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Subcellular distribution of carbonic anhydrase in the enterocyte of the rabbit ileum and the Caco-2 cell. Evidence for the presence of two isozymes bound to brush-border membranes

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The activity of CA has been determined in the membranes of enterocytes from rabbit ileum and of Caco-2 cells. No CA activity was detected in the BLM, but the activity in the BBM (43 and 7 WAU/mg protein for rabbit and Caco-2, respectively) was doubled by the addition of Triton. These two types of activity could be distinguished in rabbit ileum by their different IC_{50} in the presence of acetazolamide (10^{-5} and $5 \cdot 10^{-7}$ M) and their different sensitivities to heat. They were not modified by inhibitors of cytoplasmic isozymes and seem to correspond to two forms of CA, one situated in the extracellular leaflet of the BBM and the other one in the intracellular leaflet.

Introduction

In mammals, carbonic anhydrase (EC 4.2.1.1) is an ubiquitous zinc metalloenzyme first isolated by Meldrum and Roughton [1] from red blood cells. To date, 7 isozymes of carbonic anhydrase (CA) have been described, differing in subcellular localization, susceptibility to inhibitors, physical kinetic properties and molecular structure. Among them, CA IV has been described as a membrane bound isozyme in the endothelium of bovine [2] and human lung [3] and the epithelium of dog [4,5] and human kidney [6,7].

In the digestive tract, the cytoplasmic CAs have been studied for quantity evaluation and immunohistochemical localization [8–10] and regulation by steroids [11] and thyroxin [13]. In contrast, the membrane bound CA is poorly documented for this tissue [13]. Several authors have suggested that the metabolism of CO_2 is closely related to the cellular absorption of salt [14–17], but the role of CA is not clearly understood, although it seems implicated in the coupling of Na^+ and Cl^- absorption through Na^+/H^+ and $Cl^-/OH^-(HCO_3^-)$ antiports [13]. Nevertheless, it has been shown that CA mediates ionic absorptive responses to variations in acid-base balance [18].

The present study was designed to locate the CA activity in cells of the small intestine more precisely and to evaluate the importance of the membrane-bound enzyme in the overall CA activity of the whole cell.

Preliminary results have been presented at the 9th Réunion of the CECED (Montpellier, 1990).

Materials and Methods

Isolation of membranes from rabbit intestine. Male New Zealand White rabbits weighing 3 to 4 kg were killed by rapid intravenous injection of 6 sodium pentobarbital (0.5 ml/kg). The distal third of the intestine was carefully removed, divided into three portions and rinsed with 50 ml of cold NaCl (0.9%). After eversion, the intestine was gently blotted and the mucosa was scraped off.

Brush-border membranes were prepared according to a method modified from Kessler et al. [19]. Mucosal scrapings were homogenized using a tissue grinder (E. Bühler, Tubingen, Germany) at 25000 rpm for 2 min in 125 ml of a buffer solution containing 350 mM mannitol, 1 mM EGTA, 0.1 mM PMSF, 1 μg/ml aprotinin and 10 mM Hepes adjusted to pH 7.4 with Tris-base. After addition of 10 mM $MgSO_4$, the suspension was

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Abbreviations: BBM, brush-border membrane; BLM, basolateral membrane; CA, carbonic anhydrase; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Hepes, *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid; PMSF, phenylmethylsulfony fluoride; WAU, Wilbur-Anderson unit.

maintained at 4°C for 20 min and then centrifuged at $3000 \times g$ for 15 min. The supernatant was then centrifuged at $27000 \times g$ for 30 min. The resulting pellet was suspended using a teflon Potter (9 strokes) in 10 ml of a buffer solution (solution A) containing 30 mM sorbitol, 12 mM sodium barbital and 16 mM Hepes (pH 7.5). The last purification step was repeated after addition of 10 mM $MgSO_4$. After the last centrifugation, the vesicles were suspended in solution A at 5–10 mg protein/ml and stored in liquid nitrogen until utilization. The protein concentration was determined by the Bradford method [20] with bovine serum albumin as the standard.

Basolateral membranes were prepared according to a method modified from Del Castillo [21]. The mucosal scrapings (0.25 g/ml) were homogenized using an Ultra-Turrax T25 at 25000 rpm (6 strokes, 5 s) in a buffer solution (solution B) containing 250 mM sucrose, 0.1 mM PMSF, 1 μg/ml aprotinin and 20 mM Tris-HCl (pH 7.4). The homogenate was diluted to 0.1 g/ml and centrifuged at $2000 \times g$ for 10 min. The supernatant was then centrifuged at $27000 \times g$ for 20 min. The fluffy pellet obtained was resuspended in solution B and centrifuged at $50000 \times g$ for 45 min in a 12% Percoll gradient (rotor 50 Ti, Beckman). The upper band was resuspended in solution B and centrifuged at $60000 \times g$ for 30 min. This purification step was repeated and the membranes were resuspended in solution A and stored at a concentration of 5 to 10 mg protein/ml in liquid nitrogen until required.

Isolation of membranes from Caco-2 cells. Caco-2 cells were grown in Dulbecco's modified Eagle's medium without pyruvate, supplemented with 20% fetal calf serum, 1% non-essential amino-acid solution and 0.5% gentamycin. Cells were harvested when confluent 7 days after plating.

The Caco-2 cells were homogeneized using an Ultra-Turrax T25 (six times 5 s, 25000 rpm) in solution B (5 mg/ml) and centrifuged 10 min at $2000 \times g$. The supernatant was retained for BLM preparation and the pellet was homogenized using the T25 (twice 30 s, 25000 rpm) in solution B for BBM purification. The isolation was continued as for rabbit BBM and the membrane suspension was stored at a concentration of 2 mg protein/ml in liquid nitrogen.

The supernatant was centrifuged for 20 min at $15000 \times g$ and the fluffy pellet resuspended in solution B was then centrifuged for 30 min at $43500 \times g$ in a 14% Percoll gradient (rotor 50 Ti, Beckman). The isolation was then continued as for rabbit BLM and the membrane suspension was stored at a concentration of 2 mg protein/ml in liquid nitrogen.

Enzyme assays. The purity of the membrane suspensions was evaluated by determining the enrichment factors of enzyme markers. Alkaline phosphatase activity was determined by the method of Di Costanzo et al. [22], Na/K-ATPase activity by the method of Sall et al. [23], sucrase activity after Dahlqvist [24] and succinate–cytochrome-*c* reductase activity after Fleischer and Fleischer [25].

The CA activity was determined according to a modification of the electrometric method of Wilbur and Anderson [26]. At 0°C, 0.2 ml of membrane suspension (2 mg protein/ml) was added to 1 ml of solution containing 10.8 mM sorbitol, 25 mM sodium barbital, 9.2 mM Hepes (pH 8.3) and stirred continuously. After addition of 1 ml of CO_2-saturated water, the pH variations were registered. Since the rate of pH decrease was constant from 7.5 to 7.0, the time required for this change in pH was used to determine the enzyme activity. This time was measured in the presence (t_0) or in the absence (t) of acetazolamide (1 mM) which inhibits all CA activities. The following formula was used: CA activity $= (t/t_0 - 1$. The CA activity measured was linearly related to the amount of suspension added. The activity obtained with purified CA from Sigma (CA from bovine erythrocytes, 3000 Wilbur-Anderson units per mg enzyme) was used as the standard and CA activity was expressed in Wilbur-Anderson Units (WAU)/mg protein.

TABLE I

Specific activities and enrichment factors of marker enzymes in suspensions isolated from rabbit ileum enterocytes

Sucrase, Na/K-ATPase (determined in the presence of saponine) and cytochrome-*c* reductase are expressed in μmol substrate min^{-1} (g protein)$^{-1}$ and alkaline phosphatase is expressed in nmol substrate min^{-1} (g protein)$^{-1}$. Values are means ± S.E; n = number of samples tested.

Enzyme	n	Homogenate	BBM		BLM	
		spec. act.	spec. act.	enr. factor	spec. act.	enr. factor
Sucrase–Triton	9	38 ± 9	660 ± 50	18.9 ± 4.0	25 ± 17	0.70 ± 0.10
Sucrose + Triton	9	49 ± 9	790 ± 140	17.7 ± 2.7	39 ± 21	0.80 ± 0.40
Alk. phosphatase	4	0.3 ± 0.1	4.5 ± 0.8	16.0 ± 2.7	0.08 ± 0.06	0.30 ± 0.10
Na/K-ATPase	5	50 ± 3	35 ± 3	0.7 ± 0.1	565 ± 124	11.00 ± 2.00
Cytochrome-*c* reductase	4	36 ± 14	28 ± 3	0.7 ± 0.1	3.6 ± 0.6	0.13 ± 0.02

TABLE II

Specific activities and enrichment factors of marker enzymes in suspensions isolated from Caco-2 cells

Legends are given in Table I.

Enzyme	*n*	Homogenate	BBM		BLM	
		spec. act.	spec. act.	enr. factor	spec. act.	enr. factor
Sucrase–Triton	5	17 ±5	300 ±82	17.2±0.9	58 ±17	3.3±0.5
Sucrase + Triton	5	19 ±6	314 ±96	16.7±1.7	67 ±18	3.1±0.5
Alk. phosphatase	6	0.17±0.01	2.70± 0.17	15.8±0.8	0.40± 0.03	2.2±0.4
Na/K-ATPase	3	48 ±3	63 ±26	1.3±0.5	630 ±90	13.0±2.1

Results

Characterization of membrane microvillar vesicles

Table I shows the specific activities obtained for the enzyme markers in the crude homogenate of scrapings of rabbit intestine and in the final membrane suspensions, and their enrichment factors. The enrichment factors obtained for alkaline phosphatase (16) and sucrase (approx. 18) in BBM suspension demonstrate that the preparation was well purified in BBM. Furthermore, the enrichment factors of Na/K-ATPase (0.7) and succinate–cytochrome-*c* reductase (0.7) indicated negligible contamination by BLM and mitochondria. Since sucrase is situated in the extracellular leaflet of the BBM, one can calculate from the sucrase activities determined in the presence and in the absence of Triton X-100 that 89.3 ± 6.1% of the BBM were sealed in the normal physiological manner. In the BLM suspension, the enrichment factors obtained for Na/K-ATPase (11) and for other enzymes (< 1) revealed only trace amounts of contaminant.

The enrichment factors obtained for alkaline phosphatase and sucrase in BBM suspensions of Caco-2 cells (Table II) were of the same magnitude as in the intestine of the rabbit but the enrichment factor of Na/K-ATPase was slighdy higher than in the rabbit. The activities of sucrase in the presence and in the absence of Triton allowed us to calculate that 96.2 ±

TABLE III

Carbonic anhydrase activity of BBM and BLM from rabbit ileum determined in the presence of different permeabilizing agents

SIO, streptolysin O; 100% = 56±4 WAU/mg protein; *n* = 4). n.d., not determined

Addition	% of BBM activity	
	BBM	BLM
No	100.0	6.2±5.6
Triton	200.5±18.1	8.7±4.1
Digitonin	185.3±15.2	n.d.
SLO	143.2±11.1	7.7±9.7

TABLE IV

Carbonic anhydrase activity of BBM and BLM from rabbit ileum and Caco-2 cells (n = 4) in the absence and presence of Triton (0.1%)

	Triton	WAU/mg protein	
		Rabbit ileum	Caco-2
BBM	–	46.5±4.3	6.9±1.4
	+	92.6±7.2	15.8±3.8
BLM	–	4.6±5.1	1.1±1.7
	+	3.7±2.4	2.0±1.4

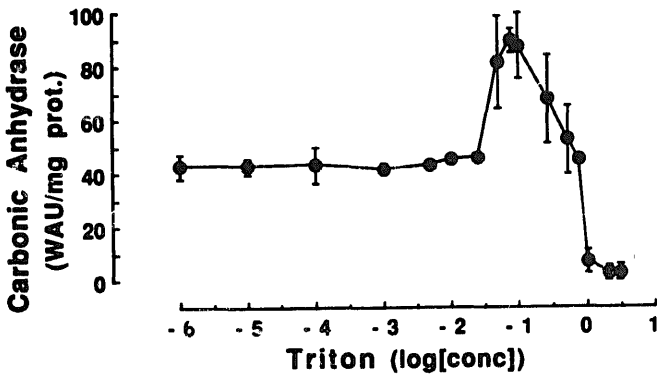

Fig. 1. Carbonic anhydrase activity of BBM from rabbit ileum determined in the presence of increasing concentrations of Triton X-100 (*n* = 5).

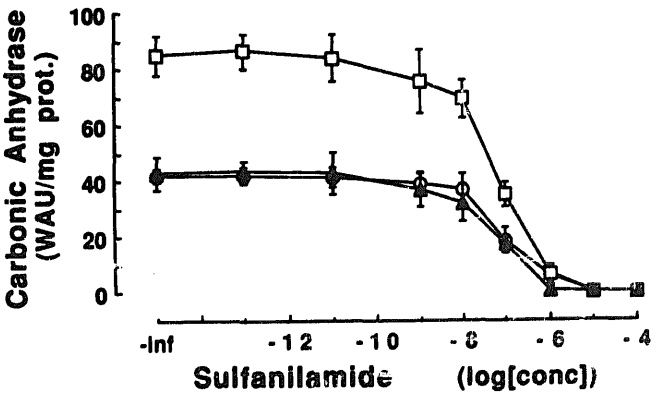

Fig. 2. Effect of increasing concentrations of sulfanilamide on the carbonic anhydrase activities of BBM from rabbit ileum in the absence (○) and in the presence (□) of Triton (0.1%), and on the difference (▲) of these two activities (*n* = 5).

3.8% of the BBM was sealed in the physiological manner. The BLM suspension was highly enriched in Na/K-ATPase (approx. 13) but contained traces of BBM, as shown by enrichment factors obtained for alkaline phosphatase and sucrase activities (approx. 3) in this preparation.

CA activity determination

The CA activity was determined in rabbit intestinal BBM in the presence of increasing Triton X-100 concentration. It can be seen from Fig. 1 that the activity obtained in the absence of Triton (42.7 ± 4.5 WAU/mg protein, $n = 5$) was not modified by the addition of the detergent below a concentration of 0.025% and doubled at concentrations ranging from 0.05–0.1%. At this concentration, the activity of the CAI or CAII obtained from Sigma was not modified. When the Triton concentration was further increased, the activity was almost completely inhibited. The addition of digitonin to the BBM suspension at a concentration known to permeabilize the membrane (20 μM) increased the enzyme activity by 85% (Table III). Similarly, in the presence of streptolysin O, which is a membrane permeabilizing toxin from *Streptococcus pyogenes*, the activity was increased by 43%.

The CA activity of the BBM purified from Caco-2 cells (Table IV) was much lower (6.9 ± 1.4 WAU/mg protein, $n = 4$) than in BBM from rabbit, but also doubled with the application of Triton. The CA activities measured in the supernatant after 100000 × g centrifugation of the crude homogenates of ileum mucosa and Caco-2 cells, considered as 'cytoplasmic' activitie, represented about 60 and 40% of the total activities, respectively.

In the BLM purified from rabbit ileum, the measured CA activity (Table IV) was 4.6 ± 5.1 WAU/mg protein ($n = 7$) and was not modified by the addition of triton (3.7 ± 2.4 WAU/mg protein). In the BLM from Caco-2 the CA activity was almost 0. These residual activities can be considered to be due to a trace contamination by BBM.

TABLE V

CA specific activities and enrichment factors determined in the homogenate and in the BBM of enterocytes from rabbit ileum in the absence and presence of Triton (0.1%) and SDS (0.2%)

Values are means ± S.E. ($n = 4$).

Detergent		WAU/mg protein		Enr. Factor
Triton	SDS	Homogenate	BBM	
–	–	12.9 ± 1.4	46.6 ± 5.2	3.6 ± 0.8
–	+	4.4 ± 0.2	45.4 ± 2.6	10.4 ± 1.1
+	–	18.4 ± 2.3	93.5 ± 11.3	5.1 ± 1.2
+	+	8.9 ± 0.9	92.9 ± 8.9	7.9 ± 1.5

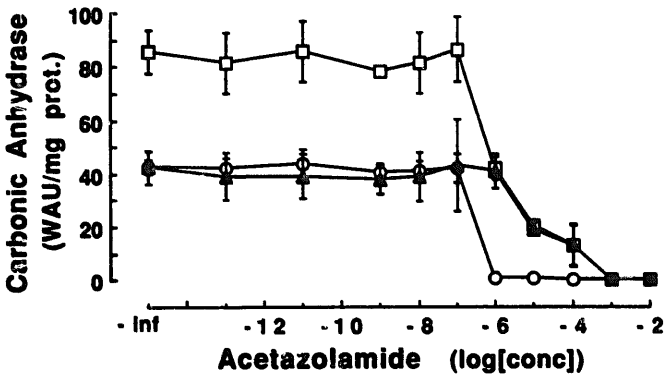

Fig. 3. Effect of increasing concentrations of acetazolamide on the carbonic anhydrase activities of BBM from rabbit ileum in the absence (○) and in the presence (□) of Triton (0.1%), and on the difference (▲) of these two activities ($n = 4$).

Since no special precautions were taken during the sampling of the mucosa, some red cells containing CAI and CAII could have contaminated the scrapings and biased the determination of the CA activity belonging to the intestinal epithelium in the crude homogenate. Since the CA activity due to red cells is proportional to the amount of hemoglobin, the hemoglobin content of the homogenate has been determined by the method of Van Kampen and Zijlstra [27] and the method of Davidsohn and Henry [28]. The results obtained by both methods were indistinguishable from their detection limit. Taking this limit (0.1 mg/ml) as the maximum value of hemoglobin concentration of the homogenate and measuring the CA activity and the hemoglobin concentration of pure blood of rabbit, the maximum contamination by the Ca belonging to the blood in the homogenate was not higher than 1.3 ± 0.5% ($n = 5$). Consequently, this contamination can be considered as negligible.

SDS (0.2%) is known to inhibit the cytoplasmic CA activity of human lung [2]. In the intestine of the

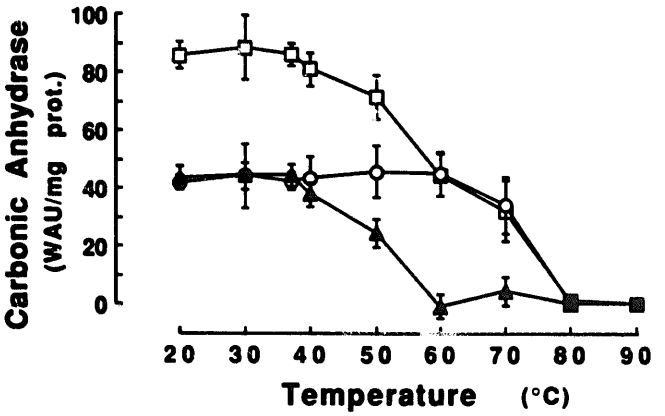

Fig. 4. Effect of incubation temperature (5 min) on the carbonic anhydrase activities of BBM from rabbit ileum in the absence (○) and in the presence (□) of Triton (0.1%), and on the difference (▲) of these two activities ($n = 4$).

rabbit, the CA activity was inhibited by SDS in the crude homogenate, but not in the BBM suspension neither in the absence nor in the presence of triton (Table V). The enrichment factors obtained for CA in the presence of SDS were underestimated since the cytoplasmic activity was not totally inhibited by SDS and, therefore, could not be equivalent to those obtained for alkaline phosphatase.

Effect of inhibitors

Sulfanilamide. The effect of increasing concentrations of sulfanilamide on the CA activity of BBM suspensions in the presence or the absence of triton is shown in Fig. 2. Both activities were completely abolished at concentrations higher than 10^{-6} M. Furthermore, the difference obtained between both activities, which will be called the Triton-revealed activity and the activity obtained in the absence of Triton have the same IC_{50}, ilo, 10^{-7} M.

Acetazolamide. Fig. 3 shows the effect of increasing concentrations of acetazolamide on the CA activities of BBM treated with Triton or not. It can be seen that at concentrations of acetazolamide higher than 10^{-6} M, an activity in the presence of Triton remains, although in the absence of Triton no activity can be detected at these concentrations. Consequently, the Triton-revealed activity and the activity obtained without Triton have different IC_{50} values (10^{-5} M and $5 \cdot 10^{-7}$ M, respectively). The same results were obtained in the presence of increasing concentrations of ethoxzolamide which is an analog of acetazolamide.

Chlorine. The CA of BBM (55.0 ± 2.9 WAU/mg protein, $n = 5$) was not modified by the addition of 50 mM chlorine (54.3 ± 3.5 WAU/mg protein, $n = 5$).

Effect of temperature

The CA activity of rabbit BBM previously incubated at different temperatures during 5 min was measured before and after addition of Triton. The results obtained are shown in Fig. 4. In this experiment, the Triton-revealed activity and the CA activity obtained in the absence of Triton have different comportments. In fact, the Triton-revealed activity was much more thermo-sensitive. This activity decreased when treated at temperatures higher than 37°C, in contrast with the absence of Triton where the enzyme was still active even when the incubation temperature was 60°C.

Discussion

This study confirms the presence of CA in the BBM of the rabbit ileum as has been reported previously [7,13]. Furthermore, the BLM of the enterocytes are devoid of CA activity, in contrast with the BLM of renal tubular cells [29]. Some mitochondria have a CA activity [30] and, although our purified BBM preparation has only a very weak contamination with mitochondria, we have also isolated mitochondria from enterocytes in order to determine their CA activity. No activity was detected with or without the addition of Triton (results not shown).

In the purified BBM of rabbit ileum enterocytes and of cultured Caco-2 cells, the addition of Triton X-100 revealed a supplementary enzymatic CA activity as has been described for trout enterocytes [30]. The results obtained emphasize the importance of the Triton concentration in the tissue extract on CA activity (Fig. 1). In brain tissue, a CA activity was also revealed by solubilizing the myelin sheath with Triton [31,32]. Interestingly, the activity determined in the absence of Triton and the activity appearing after the addition of Triton can be distinguished by their comportment towards classical inhibitors and temperature inactivation. Consequently, they can be considered as two different proteins, i.e., two different isozymes of CA.

Although the cytoplasmic CA activity represents about 60% of the overall activity of ileum enterocytes, the activity revealed by Triton addition was not due to cytoplasmic CA linked to the membrane, since it was not inhibited by SDS nor by chlorine, both of which are inhibitors of cytoplasmic CA. Moreover, the activity unmasked by Triton co-purified with the BBM and was also unmasked by permeabilizing agents, such as digitonin and streptolysin O, which do not solubilize the membrane. Therefore, it seems likely that both types of CA activity are included in the membrane. They could belong to the apical membranes of different cells but this hypothesis can be excluded, since the same results were obtained with both ileum enterocytes and Caco-2 cells and it is likely that there is only one type of cell in Caco-2 cell cultures with only one type of brush-border membrane.

The question arising is the location of both CA molecules. Since approx. 90% of the BBM vesicles are sealed in the physiological manner, it can be considered that their active sites are situated on both sides of the membrane; one isozyme being situated in the extracellular leaflet of the BBM and the other (revealed by Triton) being situated in the intracellular leaflet of the membrane. An extracellular location for the CA active site has been reported [7]. However, the methods used would not have detected the intracellular membranous activity.

It has been proposed that CA could form channels in lipid bilayers able to separate protons from bicarbonate [33]. Our results suggest that the enzyme molecules do not cross the whole membrane but only one leaflet. Consequently, either they work together in a kind of cascade and under these conditions they work in opposition to facilitate CO_2 diffusion [34] or proton and bicarbonate translocation through the membrane [7], or they work independently, each modifying

uniquely its particular environment, i.e., the intracellular compartment or the lumen fluid depending on pCO_2, pH and bicarbonate concentration.

In conclusion, we have shown that the BBM from ileum enterocytes and carcinomic colonic cells possess two different molecules of CA, differentiated by response to inhibitors and different sensitivities to heat. Each CA is located in a different layer of the membrane. Furthermore, it may be a particularity of the intestine, since the presence of two CA has also been demonstrated in fish intestine [39] Work is now in progress to purify both enzymes and to determine whether this property is common to the whole digestive tract and other transporting epitheliums.

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