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Age-dependent effect of secretagogues during colonic maturation

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Abstract

Intestinal secretory response is altered during colonic development. The aim of this report was to study the developmental changes of the Ca^{2+}- and cAMP-induced regulatory pathways with special attention to the direct and indirect effect of secretagogues on the colonic epithelium. We investigated the effect of bethanechol, 5-hydroxytryptamine (5-HT), and histamine on Cl^- secretion and stimulation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) and cAMP in the distal colon of suckling, weanling and adult rats. In the presence of tetrodotoxin, immature colon of suckling and weanling rats displayed higher potency (EC_{50}) of 5-HT to stimulate Cl^- secretion, whereas the potency of histamine was not changed during development. The potency of bethanechol was reduced during weaning and partially restored in adulthood. 5-HT increased cAMP level similarly in both neonatal and adult colonic crypts, but the adults had higher basal level of cAMP than suckling rats. Also the effect of bethanechol on $[Ca^{2+}]_i$ was independent of colonic maturation. The results suggest that colonic Cl^- secretion displays developmental changes of regulation depending on the non-neural secretagogue-signalling pathway and that these developmental changes seem to be localized somewhere outside colonocytes.

Keywords: Colonic secretion; Ontogeny; Ion transport development; Bethanechol; Histamine; 5-Hydroxytryptamine

1. Introduction

During the perinatal period the gastrointestinal tract undergoes a series of developmental changes including changes of intestinal secretion (Pácha, 2000). It is assumed that structural and functional immaturities of the intestinal cells are responsible for developmental changes in diarrheal response to bacterial (Chu and Walker, 1993; Lu et al., 2003) and viral enterotoxins (Morris and Estes, 2001) that cause the death of several millions of people (largely children) each year world-wide. The secretory diarrhea results from overstimulation of the intestinal tract's secretory pathways driven by active Cl^- secretion across the apical membrane of the enterocytes (Field, 2003). This secretion is controlled by neurons of the submucosal plexus and endocrine pathways via enterochromaffin and immune cells (Cooke, 1998; Field, 2003) that release a variety of paracrine substances having secretory activity (Barrett and Keely, 2000).

Although a number of studies have identified developmental changes in intestinal secretion (Cohen et al., 1986; Potter et al., 1987; McEwan et al., 1990; Grondahl et al., 1996) the progression from the "neonate" to the "adult" status was rarely investigated. Some studies have suggested marked changes of intestinal secretion in response to secretagogue stimulants such as 5-hydroxytryptamine (5-HT), histamine, carbachol, and vasoactive intestinal peptide (Bach and Carey, 1994; MacNaughton and Gall, 1994; Grondahl et al., 1996; Erlwanger et al., 1999). However, whether secretory changes occur directly at the level of epithelial cells (changes of sensitivity or secretory capacity) or indirectly at the level of submucous nerves or other cells of lamina propria is still unknown. The consideration of indirect effects seems to be critical for the interpretation of these findings since bethanechol and histamine have recently been shown to stimulate Cl^- transport in primary culture of enterocytes isolated from adult but not weanling animals

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(Venkatasubramanian et al., 2001) even if both transmitters were shown to stimulate Cl^- secretion in intact intestine (Bach and Carey, 1994; MacNaughton and Gall, 1994).

The knowledge of age-dependent changes of colonic secretion is important with respect to the treatment and understanding of inflammatory and toxigenic diarrhea and intestinal allergy in infancy. The aim of the present study was, therefore, to examine the developmental characteristics of Ca^{2+}- and cAMP-induced secretagogues in isolated colon and colonic crypts and re-evaluate their effect with special attention to the direct and indirect effects on the colonic epithelium.

2. Materials and methods

2.1. Animals

Wistar rats were bred and maintained in an air-conditioned room at 21 °C on a 12:12-h light–dark cycle. Standard rat chow and tap water for drinking were provided ad libitum (according to guidelines of the European Community). The day of birth was considered as day 0, and approximately 24 h after birth the litter size was reduced to eight pups that were housed with their dams until the age of 30 days. Three different ages were used in the experiments: 13- to 16-day-old rats (sucklings), 21- to 28-day-old rats (weanlings) and 80- to 100-day-old adults. Animal protocols were approved by the Institutional Animal Care Committee.

2.2. Electrophysiology

The suckling and weanling rats were killed by decapitation, adult rats by cervical dislocation. After removal, the distal colon was partially stripped and mounted in a modified Ussing chamber (window area 9.6 mm^2), bathed in standard Krebs–Ringer solution (composition in mM: 140.5 Na^+, 5.4 K^+, 1.2 Ca^{2+}, 1.2 Mg^{2+}, 119 Cl^-, 21 HCO_3^-, 0.6 $H_2PO_4^-$, 2.4 HPO_4^{2-}, 10 D-mannitol, 10 D-glucose, 2.5 L-glutamine, 0.5 β-hydroxybutyrate) gassed with carbogen (95% O_2+5% CO_2) and kept at 37 °C. After an equilibration period of 20–30 min the response to serosal histamine, bethanechol, or 5-HT was tested. Potential difference, tissue resistance and the short-circuit current (Isc) were measured by a computer-controlled voltage clamp. The changes of Isc were derived as maximum (peak to baseline) value. All experiments were performed in the presence of mucosal amiloride (10^{-5} M) and serosal tetrodotoxin (10^{-6} M) to inhibit electrogenic Na^+ absorption and enteric neuronal activity, respectively. This concentration of tetrodotoxin was chosen because it had been shown to inhibit neural stimulation of Isc (Biagi et al., 1990). In some experiments the effect of theophylline (1 mM), a phosphodiesterase inhibitor, and 2′,5′di(ter-butyl)-1,4-benzohydroquinone (0.1 mM; BHQ), an inhibitor of endoplasmic Ca-ATPase was tested.

2.3. Microfluorimetry of Ca^{2+}

Intact colonic crypts were isolated from distal colon by incubating an everted segment of the distal colon in ethylenediamine-tetraacetic acid followed by mechanical vibration as described earlier (Beskid and Pácha, 2003). The isolated crypts were transferred to a poly-L-lysine coated coverglass, allowed to settle and loaded with Fura-2/AM (2 μM) at room temperature. The Fura-2/AM was then washed away and the coverglass was mounted in an inverted microscope (Olympus IX 50) attached to CCD camera (Micromax, Princeton Instruments Inc., New Jersey, USA). Changes of $[Ca^{2+}]_i$ in isolated crypts were measured by ratiometry using dual wavelength excitation. The crypts were excited with a light beam from 100 W xenon lamp filtered through alternating 340 and 380 nm interference filters and their fluorescence examined under a 20× objective and directed to the camera through a 510 nm filter. Image acquisition and data analysis were performed with the software package Metamorph/Metafluor (Universal Imaging Corp., West Chester, Pennsylvania, USA).

2.4. Intracellular cAMP concentration measurement

Cyclic AMP generation was measured in isolated crypts or scraped colonic mucosa. The suspension of isolated crypts was incubated at 37 °C for 3 min with different concentrations of 5-HT (10^{-8}–10^{-4} M) in the presence of 3-isobutyl-1-methylxanthine (IBMX; 10^{-4} M) added 10 min prior to the addition of 5-HT in oxygenated MEM. In some experiments the partially stripped distal colon was incubated in the absence or presence of theophylline (1 mM) or forskolin (10 μM), then the mucosa was scraped off and frozen rapidly in liquid nitrogen. The specimens of crypts and scraped mucosa were homogenized, sonicated, 5% perchloric acid was added and the samples centrifuged. The supernatant containing extracted cAMP was neutralized with 1 M $KHCO_3$ (indicator: Bromocresol Purple) and measured by radioimmunoassay kit (Immunotech, Prague, Czech Republic) according to manufacturer's protocol. The pellet was analyzed for protein content using bovine albumin as a standard.

2.5. Morphometry of colonic surface

Semithin distal colon sections of suckling, weanling and adult rats were prepared as follows. After sacrificing the animal, a portion of distal colon just above the pelvic brim was removed, fixed in 10% buffered formalin, embedded in EPON 812 and stained with toluidin blue as previously described (Pácha et al., 2003). A stereological method of vertical sections (Baddeley et al., 1986; Vagnerová et al., 1997) was used for the estimation of the epithelial surface area. The vertical axis was chosen to be perpendicular to the colonic muscle wall (tunica muscularis externa) which was adopted as an internal "horizontal" reference. Semithin vertical sections were examined under a C.A.S.T.-Grid system for stereology (Olympus, Denmark) using an Olympus BX 50 microscope and 10× planapochromat objective. The cycloid test system was superimposed on the sections and the intersections of cycloid arcs with the epithelial surface and tunica muscularis externa wall were counted. Based on the formula for surface density estimation (Baddeley et al., 1986), the epithelial surface area factor (Fa) was estimated by the ratio of the number of intersections with the epithelial surface and the number of intersections with tunica muscularis externa wall. The factor Fa thus represented the multiplication of folded epithelial sheet in comparison with the smooth muscle tube area beneath. One microscopical specimen per animal and three to five cross-sections per specimen were evaluated (Fig. 1). The density of the cycloid system was chosen so that there were approximately 250 intersections between test lines and epithelium surface.

2.6. Data analysis

For ion transport studies, results were expressed as the maximum change in Isc response (ΔIsc, μA/cm^2) and all Isc values represent mean±S.E.M. from independent experiments unless stated otherwise. The secretagogue effect was characterized by Isc_{max} (maximum Isc response) and EC_{50} (the concentration of the agonist producing 50% of the maximum response) which were determined by a non-linear curve-fitting procedure using the curve (Elmhurst et al., 1997):

$$Isc = Isc_{\min} + (Isc_{\max} - Isc_{\min}) / \left(1 + e^{-k(\log\ c - \log EC_{50})}\right)$$

where Isc means estimation of the agonist's effect, k is a power coefficient and c is a molar concentration of the agonist. Comparison of concentration–response curves was achieved by repeated-measures analysis of variance (ANOVA) with one grouping factor (age) and one within factor (concentration). One-way ANOVA followed by Newman–Keuls multiple range test was used to analyze the effect of age on ISC_{max}, EC_{50}, amplification of mucosal surface, and effect of BHQ and theophylline. Student's t-test was used for further analysis. In all cases a value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Effect of agonists on Isc

In the presence of tetrodotoxin that eliminated the involvement of action potential-dependent release of neurotransmitters, the serosal addition of the cholinergic agonist bethanechol evoked a concentration-dependent increase of Isc (Fig. 2) in all age groups studied. However, the bethanechol potency to stimulate Isc (as reflected by EC_{50}) was the highest in the immature colon of suckling rats and decreased later (Table 1). In contrast, the maximum response (Isc_{max}) evoked by bethanechol was higher in weanlings compared to both sucklings and adults (Table 1). The potency of 5-HT to increase Isc was also lower in adulthood (Fig. 3), but the drug was similarly effective in suckling and weanling rats. Half-maximal effective concentration of 5-HT was fourfold higher in mature than in immature colon (Table 1). The maximum response to 5-HT was not changed during the development. In contrast to bethanechol and 5-HT, the effect of the inflammatory mediator histamine was identical in both immature and mature colon (Fig. 4, Table 1).

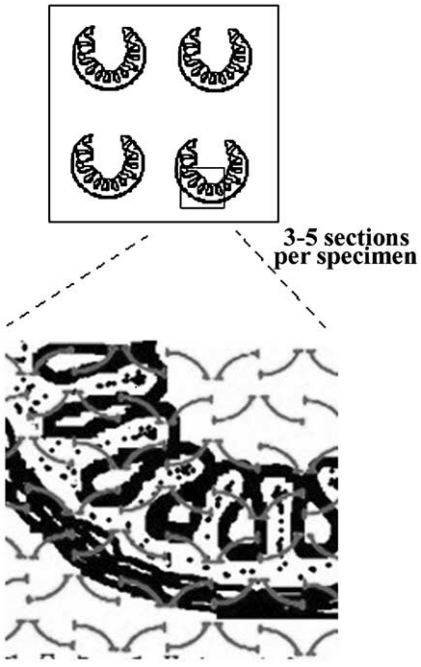

Fig. 1. Illustration of colonic vertical section with a superimposed test system. The epithelial surface area amplification factor was estimated by counting intersections of cycloid arcs with the epithelial surface and with the muscle wall (*tunica muscularis externa*).

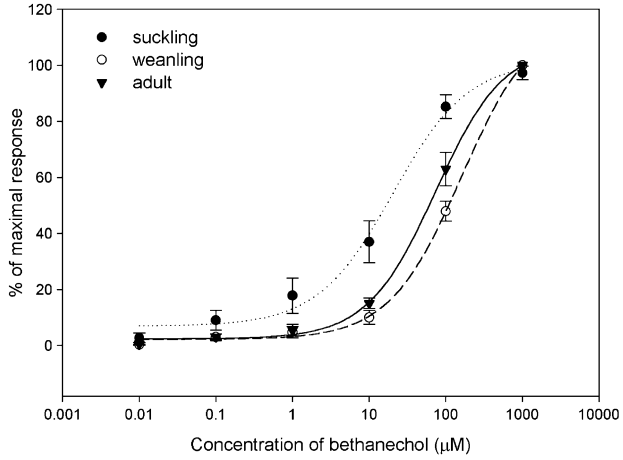

Fig. 2. Concentration-dependent response of the colonic short-circuit current (ΔIsc) to bethanechol in distal colon of suckling, weanling, and adult rats. In each case the maximum response in particular stage of development was expressed as 100%. Age of animals was associated with significant changes of Isc response to bethanechol (repeated-measured ANOVA, $P<0.002$; 6–9 experiments in each group).

Table 1
Summary of the effects of secretagogues on distal colon of suckling, weanling and adult rats

	Suckling		Weanling		Adult	
	EC_{50}	Isc_{max}	EC_{50}	Isc_{max}	EC_{50}	Isc_{max}
Bethanechol	38±7	93±8	411±101[a]	287±19[a]	96±29[b]	134±18[b]
5-HT	2.1±0.9	90±11	1.8±0.4	74±10	8.6±1.6[a,b]	119±15
Histamine	36±14	98±16	20±6	96±14	25±6	82±6

Values are expressed as means±S.E.M. EC_{50}, concentration of the agonist that produces a half-maximal response (in μM); Isc_{max}, the maximum response of Isc to the agonist (in μA/cm^2). Both parameters were calculated according to the equation described in Materials and Methods from concentration–response curves using absolute values (in μA/cm^2) of peak changes of Isc induced by the secretagogues. The mean values of power coefficient k were similar in suckling, weanling, and adult animals, respectively (bethanechol: 2.48, 1.72, 2.24; 5-HT: 2.04, 1.99, 1.75; histamine: 3.86, 2.90, 3.63). Comparison was made by one-way ANOVA (bethanechol: Isc_{max}, EC_{50}, $P<0.001$; 5-HT: EC_{50}, $P<0.001$) and the significance of the difference between individual means was determined by Newman–Keuls test; [a]significantly different from sucklings ($P<0.005$) and [b]weanlings ($P<0.005$). There was not any significant difference in power coefficient k during development.

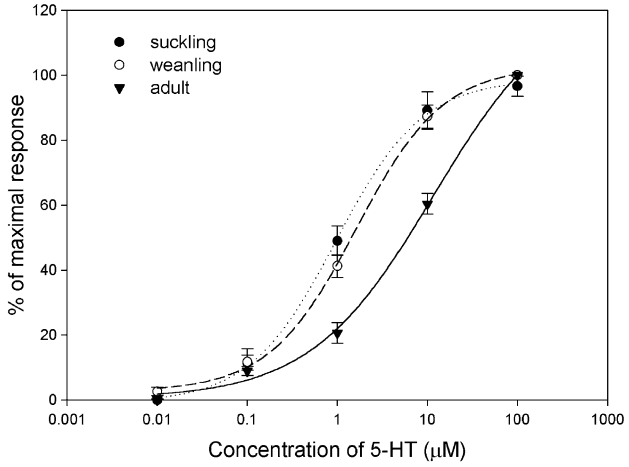

Fig. 3. Concentration-dependent response of the colonic short-circuit current (ΔIsc) to 5-hydroxytryptamine (5-HT) in suckling, weanling, and adult rats. In each case the maximum response in particular stage of development was expressed as 100%. Age of animals was associated with significant changes of Isc response to 5-HT (repeated-measured ANOVA, $P<0.001$; 6–8 experiments in each group).

To identify the charge-carrying ions involved in response to bethanechol, 5-HT and histamine, the furosemide treatment and Cl^- substitution experiments were performed in suckling and adult rats. As shown in Table 2 the replacement of chloride salts with their equivalent gluconate salts as well as the presence of the blocker of $Na^+/2Cl^-/K^+$ cotransporter (furosemide), reduced significantly the response to all three secretagogues. This indicates that the electrical response is primarily a result of electrogenic Cl^- secretion.

To evaluate whether the changes of the secretory effect of bethanechol and 5-HT reflect the developmental changes of cAMP and $[Ca^{2+}]_i$ regulatory pathways in colonocytes we measured Isc effects of theophylline (1 mM), a phosphodiesterase inhibitor, or 2′,5′-di(ter-butyl)-1,4-benzohydroquinone (BHQ, 0.1 mM), the inhibitor of endoplasmic reticulum Ca^{2+}-pump. As shown in Fig. 5 both drugs stimulated Isc in all three age groups and the response was highest in fully matured colon. Cyclic AMP content in colonic mucosa exhibited a developmental pattern. Basal cAMP content was 40% lower in immature colon than in adulthood and the inhibition of cAMP hydrolysis by theophylline accounted for more than two-fold increase of cAMP in immature colon and less than 50 % in fully matured colonic mucosa. In contrast, the effect of forskolin was comparable in both mature and immature mucosa (Table 3).

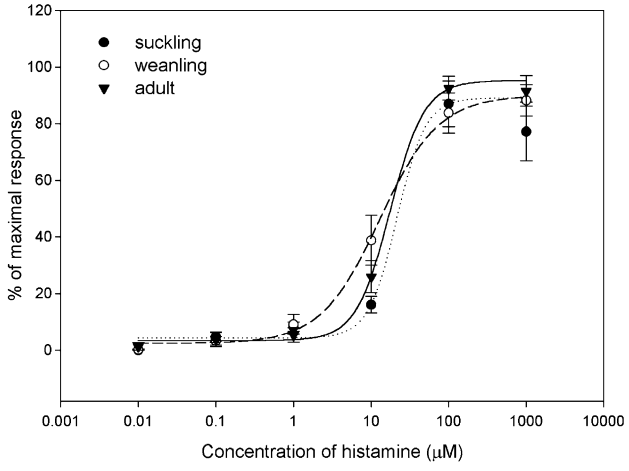

Fig. 4. Concentration-dependent response of colonic short-circuit current (ΔIsc) to histamine in suckling, weanling, and adult rats. In each case the maximum response in particular stage of development was expressed as 100%. No significant effect of age (repeated-measured ANOVA, $P>0.5$; 6–8 experiments in each group).

Table 2
Short-circuit current response (ΔIsc) of distal colon to secretagogues in the presence and absence of Cl^- and furosemide

	Controls		Cl^--free		Furosemide	
	Suckling	Adult	Suckling	Adult	Suckling	Adult
Bethanechol	58±7	66±10	13±5[a]	10±5[a]	22±4[a]	18±5[a]
5-HT	65±8	59±8	9±3[a]	12±4[a]	27±4[a]	21±4[a]
Histamine	65±10	74±9	8±3[a]	13±3[a]	30±4[b]	24±5[a]

Data are expressed as changes of the short-circuit current in μA/cm^2 after administration of the secretagogue (0.1 mM). Furosemide was added to the serosal compartment to reach a concentration 0.1 mM. Data are given as means±S.E.M.; numbers of experiments were 4–6 in each group. [a]$P<0.01$ or [b]$P<0.05$ from control values.

3.2. Morphometry of colonic epithelium

The colonocytes involved in secretion are the cells of the colonic crypts that undergo rapid growth in early postnatal life (Pácha et al., 2003). Therefore, the comparison of colonic secretory capacity must consider the development of colonic surface due to crypt growth and not only the surface roughly corresponding to that of a smooth-bore tube. We found that the relationship between mucosal surface (envisaged as the surface displayed by folding epithelium due to crypts) and colonic surface (envisaged conventionally as the tunica muscularis externa), which is used in normalization of Isc, differs during the development. The enlargement factor (mucosa surface/colonic surface) was similar in weanling and adult rats (weanlings: 3.17±0.04; adults: 3.15±0.07) but was significantly lower in suckling rats (2.54±0.09; $P<0.001$, ANOVA). The crypt epithelium of suckling rats is therefore only 80% of that of weanling or adult animals. Thus the values of the electrophysiological data obtained in sucklings should be taken to be 1.25 times higher.

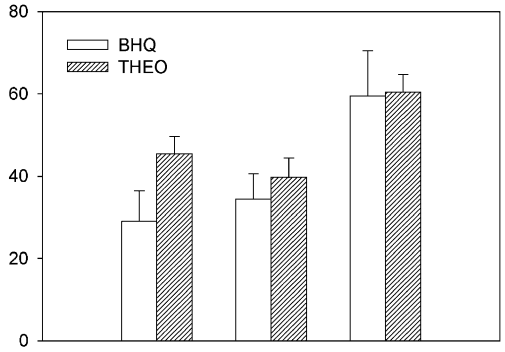

Fig. 5. Developmental pattern of the effect of 0.1 mM BHQ and 1 mM theophylline (THEO) on short-circuit current (ΔIsc) in rat distal colon. Values are means±S.E.M. of 6–9 (BHQ) or 18–26 animals (theophylline). *Significantly different from adult animals ($P<0.05$).

Table 3
Effect of theophylline and forskolin on cAMP in colonic mucosa

	Suckling	Adult
Basal	7.1±1.4 (10)	12.2±1.8 (8)[a]
Theophylline (1 mM)	15.8±2.4 (9)[b]	17.8±2.2 (8)
Forskolin (10 μM)	80.0±20.4 (10)[b]	94.5±16.0 (8)[b]

Values are given as means±S.E.M. The level of cAMP is given in pmol/mg protein. Student's *t*-test was used to compare basal and stimulated values. [a]$P<0.05$ compared with suckling rats; [b]$P<0.01$ compared with basal cAMP values of untreated tissue.

3.3. Effect of bethanechol on intracellular Ca^{2+}

We next investigated whether the age-specific differences in stimulation of colonic secretion by bethanechol could be due to the developmental changes of Ca^{2+}-response pathway. We therefore compared the effect of bethanechol on $[Ca^{2+}]_i$ in crypts isolated from weanling and adult distal colon. In the resting state, $[Ca^{2+}]_i$ was low (below 100 nM) and a gradient of intracellular Ca^{2+} distribution was observed in colonocytes of mature crypts along the crypt–villus axis (Table 4). The application of bethanechol evoked significant but transient $[Ca^{2+}]_i$ response with a rapid onset and subsequent return to the resting level within several minutes despite the presence of the drug. The effect of the drug was most obvious in the basal region of the crypts (Table 4) and the pattern of axial heterogeneity was similar in both weanling and adult rats. The weanling crypts failed to exhibit higher $[Ca^{2+}]_i$ response to bethanechol than the crypts of adult animals (Table 4). Similarly to our data summarized in Fig. 2 and the data of Nitschke et al. (1993) on HT-29 colonic cells, the distinct effect of bethanechol on $[Ca^{2+}]_i$ was seen only at bethanechol concentrations above 10^{-5} M.

3.4. Effect of 5-HT on intestinal crypts

To determine whether the developmental changes in the action of 5-HT depend on the transduction system, the effect of 5-HT on $[Ca^{2+}]_i$ and cAMP production was examined. Although 5-HT receptors coupled with $[Ca^{2+}]_i$ were reported in intestinal cells, we did not find any modulation of intracellular Ca^{2+} in colonic crypt cells by 5-HT (data not shown). In contrast, 5-HT stimulated cAMP level in colonic crypts in a concentration-dependent manner

Table 4
Effect of bethanechol on $[Ca^{2+}]_i$ in the crypts isolated from weanling and adult rats

		$[Ca^{2+}]_i$	$\Delta[Ca^{2+}]_i$		
			10^{-5} (M)	10^{-4} (M)	10^{-3} (M)
Weanling	Base	61±8 (13)	64±5 (6)[a]	174±22 (10)[a]	344±60 (8)
	Middle	53±5 (13)	34±4 (6)[b]	85±13 (9)	139±38 (9)[c]
	Mouth	47±4 (12)	37±5 (5)	61±7 (10)	93±19 (9)[c]
Adult	Base	84±7 (24)	92±9 (6)[a]	177±17 (14)	467±47 (15)[e]
	Middle	63±6 (20)[d]	61±10 (6)[a]	105±15 (11)	197±26 (11)[c]
	Mouth	52±6 (18)[d]	51±9 (5)	72±16 (11)	158±21 (10)[c]

Values are given as means±S.E.M. $[Ca^{2+}]_i$, resting level of intracellular Ca^{2+} in the lower, middle and upper part of the crypt in nM; $\Delta[Ca^{2+}]_i$, the peak increase of intracellular Ca^{2+} (in nM) in response to various concentrations of bethanechol. ANOVA showed a significant effect of age ($P<0.011$), spatial distribution along the crypt ($P<0.0001$), and bethanechol concentration on $[Ca^{2+}]_i$ ($P<0.0001$). [a]$P<0.01$ or [b]$P<0.05$ compared with the effect of 10^{-3} M bethanechol; [c]$P<0.01$ or [d]$P<0.05$ compared with the crypt base; [e]$P<0.01$ compared with weanlings.

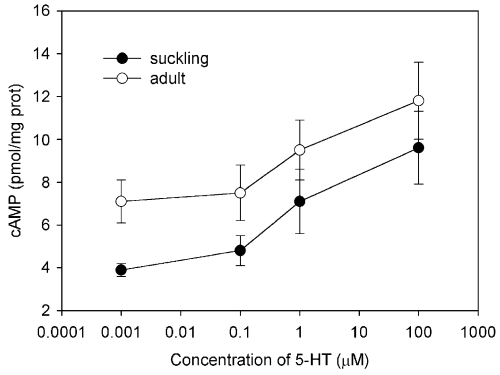

Fig. 6. Concentration–response curve for 5-hydroxytryptamine-induced cAMP level in isolated crypts of suckling and adult rats. Basal concentration of cAMP in untreated crypts of suckling and adult crypts were 3.9±0.3 and 7.3±1.0 pmol/mg prot., respectively (ANOVA: $P>0.38$; 6–7 experiments in each group).

compared to cells incubated with IBMX alone; the stimulatory effect of 5-HT was similar in colonic crypts of both immature and mature rats (Fig. 6; $P>0.38$). Similarly as colonic mucosa the colonic crypts had also significantly increased content of cAMP in adult rats ($P<0.05$).

4. Discussion

This study was designed to elucidate ontogenetic changes of epithelial ion secretion in rat intestine. A comparison of three secretory agents, the muscarinic agonist bethanechol, the mediator of enterochromaffine cells 5-HT and the immunomodulator histamine in the colon of suckling, weaning, and adult rats demonstrated that the neuroimmune regulation of colonic secretion depends on colonic maturation. Whereas histamine effect was not changed during the development, the effect of other two secretagogues has been clearly shown to be age-dependent in both potency and efficacy. The potency for 5-HT was particularly high in immature colon and decreased later when 5-HT was 4–5 times less potent in stimulating Cl^- secretion. Similar developmental decrease was described earlier for the stimulation of colonic smooth muscle contraction by 5-HT (Yagi et al., 1991). In contrast to 5-HT, the potency for bethanechol was dramatically decreased during weaning and partially restored in adulthood and its efficacy in stimulating Isc was significantly increased during weaning period.

However, in view of the functional relevance of the crypt epithelium as the principal compartment responsible for colonic secretion and crypt growth during early postnatal development (Pácha et al., 2003), it is obvious that the efficacy of all three secretagogues in stimulating Isc in immature colon is higher due to the smaller area of crypt epithelial surface over which secretion occurs. Because the crypt length increases during early postnatal period the mucosal surface cannot be defined as the surface roughly corresponding to that of a smooth tube. Considering the

time-dependent crypt amplification of the mucosal surface due to crypt growth the secretory capacity in immature colon must be higher than in adulthood, i.e. the ratio ΔIsc/ functional epithelial surface must be higher in immature than in mature colon. Consequently, the values given in Table 1 are 1.25 times higher in young rats than in adult animals. Our morphometric analysis proved that the colonic surface usually considered as an unmodified (smooth) intestinal tube should be amplified by a factor of 2.5 in suckling and 3.2 in adult animals. It is not necessary to consider further enlargement of the mucosal surface due to microvillous surface area because our earlier experiments revealed that this area does not undergo developmental changes (Vagnerová et al., 1997).

The heterogeneity of the responses to the secretagogues tested indicates that the developmental changes of their effects are not due to general alterations of colonic transport during postnatal development but represent more likely developmental changes of specific regulatory networks associated with intestinal secretion. Because our data were recorded in the presence of tetrodotoxin, the observed developmental changes imply that the responses are not mediated by neural receptors and do not involve activation of enteric neuronal pathway (Biagi et al., 1990). This suggests that the changes of the tetrodotoxin-resistant response to secretagogues might be mediated by developmental changes at the level of enterocytes or indirectly by developmental changes associated with other cells of *lamina propria* releasing paracrine messengers to amplify or restrain secretory activity. Such effect of proinflammatory cells of lamina propria or myofibroblasts of the pericryptal sheath has already been demonstrated (Beltinger et al., 1999).

The developmental changes of the secretory pathways regulated by bethanechol or 5-HT may therefore involve a direct effect of the secretagogues at the level of the secretory epithelial cells or an indirect effect via non-neural indirect component. In enterocytes, bethanechol has been shown to utilize $[Ca^{2+}]_i$-signalling pathway whereas 5-HT acts via the cAMP-pathway (Budhoo et al., 1996; Albuquerque et al., 1998; Field, 2003). If changes in receptor–effector coupling are responsible for the developmental changes of Cl^- secretion, then Ca^{2+} mobilization by bethanechol or cAMP stimulation by 5-HT would be expected to be age-dependent. We demonstrated the presence of functional Ca^{2+} stores in colonic enterocytes throughout postnatal life by addition of BHQ and showed that the release of Ca^{2+} in isolated crypts exposed to bethanechol did not correlate with developmental changes of bethanechol-induced Isc. The drug induced a diffuse release of Ca^{2+} throughout the crypts, albeit more prominent at the crypt base; hence the gradient of responsiveness to cholinergic stimulation in immature colon is similar to that in adult rats (Lindqvist et al., 1998). Since BHQ, which bypasses the muscarinic signalling components and directly increases cytosolic Ca^{2+}, proved a developmental increase of Isc and the response of $[Ca^{2+}]_i$ to bethanechol was not increased in weanling animals, it is obvious that the developmental pattern of bethanechol-induced secretion is not due to the changes of cytosolic Ca^{2+}-signal in intestinal cells.

Several groups have proposed a model where the cholinergic secretory effect is mediated through Ca^{2+}-activated K^+ channels with subsequent enterocyte hyperpolarization and increase in the driving force for Cl^- exit through non-stimulated cAMP-dependent apical Cl^- channels (CFTR), (Schultheiss and Diener, 1998). Using patch–clamp analysis we have recently shown that the effect of bethanechol on membrane potential of crypt cells is similar in immature and mature crypts but the maturation of crypts is associated with decreasing K^+ conductance (Beskid and Pácha, 2003). Thus the developmental changes of the driving force can be excluded. As Cl^- secretion requires cooperativity of Ca^{2+}- and cAMP-activated membrane conductances, the actual effect of cholinergic mediators in enterocytes is affected by cAMP production and breakdown (Mall et al., 1998) and the expression of CFTR channels. Unfortunately, the data about CFTR and cAMP levels in colonocytes during intestinal maturation are absent. Our measurement of cAMP values in intestinal mucosa and in isolated crypts indicated that intracellular cAMP levels are significantly lower in the immature colon than in the adult one. Therefore, it is unlikely that cAMP participates in developmental changes of bethanechol-induced secretion. The decreased cAMP level in our experiments seems to reflect the increased activity of intestinal phosphodiesterases (PDE) because their inhibition by theophylline produced much higher increase of cAMP in mucosa of immature colon than in adulthood. A similar pattern of developmental decrease of cAMP–PDE activity was found in enterocytes of the small intestine (Chastre et al., 1987).

Similarly, the developmental changes of 5-HT-dependent Cl^- secretion can hardly be explained by coupling of receptor and second messenger production system. Although the existence of $5HT_2$ receptors coupled to inositol 1,4,5-trisphosphate/diacylglycerol (IP_3/DAG) was proved in intestinal mucosa (Siriwardena et al., 1993), we have not observed any increase in cytosolic Ca^{2+} level even if bethanechol elicited an increase of $[Ca]_i$ in the same crypt similar to the findings of Hardcastle et al. (1999) in mature mouse crypts. In contrast, our data indicate that 5-HT produces an increase of cAMP. It has been recently shown that $5\text{-}HT_4$ receptors activate cAMP production in rat colonocytes (Albuquerque et al., 1998) and are responsible for tetrodotoxin-insensitive stimulation of Isc by 5-HT in mature rat colon (Budhoo et al., 1996). To determine whether maturation alters sensitivity of the colon to 5-HT and the magnitude of cAMP response we examined the dose–response effect of 5-HT on cAMP in both immature and mature crypts. Even if the basal levels of cAMP were lower in immature epithelium, the sensitivity of cAMP production to 5-HT was not changed during postnatal development. Although higher level of intracellular cAMP was described to increase the sensitivity of the colonic

mucosa to 5-HT (Scott et al., 1992), it is obvious that this is not the mechanism operating in colonocytes of young animals and the developmental shift of 5-HT concentration–response curve observed in our experiments cannot be explained by developmental changes of cAMP metabolism.

Even if CFTR expression is unknown in colonocytes during maturation, the similar efficacy of histamine together with the dissimilar efficacy of bethanechol and 5-HT during postnatal development indicate that the developmental amplification/attenuation of the secretory effect of bethanechol and 5-HT must be localized somewhere out of enterocytes via some paracrine secretory stimuli. Since stripped colonic tissue which has no myenteric plexus was used, it is likely that bethanechol and 5-HT interact not only with enterocytes but also with the submucosal plexus or other cells of lamina propria. The secretory response of enterocytes has been shown to be enhanced by the presence of fibroblasts that possess both cholinergic and serotonergic receptors (Powell et al., 1999; Beltinger et al., 1999) and are the major site of intestinal synthesis of prostaglandins (Craven and DeRubertis, 1983). Similarly, cholinergic regulation has also been described in gut immune cells including mast cells (Blandina et al., 1980) and lymphocytes (Qiu et al., 2004). In addition, the interaction of secretagogues with some enteric neurons cannot be completely excluded. Tetrodotoxin-insensitive, likely secretomotor neurons, have been recently described in guinea-pig distal colon similarly as the action potential-independent release of transmitters (Schneider and Galligan, 2000; Lomax et al., 2001).

In summary, our data show that non-neural regulatory pathway of colonic secretion is present already in immature colon and that developmental patterns for particular secretagogues are not identical. It seems reasonable to suggest that these differences are determined not only by the ontogeny of colonic epithelium but also by indirect paracrine signals that may cooperate with direct effect of secretagogues at the level of secretory enterocytes.

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