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CHANGES OF ION AND WATER CONTENT OF MOUSE INTESTINAL CELLS AFTER PILOCARPINE AND ISOPROTERENOL STIMULATION

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Abstract

Cytoplasmic monovalent ion and water contents in morphologically defined mice jejunal cells were measured by X-ray microanalysis in order to gain insight into the cell-type specificity of intestinal electrolyte transport mechanisms. Ion and water contents were measured independently. It was found that in some cases net fluxes of ions and water do not correspond to the assumption of constant osmotic activity of cytoplasmic Na and K ions. Stimulation of secretion with the cholinergic secretagogue pilocarpine resulted in efflux of Cl^- from and influx of K^+ into crypt enterocytes containing small secretion granula (crypt A cells). No significant changes in ion concentrations were found in crypt enterocytes without secretion granula (crypt B cells). Crypt A cells were more likely to be stimulated by pilocarpine than crypt B cells, and the basolateral K^+ efflux pathway in crypt A cells appeared to be rate-limiting. In villus enterocytes, pilocarpine stimulated Cl^- efflux. Isoproterenol caused marked changes in the cytoplasmic Cl content of all epithelial cells. These changes were reversed by inhibition of adenylate cyclase by alloxan, with the sole exception of Cl increase in villus absorptive cells. The results are consistent with an cAMP-mediated stimulated secretion in crypt epithelial cells and a predominantly cAMP-independent stimulation of absorption in villus cells. The results obtained suggest a transcellular route of Cl absorption in the mouse jejunum.

Key Words: Intestine, ion transport, electrolytes, X-ray microanalysis, pilocarpine, isoproterenol, alloxan, secretion, absorption.

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Introduction

The regulation of electrolyte and water transport in the intestine is of considerable physiological and pathophysiological interest. The principal mechanisms of epithelial transport are well known. However, epithelial transport is more complicated in the intestine than in other epithelia mainly due to the interplay of its absorptive and secretory functions. These functions are spatially separated with secretion taking place in the crypts and absorption occurring in the villus epithelium. This separation is small enough to limit the possibilities of conventional electrophysiological techniques considerably.

The combination of X-ray microanalysis and cryoelectron microscopy offers an interesting alternative because it allows the measurement of ion and water contents in single, morphologically well defined cells within a complex tissue (Roomans, 1988; von Zglinicki and Roomans, 1989). The preparation of tissues can be optimized to retain the *in vivo* distributions of ions and water at the organelle and all higher levels (von Zglinicki *et al.*, 1986), even if artificial redistribution during preparation occurs on a macromolecular scale.

Changes in cytoplasmic ion concentrations in crypt enterocytes after secretory stimulation with Vasoactive Intestinal Polypeptide (VIP) were demonstrated and it was concluded that the Na extrusion from secretory cells via the Na^+/K^+ pump is the rate-limiting step in this system (von Zglinicki and Roomans, 1993). It seems to be of interest to compare these results with the effects of other mediators of intestinal transport.

While VIP is a nonadrenergic, noncholinergic neural stimulator of intestinal secretion, acting mainly by increasing the intracellular cAMP concentration (Donowitz and Welsh, 1987), pilocarpine is known to be a cholinergic secretory stimulator in many exocrine glands. Its main mode of action appears to be via an increase of cytoplasmic Ca^{2+}, however, systemic effects cannot be ruled out (Donowitz and Welsh, 1987).

On the other hand, isoproterenol is known to stimulate the adenylate cyclase in susceptible cells (Rick

et al., 1986). The resulting increase in cytoplasmic cAMP should inhibit absorption and stimulate secretion in the intestine. However, isoproterenol as a β-adrenergic agonist stimulates in fact absorption in the small intestine (Morris and Turnburg, 1981). More insight into this apparent contradiction appears possible if stimulation by isoproterenol is accompanied by an inhibition of the adenylate cyclase. Alloxan is a potent blocker of adenylate cyclase (Sagström *et al.*, 1987). The effects of these drugs on ion and water content on the cells of the mouse small intestine will be dealt with here.

Materials and Methods

Mice (of either sex, weighing 20-25 g) were injected with either 0.1 ml isoproterenol or pilocarpine in saline, to obtain a dose of 20 mg/kg body weight of isoproterenol, or 8 mg/kg body weight pilocarpine. Some of the isoproterenol-treated animals received also 0.1 ml alloxan in saline to obtain a dose of 175 mg/kg body weight 1 min after the isoproterenol injection (Sagström *et al.*, 1987). Untreated animals were used as controls (von Zglinicki and Roomans, 1989). 5 min after the last injection the mice were killed by cervical dislocation and samples from the proximal part of the jejunum were taken by a cryobioptical method capable of minimizing artificial ion redistribution (von Zglinicki *et al.*, 1986). Briefly, small cylinders of intestinal tissue were punched out and simultaneously frozen to liquid nitrogen temperature. Only one sample per animal was taken in order to minimize post-mortal ionic changes. Cryosections about 0.5 μm thick were cut dry in a LKB CryoNova cryoultramicrotome at a temperature of about 170K. Sections were collected on copper slot grids, freeze-dried overnight in a vacuum evaporator at a pressure of less than 10^{-3} Torr, carbon-coated and examined in a Philips EM 400 electron microscope in the transmission mode at 100kV. X-ray spectra were collected with a LINK AN10000 energy-dispersive detector system.

Measurements were performed in the cytoplasm of, respectively, smooth muscle cells, Paneth cells, villus absorptive cells and in crypt epithelial cells. It was shown before (von Zglinicki and Roomans, 1989) that these so-called "undifferentiated" crypt enterocytes might be differentiated into two subtypes of cells, one containing small secretion granula (termed crypt A cells) and one lacking secretion granula but with a significantly higher K concentration in the cytoplasm of control cells (crypt B cells). Spectra from both cell types were taken separately. However, because both cell types could not be discriminated by morphological criteria alone, measurements of the cytoplasmic water content had to be pooled together.

Quantitative evaluation of spectra was done according to Hall *et al.* (1973) by using the QUANTEM software (Gupta and Hall, 1982). A correction for extraneous background was included in the procedure (Roomans, 1988). The spectra were standardized against those obtained from frozen-dried cryosections of gelatine containing known amounts of salts (Roomans and Sevéus, 1977).

The water, respectively dry mass, content of cells was estimated by one of the two following methods:

[1] In some cases, frozen-hydrated cryosections were transferred into the microscope at low temperature (less than 140 K) and photographed both in the frozen-hydrated and in the dried state with a cumulative electron dose on the frozen-hydrated section less than 10^4 e/nm^2. The irradiated area was not smaller than about 100 μm^2 and the cumulative irradiation time before taking a picture was less than 100 sec. The dry mass content (dry mass per wet mass) was then estimated as the ratio of the optical transmissions of the respective areas in the micrographs taken from dried and hydrated sections, corrected for differences in film response and shrinkage of sections during freeze-drying (von Zglinicki, 1991a; von Zglinicki, 1993).

[2] The water content was also analyzed in parallel samples by X-ray microanalysis of bulk specimens in the frozen-hydrated and dried state (von Zglinicki, 1991a). The experimental procedure was as follows: Whole cryobioptical samples were mounted onto aluminum stubs at 195 K using n-heptane as a cryo-glue (Steinbrecht and Zierold, 1984). They were cryofractured and carbon-coated on the cold stage of a Polaron E7400 cryotransfer system fitted to a Phillips 525 scanning electron microscope. After cryotransfer onto the precooled specimen stage of the scanning microscope X-ray microanalysis was carried out first in the frozen-hydrated and then in the frozen-dried state. Analysis was done at an accelerating voltage of 20 kV and at a temperature below 140 K using a LINK AN10000 microanalyzer. Different cell types in the crypts could not be discriminated with certainty in these bulk samples, therefore, smooth muscle cells, villus cells, and crypt cells were measured without further differentiation. Dry mass fractions F_i were obtained from the concentrations of an element i in the hydrated ($C_{h,i}$) and dried ($C_{d,i}$) state, according to

$$F_i = \frac{C_{h,i}}{C_{d,i}} \qquad (1)$$

(Roomans, 1988). Weighted means F of these estimates for all elements i were calculated from:

$$F=\frac{\sum C_{d,i}F_i}{\sum C_{d,i}}=\frac{\sum C_{h,i}}{\sum C_{d,i}} \qquad (2)$$

omitting the values for Na due to their high variation. The respective advantages and disadvantages of these two techniques to measure the intracellular water content were recently reviewed and the agreement of results obtained by both methods was confirmed (von Zglinicki, 1991a).

The data obtained from both methods were pooled together and used for the calibration of dry mass fraction measurements of one cell type relative to the other obtained from densitometrical measurements using frozen-dried cryosections alone (von Zglinicki, 1991a).

The concentration of an element i per local water $C_{w,i}$ is calculated as

$$C_{w,i}=C_{d,i}\frac{F}{1-F} \qquad (3)$$

and the osmolarity of the intracellular ions O is estimated from the molar concentrations of Na and K ($C_{w,Na}$ and $C_{w,K}$):

$$O=2(C_{w,Na}+C_{w,K}) \qquad (4)$$

Statistical comparison of the data was done by an analysis of variance. Critical differences between means (CDM) were computed at the 5% significance level, i.e., means differing by more than the CDM are taken as significantly different.

Results

The morphology of the intestine as seen in frozen-dried cryosections was similar to that found in earlier studies (von Zglinicki and Roomans, 1989, 1993). In bulk samples examined in the scanning electron microscope, individual villi could be seen, at least after freeze-drying. However, discrimination between different cell types within the crypts was not possible with certainty in these samples.

Element concentrations were estimated using both thin sections and bulk specimens. Typical differences between those estimates are illustrated in Fig. 1. In muscle cells, considerably higher P concentrations were measured in bulk specimens. In villus enterocytes, the prominent difference between both methods is in Na and Cl.

Table 1 shows the cytoplasmic concentrations of the monovalent ions Na, K, and Cl as measured in thin

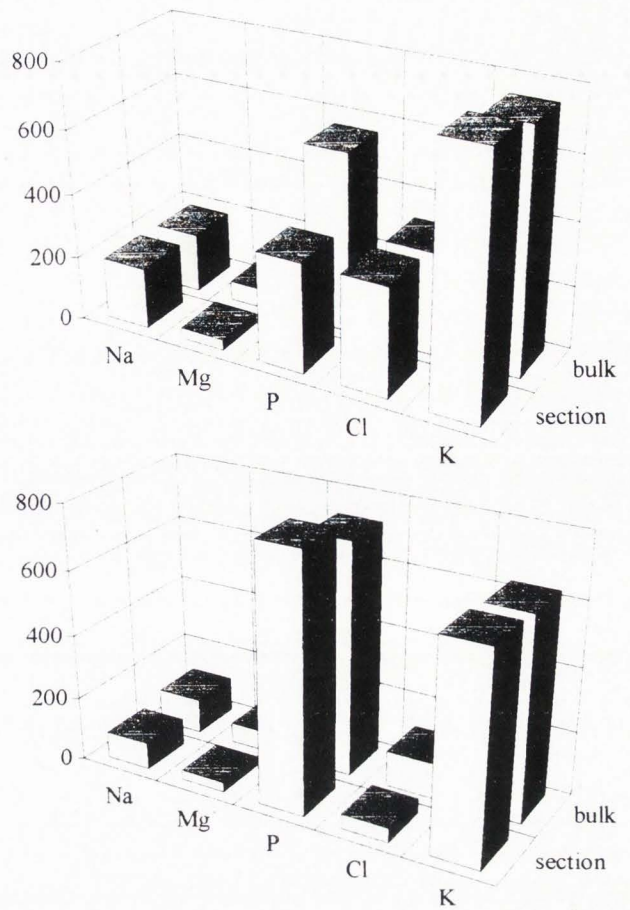

Fig. 1: Element concentrations (in mmol/kg dry weight) in muscle cells (a) and villus enterocytes (b) as measured by bulk specimen X-ray microanalysis (bulk) or by using thin sections (cytoplasmic measurements, section). Standard errors of means are 15 - 20% (Na, Mg), respective less than 10% (P, Cl, K). Differences between Mg und P (Fig. 1a) resp. Na, Mg, and Cl (Fig. 1b) estimates are significant.

sections and the dry mass content of the cytoplasm of crypt A, crypt B, villus, Paneth, and muscle cells. From these data, cytoplasmic osmolarities can be calculated according to eq. (4). An overview of these osmolarities is given in Fig. 2. Although most of the data are reasonably close to the expected value around 300 mOsmol, some prominent and significant exceptions must be noted. Except in villus cells, osmolarities increase significantly in all examined cell types after isoproterenol stimulation. In addition, osmolarities are higher than expected in Paneth cells under control conditions, but lower than expected in both types of crypt enterocytes after stimulation with pilocarpine and after combined treatment with isoproterenol plus alloxan. Generally, the calculated osmolarities mirror rather closely the changes

Table 1: Cytoplasmic total concentrations of Na, K, and Cl.

	Control	*Pilocarpine*	*Isoproterenol*	*Isoproterenol + Alloxan*	*CDM*
Paneth cells:					
Na	123 ± 19	102 ± 16	109 ± 26	61 ± 8	57
Cl	133 ± 13	102 ± 18	191 ± 28	90 ± 9	42
K	1030 ± 68	784 ± 57	955 ± 68	707 ± 32	191
n	22	13	14	14	
DM	19.6 ± 2.8	15.6 ± 1.9	27.5 ± 3.3	18.9 ± 1.7	7.4
n	18	10	18	14	
crypt A cells:					
Na	68 ± 6	87 ± 11	89 ± 9	50 ± 12	29
Cl	100 ± 9	24 ± 7	64 ± 10	106 ± 12	36
K	611 ± 42	814 ± 30	656 ± 28	609 ± 38	153
n	22	15	19	7	
crypt B cells:					
Na	114 ± 7	80 ± 10	139 ± 17	61 ± 10	34
Cl	106 ± 6	112 ± 14	189 ± 13	97 ± 9	30
K	873 ± 20	841 ± 53	935 ± 36	633 ± 44	113
n	37	29	22	32	
crypt enterocytes:					
DM	15.3 ± 1.9	9.9 ± 0.8	19.3 ± 1.7	12.9 ± 1.0	4.1
n	14	16	18	26	
villus cells:					
Na	84 ± 8	95 ± 8	90 ± 8	70 ± 18	ns
Cl	46 ± 4	24 ± 3	108 ± 9	116 ± 10	25
K	645 ± 17	904 ± 44	674 ± 17	701 ± 35	109
n	15	36	26	10	
DM	17.4 ±1.3	11.6 ± 0.7	19.3 ± 1.3	17.4 ± 1.1	3.3
n	15	21	18	26	
muscle cells:					
Na	192 ± 24	126 ± 15	146 ± 12	134 ± 19	50
Cl	342 ± 14	117 ± 27	135 ± 30	248 ± 27	83
K	787 ± 65	730 ± 65	805 ± 37	963 ± 37	136
n	15	18	23	15	
DM	14.0 ± 1.7	16.4 ± 1.4	20.7 ± 1.9	12.6 ± 1.2	5.5
n	8	14	7	21	

Data given as mean ± SEM, mmol/kg dry weight, as measured using thin sections and dry mass content DM (in % of wet mass) in cells of the mouse intestinal mucosa after different treatments.
n: number of cells measured, CDM: critical difference between means at the 5% significance level.

in water content between cell types and treatments.

To facilitate the comparison of treatment effects in the different cell types, ionic concentrations per dry mass as given in Table 1 are also displayed in Figures 3 (crypt enterocytes), 4 (Paneth and villus cells) and 5 (muscle cells). The results are as follows:

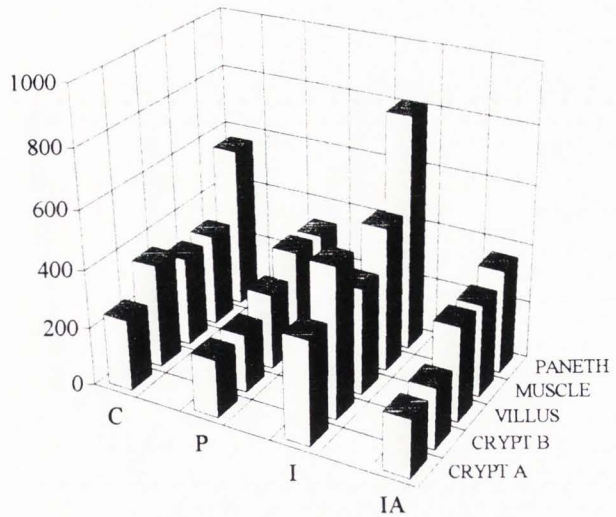

Fig. 2: Cytoplasmic osmolarities (in mOsmol) in the different cell types after the different treatments as calculated according to eq. 4. C: controls, P: pilocarpine, I: isoproterenol, and IA: mice treated with isoproterenol followed by alloxan. Standard errors of the estimates are around 20%.

Pilocarpine stimulation

Enterocytes react differently to pilocarpine stimulation: A significant increase in K and a decrease in Cl is found in crypt A cells and in villus cells (by direct comparison of control and pilocarpine treated groups), while a decrease in cytoplasmic Na is measured in crypt B enterocytes. K is lost from Paneth cells. In muscle cells, both Na and Cl are decreased. Pilocarpine stimulation leads to a significant increase of the water content in crypt and villus enterocytes, but not in Paneth or muscle cells.

Isoproterenol stimulation

In this case, crypt B, villus cells, and even Paneth cells react similarly by increasing their cytoplasmic Cl content. On the contrary, cytoplasmic Cl decreases significantly in crypt A and in muscle cells. Changes in Na and K are not significant. There is a water loss after isoproterenol stimulation which is significant in Paneth and muscle cells.

Action of alloxan

Treatment of the mice with alloxan after isoproterenol stimulation reverses most of the changes brought about by isoproterenol alone. The water content of all cell types is the same as in the controls within the experimental error. The prominent changes in cytoplasmic Cl concentrations due to isoproterenol stimulation are fully reversed in the crypt epithelial cells and even partly reversed in muscle cells. However, Cl remains

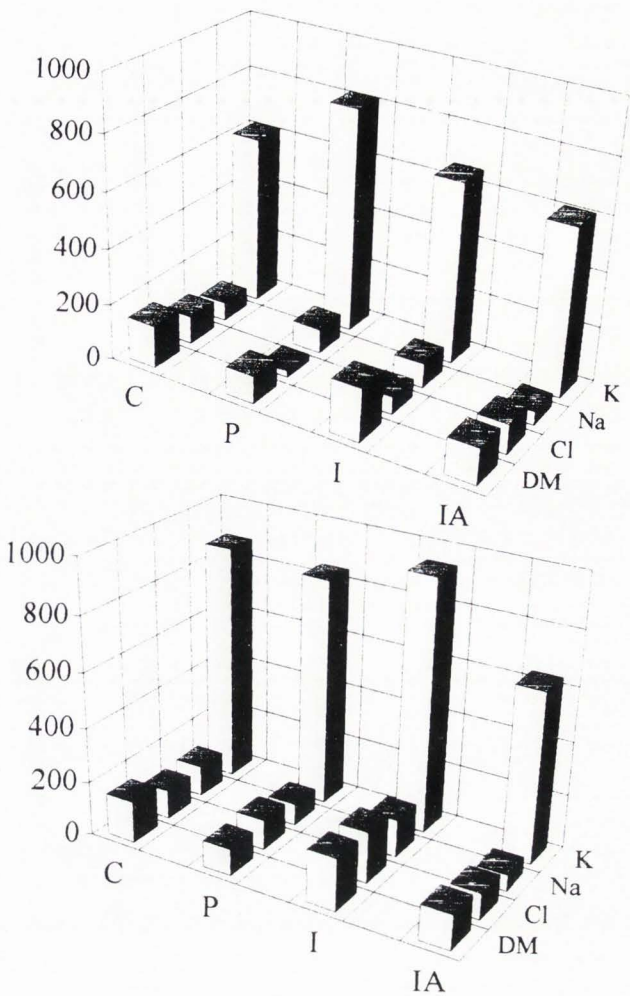

Fig. 3: Concentrations of K, Na, Cl (in mmol/kg dry weight) and the dry mass fraction DM (in per mille of wet mass) in the cytoplasm of crypt A cells (a) and crypt B cells (b) under different treatments. C: controls, P: pilocarpine, I: isoproterenol, and IA: mice treated with isoproterenol followed by alloxan. Standard errors of means are given in Table 1.

increased as after isoproterenol stimulation alone in villus cells. Moreover, there is a decrease of Na and K concentrations beyond the levels found in controls in crypt B and Paneth cells.

Discussion

Some significant differences were found between element concentrations as measured in thin sections and those measured in bulk specimens. However, in most cases these differences are simply due to the lower resolution of bulk specimen X-ray microanalysis. Muscle nuclei contain high P concentrations (von Zglinicki and Roomans, 1989) and are included in the measuring

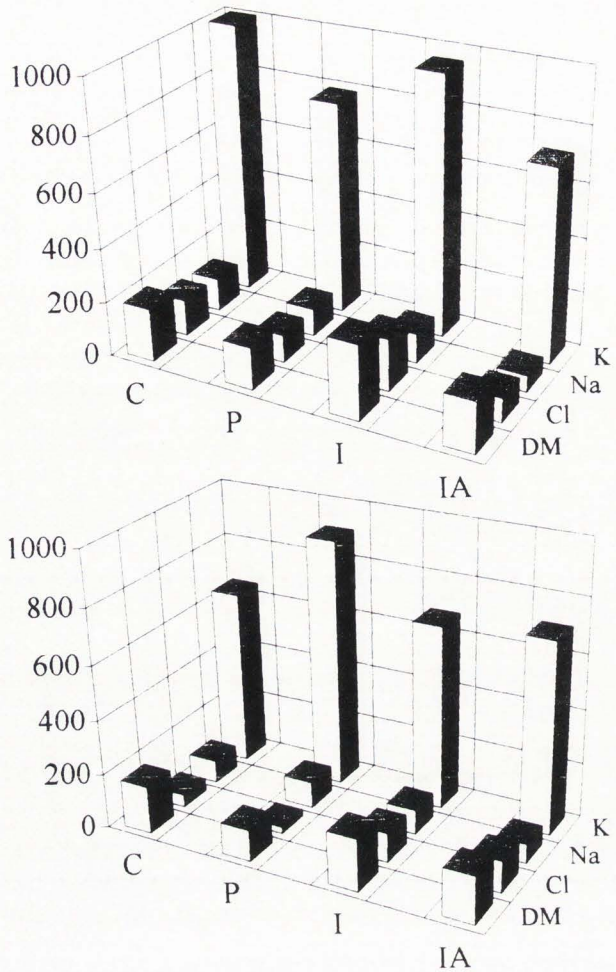

Fig. 4: Concentrations of K, Na, Cl (in mmol/kg dry weight) and the dry mass fraction DM (in per mille of wet mass) in the cytoplasm of Paneth cells (a) and villus enterocytes (b) under different treatments. For explanation, see legend to Fig. 3.

volume, leading to an increased average cellular P concentration (Fig. 1a). On the other hand, the luminal volume is largest in the villus region. Precipitation of NaCl from the extracellular space, especially onto the brush border membrane, is most probable in the villus and will be included in the measurements as soon as the slightest overpenetration of the cells by the beam occurs.

Different cell types within the crypts could not be differentiated with certainty in the scanning image. Therefore, ion concentrations as used to compare the effects of the different treatments are measured as truly cytoplasmic concentrations by thin section X-ray microanalysis. Bulk specimen X-ray microanalysis is used to measure the water content of villus enterocytes and smooth muscle cells. These data serve as an additional

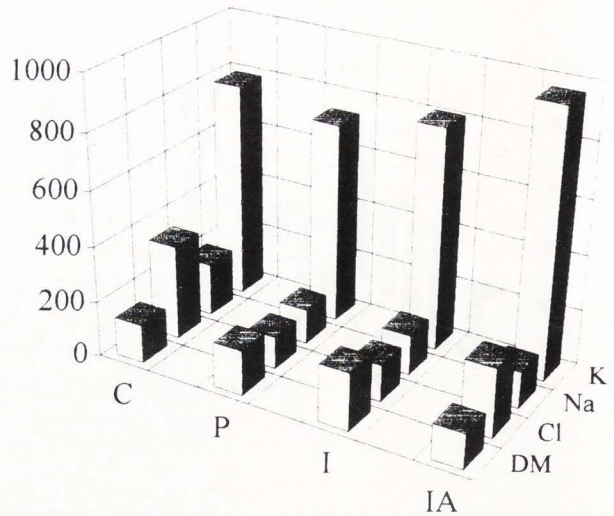

Fig. 5: Concentrations of K, Na, Cl (in mmol/kg dry weight) and the dry mass fraction DM (in per mille of wet mass) in the cytoplasm of muscle cells under different treatments. For explanation, see legend to Fig. 3.

standard for the water content measurements carried out in cryosections.

If measurements of cytoplasmic ion concentrations per dry weight and cytoplasmic water fractions are combined to estimate molar ion concentrations and osmolarities according to eq. (4), some significant differences from the expected value of about 300 mOsmol are found (Fig. 2): The cytoplasmic osmolarity is overestimated in all cell types except villus enterocytes after isoproterenol stimulation as well as in Paneth cells under control conditions. The osmolarity is somewhat underestimated in both types of crypt enterocytes after pilocarpine stimulation and after treatment with isoproterenol plus alloxan. There are at least two possible reasons for these deviations:

First, measured ion concentrations or water contents might be wrong. However, the water content of villus and muscle cells in the very same intestinal samples was measured using two independent techniques, namely densitometry of frozen-hydrated, then frozen-dried sections and X-ray microanalysis of bulk specimens. Results of both techniques were in very good agreement in all four treatment groups (von Zglinicki, 1991a). Moreover, it was demonstrated that the preparation technique used here is well suited to preserve the *in situ* ion and water distribution even on a subcellular scale (von Zglinicki *et al.*, 1986; von Zglinicki, 1991a). The possibility of arteficial ion and water shifts during "slow" freezing (i.e., any freezing regime not resulting in vitrification) was discussed and was regarded as

highly improbable for a diffusion length of cellular dimensions (von Zglinicki, 1991b). Confirmation has been obtained by experiments using high pressure frozen erythrocytes (Zierold *et al.*, 1991).

Second, an apparently increased osmolarity could result from a decrease in the osmotic activity of potassium and sodium. A decreased osmotic activity could most easily be explained by cooperative electrostatic binding of ions to polyelectrolyte charges. Such counterion condensation has been clearly established for the interaction of ions with nucleic acids (Manning, 1978). We could confirm experimentally the cooperative binding of K and Cl to the chromatin in isolated lymphocyte nuclei (von Zglinicki *et al.*, 1989) as well as non-Donnan type binding of monovalent ions to muscle proteins (von Zglinicki, 1988). There are many other reports supporting the idea that a significant fraction of cellular monovalent ions might not always be free in solution (for review, see Ling, 1992).

If the experimental results suggest ion and water fluxes apparently independent from each other, net fluxes of ions cannot be deduced from concentrations per wet weight or from molar concentrations. On the other hand, dry weight is still a valid reference value because it can safely be assumed that the dry mass of cells has not changed significantly through the few minutes of treatment. Concentrations of ions per dry weight give a good estimate of total cellular ion content, allowing the estimation of net ion fluxes. If water fluxes evidently do not equilibrate these ion fluxes, changes in the osmotic activity of the major monovalent ions are suggested.
There are a number of membranous ion transporters in each epithelial cell. Changes in cytoplasmic ion content reflect relative changes of the activity of these transporters after stimulation. The four major ion transport systems known in secretory cells of the small intestine are a luminal Cl^- efflux pathway and a $Na^+/K^+/2Cl^-$ cotransporter, the Na^+/K^+ pump and a K^+ efflux pathway on the basolateral side (Donowitz and Welsh, 1987; see upper part of Fig. 6).

The situation appears to be more complex in the absorptive cells (Fig. 6, lower part). A number of Na^+-dependent transport systems exists in the apical membrane, including Na^+/H^+ exchange, Na^+/phosphate and Na^+/sulfate cotransport and Na/substrate cotransport systems. Na^+/Cl^+ cotransport via linked Na^+/H^+ and Cl^-/HCO_3^- exchangers has been demonstrated in the ileum (Liedtke and Hopfer, 1982), but was excluded in the rabbit jejunum (Gunther and Wright, 1983). During absorption in the jejunum, Cl^- is thought to follow the electrical gradient passively by a paracellular route (Donowitz and Welsh, 1986).

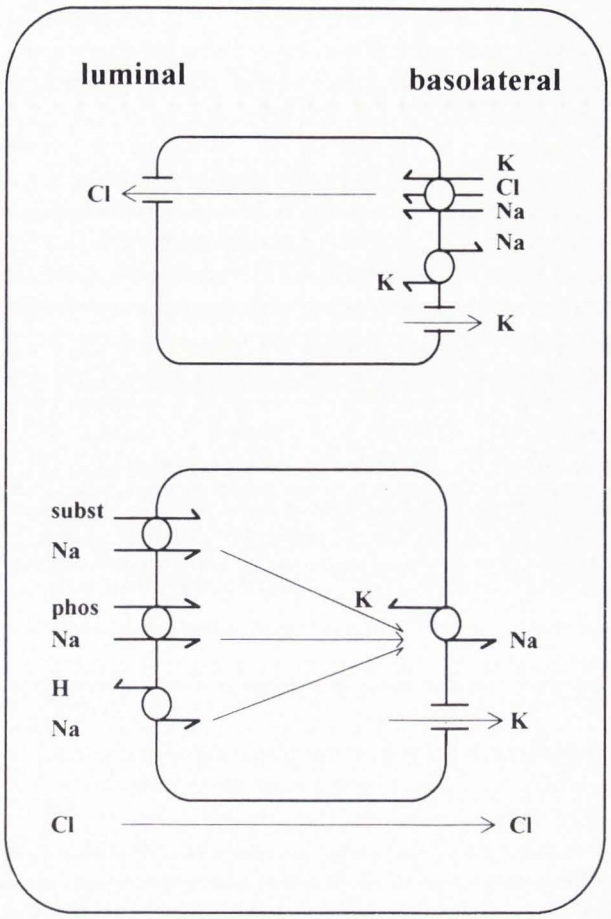

Fig. 6: Ion transport systems involved in secretion (upper part) and absorption (lower part) of electrolytes in the intestine. Modified after Liedtke and Hopfer (1982), Gunther and Wright (1983), Donowitz and Welsh (1986,1987).

Stimulated secretion - pilocarpine versus VIP

Changes of intracellular ion concentrations after stimulation with the secretagogue Vasoactive Intestinal Polypeptide (VIP) were measured in an earlier study (von Zglinicki and Roomans, 1993). It was found that an increase of the cytoplasmic Na concentration was the main effect of VIP in both subtypes of crypt enterocytes and it was concluded that the Na^+ extrusion via the basolateral Na^+/K^+ pump was the rate-limiting step of electrolyte secretion in this system. Moreover, a depolarization of the smooth muscle cells by VIP appeared reasonable from the measured increase of Na and Cl and decrease of K in those cells.

In contrast to VIP, pilocarpine is a cholinergic secretagogue. Its main intracellular second messenger is thought to be Ca^{2+} rather than cAMP (Yule *et al.*, 1993). Pilocarpine stimulates secretion of mucus, electrolytes and water in the rat small intestine (Freier

et al., 1991; Kemper and Specian, 1991). Its effects on intracellular ion concentrations in the small intestine are completely different from those of VIP: In the smooth muscle cells, pilocarpine treatment results in a decrease of Na and, especially, Cl, i.e., most probably in a hyperpolarization of the cell membrane rather than a depolarization. More important, the reaction patterns of crypt A and B enterocytes, which were more or less the same under VIP stimulation, are completely different after stimulation with pilocarpine. A decrease of intracellular Cl as found in crypt A cells is as expected if stimulation of secretion occurs mainly via the opening of a luminal Cl^- channel. The increase of K in pilocarpine-stimulated crypt A cells indicates the basolateral K efflux pathway as the ultimate rate-limiting step of electrolyte secretion in this system. The net K influx in crypt A cells is fairly balanced by an influx of water.

In crypt B cells, monovalent ion contents remain about constant. Together with the increase of cytoplasmic water content measured in crypt enterocytes, a significantly decreased apparent osmolarity is obtained (see Fig. 2). However, this result is due to the inability to discriminate between crypt A and crypt B cells in the course of water content measurements. In the case of significantly different ion fluxes the assumption of the same water content in crypt A and crypt B cells is probably wrong. However this might be, from the rather good constancy of ion contents it is tempting to speculate that the Cl^- transport rate in crypt B cells is not influenced at all, i.e., that pilocarpine stimulates electrolyte secretion in crypt A cells only.

The increase in K found in villus absorptive cells is in accord with the expected antiabsorptive action of pilocarpine which might result from an inhibition of the basolateral K^+ efflux pathway. The net K^+ influx is compensated by a water influx. A basolateral Cl^- efflux after pilocarpine treatment appears not very probable. The measured decrease in cytoplasmic Cl might, therefore, indicate that an inward Cl transport is involved in absorption in the mouse jejunum. This Cl^- transport would be active under control conditions, but would be blocked by pilocarpine.

By stimulating at the same time the influx of water and the efflux of K^+, pilocarpine causes a decrease of the apparent osmolarity in Paneth cells down to "normal" values (Fig. 2). It is not clear whether this should be seen in connection with the known stimulation of mucus secretion by pilocarpine (Freier *et al.*, 1991; Kemper and Specian, 1991).

Isoproterenol treatment and the action of cAMP

Isoproterenol causes marked changes in the cytoplasmic Cl content of all epithelial cells. Moreover, it causes an efflux of water from all cell types without profound changes in the contents of Na and K. All these changes are reversed by alloxan, with the sole exception of a Cl increase in villus absorptive cells. Isoproterenol is known to stimulate Cl^- secretion in airway epithelia by activation of both an apical Cl^- efflux and by stimulation of a basolateral $Na^+/K^+/2Cl^-$ cotransport system (Haas *et al.*, 1993). However, in the small intestine isoproterenol stimulates overall absorption (Morris and Turnburg, 1981).

Alloxan antagonizes the action of isoproterenol in alloxan-induced diabetic rats by decreasing the number of β-adrenergic receptors (Ozturk *et al.*, 1990). This, however, cannot be the mechanism of alloxan action in our system due to the short incubation times. In short-time experiments, alloxan is a potent inhibitor of adenylate cyclase (Sagström *et al.*, 1987). Whether this is due to generation of free radicals by alloxan (Abdel Raman *et al.*, 1992; Zhang *et al.*, 1992), or by some other mechanism, is not known at present.

With these results in mind it becomes evident that an *in vitro* injection of isoproterenol stimulates both secretion in crypt cells and absorption in the cells of the intestinal villi, but by two different mechanisms. The action on the crypt cells is via an increase of cAMP, because it is reversed by alloxan. On the contrary, the action on villus cells is independent of cAMP and must, therefore, be due to a systemic regulation.

The fact that the sole change measured in villus enterocytes is an increase of Cl suggests again that absorption of Cl^- in the jejunum of mice takes place by a transcellular route, contrary to results found in the rabbit jejunum (Gunther and Wright, 1983; Donowitz and Welsh, 1986).

The changes in ion contents measured in crypt cells are not conclusive as to whether the one or the other cell type is involved in isoproterenol-stimulated electrolyte secretion. Crypt A and B cells react in a different, in fact opposite, manner. A decrease of cytoplasmic Cl as found in crypt A cells is more consistent with a simple model of secretion, however, neither the relative secretory activity of crypt A and B cells nor the involvement of Paneth cells in electrolyte secretion can be deduced from these data.

The water loss from all crypt epithelial cells without large changes in total cytoplasmic ion contents after isoproterenol stimulation is reversed by alloxan. The water loss is cAMP-dependent, therefore. It appears that cAMP-mediated stimulation by isoproterenol influences the osmotic activity of cytoplasmic ions in crypt epithelial cells. The same conclusion was drawn from a study of the cAMP-mediated stimulation of intestinal secretion by VIP (von Zglinicki and Roomans, 1993). However,

it should be noted that there are major differences in the reaction patterns of crypt cell ion and water contents to VIP and isoproterenol stimulation. A clear conclusion concerning a role of cAMP content for the osmotic activity of ions in these cells can therefore not be drawn from these data.

In addition to reversing an increase of Cl content and an efflux of water in Paneth and crypt B cells, alloxan treatment also decreases Na and K contents in the same cells. This result suggests the existence of a cAMP-dependent $Na^+/K^+/Cl^-$ cotransporter in the membrane of crypt B and Paneth cells.

Acknowledgements

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Discussion with Reviewers

A. Dörge: Cellular element concentrations and water content were determined 5 min after the injection of the secretagogues. Is there any evidence that maximal stimulatory effects are already achieved at this time?
Authors: We have not studied this extensively in our experiments on intestine, but considering our experience with other tissues, such as salivary glands under similar conditions, and considering the general literature on stimulation of intestinal secretion, we would estimate that the stimulatory effect is close to maximal after 5 min.

A. Dörge: The main problem I have with the present paper derives from the interpretation that isoproterenol leads to a decrease in the osmotic activity of cytoplasmic ions. The opposite effect occurs in Paneth cells after the action of pilocarpine. Is there any basic mechanism known which would explain the phenomenon? In this respect the question of the reliability of the dry weight content measurements also arises.
N. Pivovarova: It is known that at normal conditions the main part of K and probably of Na is osmotically active. Pilocarpine seems to cause a significant osmotic imbalance in Paneth cells and crypt B cells. The sum of K and Na per cell water decreases by more than 40 %. Do the authors have any explanation for this phenomenon? Could methodological problems with the bulk specimen analysis, especially overpenetration and/or arteficial freeze-drying, play a role?
Authors: The apparent osmotic imbalance in crypt B and Paneth cells occurs already in the controls and is reversed by pilocarpine (see Fig. 2). To our best knowledge, we have excluded sources of possible artefacts (see discussion and references cited there as well as von Zglinicki and Uhrik (1988) and von Zglinicki and Zierold (1989) regarding the problems of cryotransfer and radiation damage, and von Zglinicki and Lustyik (1986) regarding the question of arteficial freeze-drying during bulk specimen analysis). Overpenetration occurs to a certain degree in the villi in freeze-dried bulk specimens. However, because Na is excluded from the averaging (eq. 2), the effect of overpenetration is too small to influence the water fraction measurements significantly (von Zglinicki, 1991a). Cooperative binding [counterion condensation, see Manning (1978)] provides at least one theoretical framework to understand a decreased apparent osmotic activity. It appears easily possible that pilocarpine or isoproterenol cause changes in the conformation of major cellular polyelectrolyte macromolecules, for instance by calcium binding. Those conformational changes would influence the availability of surface charge clusters to cytoplasmic monovalent ions.

B.L. Gupta: Why have you not included the values for P and S in the results? Since P and S are mostly part of the macromolecules, changes in these elements would additionally reflect changes in cell volume.
Authors: S was not measured because thin section X-ray microanalysis was done at room temperature. It is known that at ambient temperature a preferential loss of S is possible (Rick *et al.*, 1982; von Zglinicki and Zierold, 1989). P concentrations under control conditions were given in a previous paper (von Zglinicki and Roomans, 1989). The sole significant effect of the different treatments is a decrease of P in crypt B and Paneth cells after the combined action of isoproterenol and alloxan. This decrease might be due to an effect of alloxan on the cellular phosphates or to interindividual differences. However, as concentrations are measured as concentrations per dry weight, they are independent on any change in cell volume.

N. Pivovarova: The concentration of Cl is rather low in the cells of all types. Cl probably could not play the role of the counter-ion to compensate for the cation charge. Might Cl be lost during analysis if any significant mass loss takes place? If not, what other ions could provide electroneutrality of the cytoplasm?
Authors: Although there is surely mass loss from our frozen dried cryosections especially during the first few seconds of irradiation as in any other biological X-ray microanalysis (von Zglinicki, 1993), there is no reason to assume preferential loss of Cl. In fact, the measured Cl concentrations are well in the expected range. It is well known that phosphates, net negative macromolecular charges and carbonate ions are the major intracellular negative charges.

N. Pivovarova: When the ion transport process across

epithelia is studied it would be most interesting to measure the ion composition both of cells and of secreted fluid in the lumen. Frozen-hydrated samples seem to be appropriate in this case. Why did the authors not take the advantage of the specimen?

Authors: According to our experience, the lumina in the cryobioptically prepared mouse jejunum are too narrow for reliable X-ray microanalysis using bulk specimens, even in the frozen-hydrated state. Cryosections a few μm thick would be appropriate for this purpose.

B.L. Gupta: In your model for absorption (Fig. 6, lower part) there is no provision for the absorption of K from the lumen. Is there not a $Na^+/K^+/2Cl^-$ cotransporter in the apical membrane?

Authors: K^+ absorption and secretion has been shown to exist in the rabbit and rat descending colon, and a apical $Na^+/K^+/2Cl^-$ cotransport was demonstrated in the flounder intestine, but not in the mammalian small intestine (Donowitz and Welsh, 1986).

B.L. Gupta: In your discussion you suggest that Cl^- may be absorbed transcellularly but your model shows a paracellular route. Why?

Authors: Paracellular Na^+/Cl^- cotransport as known in many ion-transporting epithelia (Kinne 1988) was excluded for the rabbit jejunum (Liedtke and Hopfer, 1982; Gunther and Wright, 1983). We are not aware of other results from different mammalian species. To our knowledge, the present results suggest for the first time the possibility of paracellular Cl absorption in the mouse jejunum.

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