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Postnatal changes in response to adenosine and adenine nucleotides in rat duodenum

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1 The effects of adenosine and adenine nucleotides were studied in rat duodenum from postnatal day 1 to day 70. The mechanical activity of duodenal segments was recorded through an isotonic transducer connected to a polygraphic recorder.

2 In rat duodenal segments, adenosine-5′-triphosphate (ATP, 10^{-4} M) and adenosine-5′-diphosphate (ADP, 10^{-4} M) produced a contractile response on postnatal day 1. This response increased with age, peaking on day 7, followed by a gradual decrease and was non-existent by day 21. In contrast, a relaxant response to ATP and ADP was apparent on day 21, and continued to increase up to day 70.

3 The contraction caused by ATP was inhibited by indomethacin or the P_{2y}-purinoceptor antagonist, reactive blue-2 but not by tetrodotoxin or hyoscine. Thus, it may be mediated by production of prostaglandin through the P_{2y}-purinoceptor. The relaxation produced by ATP was inhibited by reactive blue-2 but not by tetrodotoxin, guanethidine or the P_1-purinoceptor antagonist, 8-phenyltheophylline indicating that ATP acts on smooth muscle directly through the P_{2y}-purinoceptor. The pD_2 for the contractile response to ATP was 5.15 on day 7 and that for the relaxant response, 6.64 on day 70.

4 Adenosine (10^{-4} M) and adenosine-5′-monophosphate (AMP, 10^{-4} M) elicited no response before day 14. On day 14, both adenosine and AMP produced a small relaxant response which increased with age. The relaxation produced by adenosine was inhibited by 8-phenyltheophylline but not by tetrodotoxin or guanethidine, indicating that it is mediated by an action on the P_1-purinoceptor of smooth muscle.

5 It is evident from these results that in neonatal rat, a contractile response to ATP and ADP occurs initially in the duodenum, followed by a relaxant response to adenosine and AMP on day 14 and to ADP and ATP on day 21.

6 The smooth muscle of rat duodenum may tentatively be concluded to contain separate purinoceptors for adenosine and AMP (P_1) and ADP and ATP (P_2) and the responses to P_1- and P_2-agonists change during the course of development.

Introduction

Non-adrenergic, non-cholinergic (NANC) nerves are known to be widespread throughout the autonomic nervous system of vertebrates. According to Burnstock (1972), adenosine-5′-triphosphate (ATP) or a related nucleotide may be a neurotransmitter released from some of these nerves which he designated as 'purinergic'. He also proposes that there are two types of receptors for purine compounds, the P_1-receptor for adenosine and adenosine-5′-monophosphate (AMP) and the P_2-receptor for adenosine-5′-diphosphophosphate (ADP) and ATP (Burnstock, 1978). This hypothesis has been supported and extended by many experiments including those on the gastrointestinal tract (Burnstock & Buckley, 1985). Manzini *et al.* (1985, 1986) have presented evidence for a NANC inhibitory neuronal mechanism in rat duodenum and suggest that ATP may possibly be a neurotransmitter released from inhibitory NANC nerves in rat duodenum.

The response of rat duodenum to certain neuropeptides including thyrotropin-releasing hormone (Tonoue *et al.*, 1981), methionine[5] enkephalin (Furukawa *et al.*, 1982), and calcitonin-gene related peptide (Furukawa & Nomoto, 1988) was found, in a

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previous study, to change within several weeks following birth and the physiological role of neurotransmitters or neuromodulators in the intestine was thought to change with the progress of development after birth. No data are presently available on the postnatal ontogenesis of purinoceptors. It thus seemed worthwhile to examine the response of rat duodenum to purine nucleosides and purine nucleotides in the developing neonatal rat duodenum.

In the present study, P_2-receptors, which induce contraction through prostaglandin production, were noted in the duodenum of neonatal rats, followed by development of P_1- and P_2-receptors which caused relaxation through a direct action on smooth muscle.

Methods

Animals and preparations

Wistar-Imamichi strain rats aged 1, 3, 7, 14, 21, 28 and 70 (adult) days were used. The day of birth was designated as day 0, with day 1 as 12–24 h after birth. At an age less than 21 days, the duodena of both male and female rats were used and after day 28, only those of male rats were used. The animals were stunned by a blow on the head and bled by cutting the vessels in the neck. Each duodenum was then dissected out. About one third the duodenum from the proximal area was used. The length of a duodenal segment was about 1 cm on days 1 and 3; 1.5 cm on days 7 and 14; 2 cm on days 21 and 28 and 2.5 cm in adult rats.

Mechanical studies

All segments were placed in an organ bath containing 25 ml of modified Locke solution (composition in mM: NaCl 154, KCl 4.02, $CaCl_2$ 1.36, $MgCl_2$ 0.9, $NaHCO_3$ 2.97, glucose 5.56, pH 7.0) saturated with 95% O_2 and 5% CO_2. Experiments were performed at 32°C with continuous bubbling of air. The mechanical activity of the duodenum was recorded through an isotonic transducer (Nihon Koden, TD 112S) attached to a polygraphic recorder (Nihon Koden, WI-640GS); a load of 0.5 g was used for segments of days 1 to 7, 0.75 g for days 14 to 28 and 1 g for segments from adult rats.

Drugs

The following drugs were used: adenosine (Sigma), adenosine-5′-monophosphate sodium salt (AMP, Sigma), adenosine-5′-diphosphate sodium salt (ADP, Sigma), adenosine-5′-triphosphate disodium salt (ATP, Sigma), tetrodotoxin (TTX, free base, kindly supplied by Sankyo), hyoscine (Scopolamine hydrochloride, Sigma), indomethacin (Sigma), reactive blue-2 (RB-2, Sigma), guanethidine sulphate (Ciba-Geigy) and 8-phenyltheophylline (8-PT, Sigma). The drugs were usually dissolved in saline to make a stock solution, 0.5 ml of which was added to the 25 ml organ bath. Indomethacin was dissolved in 70% ethanol and 37.5 μl of the solution thus obtained were added to the organ bath. 8-PT was dissolved in dimethylsulphoxide (DMSO) and 5 μl was added to the organ bath. RB-2 was dissolved in distilled water which was then added in a volume of 25 μl to the organ bath.

Results

Experiments were repeated 5 or more times, and essentially the same results were obtained in each case. Table 1 shows the body weights of the rats and those of the duodenal segments.

Mechanical response to adenosine and adenine nucleotides

Figure 1 and Table 1 show postnatal changes in the duodenal response to adenosine, AMP, ADP and ATP (all at 10^{-4} M). Duodenal preparations from 1 to 7 day-old rats showed no response to the P_1-agonists, adenosine and AMP. However, a weak but distinct contraction, was induced by the P_2-agonists, ADP and ATP in duodena of 1 day-old rats. The contractile response to ADP and ATP increased in intensity with age, peaking on days 7 to 14. The magnitude of this response per mg wet weight of tissue was maximal at day 7 (Table 1). The contraction induced by ATP appeared without a time lag after adding the nucleotide to the bath on day 7. On day 14, a short time lag (14.2 ± 1.1 s, $n = 6$) before the contractile response to ATP was observed and some preparations (5 out of 20) showed a slight relaxant response to ATP before a contraction appeared (Figure 3f). After day 14, contractile response to ATP decreased and in its place, a slight but distinct relaxant response was noted. This relaxation, consistent on day 21 for the first time, continued to increase until day 70 (Figure 1). By day 14, both adenosine and AMP produced a slight relaxant response which increased in segments taken from older rats (Figure 1). On day 70, the magnitude of the relaxant response to adenosine and AMP became about half that of the ADP- and ATP-induced relaxation (Table 1).

Features of the contractile and relaxant responses to ATP

The pD_2 s for the contractile and relaxant responses to ATP were determined on days 7 and 70, respectively. A linear concentration-contractile response

Table 1 Postnatal changes in response to adenosine, AMP, ADP and ATP (each at 10^{-4} M) in rat duodenal segments

Age (days)	*1*	*3*	*7*	*14*	*21*	*28*	*70*
n	8	4	12	8	8	6	6
Body weight (g)	5.7 ± 0.2	8.5 ± 0.2	14.0 ± 0.4	25.1 ± 0.6	36.9 ± 0.8	65.2 ± 0.7	368 ± 7
Preparation weight (mg)	19.7 ± 1.3	24.9 ± 0.8	29.5 ± 1.5	61.1 ± 1.9	115.9 ± 0.5	169.6 ± 4.7	403 ± 21
				Responses (mm)			
Adenosine	0	0	0	−0.45 ± 0.09 (−0.74)	−0.41 ± 0.04 (−0.35)	−0.78 ± 0.18 (−0.46)	−1.30 ± 0.42 (−0.32)
AMP	0	0	0	−0.60 ± 0.10 (−0.98)	−0.41 ± 0.05 (−0.35)	−1.07 ± 0.22 (−0.63)	−1.50 ± 0.31 (−0.37)
ADP	+1.00 ± 0.22 (+5.07)	+2.37 ± 0.37 (+9.5)	+3.29 ± 0.29 (+11.2)	+3.25 ± 0.25 (+5.32)	−0.43 ± 0.05 (−0.37)	−0.77 ± 0.08 (−0.45)	−3.20 ± 0.62 (−0.79)
ATP	+0.82 ± 0.20 (+4.16)	+2.33 ± 0.45 (+9.4)	+3.15 ± 0.30 (+10.7)	+3.18 ± 0.44 (+5.20)	−0.54 ± 0.05 (−0.47)	−0.76 ± 0.07 (−0.45)	−3.20 ± 0.64 (−0.79)

Symbols (+) and (−) indicate contractile and relaxant responses, respectively.
Values are the mean ± s.e.mean. Figures in parentheses were estimated from the equation: (Response (mm)/preparation wet weight (mg)) × 100.

curve was obtained in the range 2.5×10^{-6} to 10^{-4} M ATP and pD_2 was 5.15 ($n = 7$) (Figure 2). In the range 10^{-7} to 10^{-5} M ATP, a linear concentration-relaxant response curve was constructed again and pD_2 for the relaxation was 6.64 ($n = 6$), which was larger than that of the contraction (Figure 2).

Contractile response to ATP The contractile response to 10^{-5} M ATP of duodenal segments taken from rats of days 7 to 14 did not change on preincubation with 10^{-6} M TTX or 2.5×10^{-7} M hyoscine for 15 min, respectively (Figure 3a and b). The presence of subtypes of P_2-purinoceptors, x and y has been proposed by Burnstock & Kennedy (1985) on the basis of agonist potency orders. RB-2 was used to distinguish P_y- from P_x-purinoceptors, since it has been shown to act as a competitive antagonist against P_{2y}-purinoceptor-mediated responses within the concentration range 0.1–10 μM (Burnstock *et al.*, 1986; Burnstock & Warland, 1987). In the presence of 10^{-5} M RB-2, added 5 min before ATP, the duodenal contractile response to ATP was inhibited by 71.0 ± 3.0% (mean ± s.e.mean, $n = 5$) (Figure 3c). The contractile response to ATP of the duodenum on days 7 to 14 was inhibited in most cases in the presence of 10^{-5} M indomethacin added 30 min previously (Figure 3d, e and f) and the residual response to ATP was 22.2 ± 5.2% ($n = 8$). The addition of the vehicle alone (70% ethanol) to the bath failed to have any effect on the response to ATP in rat duodenum. Therefore, the contractile response to ATP is not mediated by cholinergic neurones, but rather by stimulation of prostaglandins synthesis through an effect on P_{2y}-purinoceptors in the duodenum.

Relaxant response to ATP As shown in Figure 4, the relaxant response to 10^{-5} M ATP on day 70 was not affected by preincubation with either 10^{-6} M TTX for 15 min, 6.4×10^{-6} M guanethidine for 30 min, 2.5×10^{-7} M hyoscine for 15 min, 10^{-5} M indomethacin for 30 min or the P_1-antagonist, 8-PT (10^{-6} M) (Griffith *et al.*, 1981) for 5 min (Figure 4a, b, d and e). However, this response was markedly inhibited by preincubation with 10^{-5} M RB-2 for 5 min by 75.5 ± 4.2% ($n = 5$) (Figure 4c). The relaxant response to ATP thus appears to involve an action on the P_{2y}-purinoceptor of smooth muscle but not on adrenergic nerves in the myenteric plexus of the duodenum.

The nature of the response to adenosine

The mechanism of the relaxant response to adenosine 10^{-4} M in the duodenum was examined in adult (about 70 days old) rats. This response to adenosine (10^{-4} M) was inhibited by 68 ± 4% ($n = 5$)

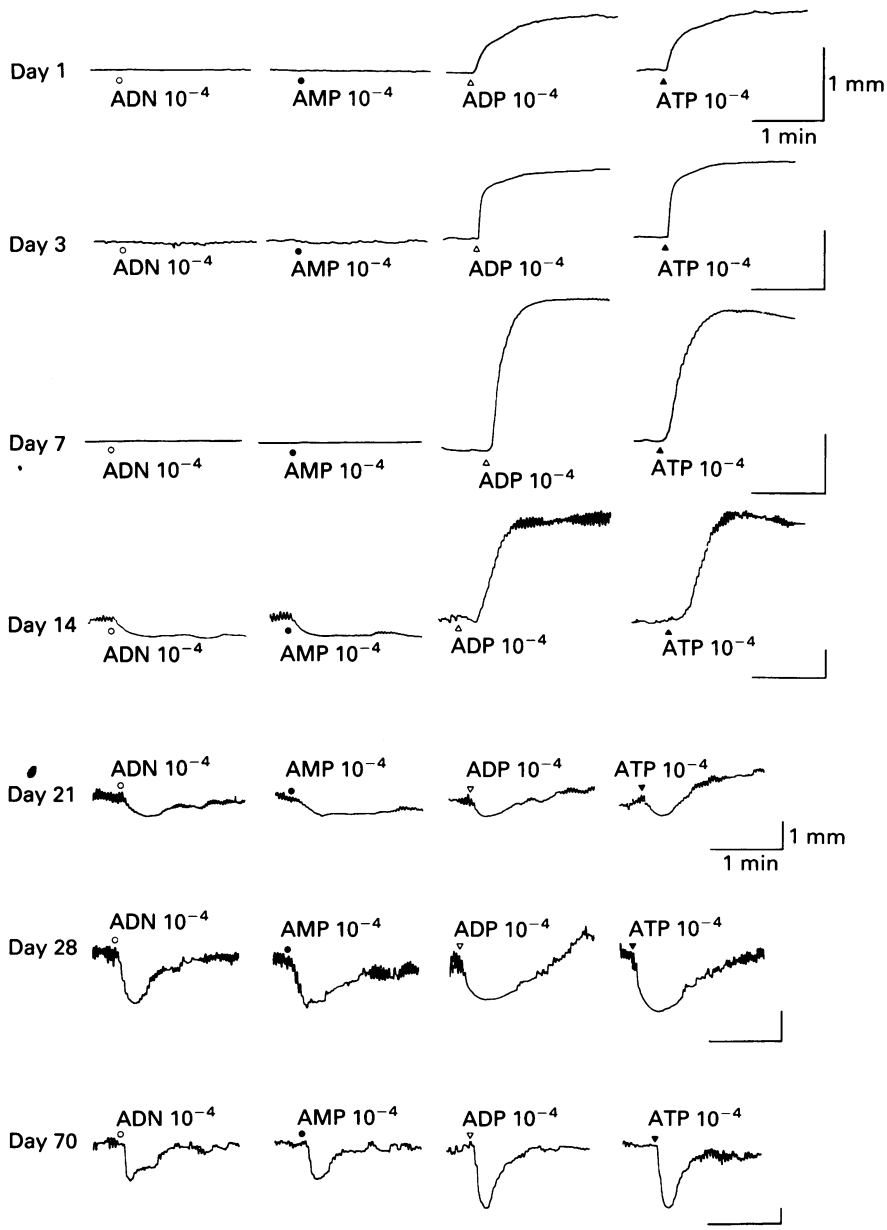

Figure 1 Mechanical responses to adenosine (ADN), adenosine-5′-monophosphate (AMP), adenosine-5′-diphosphate (ADP) and adenosine-5′-triphosphate (ATP), each at 10^{-4} M, in duodenal segments of rats from 1 to 70 days old. Both mechanical activity (mm) and time (min) scales are shown.

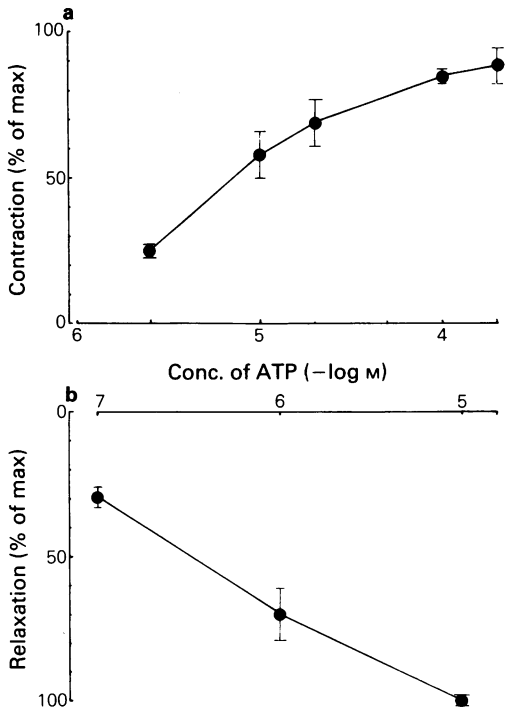

Figure 2 Log concentration-response curves for (a) contraction (day 7, $n = 7$) and (b) relaxation (day 70, $n = 6$) caused by ATP in an isolated segment of rat duodenum. Values are the mean with s.e.mean shown by vertical lines. The pD_2 of ATP for the contractile response was 5.51 and for the relaxant response, 6.46.

by 10^{-6} M 8-PT added 5 min previously (Figure 5c). Preincubation with 10^{-6} M TTX for 15 min or both 6.4×10^{-6} M guanethidine for 30 min and 2.5×10^{-7} M hyoscine for 15 min failed to affect this response (Figure 5a and b). Neither did the vehicle of 8-PT (5 μl DMSO) by itself have any effect. It would thus appear that adenosine acts directly on smooth muscle through the P_1-receptor to induce relaxation.

Discussion

It is evident from the data presented above that, in rat duodenum, the contractile response to P_2-agonists such as ATP and ADP occurs before the myogenic relaxant response. Contractile responses, detectable on day 1, became maximum at days 7 to 14, decreased markedly thereafter and then became non-existent by day 21. In contrast, on day 21, transient relaxation, due to ATP or ADP, was detectable. The relaxant response to ATP and ADP continued to increase until adulthood. This postnatal change in the response of rat duodenum to ATP or ADP is in contrast to the consistent direct myogenic effects of the P_1-agonists, adenosine and AMP, seen on day 14 for the first time as well as in adult rats.

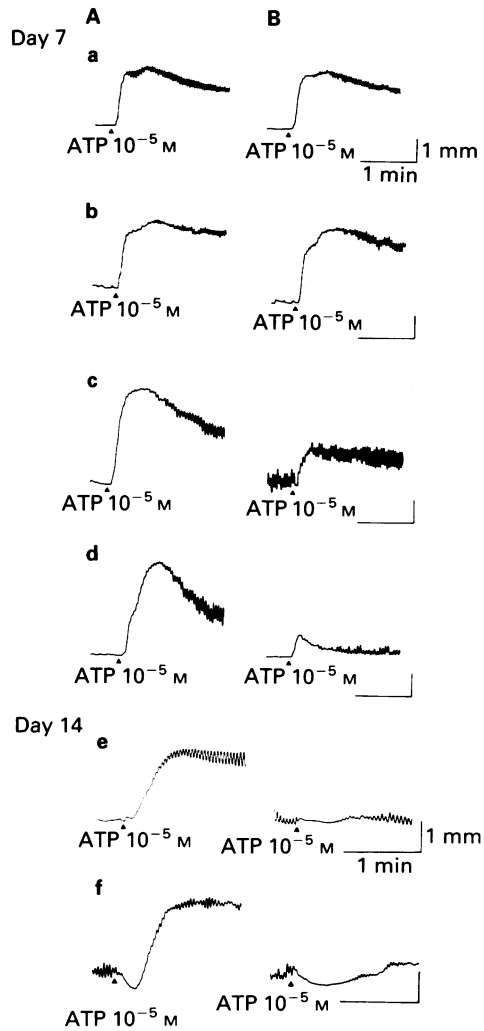

Figure 3 Effects of preincubation with tetrodotoxin (10^{-6} M for 15 min) (a), hyoscine (2.5×10^{-7} M for 15 min) (b), reactive blue-2 (10^{-5} M for 5 min) (c) and indomethacin (10^{-5} M for 30 min) (d, e and f) on the contractile response of rat duodenal segments to ATP on postnatal days 7 (a–d) and 14 (e and f). Contraction caused by ATP was not affected in the presence of tetrodotoxin or hyoscine. The response to ATP was inhibited by reactive blue-2 or indomethacin. Responses before (A) and after (B) incubation with the inhibitors are shown.

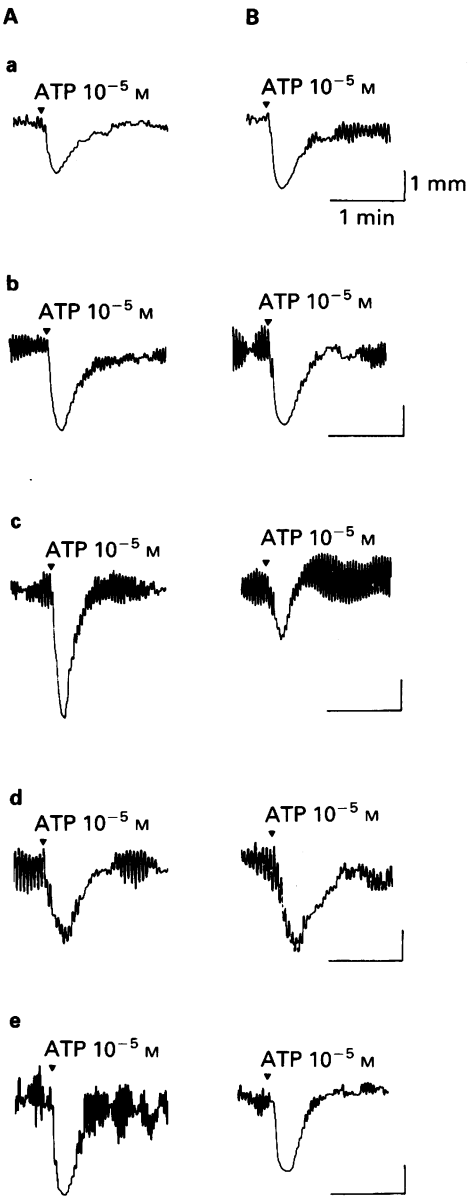

Figure 4 Effects of preincubation with tetrodotoxin (10^{-6} M, 15 min) (a), guanethidine (6.4×10^{-6} M, 30 min) and hyoscine (2.5×10^{-7} M, 15 min) (b), reactive blue-2 (10^{-5} M, 5 min) (c), indomethacin (10^{-5} M, 30 min) (d), and 8-phenyltheophylline (10^{-6} M, 5 min) (e) on the relaxant response to ATP of duodena on day 70. Relaxation produced by ATP showed no change in the presence of tetrodotoxin, guanethidine plus hyoscine or 8-phenyltheophylline, but was inhibited on adding reactive blue-2. Responses to ATP before (A) and after (B) incubation with the inhibitors are shown.

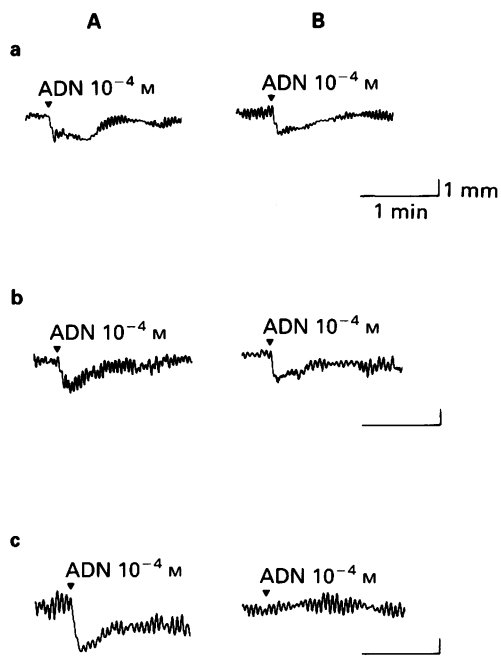

Figure 5 Effects of preincubation with tetrodotoxin (TTX, 10^{-6} M, 15 min) (a), guanethidine (Guan, 6.4×10^{-6} M, 30 min) and hyoscine (Hyos, 2.5×10^{-7} M, 15 min) (b) and 8-phenyltheophilline (8-PT, 10^{-6} M, 5 min) (c) on the relaxant response to adenosine (ADN) of duodena on day 70. The relaxant response to adenosine failed to change in the presence of TTX or Guan plus Hyos, but was inhibited by the addition of 8-PT. Responses to adenosine before (A) and after (B) incubation with the inhibitors are shown.

The contractile response to ATP in the duodena of rats may possibly be mediated by prostaglandins since the prostaglandin synthesis inhibitor, indomethacin (Vane, 1971) inhibits this response and ATP and ADP are potent stimulators of prostaglandin biosynthesis in various organs in mammals (Needleman *et al.*, 1974; Burnstock *et al.*, 1975; Burnstock, 1978; Gordon, 1986). RB-2, a P_{2y}-antagonist, has also been found to inhibit the contractile response to ATP, indicating that ATP stimulates prostaglandin synthesis through an effect on the P_{2y}-purinoceptor in neonatal rat duodenum. The absence of any effect of hyoscine or TTX on the response to ATP indicates that the contractile response is not mediated by cholinergic neurones.

The relaxant as well as the contractile response to 10^{-5} M ATP was markedly inhibited by RB-2 10^{-5} M but not by guanethidine or TTX. The relaxant response to ATP is thus myogenic through postsynaptic P_{2y}-receptors and is not induced by an

effect on adrenergic nerves. The conversion of ATP and ADP to AMP or adenosine, resulting in a relaxant response, would not be possible since the P_1-antagonist, 8-PT, failed to inhibit the response to ATP. The relaxant response to ATP has been found to arise mainly from an increase in the potassium permeability of the smooth muscle membrane (Brown & Burnstock, 1981). Both relaxant and contractile responses to ATP may be mediated through P_{2y}-receptors since each is inhibited by RB-2. From the above findings, the P_{2y}-receptor appears to be linked to two different transduction mechanisms, the early one to prostaglandin synthesis and the later, to potassium ion channels. The affinity of the relaxant response to ATP (pD_2: 6.64) was higher than that for the contractile response (pD_2: 5.51). The proposed different transduction mechanisms may perhaps account for the observed differences in pD_2 values. However, the presence of two subtypes of P_{2y}-receptor in the duodenum of the developing rat remains a possibility. Change in the features of P_{2y}-purinoceptors in the duodenum seems to occur between postnatal days 14 and 21. It is unlikely that the responsiveness of the duodenum to prostaglandins is lost by about day 21, since prostaglandins E_1 and E_2 have been shown to contract the longitudinal muscle of adult rat small intestine through a direct action on it (Bennett *et al.*, 1968).

An increase in muscle tone of the duodenum may be responsible for the change in response to ATP, causing contraction instead of relaxation. The response to ATP has been found to vary according to the tone of the preparation (Burnstock *et al.*, 1970). Cocks & Burnstock (1979) classified this tone in the case of the taenea coli of guinea-pig, based on the response to non-adrenergic, non-cholinergic inhibitory neurone stimulation and exogenous ATP as follows. Preparations have high tone if the response to either stimulus is relaxation without 'rebound contraction', those having medium tone give relaxation followed by 'rebound contraction' to each stimulus, while with low tone preparations, 'rebound contraction' occurs with cessation of the stimulus, with little or no relaxation during stimulation. Based on this classification, it may be concluded that with the progress of postnatal growth, duodenal muscle tone becomes higher and reaches a critical level, where a relaxant response to ATP would be evident, on about day 21. In this study, a contractile response to ATP, preceded by a relaxant response on day 14, was blocked by indomethacin. This seems to be related to the observation of a previous study that indomethacin inhibits rebound contraction on cessation of stimulation of non-adrenergic, non-cholinergic nerves or by the exogenous application of ATP to guinea-pig taenia coli (Burnstock *et al.*, 1975). However, contractile responses to ATP of rat duodenum on days 1 to 7 are not a rebound phenomenon, since exogenous ATP caused contraction without a time lag after adding ATP to the bath, and the contractile response to ATP always occurred in the presence of the nucleotide in the bath but not following its removal. Thus, ATP-induced contraction on day 14 is not a rebound phenomenon but a remnant of that observed at the neonatal stage.

Relaxant responses to adenosine were blocked by the P_1-antagonist, 8-PT, which indicates that adenosine stimulates the P_1-purinoceptor in rat duodenum, leading to relaxation. The involvement of adenosine or P_1-purinoceptors in the regulation of transmitter release from adrenergic, cholinergic and non-adrenergic, non-cholinergic nerves has been reported (Jager & Den Hertog, 1985). We showed that TTX and guanethidine did not affect the response to adenosine, which confirms that adrenergic nerves are not involved in the response to adenosine in rat duodenum. However, a further study should be performed to determine whether adenosine actually regulates transmitter release from myenteric neurones in rat duodenum.

Duodenal responses to AMP and ADP were essentially the same as those to adenosine and ATP, respectively, with respect to ontogenetic change and magnitude of response. This is consistent with the notion that adenosine and AMP are P_1-agonists and ADP and ATP, P_2-agonists (Burnstock, 1978). We thus propose that the smooth muscle of rat duodenum may possess separate purinoceptors for adenosine and AMP (P_1) and ADP and ATP (P_2), as has been indicated in a previous study with the smooth muscle of guinea-pig caecum (Brown & Burnstock, 1981; Satchell & Maguire, 1982; Ferrero & Frischknecht, 1983), and that the response to P_1- and P_2-agonists changes with the progress of development.

A remarkable degree of coordination has been found between the development of various gastrointestinal functions in rats and major developmental changes that occur during the third week following birth (Henning, 1981; Thomson & Keelan, 1986). The present study indicates that changes in the P_2-receptor-mediated response to purine nucleotides in rat duodenum also occur in accordance with this timing. Although the ontogenesis of nerves containing purine nucleotides has not been studied, the present results indicate that P_2-agonists such as ATP and ADP may affect the contractility of smooth muscle of the rat duodenum through synthesis of prostaglandins in neonatal rats before day 14, while at about postnatal day 21 they begin to act as transmitters of non-adrenergic, non-cholinergic inhibitory neurones, causing the appearance of myogenic relaxant responses.

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