Isolation of cells from rabbit renal proximal tubules by using a hyperosmolar intracellular-like solution

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A novel method of isolation of cells from rabbit kidney proximal tubules by using an intracellular-like solution (ICS) and gentle mechanical agitation in the absence of enzymes or chelators is described. Metabolic and functional characteristics of these cells were studied after washing and resuspension in modified Hanks medium, and the results were compared with those obtained in cells similarly prepared in extracellular-like solution (ECS). Trypan Blue exclusion and protein content were not different between the two preparations. However, oxygen consumption, ATP content and time- and concentration-dependent rates of uptake of phosphate, α -methyl glucoside and L-alanine were severalfold higher in cells prepared in ICS. Na^+ -dependent uptake of these solutes was 95% and 80% of total uptake in cells prepared in ICS and ECS respectively. Maximum transport rates (T_{max}) of phosphate, α -methyl glucoside and L-alanine were significantly higher in cells prepared in ICS. We propose that the use of ICS in the isolation procedure would yield a functionally more viable cell preparation, and therefore provides an ideal model for transport and metabolic studies at a cellular level.

Attempts have been made in the past to isolate cells from chick (Larkins *et al.*, 1974; Liang *et al.*, 1982), rat (Jones *et al.*, 1979), rabbit (Thimmapayya *et al.*, 1970; Eveloff *et al.*, 1980), dog (Michelakis, 1975) and human (Bear *et al.*, 1975) kidneys by employing digestive enzymes and/or chelating agents. Since these preparations utilized renal cortex or medulla or the whole kidney as the starting material, the resulting cells are heterogeneous in nature. To lessen the heterogeneity, separation of proximal-tubule cells from rat (Kreisberg *et al.*, 1977) and rabbit (Heidrich & Dew, 1977) kidney cell suspension by free-flow electrophoresis has been reported. However, the suitability of these cells for transport and metabolic studies was not examined.

Since better functional preservation has been achieved in dog and human cadaver kidneys for

transplantation by using hyperosmolar ICS (Collins *et al.*, 1969; Sacks *et al.*, 1973; Diethelm *et al.*, 1975; Ross *et al.*, 1976), we postulated that the use of such a solution during the isolation procedure could lessen cell damage and thus better preserve the functional characteristics of the cell. To examine this possibility, attempts were made to prepare cells from rabbit proximal tubules by using either ICS or the conventional ECS during the isolation procedure.

In the present paper, a simple and rapid method is described for isolation of cells from purified rabbit proximal tubules by gentle mechanical agitation in the absence of digestive enzymes and chelators. Metabolic and transport properties of cells isolated with ICS are compared with those isolated with ECS. Evidence in support of the superiority of the use of ICS is presented.

Materials and methods

Chemicals

Carrier-free $\text{H}_3^{32}\text{PO}_4$, [$\text{U}-^{14}\text{C}$] α -methyl D-glucopyranoside (275 Ci/mol) and L-[$\text{U}-^{14}\text{C}$]alanine

Abbreviations used: ICS, intracellular-like solution; ECS, extracellular-like solution; ICS cells, cells prepared in ICS; ECS cells, cells prepared in ECS; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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(176Ci/mol) were purchased from New England Nuclear Co. All other chemicals of analytical grade were obtained from Sigma or J. T. Baker Chemical Co.

Solutions

(1) ICS is a modified Collins solution (Collins *et al.*, 1969), with the following composition (mM): KCl 14, K_2HPO_4 44, $NaHCO_3$ 9, glucose 180; pH 7.4 adjusted with KH_2PO_4 ; osmolality 330 mosmol/kg. (2) ECS is a modified Hanks solution, with the following composition (mM): NaCl 137, KCl 5, Na_2HPO_4 0.33, KH_2PO_4 0.44, glucose 5.5, Tris/HCl 10, glutamine 2; pH 7.4 adjusted with Tris base; osmolality 285 mosmol/kg. (3) Uptake medium (mM): NaCl 137, KCl 5, $CaCl_2$ 1.3, $MgCl_2$ 0.5, $MgSO_4$ 0.4, $NaHCO_3$ 4, Hepes 10, glutamine 2, β -hydroxybutyrate 1; pH 7.4 adjusted with Tris base. (4) 'Stop solution' (mM): NaCl 140, Na_2HAsO_4 10, Hepes 5; pH 7.2 adjusted with KOH.

Preparation of cells

New Zealand White male rabbits (1.3–1.7 kg), fed *ad libitum* on standard rabbit chow, with tap water as the drinking water, were used. Isolation of proximal tubules from rabbit kidneys was similar to methods previously described (Brendel & Meezan, 1975; Chung *et al.*, 1982). All procedures were carried out at room temperature. Rabbits were killed by cervical dislocation, and the kidneys were rapidly excised with intact renal arteries. From this point until the cells were resuspended in the uptake medium, either ICS or ECS medium was used consistently in each of the preparatory steps. Kidneys were perfused through the renal artery till they became pale and the venous outflow was clear. This was followed by perfusion with medium containing iron oxide until the kidney became grey-black. The cortex was removed, minced into approx. 0.5 cm pieces and homogenized with a Dounce hand-driven homogenizer by giving five full strokes with the B pestle. The homogenate was suspended in 500 ml of the preparatory solution and filtered through 250 μ m- and 80 μ m-pore-size nylon meshes (Nytex; Tetco, Elmsford, NY, U.S.A.). The contents, left on the 80 μ m mesh, were washed, removed and suspended in 100 ml of the fresh medium. The iron oxide associated with the glomeruli was carefully removed from the suspension with a magnet. Microscopic examination of this suspension revealed predominantly proximal tubules, with only few glomeruli and single cells. The tubule suspension was transferred into a suspension-culture flask (Celstir; Wheaton Instruments, Millville, NJ, U.S.A.) and stirred at moderate speed for 30 min to release the cells from the tubules. The contents of the flask were then

passed through 80 μ m- and 41 μ m-pore-size nylon meshes to separate the isolated cells from the tubules. The filtrate was centrifuged at 1315g (Clays Adams model 0131) for 1 min, and the cell pellet, prepared in either ICS or ECS up to this point, was then suspended in the uptake medium. The cells were washed three times with the fresh uptake medium by repeated centrifugation (30s) and suspension. The cell pellet was finally suspended in the uptake medium to yield approx. 15×10^6 cells/ml and counted with a haemocytometer after appropriate dilution. About 95% of the cells in the final suspension were found as single cells, and the remainder as pairs. No tubule fragments or glomeruli could be detected. At least 30 min elapsed between final suspension of cells in the uptake medium and subsequent use for experimental studies.

Viability studies

Trypan Blue exclusion was determined by differential counting of stained and unstained cells in the presence of the dye (Patterson, 1979). Oxygen consumption by cells was measured with an Oxygraph (Gilson model 5/6). Cells (2×10^7) suspended in 0.5 ml of uptake medium containing 1 mM-sodium phosphate were injected into a water-jacketed glass chamber (1.8 ml volume) filled with similar solution and fitted with a platinum electrode polarized to 0.8 V. The temperature of the chamber was maintained at 37°C by continuous circulation of water. O_2 consumption was linear with time for at least 15 min. Changes in O_2 content in 10 min were used for the calculation of the rate of O_2 consumption by the method of Estabrook (1967). Experimental zero O_2 concentration in the chamber was obtained by adding sodium dithionite. Cellular ATP content was determined in $HClO_4$ extracts of the cells, neutralized with KOH, by a fluorimetric method using hexokinase and glucose-6-phosphate dehydrogenase (Greengard, 1974). Protein was measured in cell pellets dissolved in 1 M-NaOH by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Uptake studies

Cells were separated from the incubation medium by centrifugation, as this method gave more consistent results than with the Millipore-filtration technique. The uptake of the labelled solutes was linear with the concentration of cells in the range 0.8×10^6 – 2.4×10^6 cells in a final incubation volume of 200 μ l. After final washing, cells were suspended in uptake medium containing the indicated concentrations of the appropriate solute. Approx. 1.5×10^6 cells, suspended in 100 μ l of uptake medium in an uncapped borosilicate-glass

test tube, were preincubated in a shaking water bath at 37°C for 2 min. An additional 100 μ l of similar uptake medium (prewarmed to 37°C) containing unlabelled and a trace amount of labelled solute under study was then added, and the incubation was continued for different time intervals as indicated in the text. The reaction was arrested by adding 1 ml of ice-cold 'stop' solution into the test tube, which was then left on ice for 2 min. The test tubes were then centrifuged (1315g) for 1 min and the supernatants discarded. The cell pellets were again washed with 1 ml of the ice-cold stop solution and centrifuged. The final cell pellets were dissolved in 0.5 ml of 1 M-NaOH overnight at room temperature. The solubilized cells were transferred into a scintillation cocktail (ACS; Amersham), to which another 0.5 ml of the water used to rinse the tubes was added. The radioactivity was counted in a liquid-scintillation counter (Beckman model LS9800). Appropriate zero-time controls were made for all the samples by adding uptake medium containing labelled solute after the addition of ice-cold 'stop' solution to the cell suspension and cooling on ice. The procedure described above was similar to that used by Liang *et al.* (1982) for chick renal cells. For inhibitor studies, cells were suspended in uptake medium containing inhibitor before incubation. Kinetic parameters $T_{\max}$ and K_m were calculated, from the values of solute concentration and the corresponding uptake rates, by linear-regression analysis.

Results and discussion

The light-microscopic observation of ICS and ECS cells indicated heterogeneity in cell size and shape. However, no morphological differences could be detected between the two cell preparations. Representative light-microscopic pictures of ICS cells are shown in Fig. 1(a). Examination of the scanning electron micrographs of both cell preparations suggested well-preserved morphological integrity of the individual cells. In some projections, the presence of microvilli was observed (Fig. 1b).

The yield and the viability characteristics of isolated cells are summarized in Table 1. To our knowledge, no similar data are available for cell preparations from isolated rabbit proximal tubules for comparison. Neither Trypan Blue exclusion nor protein content was different between the two preparations, suggesting that the cells excluded vital dye, and loss of cellular protein, if any, was similar in the two cell preparations. The O_2 -consumption rate of ICS cells was 4-fold higher than for ECS cells. The O_2 consumption of the cells, when left at room temperature, did not decrease

Fig. 1. Representative light-microscopic (a) and scanning-electron-microscopic (b) pictures of ICS cells

For scanning electron microscopy, cells were pelleted by centrifugation and were fixed in 2% (v/v) glutaraldehyde/2% (w/v) paraformaldehyde in 0.1 M-cacodylate, pH 7.4, for 24 h at 4°C. After dehydration in graded series of alcohols, specimens were critical-point-dried with CO_2 as transition fluid and then sputter coated by using a hummer 1. Specimens were examined and photographed with an ETEC Autoscan microscope. Magnification: (a) $\times 160$; (b) $\times 6800$.

significantly for up to 3 h (results not shown). Addition of 0.5 mM-ADP did not stimulate respiration rates in either cell preparation, suggesting that the cell membrane is impermeable to this nucleotide. Addition of antimycin A (50 μ M) inhibited the O_2 -consumption rates by approx. 46 and 27% in ICS and ECS cells respectively. The higher ATP

Table 1. *Yield and viability parameters*

Experimental details are given in the text. Values are means $\pm$ S.E.M. for the numbers of individual preparations indicated in parentheses.

	ICS cells	ECS cells
Yield (10^6 cells/2 kidneys)	56.8 ± 3.9 (25)	34.5 ± 2.4 (20)
Trypan Blue exclusion (%)	91 ± 0.5 (19)	89 ± 0.8 (13)
Protein ($\mu\text{g}/10^6$ cells)	134.1 ± 4.2 (13)	134.6 ± 7.0 (9)
O_2 consumption ($\text{nmol}/10^7$ cells per min)	3.4 ± 0.3 (14)	0.9 ± 0.1 (10)
ATP ($\text{nmol}/10^7$ cells)		
(a) Initial	8.5 ± 0.7 (8)	2.1 ± 0.2 (9)
(b) After 30 min incubation at 37°C	10.0 ± 0.8 (8)	2.2 ± 0.2 (8)

Table 2. *Kinetic parameters of uptake of P_i , α -methyl glucoside and L-alanine by ICS and ECS cells*

Incubation time was 1 min. K_m and $T_{\max}$ values were calculated, from the values of solute concentration and the corresponding uptake rates, by linear-regression analysis. Apparent K_m values are given in mM and $T_{\max}$ values as nmol/min per 10^6 cells. Results are means $\pm$ S.E.M. for three individual cell preparations.

	$T_{\max}$		K_m	
	ICS	ECS	ICS	ECS
P_i	0.337 ± 0.046	0.068 ± 0.017	0.427 ± 0.069	0.407 ± 0.054
α -Methyl glucoside	3.36 ± 0.80	1.22 ± 0.25	2.60 ± 0.19	5.17 ± 0.61
L-Alanine	12.41 ± 1.70	2.76 ± 1.12	13.72 ± 2.08	19.00 ± 3.05

content observed in ICS cells correlates well with the O_2 -consumption rates. Maintenance of ATP content during 30 min incubation at 37°C indicates that the energy metabolism was regulated. Lower ATP content and O_2 consumption in ECS cells might possibly be due to loss of adenine nucleotides or to mitochondrial damage. Since ATP is the major fraction of the total adenine nucleotides under normal conditions in the cell, the higher ATP content in ICS cells is unlikely to be the result of an augmented synthesis of adenine nucleotides *de novo* within the short period of time of isolation. The ATP contents found in ICS cells are closer to the reported values of about 9.0 nmol/mg dry wt. for rat proximal tubules (Burch *et al.*, 1980) and 7.5 nmol/mg of protein for rabbit proximal tubules (Balaban *et al.*, 1980). The fact that in ECS cells Trypan Blue exclusion and morphology were not different, whereas the metabolic and transport rates were lower than in ICS cells, would suggest that Trypan Blue exclusion may not accurately represent the functional integrity of the isolated proximal tubular cells, a conclusion in agreement with another report (Heidrich & Dew, 1977).

The time course of uptake of P_i (0.1 mM), α -methyl glucoside (1 mM) and L-alanine (1 mM) (Fig. 2) revealed that the accumulation of each solute in the cells was a time-dependent and saturable process. Although most of the solute uptake was Na^+ -dependent in both cell preparations, the percentage of Na^+ -dependent uptake was higher in ICS cells (95%) than in ECS cells (80%). The accumulation of solutes continued to increase up to

30 min in ICS cells, whereas it tended to decrease after 10 min in ECS cells. The lower uptake rates of solutes and higher Na^+ -independent transport seen in ECS cells could be due to a decreased ability to maintain the ionic gradients, as a consequence of inadequate energy metabolism or changes in the membrane properties, or both.

To see whether the differences in time-dependent concentrative uptake rates of solutes between ICS and ECS cells were due to the loss or masking of specific transporters in the membrane, or to changes in the binding affinities, kinetic studies were undertaken. Since the uptake rate of each solute was linear with time up to at least 2 min, the initial uptake rates as a function of solute concentrations were studied at 1 min incubation time. At all the concentrations of each solute studied, the uptake rates were higher in ICS cells (Fig. 3). The kinetic parameters of three such experiments (Table 2) revealed that the maximum transport rates ($T_{\max}$) of all the three solutes were higher in ICS cells. This observation suggests the possibility that either there was a loss of transporters from the membrane or the accessibility of the transporters to solutes became limited in ECS cells. The apparent K_m values (Table 2) were higher for α -methyl glucoside and L-alanine in ECS cells, but were not different for P_i . Thus the affinity of the transporters for α -methyl glucoside and L-alanine, but not for P_i , was also lower in ECS cells.

The effects of some known inhibitors on the rates of uptake of P_i and α -methyl glucoside by cells were studied in an attempt to obtain a possible clue

Fig. 2. Time course of solute uptake by ICS and ECS cells in the presence and absence of Na^+

Concentrations of the solutes in the final incubation medium were (a) P_i (0.1 mM), (b) α -methyl glucoside (1 mM) and (c) L-alanine (1 mM). NaCl was replaced by KCl in Na^+ -free medium. Data presented for each solute were obtained from duplicate samples of a single preparation and are representative values of three such preparations. ICS, ICS cells; ECS, ECS cells; +Na, Na^+ -containing medium; -Na, Na^+ -free medium.

Fig. 3. Effect of (a) P_i , (b) α -methyl glucoside and (c) L-alanine concentrations on their respective uptakes by ICS cells and ECS cells in the presence of Na^+

Incubation time was 1 min. Inset shows Lineweaver-Burk plots. Data shown for each solute were obtained from duplicate samples of a single preparation. Calculated kinetic parameters of three such experiments are given in Table 2. ICS, ICS cells; ECS, ECS cells.

to the differences in the uptake of solutes by the two cell preparations. The effect of ouabain on uptake of P_i and α -methyl glucoside was studied in K^+ -free medium, because K^+ is known to decrease

Table 3. Effect of inhibitors on uptake of P_i (0.1 mM) and α -methyl glucoside (1 mM) by ICS cells and ECS cells. KCl was replaced by NaCl in K^+ -free medium. Incubation time was 1 min. Values are expressed as percentages of control (100%). Results are means $\pm$ S.E.M. for duplicate samples of three cell preparations.

Inhibitor	Concn. of inhibitor (mM)	P_i uptake		α -Methyl glucoside uptake	
		ICS cells	ECS cells	ICS cells	ECS cells
Control	—	100.0 $\pm$ 27.0	100 $\pm$ 5.0	100.0 $\pm$ 10.2	100 $\pm$ 29.0
Phlorrhizin	0.1	—	—	2.1 $\pm$ 0.3	5.5 $\pm$ 2.6
Phloretin	0.1	—	—	84.0 $\pm$ 6.8	73.0 $\pm$ 24.0
Arsenate	1.0	40.0 $\pm$ 21.0	31.0 $\pm$ 12.6	—	—
Arsenate	5.0	14.0 $\pm$ 1.8	11.5 $\pm$ 2.1	—	—
K^+ -free medium	—	102.0 $\pm$ 28.0	87.5 $\pm$ 6.4	96.0 $\pm$ 4.9	141.0 $\pm$ 29.0
Ouabain in K^+ -free medium	1.0	74.0 $\pm$ 12.0	39.5 $\pm$ 21.0	76.8 $\pm$ 6.8	113.0 $\pm$ 5.7

the Na^+ -facilitated binding of ouabain to the ($Na^+ + K^+$)-activated ATPase. There is no significant difference in percentage inhibition of uptake of P_i and α -methyl glucoside between these two cell preparations (Table 3). The rate of inhibition of uptake of these solutes in both cell preparations was similar to those observed in chick renal cells (Liang *et al.*, 1982) and in cultured rabbit proximal-tubular cells (Chung *et al.*, 1982).

There is no previous report of preparation of cells from isolated proximal tubules which are suitable for metabolic and functional studies. We have now prepared structurally intact and functionally viable cells from rabbit renal proximal tubules without using enzymes or chelators. The use of hyperosmolar intracellular-like solution appears to enhance further the functional characteristics of the isolated cells. We suggest that ICS cells may be a more ideal model in which to study transport and metabolic functions at a cellular level.

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