An approach to intestinal permeability

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Section II

MODULATION OF ELECTROLYTE AND MACROLECULAR PERMEABILITY IN ANIMAL MODELS

Chapter 7

COMBINED AND SEPARATE EFFECTS OF CARBACHOL AND ANTIGEN ON MACROMOLECULAR PERMEABILITY IN VITRO IN THE JEJUNUM OF COW'S MILK SENSITIZED GUINEA PIGS

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ABSTRACT

It has been shown in isolated rat ileum that carbachol, via the muscarinic receptor, induced an increase of the permeability for macromolecules via the paracellular pathway. Increased horseradish peroxidase (HRP) transport through intestinal tissue from cow's milk sensitized guinea pigs after *in vitro* challenge with β-lactoglobulin has been documented as well.

The aim of the present study was to determine whether carbachol could also increase permeability for HRP in isolated small intestine of the guinea pig and if so, whether the presence of carbachol could increase the effects of sensitizing antigens at the mucosal side of sensitized intestinal tissue. Furthermore we asked whether the effects of carbachol and antigen challenge on macromolecular permeability could be cumulative. We used isolated and stripped jejunum of guinea pigs that were i.p. sensitized for cow's milk and *in vitro* challenge at the mucosal or serosal side with β-lactoglobulin with or without previous activation with serosally added carbachol. We measured the transepithelial short circuit current, the conductance and the flux of enzymatically active HRP taken as a bystander protein. We found that carbachol can increase the permeability for HRP in guinea pig small intestine by increasing paracellular permeability and endosomal uptake. Application of β-lactoglobulin also activated these pathways. No additive effect was found for carbachol (10^{-4} M) and β-lactoglobulin (0.4 mg/ml) at the used concentrations.

We conclude that the epithelium of the isolated guinea pigs jejunum is spontaneously activated by endogenous release of acetylcholine, leading to increased transepithelial permeability for macromolecules and increased short circuit current. We suggest that this high cholinergic tonus may underly the susceptibility of guinea pigs to oral sensitization for food allergens.

INTRODUCTION

The guinea pig is frequently used as an animal model in studies concerning food allergy, in particular cow's milk allergy, since its introduction as such by Baird and co-workers (Cuthbert et al. 1983, Baird et al. 1984, Baron et al. 1988). One of the reasons is their finding that it is possible to induce immunological sensitization to cow's milk proteins in this species by simply feeding them cow's milk for about 20 days. Repeated intra-peritoneal (i.p.) injection with cow's milk is another way to induce sensitization in guinea pigs (like in other laboratory animals as mice and rats, in which oral sensitization procedures are hardly successful).

Both the oral and i.p. sensitization to cow's milk can be detected by an immediate hypersensitivity reaction of the guinea pigs' small intestine and colon. Isolated, muscle-free preparations challenged at the serosal side with the cow's milk protein β-lactoglobulin, show a transient increase of the short circuit current (Isc) in the Ussing chamber. The primary step in the hypersensitivity reaction is the ß-lactoglobulin-induced crosslinking of immunoglobulins bound to mucosal mast cells, which results in degranulation of these cells. Released mast cell products can induce the epithelial secretory response by a direct action on epithelial receptors, but also via activation of enteric nerves. The latter is deduced from the fact that part of the secretory response could be blocked by tetrodotoxin, a blocker of neuronal action potentials (Baird and Cuthbert, 1987). In case of mucosal application of the antigen, the epithelial barrier will limit the permeation of the antigen and therefore mast cell degranulation and secretory response will be reduced as compared to serosal application.

Another effect of β-lactoglobulin induced mast cell degranulation is the increased permeability to macromolecules as demonstrated by Heyman et al. (1990) with guinea pig jejunum *in vitro*, for the intact macromolecular probe horseradish peroxidase (HRP, MW 40kD).

In rats sensitized to ovalbumin, *in situ* jejunal challenge induced an increased permeability to the antigen itself (MW 45 kD) and to the permeability marker ^{51}Cr-EDTA with a MW of 342 D (Ramage et al. 1988). The increased permeability upon luminal ovalbumin challenge could partly be blocked by TTX, indicating that nerves are also involved in antigen-induced hypersensitivity reactions in rats. Interestingly, in Ussing chamber experiments with isolated jejunum of rats, it appeared that the Isc-response to serosal challenge was not affected by TTX while the response to luminal challenge was strongly inhibited (Crowe et al. 1990). More specific, cholinergic neurons are thought to play a role in the anaphylactic response (Javed et al. 1992).

With rat ileum *in vitro*, we (Bijlsma et al. 1996) have shown that carbachol, a muscarinic analogue of the neurotransmitter acetylcholine, caused an increased permeability to intact HRP, even in the presence of TTX. This occurred via an increased endocytosis as well as via an induction of paracellular leak to this protein. We speculated that cholinergic stimuli may cause an increased permeability for luminal food antigens, possibly leading to sensitization to luminal antigens in naive animals and to increased mast cell degranulation in sensitized animals. This in turn may lead to an amplified and/or sustained loss of barrier function to potentially antigenic luminal substances via a direct effect of the mast cell products on the epithelial cells or via stimulation of the release of acetylcholine and probably other transmitters from neuronal tissue.
Therefore, it was of interest to study the effect of carbachol on intestinal barrier function and whether it could modulate the responses to luminally or serosally applied antigens in sensitized guinea pigs. The findings that carbachol addition (Bijlsma et al. 1996) and mast cell degranulation (Cooke et al. 1984) may cause the release of neurotransmitters, prompted us to do these experiments in the presence of TTX in order to limit neuronal activity as far as possible.

METHODS

Immunization procedure Female guinea pigs (Dunkin-Hartley) aged 6 weeks were sensitized to cow's milk by i.p. injection of 0.5 ml fresh pasteurized cow's milk, followed by a booster with the same amount of cow's milk on day 15. Experiments were conducted from days 25 to 30.

Electrophysiological studies Animals were killed by i.p. injection of Na-pentobarbital (120 mg/kg), the small intestine was rapidly removed and rinsed of intestinal contents with ice-cold Ringer's. Segments of proximal jejunum free of Peyer's patches were stripped of muscle layers and mounted in Ussing chambers with an exposed surface area of 0.5 cm^2. Both sides of the epithelium were perfused with Ringer's solution, gassed with humidified 5% CO_2 + 95% O_2. The Ringer's composition was (in mM) Na^+ 140, K^+ 5.2, Ca^{2+} 1.2 Mg^{2+} 1.2, HCO_3^- 25, HPO_4^{2-} 2.4, $H_2PO_4^-$ 0.4, l-glutamine 2. After carbogenation the pH was 7.4 and the osmolality 290 mOsm/kg. Solutions were maintained at 37 °C with water jackets and recirculated (total volume 12 ml on either side) by a gas lift. The mucosal and serosal bathing solutions were connected

via 3M KCl-agar bridges to calomel electrodes for measurement of transepithelial potential difference and to Ag-electrodes for current passage through the system. The tissue was kept under short-circuit conditions by an automatic clamping device that corrected for fluid resistance (World Precision Instruments DVC 1000, New Haven, Connecticut, USA). The short-circuit current was continuously recorded. The ionic conductance was calculated from current deflections evoked by voltage pulses of 0.5 mV of 1 second duration every 60 seconds.

Flux measurements After a 20 minutes equilibration period, TTX (Sigma) was added to the serosal compartment to a final concentration of 10^{-6} M. Carbachol (final concentration 10^{-4} M) was added serosally to some of the tissues 30 minutes later (t = 50 min). Atropine (Sigma) was added to the serosal compartment to a final concentration of 10^{-5} M. Flux studies were started at t = 110 minutes. HRP (Sigma, type VI) was added to a final concentration of 10^{-5} M (0.4 mg/ml) mucosally. β-lactoglobulin (Sigma, type V) was simultaneously added to either the mucosal or the serosal side to a final concentration of 0.4 mg/ml. Serosal samples of 200 µl were taken at t = 120, 150 and 180 minutes for HRP detection and were replaced by oxygenated Ringer. The appearance of HRP in the serosal bath was measured enzymatically as described previously (Heyman et al. 1982).

Electron microscopy In some of the flux experiments tissues were fixed immediately after the flux measurements in 2,5 % glutaraldehyde in 0.1 M sodium-cacodylate buffer (pH 7.4) for 2 h at room temperature and rinsed overnight (4 °C) in 0.1 M sodium-cacodylate buffer (pH 7.4) and 3 times for 5 min each in 0.05 M TRIS buffer (pH 7.6). Peroxidase activity was histochemically demonstrated using the method of Graham and Karnovsky (1966). The tissue was preincubated for 15 min in 5 mg diaminobenzidine in 10 ml 0.05 M TRIS buffer (pH 7.6, 22 °C) and subsequently incubated 15 min in the same buffer containing 0.01% H_2O_2. Tissues were then processed for routine electron microscopy.

Statistical analysis Statistical significance was tested by Wilcoxon's signed rank test for paired observations or by the Mann-Whitney U test for unpaired observations as indicated, using one-tailed P-values.

RESULTS

The short-circuit current (Isc) and the conductance (G) of the jejunal segments stabilized at 30 minutes after the addition of 10^{-6} M TTX to respectively 8.4 ± 2.9 $\mu A/cm^2$ and 25.0 ± 2.7 mS/cm^2 (mean ± s.e.m.; N = 11 animals). After the addition of 10^{-4} M carbachol a peak value in Isc was reached within 2 minutes: Isc = 63.2 ± 7.8 $\mu A/cm^2$. Thereafter Isc values gradually returned to baseline values within 60 minutes. At this timepoint Isc and G were respectively 13.9 ± 3.3 $\mu A/cm^2$ and 26.4 ± 3.3 mS/cm^2 in carbachol-exposed tissues. In control tissues Isc at this time point was somewhat lower (6.6 ± 2.6 $\mu A/cm^2$). The G values at this timepoint were not different: 25.6 mS/cm^2.

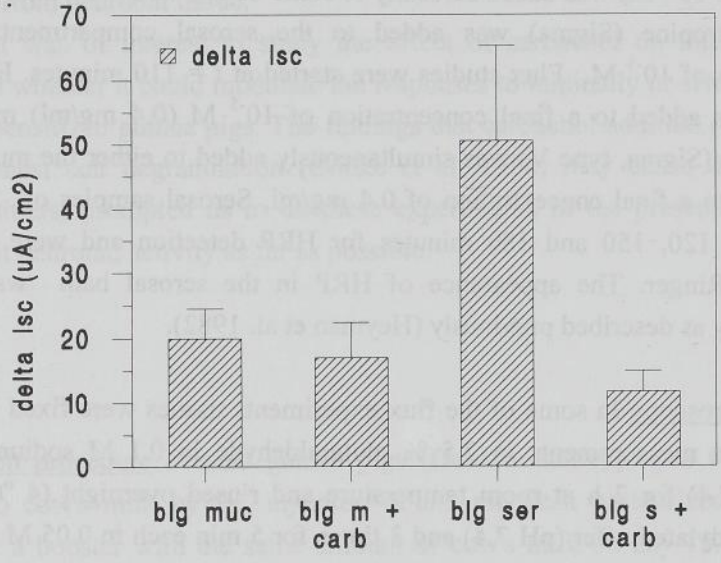

Fig 1. Changes in Isc induced in isolated and stripped jejunum by β-lactoglobulin at the mucosal side, by β-lactoglobulin at the mucosal side 60 min after serosal carbachol, by β-lactoglobulin at the serosal side, and by β-lactoglobulin at the serosal side 60 min after serosal carbachol. For significance of differences: see text.

The peak Isc values after mucosal or serosal addition of ß-lactoglobulin are shown in Fig. 1. In the absence of carbachol, serosal addition of ß-lactoglobulin caused a significantly larger Isc respons than mucosal addition ($p < 0.01$). In contrast, in carbachol treated tissues, serosal addition of β-lactoglobulin caused an Isc response comparable to mucosal addition ($p > 0.1$). Isc responses to mucosal addition of ß-lactoglobulin without or after previous application of carbachol were comparable ($p > 0.1$), but Isc responses to serosal addition of ß-lactoglobulin after carbachol were much smaller than without carbachol ($p < 0.001$). Irrespective of the absence or

presence of carbachol, peak Isc responses to serosal ß-lactoglobulin were reached in about 2 minutes and to mucosal ß-lactoglobulin in about 5 minutes.
Prior addition of carbachol may reduce the epithelial secretory response upon subsequent stimulation by the mast cell mediator histamine, as reported in T-84 intestinal cell lines (Kachintorn et al. 1993). Therefore, serosal application of histamine in tissues that were not exposed to ß-lactoglobulin was performed without or after induction of the electrophysiological response to carbachol. (These experiments were done between t = 180 and t = 240 minutes with tissues that were used for measurements of the HRP flux under control conditions.) Isc responses to serosally added histamine (10^{-4} M) after Isc was recovered from prior application of carbachol was 3.4 ± 1.0 $\mu A/cm^2$, while without carbachol the response was significantly larger: 25.1 ± 5.5 $\mu A/cm^2$ ($p < 0.002$, n=10).

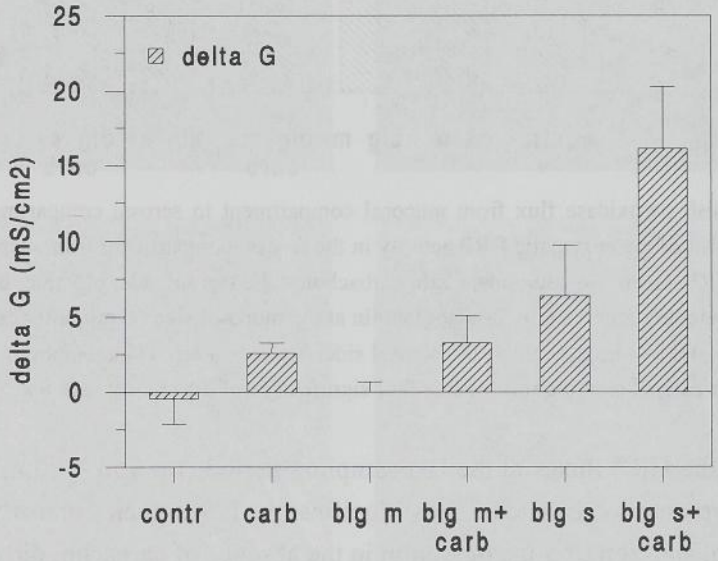

Fig 2. Time dependent change in conductance from t =110 min to t =180 min. Contr: no additions; Carb: carbachol at the serosal side; blg muc: β-lactoglobulin at the mucosal side; blg muc + carb: β-lactoglobulin at the mucosal side 60 min after carbachol at the serosal side; blg ser: β-lactoglobulin at the serosal side; blg ser + carb: β-lactoglobulin at the serosal side 60 min after carbachol at the serosal side. For significance of differences: see text.

Fig. 2 shows the change in conductance from the start to the end of the HRP-flux measurements (from t = 110 to t = 180 minutes). Values in the presence of carbachol were comparable to controls and to values after mucosal addition of ß-lactoglobulin. Serosal application of ß-lactoglobulin showed a tendency to increase the conductance, but this did not reach significance. However, serosal addition of ß-lactoglobulin in the

presence of carbachol showed a significant increase in conductance, compared to controls ($p < 0.05$), to carbachol alone ($p < 0.05$) and also to serosal ß-lactoglobulin in the absence of carbachol ($p < 0.05$).

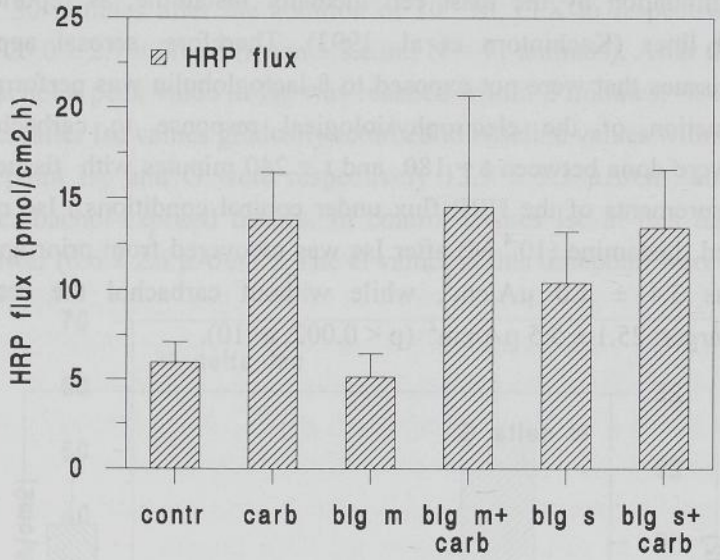

Fig 3. Horseradish peroxidase flux from mucosal compartment to serosal compartment calculated from the increment of the enzymatic HRP activity in the serosal compartment from 40 to 70 min after application of HRP. Contr: no additions; Carb: carbachol at the serosal side; blg muc: ß-lactoglobulin at the mucosal side; blg muc + carb: ß-lactoglobulin at the mucosal side 60 min after carbachol at the serosal side; blg ser: ß-lactoglobulin at the serosal side; blg ser + carb: β-lactoglobulin at the serosal side 60 min after carbachol at the serosal side. For significance of differences: see text.

Fig. 3 shows the HRP fluxes at the last sampling period (t = 150 -180 minutes). The flux in the presence of carbachol was significantly larger than control values ($p < 0.05$). Mucosal addition of ß-lactoglobulin in the absence of carbachol did not increase the HRP flux, and in the presence of carbachol it was not different from the flux with carbachol alone. Serosal addition of ß-lactoglobulin caused a significant increase in HRP flux compared to control and to mucosal addition of ß-lactoglobulin ($p < 0.05$). The HRP flux after serosal addition of ß-lactoglobulin in the presence of carbachol was significantly increased ($p < 0.05$) compared to control, but was not different from the flux with carbachol or the flux with carbachol plus mucosal ß-lactoglobulin. Thus, in the presence of carbachol, neither mucosal nor serosal addition of ß-lactoglobulin caused a further increase in HRP flux, compared to the effect of carbachol itself. Moreover, there was no significant difference in the HRP flux increase in the presence of serosal ß-lactoglobulin, with or without carbachol.

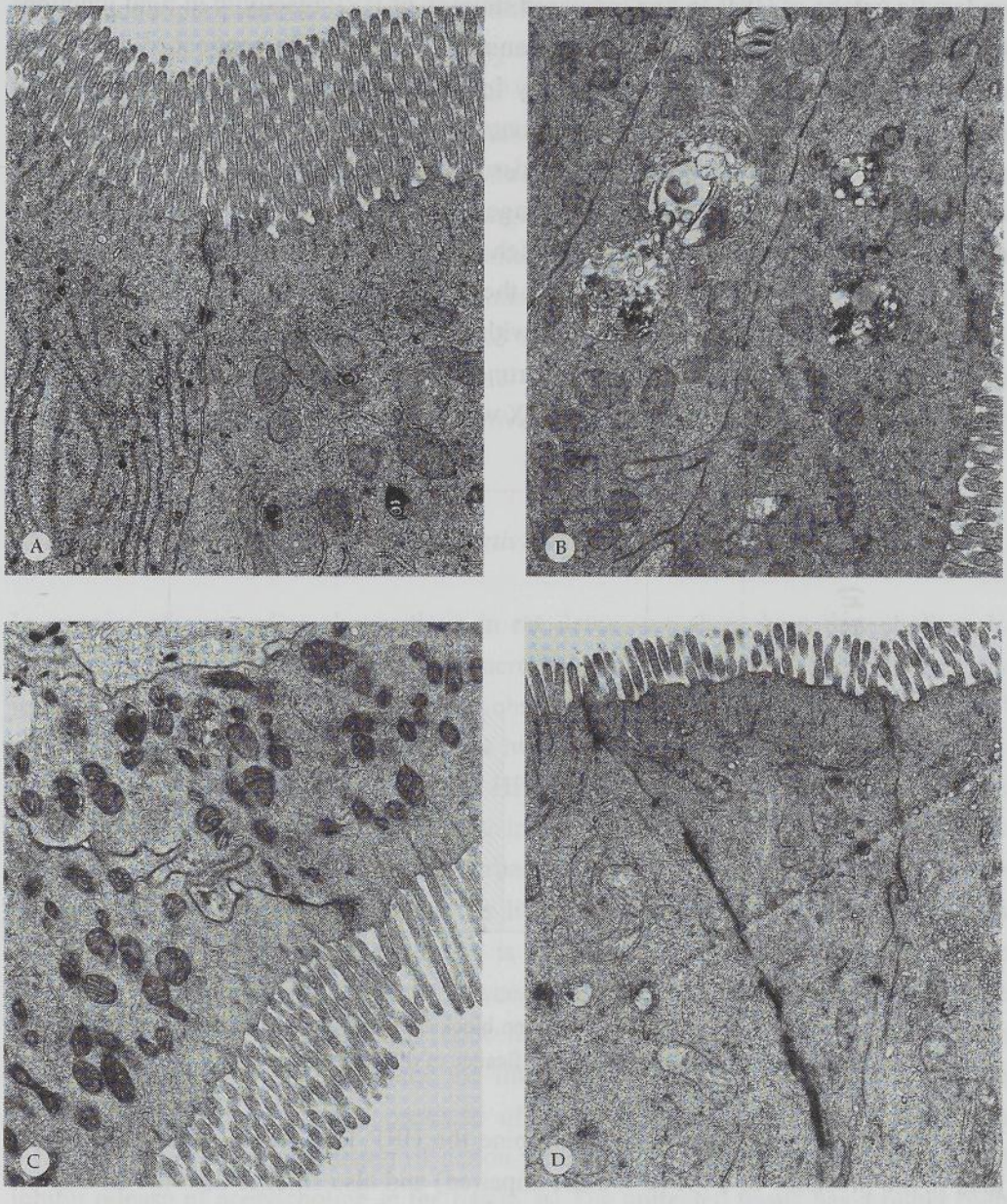

Fig 4. Electronmicrographs of guinea pig jejunum. A: Control: HRP at mucosal side and no further additions. The lateral intercellular spaces are empty. B: Control showing HRP product in the lateral intercellular spaces. C: HRP product in lateral intercellular spaces of carbachol activated tissue. D: HRP product in lateral intercellular spaces of β-lactoglobulin (serosal side) activated tissue.

Electronmicroscopy was performed on tissues from 6 animals. From the micrographs it was clear that carbachol and serosally added ß-lactoglobulin caused an increase in endocytic uptake of HRP and an increased staining of HRP reactivity in tight junctions and lateral intercellular spaces in all six animals. However, in contrast to our earlier observations in rat ileum, HRP reactivity in tight juntcions and lateral intercellular spaces was occasionally also seen in control tissues from two of the six studied animals. The HRP fluxes in the control tissues of these two animals were high:11.5 and 11.7 pmol/cm^2 .h and in the same range as HRP fluxes of the tissues from these animals in the presence of carbachol, which were 13.8 ± 2.6 pmol/cm ^{2}.h. Therefore, in additional experiments we compared the fluxes of HRP under standard control conditions (i.e. in the presence of TTX) with the flux when instead of TTX, atropine (10^{-5} M) was added to the serosal compartment 30 min after mounting the tissue and with the flux when no atropine and no TTX was used.

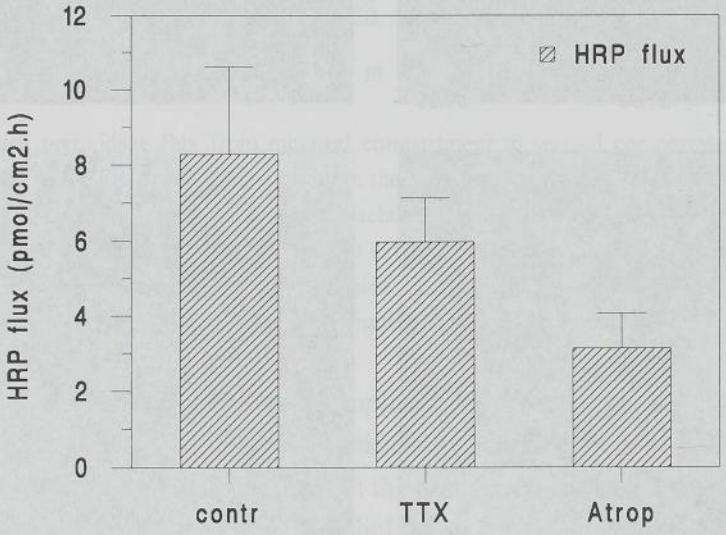

Fig 5. Mucosal to serosal flux of HRP. Contr: no blockers added; TTX: tetrodotoxin at the serosal side; Atrop: atropine at the serosal side. For significance of differences: see text.

Fig 5 shows that in the presence of atropine the HRP flux was significantly smaller than in the presence of TTX ($p < 0.05$, unpaired) and also smaller than the flux in the absence of both TTX and atropine ($p < 0.05$, unpaired). Moreover, the addition of TTX or atropine caused a decrease in Isc (mean value: 60 µA/cm^2 at t = 30 minutes) of about 30 µA/cm^2 within 10 minutes. This suggests that the tissue can have a spontaneous high release of acetylcholine and possibly other neurotransmitters.

DISCUSSION

In previous experiments it has been shown that carbachol, via the muscarinic receptor, induces an increase of the permeability for macromolecules via the trans- and paracellular pathway exemplified by HRP transport (Bijlsma et al. 1996). Although the route of HRP transport was not studied in detail, Heyman et al. (1990) have documented increased HRP transport through intestinal tissue from cow's milk sensitized guinea pigs after *in vitro* challenge with ß-lactoglobulin.
The aim of the present study was to determine whether carbachol could also increase permeability for HRP in isolated small intestine of the guinea pig and if so, whether the presence of carbachol could increase the effects of sensitizing antigens at the mucosal side of sensitized intestinal tissue. Furthermore we asked whether carbachol and antigen challenge could have an additive effect on the increase in macromolecular permeability.

Muscarinic activation and endogenous activity

In support of our earlier observations in rat ileum, we show here that cholinergic stimulation also causes an increase in macromolecular permeability in the guinea pig jejunum. The increased leak can still be observed after the Isc response to carbachol was fully declined and was still detectable two hours after application of carbachol.
Although we have not quantified the HRP in endosomes, the EM observations indicated an increased endocytic uptake in the presence of carbachol, like in rats (Bijlsma et al.1996). Furthermore, in carbachol treated tissues HRP was detected in the lateral intercellular space. Thus, the likeness of results in rat and guinea pigs indicates that the increased transport is a general consequence of cholinergic activation. An interesting difference between small intestine of the guinea pig and the rat is the occasional observation of paracellular HRP in control tissue of guinea pigs and the susceptibility of the Isc and the flux of HRP for atropine. This leads to the following conclusions: i) The presence of TTX, which in the used concentration effectively blocks the generation of action potentials in nerves, does not completely inhibit release of acetylcholine in the tissue. ii) The untreated tissue has a rather high cholinergic tone, reflected by a relatively high Isc and large permeability for macromolecules, which can both be reduced by atropine and to a lesser degree by TTX. The high cholinergic tone and Isc may partly be a result of the sensitization of the animals to a foreign antigen, as this procedure itself leads to an increase of the Isc

in the jejunum of oral β-lactoglobulin sensitized guinea pigs (Heyman et al. 1990). In orally sensitized guinea pig ileum TTX also reduced the high initial Isc values (Baird and Cuthbert, 1987). However, a high Isc was also reported in the ileum of non-sensitized guinea pigs (Cooke et al. 1984a, b), which could be modulated by the acetylcholinesterase inhibitor eserine and by atropine. This suggests that non-sensitized guinea pigs also have a high cholinergic tone. We never observed a reduction by atropine of the low (10-20 $\mu A/cm^2$) baseline Isc values in the small intestine of Wistar rats and Balb C mice (Bijlsma, van Kalkeren: unpublished observations), and rats and mice are in general rather insensitive to oral sensitization procedures.

Taking these findings together, it is tentative to hypothesize that the susceptibility of guinea pigs to oral sensitization is related to a high cholinergic tone in the small intestine, which may cause an increased transepithelial passage of luminal antigens. Interestingly, in the Hooded-Lister rat strain with a spontaneous Isc of about 60 $\mu A/cm^2$ atropine as well as TTX also caused a decrease of the Isc and of the jejunal permeability for macromolecules (Kimm et al. 1994), comparable to our present findings in guinea pigs. If our hypothesis makes sense, this rat strain should also be more susceptible to oral sensitization than e.g. Wistar rats and most other rat strains.

Luminal and serosal antigen challenge

The present findings confirm earlier observations (Heyman et al. 1990), of increased transport of intact HRP in the presence of serosal ß-lactoglobulin in cow's milk sensitized guinea pigs. In addition we now show that the release of mast cell products can augment, directly or indirectly, the endocytotic activity of transport cells and can modify the properties of tight junctions. In contrast to the effect of serosal addition of ß-lactoglobulin, the mucosal application increased Isc with only 40 % of the effect of serosal challenge and did not increase the transepithelial flux of HRP. This differs from observations in rat jejunum, where mucosal application of HRP to isolated tissue of HRP-sensitized rats in Ussing chambers induced an increase of the flux of the specific antigen itself but also of a bystander protein (Berin et al. 1997). An explanation for this difference can not be given unequivocally at this moment. However, one difference is that the present experiments were performed in the presence of TTX. This would reduce the influence of mast cell products on epithelial cells via neuronal activation. Application of TTX to rat jejunum also inhibited the lumen to blood transport of ovalbumin in ovalbumin sensitized rats (Crowe et al.

1993). Berin et al. (1997) have divided the transepithelial transport of antigens from the luminal side to the blood side into two phases. The first is the uptake into the epithelial cells and the transcytosis of the antigen. The second phase is the recognition of the antigen by the mast cells, their degranulation and the induction of increased endocytosis and paracellular leak for the antigen but also for other luminal molecules. Sensitization of the rat is required for the first phase, but it also occurred in mast cell deficient Ws/Ws rats, whereas the second phase is dependent on sensitization and the presence of mast cells (Berin et al.,1998). Studies from Bockman and Winborn (1965) and Berin et al. (1998) strongly suggest that sensitization also provides the epithelial cells of hamsters and rats with an antigen recognizing mechanism and may be part of the answer to the question how the tissue can generate the fast response to mucosal challenge with specific antigens.

In the present study the magnitude of the Isc induced by mucosal challenge did not differ in the presence and absence of carbachol. This contrasts with the finding that after a carbachol induced secretory response, histamine and serosal ß-lactoglobulin induced increases of Isc are suppressed by 86 % and 77% respectively. Recently we reported qualitatively identical effects on jejunal Isc responses to mucosal and serosal antigen application in ovalbumin-sensitized mice and similar effects of pretreatment with carbachol (Bijlsma et al. 1996). The carbachol-induced inhibition of Isc responses to histamine and serosal antigen is most probably due to heterologeous desensitization, as shown for histamine in the intestinal cell lines T84 and HT29cl.19A (Kachintorn et al. 1993 and Bouritius et al. unpublished observations). Thus, the relatively large Isc-response to mucosal challenge in the presence of carbachol may be due to an increased release of mast cell products, presumably because of increased transport of ß-lactoglobulin from the luminal side to the mast cells.

From the combined effects of carbachol and serosal or mucosal addition of ß-lactoglobulin on HRP fluxes we conclude that mast cell degranulation by either serosal or mucosal antigen does not cause a further increase of the permeability for macromolecules than that evoked by carbachol or serosal ß-lactoglobulin alone. The non-additiveness of the permeability effects of maximal stimulation of cholinergic activity and of mast cell degranulation suggests that both stimuli exert their permeability effects on the epithelial cells via a similar intracellular pathway. The observation that addition of carbachol or serosal antigen can further increase the already existing spontaneous high permeability for macromolecules in sensitized

guinea pigs implies that at the postulated spontaneous level of cholinergic activation of the enterocytes, further stimulation by carbachol or mast cell products can have an additive effect on macromolecular permeability.

The finding that carbachol amplifies the increase in ionic conductance of about 10 % of baseline conductance caused by serosal antigen to a value of about 40 % was rather unexpected, as the presence of carbachol alone did not influence the baseline conductance. This observation indicates the possibility that carbachol can potentiate the effect of one or more mast cell products on tight junction permeabilty for ions and possibly also for molecules smaller than HRP. This requires further study. The comparison of the conductance changes in Fig. 3 to the HRP fluxes in similar experimental conditions in Fig. 2 leads to one highly relevant conclusion: It clearly shows that conductance changes in general are not correlated to increases in macromolecular permeability, even if these permeability increments are partly due to paracellular leak.

In conclusion: the present results confirm the observations in rat that muscarinic activation can increase the intestinal permeability for macromolecules via both the paracellular and the transcellular route. Futhermore, they show that serosal antigen challenge of isolated intestinal tissue from sensitized guinea pig likewise increases the permeability for macromolecules. Finally, we hypothesize that the susceptibility of the guinea pig to oral sensitization could be related, at least in part, to the high cholinergic tone in its small intestine.

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