contracted the tissue to 73 ± 4, 78 ± 3 and 80 ± 5% ($n = 10$–12 out of 4 rats in each age group) of the maximal response to prostaglandin $F_{2\alpha}$ for young, adult and old rats, respectively. In the experiments, the

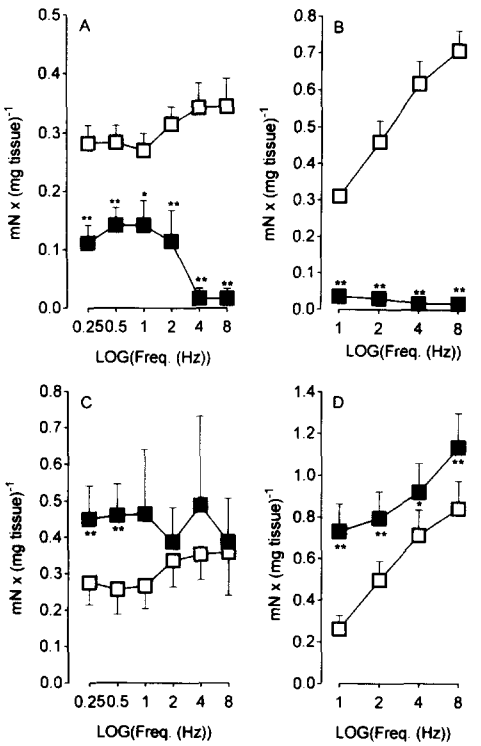

Fig. 3. The mean effect of 10^{-6} M atropine (upper panel) and 1.82×10^{-8} M physostigmine (lower panel) on the primary (left) and secondary (right) contractions induced by electrical stimulation (supramaximal voltage, 0.3 ms duration, 10-s trains) of the longitudinal muscle-myenteric plexus preparation of the ileum of young rats. Open and closed symbols indicate the contractions before and after addition of atropine and physostigmine, respectively. Contractions are expressed as mN×(mg mass of the tissue)$^{-1}$. Mean ± S.E.M.; $n = 6$–8. *,** $P < 0.05$, $P < 0.01$, significantly different from the response before addition of atropine or physostigmine.

contraction induced by 3×10^{-7} M prostaglandin $F_{2\alpha}$ was 0.69 ± 0.05, 0.62 ± 0.05 and 0.70 ± 0.04 mN × (mg tissue mass)$^{-1}$ for young, adult and old rats ($n = 24$–33). The responses elicited by electrical stimulation were very complex and have been described in Smits and Lefebvre (1996a): in short, they consisted of a primary contraction followed by a primary relaxation during the stimulation period of 10 s, and a postrelaxation followed by a rebound contraction on ending stimulation (Fig. 1B). All these responses were abolished by 10^{-6} M tetrodotoxin ($n = 2$–3 for each age group). The primary contraction and the postrelaxation decreased with increasing frequency, while the primary relaxation and rebound contraction increased with increasing frequency (Fig. 4). The primary contraction was not influenced by age (Fig. 4A), while the primary relaxation and rebound contraction were more pronounced in young rats (Fig. 4B,D). The postrelaxation was similar in the three age groups, except for old rats at 2 Hz, where they showed a significantly more pronounced postrelaxation compared to young rats (Fig. 4C).

NO (10^{-7}–10^{-5} M) induced short-lasting relaxations which were similar in shape and amplitude in the three age groups ($n = 7$ in each age group, experiments not shown). 3×10^{-4} M L-NAME abolished the primary contraction and the postrelaxation induced by electrical stimulation in the three age groups. In young but not in adult and old rats, 3×10^{-4} M L-NAME slightly reduced the primary relaxation at the higher frequencies of stimulation (Fig. 5A,E); in adult rats the primary relaxation was even slightly increased by L-NAME (Fig. 5C). The rebound contraction was increased in all age groups at 4 and 8 Hz after addition of L-NAME (Fig. 5B,D,F). In the control tissues, not receiving L-NAME, an increase in rebound contraction was also observed, but this increase was smaller (young rats: 13 ± 8 and $3 \pm 9\%$ for 4 and 8 Hz, respectively; adult rats: 14 ± 13 and $18 \pm 6\%$ for 4 and 8 Hz, respectively; old rats: 5 ± 4 and $5 \pm 6\%$ for 4 and 8 Hz, respectively; $n = 8$).

ATP (10^{-5}–10^{-4} M) induced short-lasting relaxations in precontracted tissues, but contractions at basal tone. The relaxations and contractions were similar in the three age groups ($n = 5$–6 in each age group, experiments not shown). At 10^{-5} M, the ATP-induced relaxations were 61 ± 3, 49 ± 5 and $49 \pm 6\%$ for young, adult and old rats, respectively. ATP desensitization completely blocked the

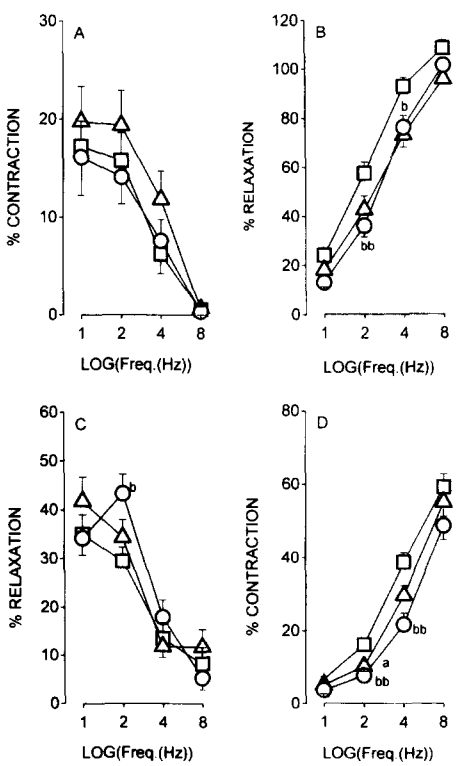

Fig. 4. Mean frequency–response curves for the primary contraction (A), primary relaxation (B), postrelaxation (C) and rebound contraction (D) induced by electrical stimulation (supramaximal voltage, 0.3 ms duration, 10-s trains) under NANC conditions of the precontracted ileal longitudinal muscle-myenteric plexus preparation of young (□), adult (△) and old (○) rats. Contractions and relaxations were expressed in relation to the contraction induced by 3×10^{-7} M prostaglandin $F_{2\alpha}$. Mean ± S.E.M.; $n = 25$–33. ^{a} $P < 0.05$, young vs. adult; ^{b,bb} $P < 0.05$, $P < 0.01$, young vs. old.

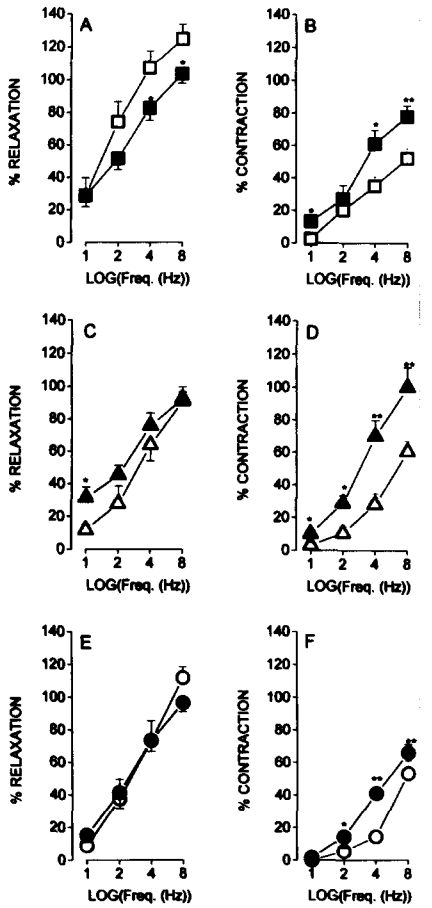

Fig. 5. Mean effect of 3×10^{-4} M L-NAME on the primary relaxation (A, C and E) and the rebound contraction (B, D and F) induced by electrical stimulation (supramaximal voltage, 0.3 ms duration, 10-s trains) under NANC conditions of the precontracted ileal longitudinal muscle-myenteric plexus preparation of young (upper panel), adult (middle panel) and old (lower panel) rats. Open and closed symbols indicate the response before and after addition of L-NAME, respectively. Mean ± S.E.M.; $n = 8$–9 in each age group. *,** $P < 0.05$, $P < 0.01$, significantly different from the response before addition of L-NAME.

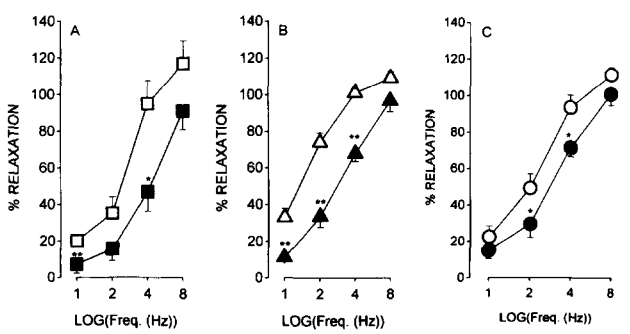

Fig. 6. Mean effect of ATP desensitization on the primary relaxation induced by electrical stimulation (supramaximal voltage, 0.3 ms duration, 10-s trains) under NANC conditions of the precontracted ileal longitudinal muscle-myenteric plexus preparation of young (A), adult (B) and old (C) rats. Open and closed symbols indicate the response before and after ATP desensitization, respectively. Mean ± S.E.M.; $n = 8$–9 in each age group. *,** $P < 0.05$, $P < 0.01$, significantly different from the response before ATP desensitization.

Table 2
The cGMP content (pmol × (mg protein)$^{-1}$) under control conditions and after electrical stimulation (supramaximal voltage, 0.3 ms duration, 2 Hz; clamped after 7 s of stimulation (ES1) or 1 (ES2) or 10 (ES3) s after a 10-s train) in young, adult and old rats

	Young	Adult	Old
Control	0.239 ± 0.041	0.269 ± 0.034	0.162 ± 0.014
ES1	0.476 ± 0.091 ^{a}	0.538 ± 0.096 ^{aa}	0.338 ± 0.030 ^{aa}
ES2	0.411 ± 0.067 ^{a}	0.405 ± 0.074 ^{a}	0.306 ± 0.026 ^{aa}
ES3	0.310 ± 0.043 ^{a}	0.296 ± 0.070	0.189 ± 0.011 ^{aa}

Mean ± S.E.M.; $n = 5$–6 in each age group. ^{a,aa} $P < 0.05$, $P < 0.01$, significantly different vs. control.

relaxations induced by ATP in young ($n = 7$), adult ($n = 4$) and old ($n = 3$) rats but did not influence the response to NO. ATP desensitization did not influence the primary contraction, the postrelaxation or the rebound contraction, but it reduced the primary relaxation partially (Fig. 6A–C); this reduction did not differ significantly between the three age groups.

3.4. Assay of cyclic nucleotides

The basal cAMP content was similar in young and adult rats (6.08 ± 1.01 and 7.20 ± 0.36 pmol × (mg protein)$^{-1}$) but decreased in old rats (4.31 ± 0.41 pmol × (mg protein)$^{-1}$, $n = 5$–6 in each age group, $P < 0.05$, between adult and old rats). Electrical stimulation did not alter the cAMP content in the three age groups.

The cGMP content under control conditions and after electrical stimulation is given in Table 2. The basal cGMP content did not differ significantly between the three age groups. After stimulation for 7 s at 2 Hz, the cGMP content increased significantly by a factor of 2. The interval of 7 s was selected to correspond with the electrically induced primary relaxation. 1 and 10 s after stimulating at 2 Hz for 10 s, the cGMP content was still significantly increased but the increase was less pronounced at 10 s; these time points correspond to the functionally observed postrelaxation and rebound contraction. The rise in cGMP content after electrical stimulation was not influenced by age.

4. Discussion

This study was designed to elucidate the possible compensatory mechanisms responsible for sustained small intestinal transit in the course of aging compensating for morphological changes that involve a pronounced decrease in the number of neurons in this tissue. As the inhibitory nonadrenergic noncholinergic and excitatory cholinergic systems are important in mediating relaxation in front of the bolus and contraction behind the bolus during peristalsis (Costa and Furness, 1982; Waterman and Costa, 1994), we investigated the effect of age on both systems.

Electrical stimulation at basal tone induced complex

responses consisting of a primary contraction, a secondary contraction and a rebound contraction, all tetrodotoxin-sensitive, suggesting a neurogenic nature. The primary contraction seems similar to that described by Barthò et al. (1992) in the same preparation being partly cholinergic, partly nitrergic. This was confirmed in our study where we found that the primary contraction, at least at the lower frequencies of stimulation, was decreased by atropine and increased by physostigmine. Moreover, the atropine-resistant primary contractions were abolished by L-NAME. At 4 and 8 Hz, atropine abolished the primary contraction completely, suggesting a fully cholinergic nature of these contractions. The secondary contraction seems to be cholinergic as this contraction was totally inhibited by atropine. The rebound contraction was probably partly cholinergic as physostigmine increased it. Surprisingly, atropine had only inconsistent effects on the rebound contractions.

Age-related changes in responses to electrical stimulation of cholinergic nerves and/or to acetylcholine receptor agonist have been observed in the rat jejunum (Kobashi et al., 1985), in the guinea-pig isolated ileum (Tsai and Ochillo, 1987), the rat proximal longitudinal small intestine (Nowak et al., 1990) and the rat colon (McDougal et al., 1984; Butt et al., 1993). In contrast, we did not observe age-related differences in the cholinergic responses induced by electrical stimulation. Morphological data indicated a 43% reduction in the number of neurons in the myenteric plexus of the rat small intestine (Santer and Baker, 1988). As the reduction of the neuron number with age seems to affect all neuron types equally (Santer and Baker, 1988) and if we assume a decreased number of cholinergic neurons with age, the persisting electrically induced cholinergic responses in older rats might be related to an increased postjunctional response to acetylcholine or a decreased breakdown by acetylcholinesterase. This seems not the case in the rat small intestine as the acetylcholine-induced concentration-response curve remained unaltered with age and as the physostigmine-induced potentialization of cholinergic responses was not influenced by age. On the other hand, spare receptors for acetylcholine might be present and if the amount of acetylcholine released in young rats greatly exceeds that required for occupation of the critical part of the receptors inducing a maximal effect, even a 40% decrease of the released amount of acetylcholine with aging will not influence the cholinergic functional responses. Still, the submaximal electrically induced cholinergic contractions were also not changed with aging, so that this possibility can also be ruled out. The longitudinal muscle-myenteric plexus preparation thus seems to be part of the group of tissues, such as the rat gastric fundus (Smits and Lefebvre, 1992), in which the functional cholinergic apparatus remains unaltered with advancing age.

Previously, we have shown that, in young rats, electrical stimulation under NANC conditions of the precontracted longitudinal muscle-myenteric plexus preparations induces complex responses consisting of primary contraction, primary relaxation, postrelaxation and rebound contraction. In young rats, the primary contraction and the postrelaxation are nitrergic, while the primary relaxation has partly nitrergic, partly purinergic and partly unknown nature and the rebound contraction is partly peptidergic (Smits and Lefebvre, 1996a). The primary contraction and the postrelaxation remained largely unchanged with aging, except for an unexplained increased amplitude of the postrelaxation at a stimulation frequency of 2 Hz in old rats. In all age groups, these responses were fully nitrergic as they were abolished by L-NAME. The increase of the rebound contractions in the presence of L-NAME in all age groups was possibly caused by the inhibition of the postrelaxation so that the rebound contractions were less functionally antagonized. The amplitude of the rebound contractions as such decreased with aging.

The primary relaxation was decreased with age. L-NAME slightly decreased this relaxation in young rats but not in adult and old rats, suggesting that NO is not involved in the primary relaxation in adult and old rats. At a low frequency of stimulation, the primary relaxation in the presence of L-NAME even increased slightly in adult rats, possibly by inhibition of the contractile nitrergic system, which is responsible for the primary contraction. The contribution of ATP to the primary relaxation seems equal in all age groups in view of the similar influence of ATP desensitization. The decrease of the primary relaxation with age might thus indeed be related to the lack of a nitrergic contribution. This is in contrast with the observations in the rat gastric fundus of an increased contribution of NO with age in the electrically induced NANC relaxations (Smits and Lefebvre, 1995). NO activates the soluble guanylate cyclase, and thus causes accumulation of cGMP (Waldman and Murad, 1987). Previously, we have shown that in the longitudinal muscle-myenteric plexus preparation of the rat small intestine, the cGMP content of the tissue also increased upon electrical stimulation and upon addition of NO (Smits and Lefebvre, 1996a). As the primary relaxation is partially nitrergic in young rats but not in adult and old rats, one could expect that the rise in cGMP content after a 7-s train stimulation, which corresponds with the moment of maximal primary relaxation, would be less in adult and old rats. This was not the case but the lack of a relationship between the degree of relaxation and the cGMP content of the tissue has been observed previously in the rat gastric fundus (Smits and Lefebvre, 1995) and in other tissues (for review, see Nakatsu and Diamond, 1988). Impairment of the responses to activation of a nitrergic pathway with aging can be due to an age-induced decrease in release of NO or a defect at the level of guanylate cyclase or further (Moritoki et al., 1988; Atkinson et al., 1994). As the NO-induced relaxation in the rat small intestine persisted with aging, defects at the postjunctional level seem less likely and the decreased

nitrergic contribution to primary relaxation seems related to a decrease in the NO release. Recently, it was shown that the relative number of nitrergic neurons, as demonstrated with NADPH-diaphorase staining, is very similar in the ileum of 6- and of 26-month-old rats (Belai et al., 1995). In view of the decrease in total neuron number (Santer and Baker, 1988), this indeed indicates a reduction in total number of nitrergic neurons. However, assuming a defect in the nitrergic neurons, it is difficult to understand why the nitrergic primary contraction and postrelaxation were maintained (unless different sets of neurons are involved).

Transit in the rat ileum is maintained with age (Varga, 1976; Smits and Lefebvre, 1996b); in the actual functional study changes in the cholinergic and the inhibitory NANC system were absent or limited. This contrasts strongly with the reduction in the number of neurons that Santer and Baker (1988) have described for this tissue. This suggests that the intrinsic nervous system of the gastrointestinal tract has a great reserve capacity or that there are compensatory mechanisms that cannot be discerned in this in vitro setup.

In conclusion, the cholinergic apparatus of the rat ileum longitudinal muscle-myenteric plexus preparation is unchanged with age, while the nitrergic contribution to NANC relaxation is decreased in adult and old rats.

Acknowledgements

This study was financially supported by Grant 3.0007.92 from the Belgian Fund for Medical Scientific Research (FGWO) and by a grant of the Investigation Fund of the University of Gent (01107393).

References

Atkinson, J., R. Tatchum-Talom and C. Capdeville-Atkinson, 1994, Reduction of endothelial function with age in the mesenteric arterial bed of the normotensive rat, Br. J. Pharmacol. 111, 1184.

Barthò, L., G. Kóczán, G. Pethö and C.A. Maggi, 1992, Blockade of nitric oxide synthase inhibits nerve-mediated contractions in the rat small intestine, Neurosci. Lett. 145, 43.

Belai, A., S. Cooper and G. Burnstock, 1995, Effect of age on NADPH-diaphorase-containing myenteric neurones of rat ileum and proximal colon, Cell Tissue Res. 279, 379.

Butt, W.G., M. Wang, S.T. Kaufman, J.P. Ryan and S. Cohen, 1993, Age-related changes in rat colon mechanisms, J. Gastrointest. Mot. 5, 123.

Costa, M. and J.B. Furness, 1982, Nervous control of intestinal motility, in: Mediators and Drugs in Gastrointestinal Motility I, ed. G. Bertaccini (Springer, Berlin) p. 279.

Desouza, R.R., H.B. Moratelli, N. Borges and E.A. Liberti, 1993, Age-induced nerve cell loss in the myenteric plexus of the small intestine in man, Gerontology 39, 183.

Gabella, G., 1989, Fall in the number of myenteric neurons in aging guinea pigs, Gastroenterology 96, 1487.

Kelm, M. and J. Schrader, 1990, Control of coronary vascular tone by nitric oxide, Circ. Res. 66, 1561.

Kobashi, Y.L., E.P. Breuing and R.P. Markus, 1985, Age-related changes in the reactivity of the rat jejunum to cholinoceptor agonist, Eur. J. Pharmacol. 115, 133.

Kupfer, R.M., M. Heppel, J.W. Haggith and D.N. Bateman, 1985, Gastric emptying and small-bowel transit rate in elderly, J. Am. Geriatr. Soc. 33, 340.

Lowry, O.H., N.J. Rosebrough and A.L. Farr, 1951, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193, 265.

Ludbrook, J., 1991, On making multiple comparisons in clinical and experimental pharmacology and physiology, Clin. Exp. Pharmacol. Physiol. 18, 379.

McDougal, J.N., M.S. Miller, T.F. Burks and D.L. Kreulen, 1984, Age-related changes in colonic function in rats, Am. J. Physiol. 247, G542.

Masoro, E.J., 1991, Biology of aging: facts, thoughts, and experimental approaches, Lab. Invest. 65, 500.

Moritoki, H., A. Tanioka, Y. Maeshiba, T. Iwamoto, Y. Ishida and H. Araki, 1988, Age-associated decrease in histamine-induced vasodilation may be due to reduction of cyclic GMP formation, Br. J. Pharmacol. 95, 1015.

Nakatsu, K. and J. Diamond, 1988, Role of cGMP in relaxation of vascular and other smooth muscle, Can. J. Physiol. Pharmacol. 67, 251.

Nowak, T.V., B. Harrington and J. Kalbfleisch, 1990, Age-related changes in enteric neuromuscular transmission, J. Pharmacol. Exp. Ther. 253, 683.

Paton, W.D.M. and E.S. Vizi, 1969, The inhibitory actions of noradrenaline on acetylcholine output by guinea-pig ileum longitudinal muscle strip, Br. J. Pharmacol. 25, 10.

Santer, R.M. and D.M. Baker, 1988, Enteric neuron numbers and sizes in Auerbach's plexus in the small and large intestine of adult and aged rats, J. Aut. Nerv. Syst. 25, 59.

Smits, G.J.M. and R.A. Lefebvre, 1992, Influence of age on responsiveness of the rat gastric fundus to agonist and to stimulation of intrinsic nerves, Eur. J. Pharmacol. 223, 97.

Smits, G.J.M. and R.A. Lefebvre, 1995, Influence of age on the signal transduction pathway of non-adrenergic non-cholinergic neurotransmitters in the rat gastric fundus, Br. J. Pharmacol. 114, 640.

Smits, G.J.M. and R.A. Lefebvre, 1996a, ATP and nitric oxide: inhibitory NANC neurotransmitters in the longitudinal muscle-myenteric plexus preparation of the rat ileum, Br. J. Pharmacol. 118 (in press).

Smits, G.J.M. and R.A. Lefebvre, 1996b, Influence of aging on gastric emptying of liquids, small intestine transit and faecal output in rats, Exp. Gerontol. (in press).

Smits, G.J.M., K. Bűyűkafşar and R.A. Lefebvre, 1995, NANC relaxation in the rat longitudinal muscle-myenteric plexus preparation: neurotransmitter(s) involved and influence of aging, Acta Gastroenterol. Belg. 57, B2.

Tovey, K.C., K.G. Oldham and J.A.M. Whelan, 1974, A simple direct assay for cyclic AMP in plasma and other biological samples using an improved competitive protein binding technique, Clin. Chim. Acta 56, 221.

Tsai, C.S. and R.F. Ochillo, 1987, The effect of aging on the responsiveness of the cholinergic receptor of the longitudinal muscle of guinea-pig isolated ileum, Res. Commun. Chem. Pathol. Pharmacol. 55, 275.

Varga, F., 1976, Transit time changes with age in the gastrointestinal tract of the rat, Digestion 14, 319.

Waldman, S.A. and F. Murad, 1987, Cyclic GMP synthesis and function, Pharmacol. Rev. 39, 163.

Waterman, S.A. and M. Costa, 1994, The role of enteric inhibitory motoneurons in peristalsis in the isolated guinea-pig small intestine, J. Physiol. 477, 459.