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ENERGY-RICH PHOSPHATES AND TRANSINTESTINAL TRANSPORT IN RAT INTESTINE INCUBATED IN VITRO AT DIFFERENT TEMPERATURES

A. FAELLI, G. ESPOSITO and V. CAPRARO

Istituto di Fisiologia Generale e di Chimica Biologica, State University of Milan, Milan (Italy)

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SUMMARY

In the present work, the transported fluid and the tissue content of ATP, ADP and AMP has been evaluated in the jejunum rat intestine which was everted and incubated in vitro both at 28 °C and at 38 °C for 1 h. The energy-rich phosphates have been measured in the tissue at the beginning and at the end of the experiment as well as in vivo. These determinations have been made in the total intestine and in the scraped mucosa. ATP and ADP content are higher in vivo and lower but constant at 28 °C in vitro; on the contrary, at 38 °C in vitro, the initial and final content of these adenilic nucleotides are both lower than at 28 °C. Under all these conditions the AMP content does not vary appreciably.

Wet weight to dry weight ratios have been reported for mucosal and submucosal tissues in unincubated and incubated intestines.

In some experiments, fluid transport (measured as an actual serosal volume increase) was determined every 20 min during a 1-h incubation. At 28 °C, fluid transport is constant throughout the time of the experiment, but at 38 °C, there is a progressive decrease of the transported fluid.

Fluid transport and ATP content of the intestine seem to be directly related. The transport activity which is lower at 38 °C than at 28 °C, seems to be due to a low availability of energy-rich phosphates.

INTRODUCTION

The intestinal transport of fluid has been widely demonstrated both in vivo and in vitro. It is usually assumed that transintestinal transport of water is principally caused by the continuous active sodium extrusion from the basolateral membrane of the enterocyte (the site of the sodium pump) [1, 2], towards the intercellular channel. The $(Na^+ + K^+)$-dependent ATPase activity seems to be associated with the sodium pump [3]. In this model a decreased activity of this pump may cause a decrease of water transport and a consequent accumulation of sodium within the intestinal cell. Such an accumulation accounts for a cellular swelling. To check the factors affecting the intestinal fluid transport, we have determined ATP, ADP and AMP levels as

well as wet weight to dry weight ratios under in vivo conditions (control) and during in vitro experiments under different conditions. These factors have been related to the simultaneous transport of fluid detected in the same experiments.

MATERIALS AND METHODS

Intestinal incubation

Albino male rats (Wistar strain Charles River Italiana) weighing 200–300 g, were used. Five groups of experiments were performed: in the control group, the intestine of the anesthetized rat was freeze-clamped in situ as described below in the section titled Adenylic nucleotide determination; whereas in the four other groups of experiments, a tract of jejunum 10 cm long was removed from the rat under barbiturate narcosis. The intestine was then everted, incubated and perfused for 1 h at 28 or at 38 °C. The mucosal side of the intestine was incubated in 50 ml of Krebs-Henseleit-bicarbonate solution with the addition of 5.56 mM glucose, and gassed with 95 % O_2 and 5 % CO_2. The serosal side was first washed for 5 min and then perfused with 3 ml of the same mucosal fluid with the addition of trace amounts (0.5 μCi/ml) of poly-[^{14}C]ethyleneglycol (New England Nuclear Corp.). This substance was used to determine fluid transport, since it is known that it does not enter the cell [4, 5]. The serosal perfusing fluid was recirculated by a peristaltic pump (2.5 ml/min, E. Bühler, Tübingen). After 5 min equilibration and at the end of 1 h experiment, samples of the serosal and mucosal fluid were taken and assayed for poly-[^{14}C]ethyleneglycol (liquid scintillation spectrometry, Tri-Carb Packard mod. 3385). In some experiments the sampling was performed every 20 min for 1 h.

At the end of the experiment, poly-[^{14}C]ethyleneglycol was never detected in the mucosal fluid. From the dilution of the isotope in the serosal medium, fluid transport could be calculated. (For further details and for the scheme of the equipment, see ref. 6.)

In two of the four experimental groups (in which the temperature was 28 and 38 °C, respectively) the experiment was stopped after 5 min of equilibration (beginning of the experiment was time zero) and the intestine was freeze-clamped (see below). In the other two experimental groups (in which the temperature was 28 and 38 °C, respectively), the intestine was incubated and perfused for 1 h, as described above, and freeze-clamped as described in the next paragraph.

Adenylic nucleotide determination

For our purposes it is essential to measure ATP, ADP and AMP content of the mucosal epithelial cells which can be easily collected by scraping off the mucosal layer [7].

Unfortunately, in tissue with a normal blood supply, the ATP content dramatically decreases within seconds [8], and therefore a quick everting and scraping does not prevent a loss in nucleotide. Since it is impossible to solve the problem, we tried to evaluate the extent of the error. The intestine was divided into two parts: the first one (total intestine = in toto) was immediately frozen by using flat tongs with aluminum jaws pre-cooled in liquid nitrogen. The other part of the intestine was, as usual, everted and separated by scraping, into two portions, i.e. mucosa and submucosa (submucosal and muscle layer); then first the mucosa and then the submucosa

were frozen. Each frozen tissue (i.e. in toto, mucosa and submucosa) was divided into two parts and immediately weighed. Part of these tissues was dried overnight at 100 °C and reweighed in order to calculate the wet weight to dry weight ratio. The other part was pulverized in a deep-cooled mortar while liquid nitrogen was added continuously. The powder was mixed with pre-cooled 0.6 M $HClO_4$, thawed, homogenized in a pestle homogenizer and centrifuged at 2 °C. The supernatant was filtered through Millipore (pore size 0.45 μm) and divided into two parts: the first part was neutralized with KOH 30 % (w/v) and centrifuged again; the supernatant obtained was assayed for ATP [9]. The second part was alkalinized with 0.5 M triethanolamine/HCl/ 2.0 M K_2CO_3 buffer, and centrifuged; ADP and AMP were subsequently determined in this supernatant [10]. By knowing the wet weight to dry weight ratio of in toto tissue, mucosa and submucosa, the amount of ATP, ADP and AMP per g dry tissue weight could be calculated in μmol. Total dry tissue weight of the intestine was also calculated, to normalize transintestinal fluid transport calculated in a 1-h long experiment.

RESULTS AND DISCUSSION

A close correlation between the transport activity and the concentration of $(Na^+ + K^+)$-dependent ATPase seems to be demonstrated in each intestinal tract [11]. These two parameters have high values in the jejunum, but they decrease in the ileum. It is known that in the caecal tract, the transport activity is drastically reduced and a strong reduction of $(Na^+ + K^+)$-dependent ATPase has been observed [12]. An increase of these two parameters has been found in the colon [11]. If we compare data reported in Table I concerning fluid transport in intestines incubated 1 h at 28 and 38 °C respectively, we can see that fluid transport is higher at 28 °C than at 38 °C and the difference between the two data is statistically significant ($P < 0.01$, not shown in Table I). If the previously cited correlation between ATPase and transport does exist, the higher transport activity at 28 °C could be partly due to the level of $(Na^+ + K^+)$-

TABLE I

FLUID TRANSPORT AND WET WEIGHT TO DRY WEIGHT RATIOS UNDER DIFFERENT EXPERIMENTAL CONDITIONS

Fluid transport expressed in ml/g total dry weight of the intestine and per hour, refers to experiments carried out at 28 and 38 °C respectively. Wet weight to dry weight ratios reported for mucosa (mucosal scrapes) and submucosa (submucosal and muscular layers) relate to in vivo unperfused (control) and to 1 h in vitro perfused intestines at 28 and 38 °C respectively. Values ±S.E.M. are reported. The number of experiments are given in parentheses. Single P values refer always to the corresponding control values of the same column.

	Fluid transport ($ml \cdot g^{-1} \cdot h^{-1}$)	Wet weight/dry weight	
		Mucosa	Submucosa
Control	—	4.43±0.37 (5)	4.15±0.14 (5)
1 h at 28 °C	2.02±0.24 (11)	6.58±0.43 (4)	5.29±0.18 (4)
P		< 0.01	< 0.01
1 h at 38 °C	0.98±0.24 (11)	8.13±0.40 (5)	5.07±0.33 (5)
P		< 0.001	< 0.05

dependent ATPase during the 1-h experiments being higher at 28 °C than at 38 °C. We have determined this enzyme under the same experimental conditions as described in the present work, both in the mucosal homogenate and in two fractions of it, namely brush border and membranes. $(Na^+ + K^+)$-dependent ATPase activity does not differ statistically under any of the conditions tested (data from this laboratory). In Table I, the wet weight to dry weight ratio is reported for the scraped mucosa and the remaining submucosa for unperfused intestines (control) and after 1 h perfusion at 28 and at 38 °C. It is possible to note that the swelling after perfusion (which is always evident, see P data of Table I) is practically the same for the submucosa. Both the data after 1 h perfusion at 28 and 38 °C, are statistically equal: $P > 0.6$ (not shown in Table I). On the contrary, in the scraped mucosa the high temperature causes a further swelling; the difference between the two data, i.e. 1 h perfusion at 28 and 38 °C, respectively, is statistically significant: $P < 0.05$ (not shown in Table I).

This swelling of the mucosal cells may cause mechanical damage to the epithelium. By using the everted sac technique, Levine et al. [13] demonstrated, by a hystological evaluation, that disruption of intestinal epithelium is more pronounced at 37 °C than at room temperature. The intestinal swelling of in vitro experiments in general, and the transport activity decrease at 38 °C vs. 28 °C in particular, might be due to low levels of ATP, ADP and AMP in the intestines incubated in vitro, if they are not due to a different $(Na^+ + K^+)$-dependent ATPase content.

In Table II, the ATP, ADP and AMP content in μmol/g dry weight is reported under different experimental conditions for the in toto tissue and for one fraction of it, i.e. the mucosa obtained from the contiguous intestinal tract. To the best of our knowledge, only few data on the ATP, ADP and AMP content of the intestine are available from the literature; the in vivo data of Lamers and Hülsmann [14] are expressed in μmol per g wet weight; by taking into account the wet weight to dry weight ratio, our in toto data for control differ slightly from theirs. Our control mucosa data are very similar to those reported by Bronk and Leese [15] obtained with a peculiar tissue extraction device and expressed in μmol/g dry weight.

TABLE II

ADENYLIC NUCLEOTIDES IN THE INTESTINE

Adenylic nucleotide content expressed in μmol/g tissue weight of total intestine (in toto) and of mucosal scrapes (mucosa) obtained from the contiguous intestinal tract, are reported. Each value ±S.E.M. refers to the following conditions: in vivo unperfused intestine (control), the beginning (time zero) and the end (1 h) of the in vitro perfused intestine, at the reported temperatures.

	Control	Time zero at 28 °C	1 h at 28 °C	Time zero at 38 °C	1 h at 38 °C
No of expts.	5	4	6	4	5
ATP in toto	7.6±0.6	3.7±0.4	3.8±0.3	2.4±0.4	0.8±0.1
mucosa	6.7±0.7	4.4±0.3	3.8±0.4	1.9±0.5	0.4±0.1
ADP in toto	4.6±0.9	3.4±0.3	3.8±0.2	2.5±0.2	1.7±0.2
mucosa	5.1±0.6	4.2±0.9	3.7±0.8	2.9±0.3	1.7±0.3
AMP in toto	1.2±0.2	2.8±0.8	2.8±0.4	2.1±0.4	1.1±0.4
mucosa	1.5±0.2	1.6±0.4	2.1±0.5	1.8±0.4	0.7±0.4

It seems that the extent of the error of control data due to delayed freezing is low; from the data of Table II, with the addition of values concerning submucosa (ATP = 5.9±0.4 μmol/g; ADP = 4.6±0.8 μmol/g; AMP = 2.7±0.5 μmol/g) we see that the ATP content decreases from in toto to submucosa; the contrary happens for AMP, while for ADP the three values are similar. As the sum of the three nucleotides in single tissue is equal, delayed freezing in control experiments seems to cause only little transformation of ATP into ADP and ADP into AMP. Practically no differences are detectable among the three data (in toto and mucosa shown in Table II, submucosa not shown) in all other in vitro experiments.

It seems therefore that the scraping time at the beginning and at the end of the experiment is not critical. Normal blood supply certainly plays an important role in maintaining high levels of ATP and low levels of AMP.

If we compare the ATP data of control and after equilibration (time zero) at 28 and 38 °C (Table II), we observe that preperfusion per se is sufficient to produce a diminution of this nucleotide; a higher temperature accentuates the phenomenon. Comparing ATP data of time zero with the corresponding data after 1 h perfusion at the same temperature, we see that at 38 °C, ATP contents are lowered further in the two tissues; on the contrary, at 28 °C, the ATP content does not vary during 1 h perfusion. Similar behaviour is detectable in the ADP content under the same experimental conditions, but the phenomenon is less evident. No consistent variations are detectable in AMP content in the tested conditions.

Aerobic and anaerobic production of ATP and energy-depending processes are probably in equilibrium at 28 °C, so that a steady-state in ATP content is maintained for almost 1 h. A few years ago we saw, by means of the apparatus described in ref. 16, a constant oxygen consumption at 28 °C through a 1-h long experiment.

At 38 °C, ATP demand probably exceeds ATP production and the steady-state is never reached. Low intestinal content of ATP and ADP and high content of AMP are reported by other authors [17] after 15 min of incubation in vitro at 37 °C. More recently, the adenylic nucleotide content of the mucosa, scraped from rat intestine in experimental conditions similar to ours has been evaluated. The results of these authors are very similar to ours [20].

The energy demand at 38 °C is certainly higher than that at 28 °C because the rate of metabolic dependent reactions, such as transport processes, is 2–3 times higher at 38 °C than at 28 °C. In the absence of blood supply, if oxygen diffusion at 38 °C is not sufficient, intestinal cells could be in an hypoxic state, as suggested by other authors [19].

A support to this hypothesis may be the fact that intestines from normal and semi-starved rats incubated in vitro at 28 °C produce the same quantity of lactic acid [20]; similar experiments performed at 37 °C [21] show that the production of lactic acid is higher in the intestines from normal than from semi-starved rats. As the thickness of the intestinal wall is higher in normal than in semi-starved rats, an insufficient oxygen diffusion may be the cause of the higher lactic acid production.

Furthermore, it seems that the oxygen supply is critical also in experiments in which the intestine is vascularly perfused in vitro at 37 °C without erythrocytes. In these experiments, the ATP level depends on the flow rate of the vascular perfusion and it reaches the in vivo level only when fluorocarbon is added to the perfusion fluid [22]. The known effect of this substance is to carry oxygen.

In experiments carried out at 28 °C, the tissue content of each nucleotide

TABLE III

INTESTINAL FLUID TRANSPORT

Fluid transport in ml/g dry weight of the total intestine is reported for three subsequent periods of 20 min and for the sum of the three periods. Data refer to experiments carried out at 28 and 38 °C, respectively. Number of experiments given in parentheses.

	Time of incubation (min)			
	0–20	20–40	40–60	sum
Expt. at 28 °C (5)	0.59±0.12	0.50±0.05	0.56±0.08	1.69±0.22
Expt. at 38 °C (5)	0.41±0.11	0.11±0.06	−0.15±0.08	0.37±0.09

remained practically constant, but at 38 °C we had a progressive diminution of the total content of nucleotides. A diffusion of these nucleotides at 38 °C into the bathing solution, as supposed by other authors [15] does not seem to take place at 28 °C. At this temperature, the nucleotide content is constant in time. An increase of the inorganic phosphate in the tissue at 38 °C which has been found by other authors [18], could also explain our results; in another substrate, bullfrog gastric mucosa incubated in vitro and gassed with N_2, ATP depletion is accompanied by a rise of the inorganic phosphate level [23].

Since the results of fluid transport (Table I) are in contrast with the well-known effect of the temperature on metabolic dependent processes, we tried to shed more light into the problem by carrying out three subsequent 20 min experiments of fluid transport in the same intestine, both at 28 °C and at 38 °C. Data are reported in Table III. We observe that 1 h perfusion data, even if lower, are not statistically different if compared with those of Table I ($P > 0.4$ at 28 °C and $P > 0.1$ at 38 °C). From these data, we can also see that at 28 °C, fluid transport is constant in time: in the three subsequent periods of 20 min, reported data are practically equal. On the contrary, at 38 °C, fluid transport decreases in time; in fact in the second 20-min period it is almost abolished and in the third period it is even negative. A tentative explanation may be that in the last period, the cell swelling draws water from the serosal compartment. Therefore, it seeems that at 38 °C, fluid transport is not in a steady state. It has been shown by other authors [24] that in the case of everted sac intestines incubated in vitro glucose absorption beyond 30 min at 37 °C diminishes progressively to zero at 60 min, even if the substrate is gassed with 100 % O_2 (phosphate buffer).

If we now compare the data concerning the ATP levels (Table II) and on fluid transport (Table III) at the two temperatures, we observe that ATP level and fluid transport are constant at 28 °C but both decrease at 38 °C. As far as we know, there are no data concerning a correlation between intestinal fluid transport and energy-rich phosphate hydrolysis, however, for example, in the case of the active gastric secretion a correlation between H^+ secretion and energy-rich phosphate hydrolysis has been demonstrated [23, 25, 26]. From our data, we cannot calculate any ratio between ATP hydrolysis and fluid transport, but we observe that after 1 h at 38 °C, the ATP level seems too low to be utilized for transport phenomena. In bullfrog gastric mucosa

H^+ secretion stops if the ATP level is only 0.3–0.6 μmol/g wet weight [23]; under in vitro conditions this tissue, if normally oxygenated, maintains nucleotide levels similar to those of our control preparation.

From our data, it seems that the everted sac of rat intestine is a good preparation, if used only at low incubation temperature (28 °C).

Furthermore, it is possible to compare the data of the intestine perfused in situ at 37 °C with those of the everted and perfused intestine in vitro at 28 °C. In the first case, fluid transport is high (10.04 ± 1.07 ml $\cdot g^{-1} \cdot h^{-1}$) and the intestine is not swollen [27]. In the second case, the fluid transport is low and the intestine is swollen. This fact could be explained by the lowered temperature. If the metabolic dependent sodium extrusion from the basolateral membrane is more affected than its passive facilitated entrance across the brush border, cell sodium accumulation causes the cell to swell, lowers the chemical gradient for the entrance of sodium, and supplies more sodium to the pump. Thus a new profile of sodium concentration among the three compartments (mucosal, cell and serosal fluid) is reached and maintained throughout the time.

At 38 °C, in in vitro conditions a sodium profile similar to that in vivo should be maintained (10–20 mM intracellular and 143 mM extracellular) for a sustained transport of fluid. If the ATP level drastically decreases, the sodium pump cannot work, fluid transport stops and the accumulated sodium causes the enterocyte to swell. In Table I, we can see that mucosal cells are more swollen than submucosal ones. The hypoxic condition of the absorbing cells may be due to a slow oxygen diffusion from the mucosal side. Many barriers may hinder this diffusion: close to the mucosal surface, unstirred layers have been demonstrated [28], further the mucopolysaccharidic fuzzy coat constitutes a second barrier and the brush border is a third barrier. Now the oxygen must reach the energy-producing organelles for production of ATP which in turn is needed at the level of the basolateral membrane for the sodium pump.

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