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DEVELOPMENT OF MOTOR RESPONSE TO INTRAMURAL NERVE STIMULATION AND TO DRUGS IN RAT SMALL INTESTINE

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1 The onset and development of functional innervation and transmitter reactivity in the small intestine, isolated from foetal and neonatal rat, was examined in relation to the development of two muscle layers.

2 About half of the preparations tested at embryonic day 15 responded to electrical field stimulation, although both acetylcholine (ACh, 1 μM) and excess K (50 mM) evoked a response in all preparations. The response to these stimuli was the extension of the preparation longitudinally. All six preparations examined at embryonic day 16 extended in response to electrical field stimulation.

3 The extension response to electrical field stimulation was observed up to embryonic day 19. Then, the response changed to a biphasic one and finally to shortening after 4-days postnatal. The responses to electrical field stimulation were blocked by tetrodotoxin (TTX, 0.3 μM) or atropine (0.3 μM). The response to ACh and excess K similarly changed and became only a shortening at embryonic day 21 and 6-days postnatal, respectively.

4 The extension and biphasic responses produced by these stimuli were invariably converted to shortening after the preparation had been opened longitudinally.

5 The pD_2 value for ACh was 6.74–7.37 during the period embryonic day 15–6-days postnatal.

6 These results suggest that in the rat intestine, functional cholinergic innervation is established at least by embryonic day 16. In the early stages, the development of the circular muscle layer precedes that of the longitudinal muscle layer in causing contraction. This results in longitudinal extension of the preparation in response to stimuli. The reverse takes place following the development of the longitudinal muscle layer.

Introduction

Enteric neurones of the intestinal tract originate from the neural crest and/or neural tube (Andrew, 1971) and migrate to the intestinal wall during middle foetal stage (rectosigmoid of the rat; Ito, Donahoe & Hendren, 1977). The intramural nerve plexus composed of these neurones is formed in a cranio-caudal fashion (human embryo; Okamoto & Ueda, 1967). Neurone density in the plexus is much higher in the newborn stage than in the adult (rat small intestine; Gabella, 1971). The physiological significance of these enteric neurones in the foetal stage is not fully understood.

The onset and development of function in the intramural nerve have been investigated in a few studies by evaluating the mechanical responses of the smooth muscle to transmural electrical stimulation. Cholinergic excitatory and non-adrenergic inhibitory neurones were both starting to function in the ileum on day 16 in the mouse and day 17 in the rabbit after insemination (Gershon & Thompson, 1973). On the other hand, the sympathetic adrenergic perivascular nerve to the intestine started to function a few days after birth in the rabbit (Burn, 1968; 1976; Gershon & Thompson, 1973; Gulati & Panchal, 1978).

In the present experiments, the onset and development of functional innervation and transmitter reactivity in the small intestine isolated from foetal and neonatal rats were investigated. Special attention was paid to differences between longitudinal and circular muscle layers in their responsiveness to nerve stimulation and to drugs since the circular muscle layer was found to develop earlier than the longitudinal layer in morphological studies (Arey, 1954; Okamoto & Ueda, 1967; Stevens & Sellers, 1977).

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Methods

Foetuses and neonates of the Wistar rat were used. The animals at various stages of development were prepared as follows. Four females were placed together with a male in a cage over night. When sperm was found in the vaginal smear the next morning, the age of the foetus was designated as embryonic day 0. The age of the neonate which was usually born on embryonic day 21 was designated as 0-day postnatal on that day. The animals examined were from embryonic day 15 to 12-days postnatal.

The foetuses were removed by Caesarean section from female rats, which had been killed by stunning and bleeding. Segments of small intestine, 1–1.5 cm long, were isolated from the region above the appendix under a binocular stereomicroscope. The isolated preparation was in some cases opened longitudinally with a razor blade.

In the foetal stage, the preparation was so fragile that it could not be set up in an organ bath with ligatures. The experiments were therefore performed using the arrangement illustrated in Figure 1. The isolated preparation was placed in a gutter formed between two parallel glass capillaries on an inclined slide glass the under surface of which was coated with a black film including a narrow transparent strip. The upper end of the muscle segment was fixed at the top of the gutter and lower part screened the transparent area. The gutter was covered by a piece of thin glass and the tissue surperfused with modified Krebs solution.

The composition of the Krebs solution was as follows (mM): NaCl 118, KCl 4.75, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 10. The solution was saturated with mixture of 95% O_2 and 5% CO_2 (pH 7.4) and kept at 32° C. When the concentration of KCl was increased to 50 mM, the

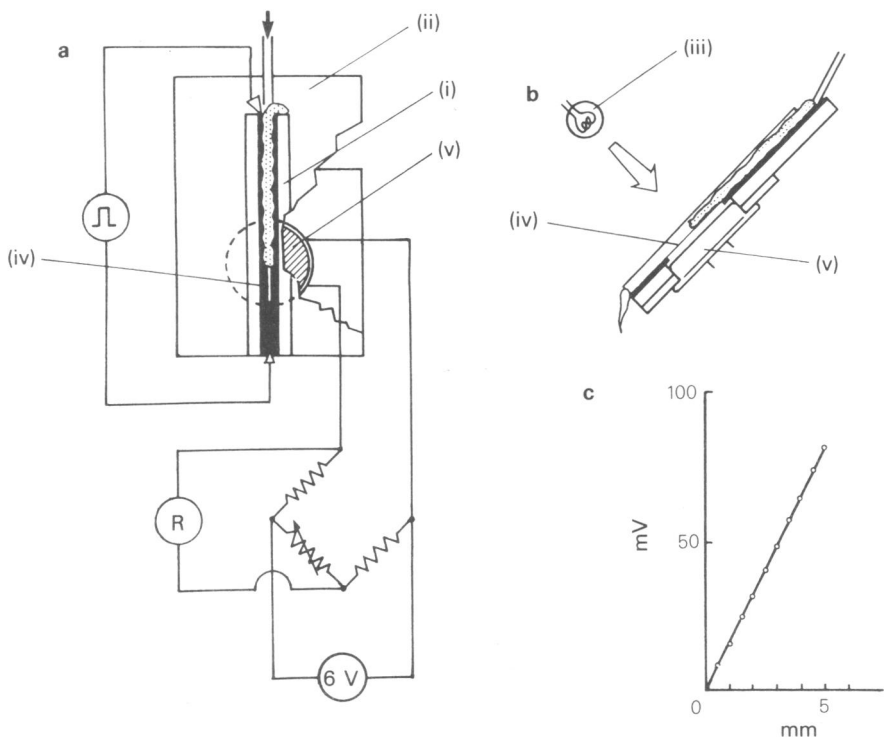

Figure 1 Experimental arrangements for recording movement of the preparation. The isolated intestinal segment was placed in a gutter (0.4–2.0 mm in width) formed by a pair of parallel glass capillaries (0.5–2.0 mm in diameter, 2–3 mm in length, (ai) on a slide glass (aii), and superfused with Krebs solution from the top of the preparation (↓). The light of a lamp (biii) was detected by a cadmium sulphide photoconductive cell (CdS cell, (a,b,v)) through a narrow transparent space (0.1–0.3 mm in width, 4–6 mm in length, (a,b,iv)) beneath a slide glass. Movement of the preparation changes the intensity of illumination projected on the CdS cell. The change in electric resistance of CdS cell was recorded as the voltage change through a Wheatstone bridge. The electrical field stimulation was applied through a pair of electrodes placed in the uppermost and lowest ends of the gutter. (c) shows that linear relationship between the length of the transparent space and the voltage change during exposure to the light.

osmolarity of the solution was maintained constant by reducing isosmolar NaCl.

The mechanical activity of the muscle segment was recorded by means of a cadmium sulphide photoconductive cell (CdS cell, Figure 1v) set parallel to the slide glass and behind the transparent area. The assembly was exposed to a light which could only reach the CdS cell through the unscreened area of the transparent strip. Therefore, according to the longitudinal movement of the muscle segment, the intensity of the illumination projected on the CdS cell varied and resulted in a change in the electrical resistance of the CdS cell. The change in the re sistance was recorded on an ink-writing oscillograph through the Wheatstone bridge. The light projected onto the assembly was guided through an optical fibre from the lamphouse and an automatic voltage stabilizer was used to keep the intensity of the light constant. The effect of ambient light was negligible in this arrangement. The change in the intensity of the light was proportional to the voltage change recorded (Figure 1c). However this recording method was not useful for preparations at embryonic day 15 and 16 because the muscle layer of the intestine at these stages was too thin to obscure the light. The change in the length of these preparations during movement was, therefore, determined every 10 s with a micrometer set in a stereomicroscope and the values were plotted on a graph against time.

The preparation was allowed to equilibrate for 60–90 min before the start of the experiment. Electrical field stimulation was applied to the muscle segment in the longitudinal direction via Ag-AgCl electrodes placed at either end of the gutter. Trains of rectangular pulses (supramaximal intensity, 0.5 ms) at 0.2–50 Hz were delivered for 10 s. Drugs were applied to the perfusing solution at the final required concentrations.

Drugs used were; acetylcholine chloride (ACh), atropine sulphate, 1-1-dimethyl-4-phenylpiperazinium iodide (DMPP), physostigmine sulphate (eserine), noradrenaline bitartrate, phentolamine methansulphonate, 5-(3-tert-butylamino-2-hydroxy) propoxy-3,4-hydrocarbostyril hydrochloride (OPC-1085) and tetrodotoxin citrate (TTX).

Results

Mechanical responses to electrical field stimulation

The youngest foetal stage examined was embryonic

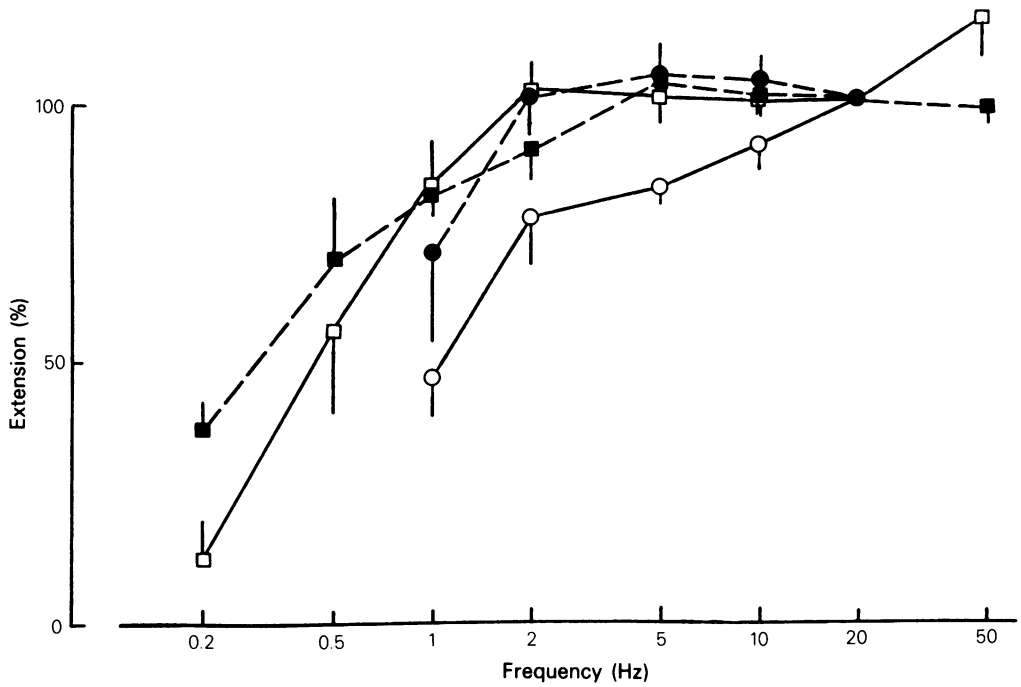

Figure 2 Relationships between frequency of electrical field stimulation and amplitude of extension response of rat intestine at four foetal stages. Ordinate scale: normalized amplitude of extension response expressed as 100% at 20 Hz. Abscissa scale: frequency of electrical field stimulation on a logarithmic scale. Each point is the mean of amplitude of extension in the preparation at embryonic day 15 (○, $n = 3$), 16 (●, $n = 3$), 17 (□, $n = 6$) and 19 (■, $n = 11$), and vertical bars show s.e.mean.

day 15. The preparation at this stage was about 0.3 mm in diameter and 10 mm in length.

Electrical field stimulation (0.5 ms duration) elicited a response in 5 out of 9 preparations at embryonic day 15, though direct electrical stimulation (20 ms duration, 20 Hz) of smooth muscle produced a response in all the preparations.

The response was an extension of the preparation in the longitudinal direction. The amplitude of the extension response increased with the stimulus frequency until it reached a maximum (4–8% of the total length of preparation) at 20 Hz (Figure 2). A ganglionic stimulant, DMPP (10 μM), also extended the preparation ($n = 3$). The addition of TTX (0.3 μM) to the perfusing solution abolished the response to electrical field stimulation, indicating that the response was brought about by neuronal excitation. These results suggest that smooth muscle can already contract at this stage, but its functional innervation is not always established.

At embryonic day 16, all 6 preparations examined extended in response to electrical field stimulation. The amplitude of the extension reached a maximum (11–14% of the total length of the preparation) at 2 Hz (Figure 2). All the preparations up to embryonic day 19 extended in response to electrical field stimulation. The response at these stages was also sensitive to TTX.

Between embryonic day 21 and 2-days postnatal, various types of response were evoked by electrical field stimulation, such as extension (Figure 3a), extension followed by shortening (Figure 3b) and *vice versa* (Figure 3c), and shortening (Figure 3d). The types of response observed varied with different stimulus frequencies and among preparations.

At 4-days postnatal, the response elicited by electrical field stimulation was predominantly shortening. In fact, only shortening was observed in 4 out of 5 preparations. The amplitude of the shortening response increased with the frequency of stimulation and it reached a maximum at 10 Hz (Figure 4). After 6-days postnatal, electrical field stimulation produced only a shortening response in all 5 preparations. All responses to electrical field stimulation were abolished by TTX (0.3 μM).

Thus, the response to neuronal excitation started

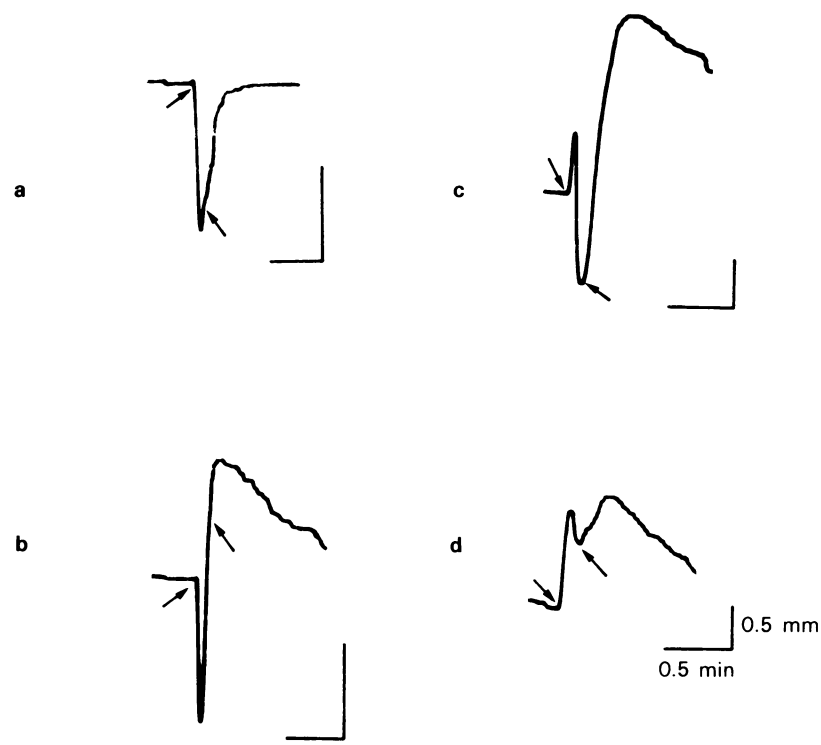

Figure 3 Various types of response to electrical field stimulation observed in the preparation at embryonic day 21. (a) An extension (at 1 Hz); (b) an extension followed by a shortening occurred during the stimulation (at 5 Hz); (c) an extension preceded by a shortening (at 20 Hz); (d) a shortening (at 5 Hz). These types of response were observed in the preparation between embryonic day 21 and 2-days postnatal. Arrows (↑ ↓) in each trace show the period of electrical field stimulation. Vertical scales 0.5 mm; horizontal scales 0.5 min.

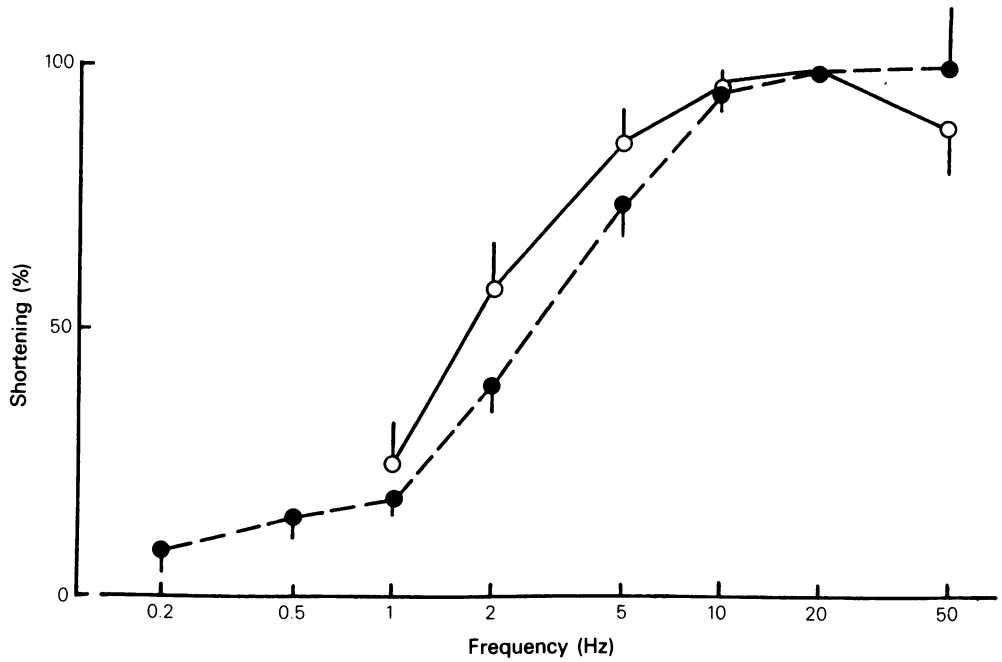

Figure 4 Relationships between frequency of electrical field stimulation and amplitude of shortening response of the preparation at 4- and 6-days postnatal. Ordinate scale: normalized amplitude of shortening response expressed as 100% at 20 Hz. Abscissa scale: frequency of electrical field stimulation on a logarithmic scale. Each point is the mean of amplitude of shortening in the preparation at 4- (○, $n = 4$) and 6-days postnatal (●, $n = 5$), and vertical bars show s.e.mean.

with extension and changed to shortening as the animal developed. Functional innervation of the intestine was already present at embryonic day 15 in about half the preparations tested and seemed to be established at embryonic day 16.

Pharmacological analysis of responses to electrical field stimulation

Phentolamine (2.7 μM) and/or OPC-1085 (a β-adrenoceptor blocking agent, 3 μM) had no significant effect on extension and shortening responses to electrical field stimulation in the preparation from embryonic day 15 to 6-days postnatal.

The extension response to electrical field stimulation was reversibly blocked by atropine (0.3 μM) and potentiated by physostigmine (1 μM) in both amplitude and duration (Figure 5). The biphasic and shortening responses observed after embryonic day 21 were also blocked by atropine (0.3 μM). These observations suggest that all the responses were caused by the excitation of intramural cholinergic neurones.

After application of atropine, the extension response during electrical field stimulation was abolished and a delayed extension response appeared in about half the preparations between embryonic day 15 and 17 (Figure 6a). In half the preparations after embryonic day 21, the delayed shortening response was observed in the presence of atropine (Figure 6b). These responses were also blocked by TTX (0.3 μM).

Analysis of extension response observed in early developmental stages

It is generally accepted that the intestinal segment isolated from a mature animal is shortened by electrical field stimulation, exogenous ACh and excess potassium ions, resulting from dominant contraction of the longitudinal muscle layer. In our recording system, the preparation in the early developmental stages was extended in response to these stimuli, although they caused shortening of the preparation in later stages. In the following experiments, we investigated why extension occurred in the early stages, and shortening in the later stages of development. Under the stereomicroscope, the preparation showed shrinkage in diameter during the extension in response to these stimuli, suggesting that the extension response in the early stages was caused by contraction of the circular muscle layer rather than relaxa-

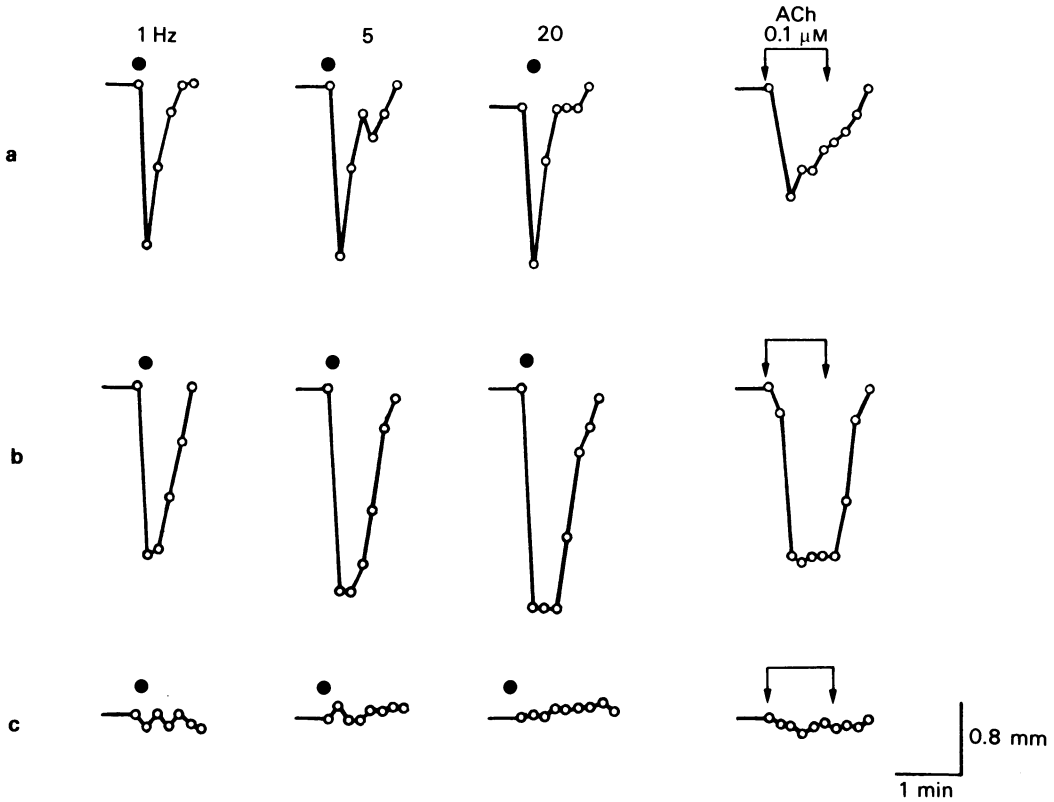

Figure 5 Effects of physostigmine and atropine on extension responses to electrical field stimulation and exogenous acetylcholine (ACh) in the preparation at embryonic day 16: (a) shows control responses to electrical field stimulation (●) at 1, 5 and 20 Hz, and to ACh (0.1 μM, 1 min, ↓ ↑). The amplitude and duration of extension responses to both stimuli, were potentiated after treatment with physostigmine (1 μM, b) and abolished by atropine (0.3 μM, c). These traces were constructed by marking the length of preparation measured at every 10 s (see Methods). Vertical scales, 0.8 mm; horizontal scale, 1 min.

tion of the longitudinal muscle layer. To test this possibility, the segments of intestine was opened longitudinally. This might be expected to reduce the influence of the circular muscle layer on the longitudinal movement of the preparation.

The opened preparation ($n = 4$) at embryonic day 19 consistently responded by shortening to electrical field stimulation, while the intact preparation was always extended as described above (Figure 7). The biphasic responses observed in the preparation ($n = 7$) between embryonic day 21 and 2-days postnatal were also consistently converted to shortening after opening. The shortening response to electrical field stimulation in these opened preparations was abolished by atropine (0.3 μM).

Development of functional activity in muscle layers

The results described above suggest that the change in response of preparations during the course of their development is due to the differences in the ability of the circular and longitudinal muscle layers to contract. The contractility of the circular muscle layer may be predominant in early stages while that of the longitudinal muscle predominates in later stages. In order to investigate this possibility, the response to high concentration of potassium ions (excess K, 50 mM) was examined at various developmental stages.

In all preparations ($n = 39$) between embryonic day 15 and 2-days postnatal, excess K induced extension. However, after embryonic day 21, the duration of the extension response to excess K became shorter and in some cases the response tended to recover even during exposure to the excess K solution. At 4-days postnatal, the response to excess K varied between preparations from a small extension to a small shortening. Finally, after 6-days postnatal, excess K almost always evoked a shortening response ($n = 5$). The extension response to excess K was also

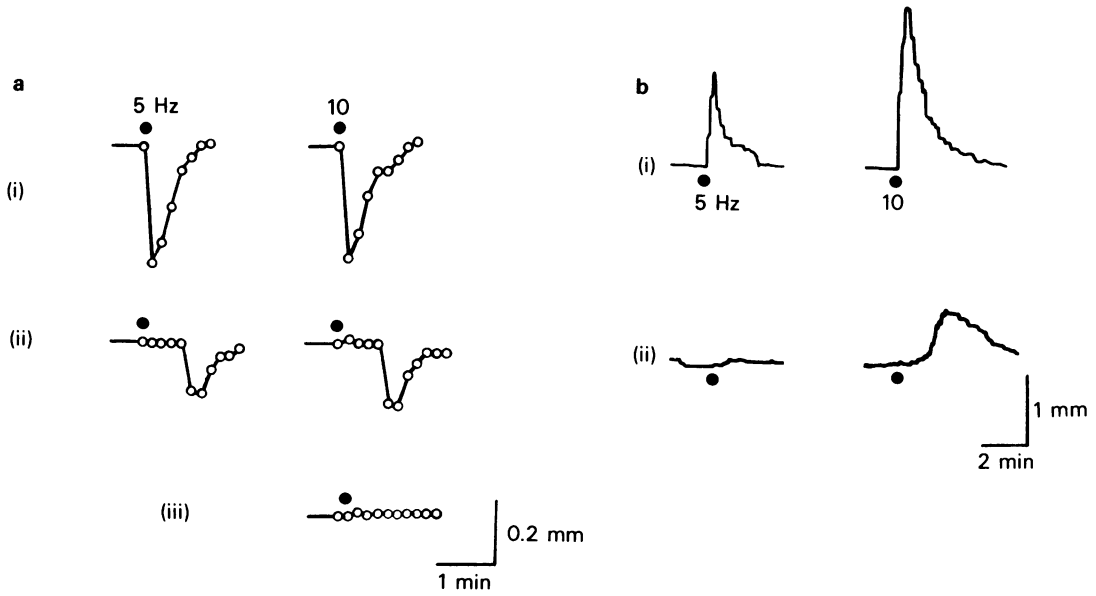

Figure 6 Delayed responses to electrical field stimulation in the presence of atropine observed at embryonic day 15 and 10-days postnatal. (a) Shows extension responses to electrical field stimulation (●) at 5 and 10 Hz in (i) the absence and (ii) the presence of atropine (0.3 μM) at embryonic day 15. After the application of atropine, the latency of the extension response was increased and the amplitude of the response was reduced. The delayed response was blocked by tetrodotoxin (TTX, 0.3 μM, (iii)). (b) Shows shortening responses to electrical field stimulation (●) at 5 and 10 Hz in (i) the absence and (ii) the presence of atropine (0.3 μM) at 10-days postnatal. Note that after the application of atropine, the response was abolished or reduced or the delayed response appeared. Vertical scales, 0.2 and 1 mm; horizontal scales, 1 and 2 min, in (a) and (b), respectively.

converted to a shortening if the preparation was opened longitudinally (Figure 7). The response to excess K was not affected by treatment with atropine (0.3 μM).

These observations suggest that, up to 4-days postnatal, the contractile activity of the circular muscle layer determines the response. Later the longitudinal muscle layer becomes dominant.

Responses to acetylcholine

ACh added to the superfusing solution caused extension in all preparations at embryonic day 15, including those which did not respond to electrical field stimulation. The ACh-induced response changed similarly to those induced by electrical field stimulation. Responses to ACh were (1) extension between embryonic day 15 and 17, (2) shortening at the lower concentration (0.01–0.2 μM) and extension followed by shortening at higher concentrations on embryonic day 19, and (3) only a shortening after embryonic day 21. These responses to ACh were abolished by atropine (Figure 5).

The sensitivity of the preparation to ACh was estimated from the dose-response curves at seven stages of development. The pD_2 values in each preparation are shown in Figure 8. Ranges of mean values were 6.74–7.36 and 7.14–7.37 for the extension and shortening responses, respectively. The pD_2 values at embryonic day 15, 21 and 4-days postnatal were 7.36 ± 0.20 (mean value ± s.e.mean), 7.20 ± 0.05 and 7.37 ± 0.05, respectively. These values among these three stages were not significantly different ($P > 0.05$), although the values tended to decrease at embryonic day 17. This suggests that the sensitivity to ACh did not change extensively between embryonic day 15 and 6-days postnatal.

Responses to noradrenaline

Noradrenaline, even at high concentrations (5 μM) failed to induce any significant response at embryonic day 17. After embryonic day 21, noradrenaline (0.5–1 μM) had only a slight effect, such as inhibition of spontaneous contraction or a small relaxation. However, these effects were not always observed in all the preparation tested, even when the tonus of the preparation was maintained by exposure to ACh. Noradrenaline-induced relaxation was obvious by 12-days postnatal and was sensitive to OPC-1085 (3 μM).

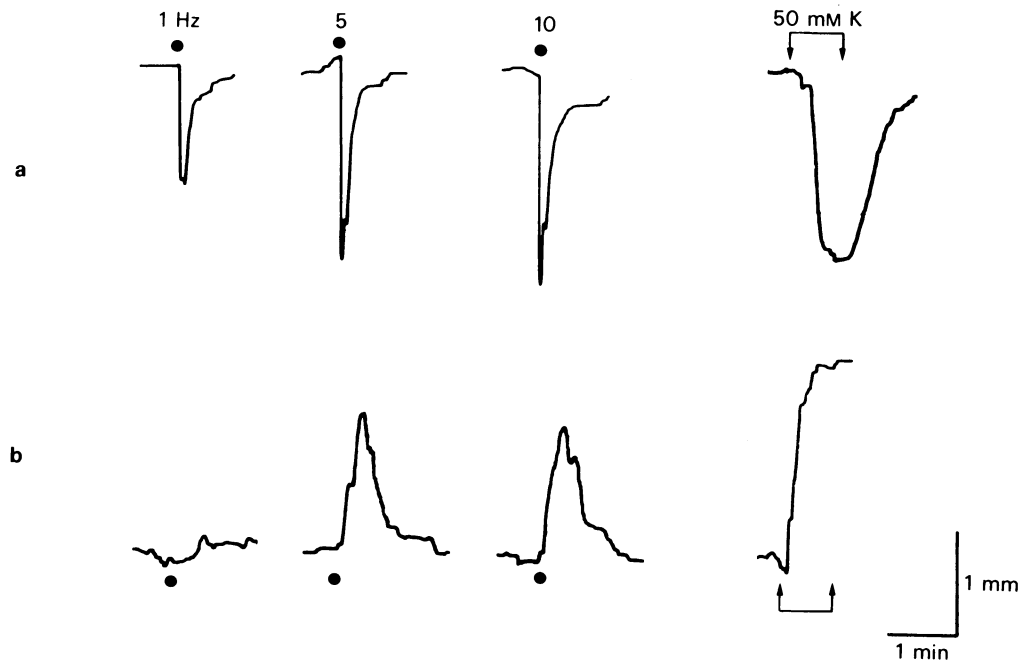

Figure 7 Effect of opening preparation longitudinally on the response to electrical field stimulation and to excess K. Responses induced by electrical field stimulation (●) at 1, 5 and 10 Hz and by exposure to excess K (50 mM, ↓ ↓) for 1 min in (a) intact and (b) opened preparation. Extension response was consistently converted to shortening by opening the preparation longitudinally. Vertical scale, 1 mm; horizontal scale, 1 min.

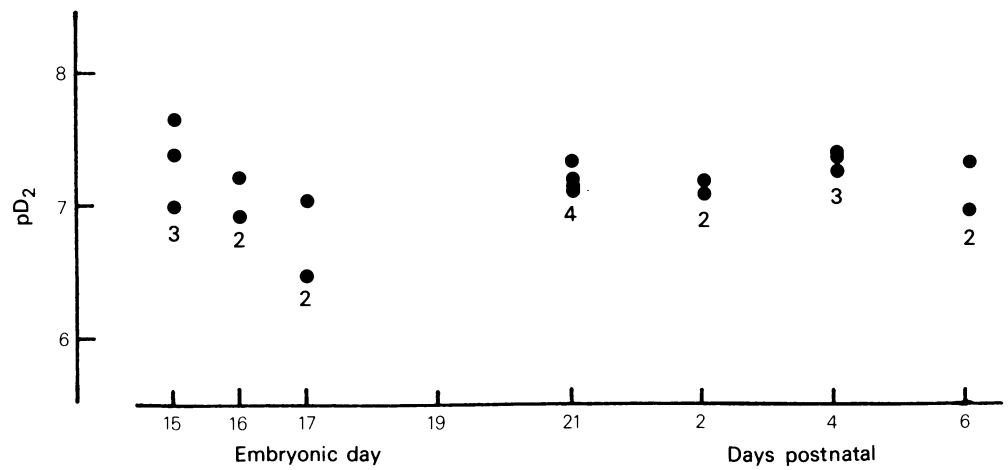

Figure 8 The pD_2 value for acetylcholine (ACh) in the preparation at different ages. The pD_2 value (●), calculated from dose-response curve is plotted as a function of age (days). Numbers below filled circles refer to the number of preparations.

Discussion

The present results suggest that the functional innervation of rat small intestine may already be present at embryonic day 15 and is fully established by embryonic day 16. It was shown in the rectosigmoid colon of the rat that the intramural ganglion cell could be first recognized morphologically on day 17 of gestation, although undifferentiated ganglion cells were present in the gut mesenchyme before day 12 of gestation (Ito *et al.*, 1977). In connection with this observation, the intramural ganglion cells may start to function at a morphologically immature stage, even if the ganglion cells develop in a cranio-caudal sequence in the rat as in the human embryo (Okamoto & Ueda, 1967).

The extension response evoked by electrical field stimulation, appeared on embryonic day 15 and was potentiated and prolonged by physostigmine and blocked by atropine. Adrenoceptor blocking agents failed to affect the extension response and noradrenaline did not induce any significant response. These results suggest that the neurone which starts to function first is cholinergic, in agreement with observations in the rabbit intestine where the cholinergic neurone functions before the adrenergic; the latter starts to function after birth (Gershon & Thompson, 1973).

Burn (1968, 1976) and Gulati & Panchal (1978) showed in the neonatal rabbit that stimulation of the periarterial nerve to the ileum at first produced a cholinergic contraction which was then replaced by adrenergic relaxation in the course of the first postnatal week. Furthermore, exogenous noradrenaline did not cause any relaxation until 2 days after birth in the rabbit intestine (Gulati & Panchal, 1978). A histochemical study in the rat duodenum (De Champlain, Malmfors, Olson & Sachs, 1970) demonstrated that adrenergic innervation did not appear until birth, although catecholamine fluorescence transiently appeared in presumptive neuroblasts within the gut at 11.5 days of gestation (Cochard, Goldstein & Black, 1978). The present results agree with these reports. Relaxation caused by electrical stimulation of the adrenergic nerve was not seen at any developmental stages tested. The effect of noradrenaline was small and was not always observed in all the preparations even after birth. These results may indicate that the adrenergic mechanism was established after 12-days postnatal. Alternatively, this may partly be due to the low tone of the preparation.

The delayed extension response in the presence of atropine, which was probably caused by contraction of the circular muscle layer, was nerve-mediated as it was sensitive to TTX. It has been reported that stimulation of the intramural inhibitory neurones (non-adrenergic) in the gut caused the after-contraction with (Holman & Hughes, 1965; Bennett, 1966) or without (Gershon & Thompson, 1973) preceding relaxation. Furthermore, it has also been observed that a non-cholinergic excitatory neurone causes an atropine-resistant contraction in avian and mammalian gastro-intestinal tracts (Ambache & Freeman, 1968; Nakazato, Sato & Ohga, 1970; Takewaki & Ohashi, 1977). In either case, the neurone which mediates the delayed response seems to start to function simultaneously with the cholinergic neurone from embryonic day 15, in agreement with findings in the rabbit intestine (Gershon & Thompson, 1973).

The extension response observed in the early stages of the development is probably determined by the contraction of circular muscle layer and not the relaxation of longitudinal muscle layer, as indicated by results with the longitudinally opened preparation. The extension response subsequently changed to shortening in older preparations, suggesting that the contractile activity of the longitudinal muscle layer develops later than that of the circular muscle layer. These observations are compatible with morphological findings that the longitudinal muscle layer develops later than the circular muscle layer (Arey, 1954; Stevens & Sellers, 1977) after myenteric ganglia have been formed (Okamoto & Ueda, 1967; Daikoku, Ikeuchi & Miki, 1975). However, the extension response to various excitatory stimuli changed to a shortening response at different stages; thus, the responses to ACh, electrical field stimulation, and excess K were converted to shortening on embryonic day 21, 4-days and 6-days postnatal, respectively. In addition to the differences in the ability of the muscle to contract, the difference in the response in developmental stages may be partly due to the unequal distribution of cholinergic innervation (Anurus, Christensen & Cooke, 1977) and ACh-sensitivity (Evans & Schild, 1953) between the two muscle layers.

The intestinal smooth muscle seems to acquire sensitivity to ACh before it is functionally innervated by cholinergic neurones, because the response to ACh was observed even in a preparation that did not respond to electrical field stimulation of the intramural cholinergic neurone. This is consistent with reports that the reactivity to transmitters precedes the functional innervation in rat vas deferens (Swedin, 1972) and chick heart (Pappano, 1977). However, in the rabbit intestine, the response to exogenous ACh appeared simultaneously with the onset of functional cholinergic innervation (Gershon & Thompson, 1973). There could be species differences. In the present experiments, pD_2 values for ACh, calculated from dose-response curves, were not significantly different during the period between embryonic day 15 and 6-days postnatal, in agreement

with the observations in the ileum of guinea-pig (Boréus & McMurphy, 1971) and human embryo (Boréus, 1967). Therefore, the sensitivity of the intestinal smooth muscle of the rat to ACh does not change during these developmental stages.

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