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Preparation of isolated brush border membranes from rabbit gallbladder epithelium: a preliminary study on the presence of the anion sensitive ATPase activity

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Fisiologia. — *Preparation of isolated brush border membranes from rabbit gallbladder epithelium: a preliminary study on the presence of the anion sensitive ATPase activity* (*). Nota di DARIO CREMASCHI, GIULIANO MEYER e SANDRA BERMANO, presentata (**) dal Corrisp. V. CAPRARO.

RIASSUNTO. — Nelle cellule epiteliali di cistifellea di coniglio sono state saggiate l'attività della ATPasi Na^+—K^+, della ATPasi anionica, della saccarasi, leucinoaminopeptidasi e fosfatasi alcalina. Si è tentato inoltre l'isolamento dell'intero orletto a spazzola e di frammenti di membrane apicali, nelle quali si è ricercata la presenza dell'ATPasi anionica (che potrebbe essere alla base dei trasporti di HCO_3^-, Cl^- e H^+ rilevati in cistifellea).

Come risultato l'ATPasi Na^+—K^+ è stata proposta come marcatore delle membrane baso–laterali e la fosfatasi alcalina delle membrane plasmatiche soprattutto apicali di cistifellea. Non si è riusciti ad isolare l'orletto a spazzola intero, ma si è giunti ad un discreto isolamento di membrane plasmatiche apicali col metodo della precipitazione con Ca^{2+}. Non è stata rilevata alcuna attività dell'ATPasi-anionica di tipo mitocondriale in questo tipo di membrane.

INTRODUCTION

A HCO_3^--activated ATPase is claimed to have been isolated from plasma membranes of many epithelia and often considered to be responsible for Cl^-/HCO_3^- or H^+/HCO_3^- exchange processes [4, 5, 13, 16, 18, 19, 20, 29, 31, 32, 33, 42], in spite of the fact that the isolation has been often controversial [14, 35, 37, 38, 39]. In the brush border membranes of rabbit gallbladder both exchange processes are present [3, 6, 28, 41], so that is seems relevant to isolate brush border membranes and to examine the possible presence of this activity in this cellular fraction.

Since gallbladder microvilli have never been isolated, we have tested many current isolation techniques used for other epithelia. The aim of this paper is to compare the results obtained and to report a preliminary assay of the ATPase.

MATERIALS AND METHODS

Gallbladders excised from New-Zealand rabbits (killed by a blow on the head) were washed free of bile with ice-cold bicarbonate Krebs-Henseleit solution (mM: Na^+ 142.9, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, Cl^- 127.7, HCO_3^- 24.9, SO_4^{2-}

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1.2, $H_2PO_4^-$ 1.2), bubbled with 95% O_2 and 5% CO_2 (pH 7.4). The mucosa was scraped off gently with a glass slide on ice, rapidly weighed and homogenized by an Omni-mixer (Ivan Sorvall Inc., Norwalk, Connecticut, USA) at 0 °C, at the speed and for the time later on reported. The saline used was different according to the procedure employed. As a first approach we looked at the HCO_3^-–ATPase, Na^+–K^+–ATPase, alkaline phosphatase, leucine-aminopeptidase and cytochrome *c* oxidase in the homogenate as, with the exception of Na^+–K^+–ATPase, very little is known about enzymes present in the gallbladder epithelial cells. The mucosae (300 mg) were homogenized twice for 30 sec at full speed in 2 ml 5mM Na_2EDTA, pH 7.4. The suspension obtained was filtered through a piece of nylon with a very coarse mesh and washed by again adding 2 ml Na_2EDTA saline (procedure 1).

Brush border isolation: When the study of these enzyme activities was completed, we looked for a fractionation technique useful for isolating entire brush borders. We started with the technique employed by Miller and Crane [24]. Some isolated and entire brush borders were observed by microscope, but the yield was negligible and purification minimal. Subsequently we tentatively used the method employed by Mircheff and Wright [26] with some modifications, but the result was again a failure. This negative outcome could be due to the slight density of microvilli in rabbit gallbladder [12], so that the techniques employed for intestine could be unsuitable.

Apical membrane isolation: The first method used to obtain fragments of apical plasma membranes was that of Schmitz *et al.* [20] as modified by Kessler *et al.* [17]. About 400 mg of mucosae were homogenized twice in 1.4 ml 50 mM mannitol + 2 mM TrisOH/Tris Cl pH 7.1 (medium A) in the Omni-mixer at maximum speed for 1 min. The homogenate was washed with a further 2 ml medium A and 0.6 ml of the final suspension were used for incubations and assays (homogenate). The remaining part was added with $CaCl_2$ (final concentration: 10 mM), left for 15 min at 4 °C and finally centrifuged at 3,000 × g for 15 min. The precipitated fraction (*a*) was processed as specified later on; the supernatant was centrifuged at 27,000 × g for 30 min. The precipitate (*b*) was resuspended in 1.4 ml 50 mM mannitol + 10 mM Tris OH/Tris Cl, pH 7.1 (medium B) and centrifuged at 27,000 × g for 30 min. The precipitate (*c*) was resuspended in 0.6 ml of medium B and analysed for enzymes (apical membrane fraction). The precipitate *a* resuspendend in 0.4 ml of medium B was pooled with supernatant *b* and *c* and processed as usual to determine enzyme activities (precipitate *a* and supernatant pool.

The second method used was similar to that of Malathi *et al* [23]. About 800 mg of mucosae were homogenized 5 times in about 16 ml medium A with the Omni-mixer at top speed for 1 min; 1 ml of the resulting homogenate was processed to determine enzyme activities (homogenate). $CaCl_2$ (final concentration: 10 mM) was added to the remaining part, mixing and shaking in the cold for 10 min. Then the suspension was centrifuged at 3,000 × g for 15 min, the

supernatant (*a*) was left in the cold for two min and again centrifuged at 43,000×g for 20 min. The consequent precipitate (*b*) was resuspended in about 16 ml medium A and centrifuged at 43,000×g for 20 min. The precipitate (*c*) was resuspended in 0.6 ml medium A and processed for enzyme determinations (apical membrane fraction). Precipitate *a* resuspended in 0.4 ml medium A was pooled with supernatant *b* and *c* and processed for enzyme determinations (precipitate *a* and supernatant pool.).

Enzyme assays. Samples were analysed in triplicate.

HCO_3^-–ATPase and Anion–ATPase. When procedure 1 was used to look tentatively at various enzymes in the homogenate, the HCO_3^-–ATPase was determined as the difference between the rates of release of phosphate from ATP in the presence and absence of 25 mM $NaHCO_3$ substituted for NaCl at pH 7.4 (solutions 1 and 2 of Table 1, 0.5 mM ouabain, 3 mM Mg^{2+}); correspondingly, the anion–ATPase was determined as the difference between the rates of release of phosphate from ATP in the absence and presence of 25 mM SCN^- (solutions 2 and 3 or 4, 0.5 mM ouabain 3 mM Mg^{2+}).

TABLE 1

Solutions used to determine ATPase activities.

Concentrations are reported as mM.

When HCO_3^- was present the solution was bubbled with adequate CO_2% to maintain pH and HCO_3^- concentration constant during the incubation period. 5 mM KCl, 3 mM $MgCl_2$ and 0.1 mM Na_2EDTA were present in all six salines.

Incubation media	NaCl	Tris buffer pH 7.4	Tris buffer pH 8.4	NaSCN	$NaHCO_3$
1	51	100	—	—	—
2	26	100	—	—	25
3	1	100	—	25	25
4	26	100	—	25	—
5	—	—	100	51	—
6	—	—	100	—	51

The medium volume was 975 µl. Reactions took place at 37 °C in a Dubnoff shaker with the suitable gas mixture; they were started by the addition of ATP (final concentration 3 mM) and of 25 µl tissue suspension (20–25 µg protein) and stopped after 20 min by the addition of 0.1 ml 50% trichloroacetic acid and by storing at —20 °C.

Blanks were set up in order to measure the rate of the non-catalysed reaction, with 25 μl tissue suspension denatured by 0.1 ml 50% trichloroacetic acid. Since SCN^- interferes with the phosphate assay, some blanks were prepared in order to make the necessary corrections.

Subsequently, when isolation methods were used, only the anion–ATPase in the homogenate, apical membrane fraction, precipitate and supernatant pools was determined, at ph 8.4, with a 3 mM Mg^{2+} concentration, as the difference between the rates of release of phosphate from ATP in the presence of 51 mM $NaHCO_3$ or of 51 mM NaSCN (solution 5 and 6 of Table 1). The specific activity was expressed as μmoles phosphate released min^{-1} mg^{-1} protein.

Na^+–K^+–ATPase. This was determined at 37 °C as the difference between the rates of release of phosphate from ATP at pH 7.4 in the presence or absence of 0.5 mM ouabain (solution 1). Details concerning incubations are the same as those reported for HCO_3^-–ATPase. The specific activity was expressed as μmoles phosphate released min^{-1} mg^{-1} protein.

Cytochrome c oxidase. This enzyme was determined at room temperature (24 °C) as reported by Smith [34]. The specific activity was expressed as sec^{-1} mg^{-1} protein.

Alkaline phosphatase. (EC 3.1.3.1.) was assayed by the method of Hausamen *et al.* [11] at room temperature (24 °C), pH 9.8 (test-combination alkaline-phosphatase opt. Boehringer). The specific activity was expressed as mU mg^{-1} protein.

Leucine-aminopeptidase (Leucine-arylamidase, α–aminoacyl-peptidehydrolase) (EC 3.4.11) was assayed by the method of Nagel *et al.* [27] at room temperature (24 °C), pH 7.2 (Test combination LAP Boehringer). The specific activity was expressed as mU mg^{-1} protein.

Inorganic phosphate: This was assayed by the method of Zilversmit and Davis [44].

Proteins. Proteins were determined by the method of Lowry *et al.* [22], with bovine serum albumin as the standard.

Statistics. Results are presented as means with standard errors (the number of experiments in parenthesis).

RESULTS AND DISCUSSION

Na^+–K^+–ATPase in the homogenate.

This enzyme is the only one involved in transports previously studied in gallbladder [40]. Table 2 shows that the ATPase specific activity in the absence of ouabain and in the presence of 5 mM KCl is 1.37 μmoles min^{-1} mg^{-1} protein;

it decreases to about 70% both when 0.5 mM ouabain is present and when K^+ is absent from the incubation media. This decrease is highly significant (paired data analysis) and is in excellent agreement with that reported by Van Os and Slegers [40]. Thus, a Na^+–K^+–ATPase specific activity of 0.43 — 0.46 μmoles min^{-1} mg^{-1} is detected.

TABLE 2

Na^+–K^+–ATPase activity in the homogenate of gallbladder epithelial cells.

Incubations for 20 min (control solution = sol. 1 of Table 1).
Specific activities are expressed as reported in Methods.

Saline	Total ATPase (A)	Mg^{2+}–ATPase (B)	$\frac{B}{A} \cdot 100$	Na^+–K^+–ATPase (C)
Control	1.37±0.26 (4)	—	—	—
+ ouabain . . . . (0.5 mM)	—	0.91±0.10 (4)	70.0±7.5 (4)	0.46±0.21 (4)
K^+–free	—	0.94±0.10 (4)	72.0±7.1 (4)	0.43±0.18 (4)

Anion–ATPase in the homogenate.

When the ATPase specific activity was determined in the homogenate (Table 3), at pH 7.4, in the presence of 51 mM NaCl (solution 1; Table 1) with 0.5 mM ouabain added, the Mg^{2+} ATPase activity so revealed was 1.08 μmoles min^{-1} mg^{-1}. It significantly increased (paired data analysis) to 1.52 μmoles min^{-1} mg^{-1} when in the incubation medium 25 mM $NaHCO_3$ and 26 mM NaCl (solution 2) were present instead of 51 mM NaCl. However, this increase was completely eliminated and the ATPase specific activity was even reduced to a value lower than the control, when NaSCN was substituted for 25 mM NaCl in the presence of 25 mM $NaHCO_3$ (solution 3) or NaCl (solution 4).

These results are in agreement with what has been reported in the literature: *a*) an anion activated ATPase should be present in the homogenate as at least the mitochondrial activity exists [7, 9, 21], *b*) it is inhibited by SCN^-, *c*) both Cl^- and HCO_3^- are activators, the latter to a larger extent than the former.

Moreover, it has been reported [20] that oligomycin largely inhibits this enzyme. In fact, when we added this antibiotic (80 μM) to the incubating media (solutions 2 and 3) we obtained a large decrease (50–80%) in the anion ATPase activity (Table 4).

TABLE 3

Anion sensitive ATPase in the homogenate of gallbladder epithelial cells.

Specific activities are expressed as reported in Methods.

Mg^{2+}–ATPase (oubain, Cl^-) (A)	Mg^{2+}–ATPase $+HCO_3^-$–ATPase (ouabain, Cl^-, HCO_3^-) (B)	$\left(\frac{B}{A} \cdot 100\right)$	HCO_3^-–ATPase (B–A)	SCN^-–insensitive Mg^{2+}–ATPase (ouabain, HCO_3^-, SCN^-) (C)	$\left(\frac{C}{A} \cdot 100\right)$	Anion–ATPase (B–C)	SCN^-–insensitive Mg^{2+}–ATPase (ouabain, Cl^-, SCN^-) (D)
1.08 ± 0.18	1.52 ± 0.29	138.4 ± 4.8	0.43 ± 0.12	0.76 ± 0.19	67.6 ± 6.0	0.75 ± 0.11	—
(4)	(4)	(4)	(4)	(4)	(4)	(4)	
0.70	0.99	141.6	0.29	0.37	53.5	0.61	0.33
(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)

TABLE 4

Effect of Oligomycin on the SCN^- *insensitive* Mg^{2+}*–ATPase and on the Anion–ATPase activities.*

Specific activities are expressed as reported in Methods.

Exp. n.	Oligomycin (μM)	SCN^-–insensitive–Mg^{2+}–ATPase (ouabain, Cl^-, SCN^-) (A)	SCN^-–insensitive–Mg^{2+}–ATPase +Anion–ATPase (ouabain, Cl^-, HCO_3^-) (B)	Anion–ATPase (B–A)
1	0	0.29	0.95	0.66
	40	0.24	0.60	0.36
	80	0.22	0.35	0.13
2	0	0.40	1.05	0.65
	40	0.28	0.83	0.55
	80	0.24	0.56	0.32

Marker enzymes for the brush border.

Sucrase activity is present in the brush border of the intestinal epithelial cells and is used as a marker of microvilli membranes because of its highly specific localization [8, 10, 26].

The presence of this enzyme in gallbladder is unlikely as the sucrose in this epithelium has a reflection coefficient (σ) equal to 1 [43]: if a sucrase was present in the brush border, sucrose should be split into glucose and fructose and the apparent σ should be lower than 1. Moreover, we have previously checked the presence of this enzyme without any positive outcome [2].

Leucine aminopeptidase is another enzyme specifically associated with intestine and kidney brush border [1, 8, 10, 36].

We have assayed it in homogenates of both gallbladder and intestine (jejunum) of the same animal. The homogenate was prepared by procedure 1, with 5 mM Na_2EDTA (pH 7.4) as a medium or an isotonic medium in which Na_2EDTA concentration was reduced to 0.5 mM (mM: sucrase 250, NaCl 12.5, Na_2EDTA 0.5, histidine/imidazole buffer 5; pH 7.4). Results are reported in Table 5. In intestine the specific activity of the enzyme is 2.5 times higher with an incubation at low Na_2EDTA (67.1 and 26.3 mU mg^{-1} respectively). On the contrary, in gallbladder this effect was not observed; however, the specific activity in gallbladder is only 1.9% with respect to intestine and its deter-

mination is affected by very large errors. The enzyme seems to be present only in negligible amounts and is not suitable for use as a marker.

TABLE 5

Leucine-aminopeptidase activity in the homogenate of gallbladder and intestine epithelial cells.

Specific activities are expressed as reported in Methods.

GALLBLADDER		INTESTINE		
5 mM Na_2EDTA (A)	0.5 mM Na_2EDTA (B)	5 mM Na_2EDTA (C)	0.5 mM Na_2EDTA (D)	$\frac{B}{D} \cdot 100$
1.9	1.3	26.3	67.1	1.9

In small intestine and in kidney tubule, alkaline phosphatase is localized in plasma membranes and in some animal species only in those of the brush border [1, 8, 10, 26]. Thus, as for leucine-aminopeptidase, we have checked for the presence of this enzyme both in gallbladder and intestine homogenates obtained from the same animals. Again, the assay was performed following procedure 1, with 5 mM Na_2EDTA (pH 7.4) as the medium or with isotonic medium containing 0.5 mM Na_2EDTA (pH 7.4). It is well known that this enzyme is activated by Zn^{2+}, so that the high concentration of the chelating agent could reduce enzyme activity; on the other hand, it is to be borne in mind that too high a Zn^{2+} concentration is inhibiting. As a matter of fact, Table 6 shows that 74.1 and 119.6 mU mg^{-1} of alkaline phosphatase were detected (experiments 1 and 2 respectively) when the 5 mM Na_2EDTA saline was used to prepare the homogenate of jejunum epithelium, but when the 0.5 mM Na_2EDTA solution was used, the corresponding activities were 352.7 and 330.7 mU mg^{-1} with an increase of 4.7 and 2.8 times. Similar high values were obtained if a saturated solution of $ZnCl_2$ with a final concentration 1 : 500 (*v/v*) was added to the incubation medium for the enzyme assay containing the homogenate prepared with 5 mM Na_2EDTA. Higher Zn^{2+} concentrations (1 : 100) were inhibiting. Thus preparation of the homogenate with 0.5 mM Na_2EDTA should be preferred; alternatively, Zn^{2+} (1 : 500) should be added.

When the assay was performed on gallbladder epithelium, similar results were obtained by the use of homogenates prepared with 5 or 0.5 mM Na_2EDTA (17.5 and 20.6 mU mg^{-1}).

TABLE 6

Alkaline phosphatase activity in the homogenate of gallbladder and intestine epithelial cells.

Specific activities are expressed as reported in Methods.

Exp. n.	GALLBLADDER						INTESTINE				
	5 mM Na_2EDTA			0.5 mM Na_2EDTA			5 mM Na_2EDTA			0.5 mM Na_2EDTA	
	(A)	With Zn^{2+} 1 : 5000 (B)	With Zn^{2+} 1 : 500 (C)	(D)	With TCA (5%) (E)	$\frac{D}{A}$	(A′)	With Zn^{2+} 1 : 500 (B′)	With Zn^{2+} 1 : 100 (C′)	(D′)	$\frac{D'}{A'}$
1	17.5	12.1	7.9	20.6	1.1	1.2	74.1	322.9	47.9	352.7	4.7
2	5.4	6.7	2.1	—	—	—	119.6	—	—	330.7	2.8

The addition of Zn^{2+}, with a final concentration of 1 : 500 (v/v), was strongly inhibiting and a similar, although smaller effect was obtained with a final concentration of 1 : 5000. All these results confirm that the two media used to prepare the bladder homogenate are equally suitable and that the Zn^{2+} concentration remaining in the homogenate is sufficient to maintain the maximal activity of the enzyme in both cases. On the other hand, in spite of the fact that the alkaline phosphatase activity is much lower in gallbladder than in intestine, it is not negligible and can be easily determined: as a confirmation that no artefact was present, the measured value was nearly completely inhibited when the homogenate was previously treated with trichloro-acetic acid (final concentration 5%).

TABLE 7

Isolation of apical plasma membranes.

Specific activities are expressed as reported in Methods.

	Alkaline phosphatase	Na^+–K^+–ATPase	Cytochrome *c* oxidase
(A)			
Apical plasma membranes			
Specific activity ..	95.3±16.6 (5)	0.5± 0.3 (5)	35.3±10.6 (5)
Enrichment factor	8.7± 2.7 (5)	4.8± 2.2 (5)	0.3± 0.1 (5)
Recovery (%) ...	6.5± 1.5 (5)	12.2± 7.6 (5)	1.2± 0.4 (5)
Total recovery...	110.9±17.2 (5)	102.9±13.8 (5)	69.0±11.3 (5)
(B)			
Apical plasma membranes			
Specific activity ..	158.0±30.3 (7)	0.01±0.05 (6)	0.9± 0.6 (7)
Enrichment factor	6.5± 1.5 (7)	0.1 ± 0.3 (6)	0.0± 0.0 (7)
Recovery (%) ...	5.9± 0.9 (7)	0.2 ± 0.5 (6)	0.0± 0.0 (7)
Total recovery...	109.4± 5.2 (7)	75.8 ±11.8 (6)	75.3±10.3 (7)

Thus we decided to use alkaline phosphatase as a marker of brush border or, at least, of all the plasma membranes. Since it has been shown that Na^+–K^+–ATPase is present in the gallbladder only at the basolateral membranes [15, 25, 40], the comparison between these two enzyme specific activities, enrichment and recoveries in the brush border or apical membrane fraction, can be

a sufficient indication of the isolation of the corresponding membranes, even if alkaline phosphatase is a marker of all plasma membranes and not only of the apical one.

Apical membrane isolation.

The results obtained by the method of Kessler *et al.* [17] are reported in Table 7 A. The enrichment factor for alkaline phosphatase was nearly 9, at least indicating that we were isolating plasma membranes. Total recovery of this enzyme was not significantly different from 100%. However, the plasma membranes so isolated were also clearly basolateral membranes as Na^+–K^+–ATPase activity was enriched by a factor equal to nearly 5 (again the total recovery was not significantly different from 100%). The fraction was not enriched but it was equally largely contaminated by cytochrome *c* oxidase (and so by mitochondria). The results obtained by the method of Malathi *et al.* [23] are reported in Table 7 B. Parallel with an enrichment factor of 6.5 for alkaline phosphatase and with high total recoveries, Na^+–K^+–ATPase and cytochrome *c* oxidase activities are negligible. Thus, the fraction seems to be enriched in apical plasma membranes and purified from basolateral plasma membranes and mitochondria.

Anion–ATPase at the apical membrane.

The anion–ATPase was assayed at pH 8.4 and with a 3 mM Mg^{2+} and ATP concentration on the apical membranes so purified and no activity was detected (7 experiments). Conversely, by the use of the method of Kessler *et al.* [17] for isolation of the apical membranes, a significant activity was revealed with an enrichment factor of 0.3 (4 experiments), comparable to the identical enrichment factor obtained for cytochrome *c* oxidase under the same conditions (Table 7 A). In conclusion, the anion–ATPase activity (at least that with properties similar to the mitochondrial activity) in gallbladder epithelium seems to have only mitochondrial origin in agreement with the results reported for other epithelial cells by Van Os *et al.* [39] and Van Amelsvoort *et al.* [37, 38]. Thus, this enzyme cannot be responsible for any transport at the apical membrane.

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