REVISTA ESPAÑOLA DE FISIOLOGIA, 42 (3), 341-348, 1986

Preparation and Properties of Isolated Epithelial Intestinal Cells from Chicken Cecum and Jejunum

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(Received on November 26, 1985)

R. FERRER, J. M. PLANAS and M. MORETO. *Preparation and Properties of Isolated Epithelial Intestinal Cells from Chicken Cecum and Jejunum.* Rev. esp. Fisiol., **42** (3), 341-348, 1986.

The isolating agents, one enzymatic (hyaluronidase) and two chemical (sodium citrate and EDTA) have been used to search for the best technique to prepare suspensions of viable cells from chicken cecum and jejunum. Viability of enterocytes was assessed in terms of cell membrane integrity (trypan blue exclusion test), metabolic activity (oxygen uptake, lactate production and ATP content) and monosaccharide cumulative capacity. Results show that: 1) In both cecum and jejunum, membrane integrity is better in cells harvested with citrate than those isolated with hyaluronidase or EDTA; 2) The best metabolic status was found in cecal cells isolated with citrate and in jejunal cells obtained with hyaluronidase; 3) The capacity to support α-methyl-D-glucoside gradients is highest in the cells harvested with citrate. The citrate-containing isolation medium is thus considered to yield epithelial cell suspensions with the best functional conditions.

Key words: Isolated enterocytes, Sugar transport, Cell viability, Cecum and jejunum, Chicken.

Suspensions of epithelial intestinal cells are helpful in studies on the mechanisms of intestinal transport and are especially suitable in the study of the cumulative capacity of enterocytes since easy access to both apical and basolateral membranes is enabled. One can also manipulate and control the cell environment, thus avoiding problems associated with the presence of connective and muscular layers.

There are several techniques described for the isolation of enterocytes from the small intestine of the rat (17, 19), guinea pig (4), chicken (8) and rabbit (1) but there is little information about isolation procedures of cells from the large intestine.

In a previous study using tissue slices (15) the cecum of the chicken was shown to be able to accumulate significant amounts of monosaccharides in birds 0- to 7-wk-old. It was thus decided to study the cecal transport properties using isolated epithelial cells. In search of the suitable technique for cell isolation from the cecum three different isolation media were tested: one containing hyaluronidase, the

second containing EDTA*, and the third containing sodium citrate. The results show that the last one is the most adequate for the isolation of epithelial cells from the chicken cecum as well as from the jejunum.

Materials and Methods

Animals. Male white Leghorn chickens 5- to 7-wk- old were used. Animals were obtained from «Cooperativa Comarcal d'Avicultura de Reus (Tarragona)» and maintained in standardized temperature and humidity conditions, with free access to a commercial diet (Gallina Blanca-Purina, Barcelona). Chickens were killed in the morning by neck fracture without previous starvation.

Cell isolation. Enterocytes were isolated from the cecal segments and from the jejunum (yolk sac region) by using three different isolation media, one enzymatic and two chemical.

a) Hyaluronidase medium, with the following composition (in mmol/l): NaCl 80, K_2HPO_4 3, $CaCl_2$ 2.5, $MgCl_2$ 1, Tris-HCl 20, mannitol 100, EGTA 0.1, hyaluronidase 0.5 g/l and 1 mg/ml BSA at pH 7.4, similar to the one described by KIMMICH (8) for the isolation of epithelial cells from chicken small intestine.

b) Sodium citrate medium, with the following composition (in mmol/l): NaCl 80, K_2HPO_4 3, Tris-HCl 20, mannitol 37, EGTA 0.1, tri-sodium citrate 27 and 1 mg/ml BSA at pH 7.4. Sodium citrate has already been used at this concentration for cell harvesting from rat small intestine by STERN and JENSEN (19).

c) EDTA medium, with the following composition (in mmol/l): NaCl 80, K_2HPO_4 3, Tris-HCl 20, mannitol 100, EGTA 0.1, EDTA 5 and 1 mg/ml BSA at pH 7.4. This chelating agent was first employed by SOGNEN (18) for cell isolation from rat small intestine.

Intestinal segments, opened lengthwise and washed with ice-cold saline, were incubated in 40 ml isolation medium at 37°C for 30 min with 2 Hz shaking. The suspension was poured through nylon stocking material and the cells were washed twice with an ice-cold medium where either the enzyme or the chelating agents were omitted. In the cases of citrate and EDTA media, osmolarity was maintained by increasing mannitol concentration. The composition of the incubation medium was the same for cells obtained by either method and was (in mmol/l): NaCl 80, K_2HPO_4 3, $CaCl_2$ 2.5, $MgCl_2$ 1, Tris-HCl 20, mannitol 100, EGTA 0.1 and 1 mg/ml BSA (pH 7.4). In experiments where cells were incubated with 10 mmol/l glucose, osmolarity was also adjusted by reducing mannitol concentration.

Viability tests. The viability of cell suspensions, prepared by using the three isolation media, was evaluated with the following tests:

a) Membrane integrity. Cell membrane integrity was estimated by determining the fraction of the population that excludes trypan blue dye (20 g/l), accordding to GIRARDI *et al.* (5). Clumps of more than three cells were discarded from counts (3).

b) Respiration measurements. Oxygen uptake was determined by the Warburg technique (21). Cells (2-10 mg protein) were re-suspended in 2.5 ml of the incubation medium supplemented with 10 mg/ml BSA, as recommended by KIMMICH (8), and placed into siliconized Warburg vessels.

* Abbreviations: EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; Tris, tris-(hydroxymethyl)aminomethane; EGTA, ethylene glycol-diaminoethylether tetraacetic acid.

Incubation was made in the presence of 10 mmol/l glucose at 37°C, with a shaking rate of 2 Hz and 35 mm of amplituded. Pure oxygen was used as phase and the CO_2 produced was trapped in the centre well with a filter paper impregnated with 0.2 ml of 5 g/l KOH. Oxygen uptake readings were made every 10 min and Q_{O_2} values are expressed as µl of oxygen consumed per mg of cell protein per hour.

c) Glycolytic activity. Glycolytic activity was assessed by measuring lactate formation in the presence of 10 mmol/l glucose. One ml of cells (2-15 mg cell protein) was incubated in 3 ml of standard incubation medium supplemented with 10 mg/ml BSA. Temperature was maintained at 37°C and the vessels were shaken at 0.5 Hz. At 30 min intervals, 0.3 ml samples were removed and the cell activity was stopped by adding 0.6 ml of 3 ml/dl of ice-cold perchloric acid. The protein was sedimented by centrifugation (5 min, 3,000 *g*) and lactate was determined on a sample of the supernatant using a Sigma Test Kit, based on the enzymatic assay of HOHORST (6). Q_L values are expressed as nanomoles of lactic acid produced per mg of cell protein per hour.

d) ATP levels. The cellular ATP content was determined after isolation, in $HClO_4$-deproteinized samples neutralized with KOH using the method of LAMPRECHT and TRATSCHOLD (11). Results are expressed as nmol ATP per mg cell protein.

e) Sugar accumulation. To evaluate sugar transport capacity, cells (6-10 mg) were incubated in 4 ml of medium at 37°C with 0.5 Hz shaking. Accumulation of ^{14}C-labelled 1-o-methyl-α-D-glucoside (α-MG) was determined by removal of 0.2 ml samples of the cell suspension added to 1.8 ml of ice-cold medium. After centrifugation (1 min, 500 *g*), pellets were re-suspended twice in the same medium to remove most of the extracellular radioactivity. The volume of water trapped in the pellet was determined in parallel experiments using labelled polyethylene glycol 4000 (PEG 4000) for the correction of the externally trapped substrate. Pellets were extracted with 0.2 ml of 3 ml/dl perchloric acid, and the radioactivity quantified by liquid scintillation procedures.

Protein content was determined by the LOWRY *et al.* (13) method. When appropriate, data were analyzed by analysis of variance and were considered statistically significant at $p < 0.05$.

Materials. 1-o-methyl-α-D - [U - ^{14}C] glucoside (α-MG) was purchased from Amersham, U.K. and [1,2-^3H] polyethylene glycol 4000 (PEG 4000) was from New England Nuclear Corp., Boston, MA. Glucose, unlabelled α-MG and phloridzin were obtained from Sigma. Enzymatic ATP determination was carried out with reagents from Boehringer Manheim GmbH.

Results

Microscopic examination of the cell suspensions prepared by the hyaluronidase,

Table I. *Trypan blue exclusion as a test of cell membrane integrity of cecal and jejunal epithelial cells isolated by three different methods.* Results are expressed as mean ± SE. Number of experiments is given in parenthesis. a: $p < 0.05$, for cecum and b: $p < 0.05$, for jejunum.

	% dye exclusion	
Isolation medium	Cecum	Jejunum
Hyaluronidase (0.5 g/l)	49.7 ± 0.76[a] (4)	46.9 ± 0.76 (3)
Sodium citrate (27 mmol/l)	67.6 ± 3.67 (3)	62.9 ± 0.05[b] (3)
EDTA (5 mmol/l)	66.8 ± 6.10 (3)	48.0 ± 3.20 (3)

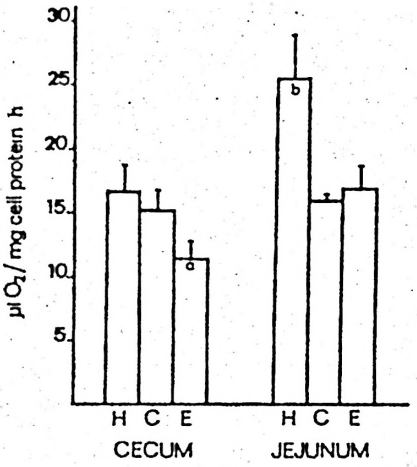

Fig. 1. *Oxygen uptake by isolated epithelial cells obtained from chicken cecum and jejunum with three different isolation agents: hyaluronidase (H), sodium citrate (C) and EDTA (E).*
Results are expressed as mean ± SE for 4-6 experiments. a: $p < 0.05$, for cecum and b: $p < 0.05$, for jejunum.

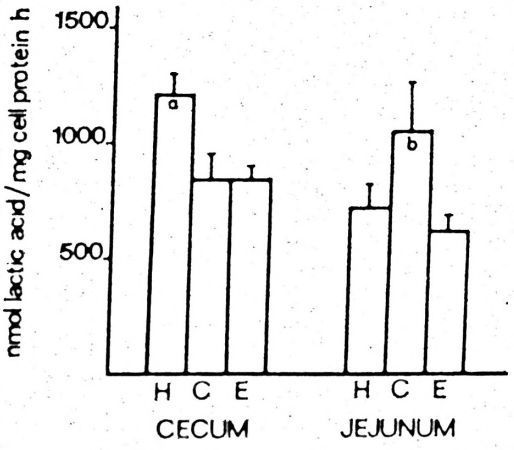

Fig. 2. *Lactic acid production by isolated epithelial cells obtained from chicken cecum and jejunum with three different isolation agents: hyaluronidase (H), sodium citrate (C) and EDTA (E).*
Results are expressed as mean ± SE for 5-8 experiments. a: $p < 0.05$, for cecum and b: $p < 0.05$, for jejunum.

sodium citrate and EDTA procedures showed morphologically identifiable intestinal epithelial cells and absence of bacterial contamination. The three methods yielded elongated columnar cells as well as some cuboidal and spherical cells. Enterocytes occurred singly, in clumps and in sheets. Cell yield was greater with chelating agents than with hyaluronidase though a similar cell dispersion was observed with the three procedures.

Table I shows cell membrane integrity estimated by the trypan blue test. The number of cells that exclude the dye is higher in cells harvested with sodium citrate followed by the cells prepared using EDTA. The lowe values were obtained with hyaluronidase. Oxygen consumption and lactic acid production were linear for at least 90 min in the six cell suspensions studied. In cecal cells, the respiratory activity was greater in those prepared with hyaluronidase and sodium citrate than with EDTA (fig. 1), while in jejunal cells the highest values were obtained with the hyaluronidase-based method. Figure 2 indicates that cecal cells harvest-

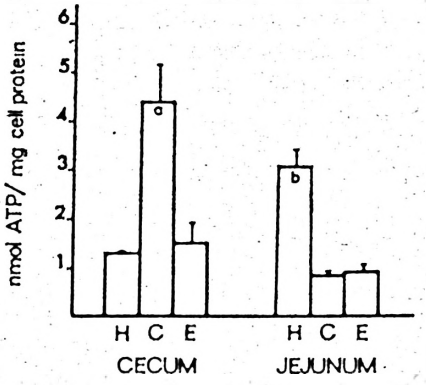

Fig. 3. *ATP levels for cecal and jejunal epithelial cells prepared by hyaluronidase (H), sodium citrate (C) and EDTA (E) treatment.*
Results are expressed as mean ± SE for 3-6 experiments. a: $p < 0.05$, for cecum and b: $p < 0.05$, for jejunum.

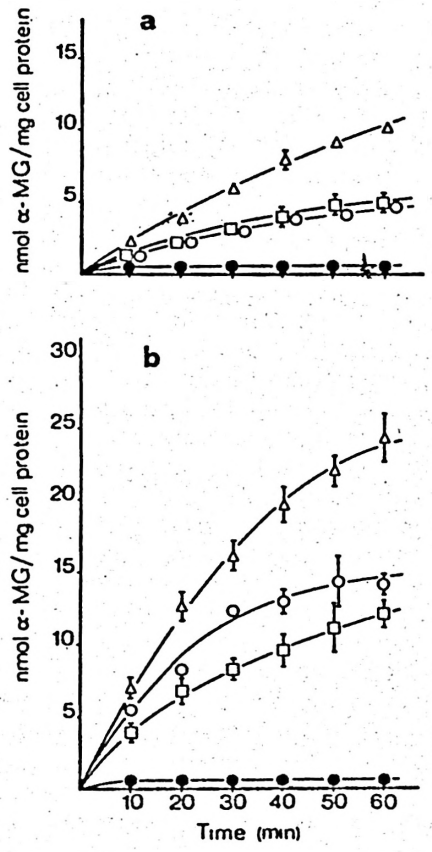

Fig. 4. *Accumulation of 0.1 mmol/l α-methyl-D-glucoside (α-MG) by isolated enterocytes from cecum (a) and from jejunum (b) with three different isolation agents:* □, *hyaluronidase;* △, *sodium citrate and* ○, *EDTA.*

Results of sugar accumulation in the presence of 0.2 mmol/l phloridzin in the three suspensions from each intestinal segment do not differ statistically and are thus plotted together (●). Each point is the mean ± SE for 6 separate experiments. Only standard errors that exceed size symbol are shown.

ed with hyaluronidase show the highest glycolytic activity (Q_L) while in jejunal cells the higher Q_L is observed in those isolated with sodium citrate. ATP levels determined immediately after the isolation process are shown in figure 3. The cecal cells isolated with citrate and the jejunal cells prepared with hyaluronidase have significantly higher ATP levels than cells isolated by the other methods. Figure 4 shows 0.1 mmol/l α-MG uptake by cecal and jejunal cells and the effect of phloridzin, a well-known inhibitor of the active hexose transport system. Both cecal and jejunal cells isolated by the sodium citrate method have the highest cumulative capacity. Phloridzin insensitive sugar uptake was the same in the six cell populations.

Discussion

Several techniques using enzymes or chelating agents —either alone or in combination— together with mechanical dissociation have been successfully employed for the isolation of intestinal epithelial cells. Such techniques have been used to dissociate epithelial intestinal cells for their use in transport studies. In most cases, cells show considerable viability and cumulative capacity depending on the animal species used and the intestinal region studied (9).

In the chicken small intestine, the hyaluronidase-based mechanical agitation procedure described by KIMMICH (8) provides a high yield of cells that are viable for 2 h and capable of supporting high sugar concentration gradients. In preliminary experiments this technique was applied to the cecal segment and found that the number of enterocytes isolated by this method was rather small. It was thus decided to try chelating agents such as sodium citrate and EDTA. Researching a method for cell isolation it is essential for isolated enterocytes to maintain most of the functional properties in the intact tissue. This was verified by membrane integrity, and metabolic and sugar uptake tests.

To determine cell membrane integrity, the trypan blue exclusion test was chosen bacause it is quick and simple to perform. Maximum viability was obtained in the

cells isolated with sodium citrate, in both cecal and jejunal segments, in agreement with what was found by EADE *et al.* (3). These authors, working with rat small intestine, observed higher viability in cells isolated with sodium citrate plus EDTA (70 %) than cells harvested with EDTA alone (48 %) or with hyaluronidase (40 %). It is noteworthy that the percentage of jejunal cells that exclude the dye in the present study, using the hyaluronidase-based isolation medium (47 %), is much lower than what is claimed for jejunal cells prepared in similar conditions (e.g. about 80 %, ref. 8). Such differences can be ascribed both to the difficulty of quantifying cell number accurately and to the criteria employed for such quantification. For instance, in the present study, aggregates of more than three cells were discarded and pale blue stained cells were considered damaged. Thus, dye exclusion methods give only a rough estimate of cell viability. The metabolic and functional studies are those which will tell what procedure yields a superior cell population.

The metabolic activity of the six cell suspensions studied was evaluated in terms of their respiratory and glycolytic capability as well as their ATP content. Epithelial cell preparations usually exhibit linear rates of oxygen consumption and lactate production for only short periods of time (20) which may indicate extensive autolytic disintegration of the cells. In contrast, in the present study, both cecal and jejunal cells obtained by the three methods show linear rates of oxygen consumption and lactate production for 90 min, in accordance with KIMMICH (8) and PRIOR *et al.* (16). Furthermore, either Q_{O_2}, Q_L or ATP levels of all suspensions studied are within the range of what has been found by others (7, 8, 14).

Oxidative phosphorylation normally supplies a significant fraction of the total cellular ATP. Accordingly, high ATP values are correlated with high oxygen consumption rates. In the intestinal mucosa, either a decrease in ATP levels or an increase in ADP (or both) may be important signals for stimulating glycolysis which results in elevated lactic acid yields (9). High lactate production rates is characteristic of the intestinal epithelium even in aerobic conditions (2, 12). The present results show that cellular ATP levels in the cells obtained using hyaluronidase and citrate are inversely correlated with lactic acid production. Thus, jejunal cells isolated with hyaluronidase and cecal cells isolated with citrate have a high ATP content and produce small amounts of lactic acid while cells obtained from the jejunum using citrate and from the cecum using the enzyme have low levels of ATP and produce large amounts of lactate. However, both cecal and jejunal cells isolated using EDTA had low ATP and lactic acid values. This is in agreement with the results obtained by HÜLSMANN (7) who observed that enterocytes from rat small intestine isolated with EDTA had half the ATP levels than those obtained with an equimolar concentration of EDTA and $MgCl_2$. Oxygen consumption was also inversely correlated with lactate production in four of the six cell populations. The exceptions were the jejunal cells isolated with EDTA —showing relatively low lactic acid production— and cecal cells isolated with EDTA —showing low Q_{O_2} and Q_L values.

The overall results of metabolic studies indicate that the superior cell populations are the jejunal enterocytes isolated with hyaluronidase and cecal cells harvested with citrate since both retain high ATP levels, have high oxygen consumption rates and low lactate production. This conclusion is also valid if metabolic data are calculated considering the different membrane integrity of each cell population.

The last test done in order to evaluate cell function was to characterize the sugar

uphill transport capability of the six cell populations. This gave an indication of membrane integrity and of the metabolic status of the cells.

Results show that jejunal cells can maintain higher sugar gradients than cecal cells. Such differences were already expected since in a previous study (15) it was shown that in 5-to 7-wk old birds most of the cecal transport ability is restricted to the proximal region of this intestinal segment, with a capacity similar to that of the jejunum. Thus, accumulation figures obtained in the present study, using cells from the whole cecal segment, are lowered by the presence of medial and distal cecal enterocytes that have very low, if any, sugar cumulative capacity. In both cecum and jejunum, the cumulative capacity of cells isolated with citrate is greater than that of cells isolated with hyaluronidase and EDTA. This cannot be ascribed to the higher membrane integrity of citrate-isolated cells because recalculation of the accumulation data, taking into account this parameter, does not affect the relative cumulative capacity of the six cell populations. This set of results indicate, first, that cells isolated with hyaluronidase and EDTA retain a lower transport capacity than citrate-isolated cells and, second, that conclusions derived from metabolic tests tell little about the transport properties of the cells (e.g., jejunal cells isolated with EDTA can support similar sugar gradients to hyaluronidase-isolated cells even though having poorer metabolic activity). Worthy of note is the observation that α-MG accumulation in jejunal cells isolated with hyaluronidase reach similar levels to those found by KIMMICH and RANDLES (10). Since our cells had lower viability than the 80 % described by KIMMICH and RANDLES, the cumulative capacity of our cell population may well be higher. Finally, phloridzin-insensitive α-MG uptake is the same in all cell suspensions studied, indicating that cellular volume is not affected by the method employed to harvest the cells.

In conclusion, the use of sodium citrate for cell disgregation from the chicken intestine yields cells with the best viability. However, jejunal cells isolated with hyaluronidase are not to be discounted for metabolic studies as they also have adequate properties.

Acknowledgements

We wish to express our sincere appreciation to Dr. J. Aguilar for his critical review of the manuscript. This work was subsidized by grant 3099-83 from «Comisión Asesora Científica y Técnica, Ministerio de Educación y Ciencia», Spain.

Resumen

Se ensayan tres agentes, uno enzimático (hialuronidasa) y dos químicos (citrato sódico y EDTA), para el aislamiento óptimo de células epiteliales de ciego y yeyuno de pollo. La viabilidad de los enterocitos se evalúa mediante el estudio de la integridad de la membrana celular (prueba de la exclusión del azul de tripán), actividad metabólica (consumo de oxígeno, producción de ácido láctico y concentración de ATP) y capacidad de las células para acumular monosacáridos. Los resultados indican: que, en ciego y yeyuno, la integridad de la membrana celular usando citrato es superior a la obtenida con hialuronidasa y EDTA; el mejor estado metabólico lo presentan las células cecales aisladas con citrato y las de yeyuno obtenidas con hialuronidasa; y las células aisladas con citrato son las que poseen mayor capacidad para establecer y mantener gradientes de α-metil-D-glucósido.

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