Biochimica et Biophysica Acta, 945 (1988) 17–22
Elsevier

BBA 74178

Evidence for the presence of carbonic anhydrase in the plasma membrane of rat hepatocytes

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(Received 22 April, 1988)

Key words: Carbonic anhydrase; Plasma membrane; Hepatocyte; (Rat)

The cellular distribution of carbonic anhydrase is a key characteristic for the role of the enzyme in cell function. In several epithelia involved in bicarbonate transport this enzyme is located in the plasma membrane. Because bicarbonate secretion is an important mechanism in bile formation by the liver, we investigated the presence of carbonic anhydrase activity in isolated plasma membranes from rat hepatocytes. Carbonic anhydrase activity was enriched 1.79-fold in plasma membrane preparations. This activity was inhibited by acetazolamide and activated by Triton X-100, but was insensitive to Cl^- or CNO^-. It is highly unlikely that the low contamination of cytoplasm and intracellular membranes could account for the presence of carbonic anhydrase activity in plasma membrane preparations. Moreover, the results from resuspension/washing of plasma membrane fractions in ionic media suggest an absence of soluble carbonic anhydrase adsorption upon plasma membrane. Accordingly, the present findings provide strong evidence for the presence of carbonic anhydrase in the plasma membrane of rat hepatocytes.

Introduction

In addition to the involvement of carbonic anhydrase (carbonate hydro-lyase; EC 4.2.1.1) in a number of physiological functions such as calcification, gas exchange and acid-base balance [1], this enzyme plays an important role in bicarbonate transport by several epithelia [1–4], including the liver [6]. A membrane-bound isoenzyme is present in most of these tissues [7] and, although the role of this isoenzyme is as yet speculative, membrane-bound carbonic anhydrase is presumably involved in a rapid buffering of OH^- or H^+ transported by the epithelium, in that it would catalyze the reversible hydration of CO_2. In the liver, most of the carbonic anhydrase activity has been described to be located within the soluble fraction, where two forms of carbonic anhydrase, CA II and CA III, are known to occur [8]. A small but significant amount is found associated to particulate fractions, including nuclei, microsomes and mitochondria [9–11]. Preliminary reports [12,13] also suggest the presence of carbonic anhydrase in the plasma membrane of rat hepatocytes. The present work provides additional evidence to confirm this point.

This work was presented in part at the XIV Meeting of the Spanish Biochemical Society, Sep-

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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tember 1987, Málaga, Spain. It has been published in abstract form in Ref. 12.

Materials and Methods

Animals

Nonfasting male Wistar CF rats (250 mg) (Faculty of Pharmacy, Salamanca, Spain) were used as donors. The animals were anesthetized by i.p. administration (50 mg per kg body weight) of sodium pentobarbital (Claudio Barcia, Madrid, Spain). After an injection of 0.5 ml 0.15 M NaCl containing heparin (250 I.U.) into the penis vein, the portal vein was cannulated. The liver was perfused with cold 4°C) 0.15 M NaCl solution until the lobes appeared free from blood and the perfusate was colorless. The liver was then removed, thoroughly washed in cold NaCl solution, weighed and used immediately.

Plasma membrane isolation

In order to obtain a plasma membrane preparation with a very low degree of contamination by other cell membranes we modified and combined the methods reported by Blitzer and Donovan [14] and Epping et al. [15]. All isolation steps were done at 4°C. Livers were minced in 20 ml buffer A (50 mM Hepes-KOH (pH 7.4)/0.25 M sucrose/1 mM EGTA/0.1 mM phenylmethylsulfonyl fluoride). After adding 20 ml buffer A, homogenization was performed with 5 up-down strokes of a motor-driven Teflon glass homogenizer (S-779, B. Braun, Melsungen, F.R.G.). The homogenate was then made up to 10-times the liver weight with additional buffer A and filtered through two layers of gauze. Filtered homogenate (H) was centrifuged at $500 \times g$ for 30 min using a Beckman L8-H centrifuge (Beckman Instruments, Madrid, Spain). The pellet (P1) was discarded and the supernatant (S1) was spun at $27\,500 \times g$ for 30 min, resulting in a pellet with a fluffy outer layer (P2) and a dark inner core (P2'), which was discarded together with the supernatant (S2). 20 ml of buffer B (50 mM Hepes-KOH (pH 7.4)/0.25 M sucrose) were added to the fraction collected (P2), before carrying out two up-down strokes of the motor-driven Teflon glass homogenizer. After making up to 40 ml with additional buffer B, 10 ml of Percoll (Pharmacia Iberica, Barcelona, Spain) stock solutions (Percoll/2.5 M sucrose, 9 : 1 (v/v)) were added. Before centrifugation at $30\,900 \times g$ for 30 min, pH was adjusted to 7.4 with 50 mM KOH and the mixture was stirred for 5 min on ice. The resulting pellet (P3) and supernatant (S3) were discarded and the easily visible band (B3), containing plasma membranes, was carefully removed and passed through a 23-gauge hypodermic needle three times before making up to 5 ml with buffer B. The resulting solution was mixed and vigorously stirred (as described above) with 12 ml of Percoll stock solution/buffer B (75% (v/v)). The mixture was transferred to the bottom of a discontinuous (0, 10, 18, 25 and 30% v/v) Percoll stock solution/buffer B density gradient, prepared as described by Epping et al. [15]. Loaded gradients were centrifuged at $48\,200 \times g$ for 2 min, excluding acceleration time. Milk-colored cellular material appeared distributed within four bands (F1, F2, F3 and F4, from top to bottom). Plasma membranes were included in fraction F1 at the buffer B/10% Percoll interphase. This fraction was collected and diluted 1 : 5 with 1 mM $NaHCO_3$. The mixture was then centrifuged at $48\,200 \times g$ for 30 min. The fluffy membrane pellet was easily washed free of the hard, glassy Percoll pellet adhering to the tube. The membranes were passed through a 23-gauge hypodermic needle three times before making up to 15 ml with additional buffer B. They were incubated for 15 min on ice and then spun at $10\,000 \times g$ for 10 min. The pellet was similarly washed once more using buffer B. The resulting pellet (PM) was resuspended in 2 ml of buffer B using a hypodermic needle as described above. In three additional experiments, we prepared a washed plasma membrane fraction (WPM) by a procedure of resuspension/washing in ionic media, as a test for adsorption of supernatant enzyme upon plasma membrane [16].

Enzyme assays

Purity of the plasma membrane preparation and contamination with other cellular materials were analyzed by marker enzyme determinations employing (Na^+/K^+)-ATPase (EC 3.6.1.37) [17], alkaline phosphatase (EC 3.1.3.1) [18], 5'-nucleotidase (EC 3.1.5.1) [19], L(+)-tartrate-sensitive acid phosphatase (EC 3.1.3.2) [20], glucose-6-phosphatase (EC 3.1.3.9) [21], succinic dehydro-

genase (EC 1.3.99.1) [22], and lactic dehydrogenase (EC 1.1.1.27) [23]. Carbonic anhydrase activity was determined by a modification of the electrometric method of Wilbur and Anderson [24], in which is determined the time required (in seconds) for a saturated CO_2 solution to lower the pH of 0.02 M Tris-HCl buffer from 8.3 to 6.3. Enzyme units for carbonic anhydrase activity are defined as 2 × (uncatalyzed time–catalyzed time)/catalyzed time, at 1°C. The method was qualitatively validated by inhibition with acetazolamide (Edemox, Wasserman, Barcelona, Spain) and quantitatively using dialyzed and lyophilized carbonic anhydrase from bovine erythrocytes (Sigma, St. Louis, MO). Carbonic anhydrase and (Na^+/K^+)-ATPase activities were determined on the day the membrane fractions were prepared. All other enzymes were assayed after overnight storage at 4°C. Protein concentrations were measured by a modification of the method of Lowry [25], using bovine serum albumin (Sigma) as standard.

Results are expressed as means ± S.E.. A paired '*t*'-test was used for calculating statistical significances of differences. Hepes was purchased from Boehringer (Mannheim, F.R.G.). Sucrose was obtained from Merck (Darmstadt, F.R.G.). Enzyme substrates and all other chemicals were from Sigma, or were of equivalent analytical grade.

Results and Discussion

Table I shows the specific activities of the marker enzymes for plasma membrane, lysosomal membrane, endoplasmic reticulum, mitochondria and cytoplasm in the different fractions obtained during the isolation process of rat hepatocyte plasma membranes (PM). The relative enrichment of these marker enzymes in the PM fraction is shown in Fig. 1. The high enrichment (23.3-times) of (Na^+/K^+)-ATPase and 5′-nucleotidase (11.7-times) together with the low contamination of glucose-6-phosphatase, succinic dehydrogenase and acid phosphatase (enrichments of 0.73- 0.13- and 0.24-times, respectively), suggest that the preparation contains mainly plasma membranes. Cytoplasmic contamination is also very low, as indicated by the lactic dehydrogenase activity found in the PM fraction (enrichment = 0.01-times). Although lower than (Na^+/K^+)-ATPase, alkaline phosphatase, assumed to be a canalicular membrane marker, was also enriched in the PM fraction (3.4-times). Thus, the preparation is presumably a mixture of basolateral and canalicular plasma

TABLE I

ACTIVITY OF MARKER ENZYMES IN THE PLASMA MEMBRANE PREPARATION (PM) AND IN THE DIFFERENT FRACTIONS OBTAINED IN THE ISOLATION PROCESS

Specific activity is defined as μmoles substrate converted/mg protein per h at 37°C. Values are means ± S.E.. Numbers in parentheses represent the number of different membrane isolation processes tests. n.d. = not detectable.

Fraction	(Na^+/K^+)-ATPase	Alkaline phosphatase	5′-Nucleotidase	Acid phosphatase	Succinic dehydrogenase	Glucose-6-phosphatase	Lactic dehydrogenase
H	1.16 ± 0.22 (12)	0.66 ± 0.17 (3)	0.38 ± 0.07 (8)	1.89 ± 0.31 (4)	0.72 ± 0.11 (4)	1.97 ± 0.16 (4)	181 ± 17 (8)
S1	0.87 ± 0.09 (4)	0.52 ± 0.17 (2)	0.27 ± 0.03 (5)	2.14 ± 0.55 (3)	0.58 ± 0.09 (3)	1.61 ± 0.10 (3)	185 ± 16 (3)
P1	1.57 ± 0.31 (4)	0.50 ± 0.08 (2)	0.49 ± 0.07 (6)	1.24 ± 0.12 (3)	1.26 ± 0.13 (3)	1.61 ± 0.16 (3)	41 ± 7 (3)
S2	2.82 ± 0.40 (3)	0.50 ± 0.18 (2)	0.15 ± 0.04 (6)	0.20 ± 0.05 (3)	n.d.	0.33 ± 0.04 (3)	239 ± 5 (4)
P2	2.76 ± 0.45 (4)	0.62 ± 0.01 (2)	0.66 ± 0.08 (6)	4.13 ± 1.17 (3)	0.91 ± 0.30 (2)	2.58 ± 1.10 (3)	29 ± 3 (2)
S3	1.10 ± 0.42 (3)	1.22 ± 0.15 (2)	0.51 ± 0.06 (5)	1.24 ± 0.02 (3)	0.12 ± 0.01 (2)	4.22 ± 0.84 (3)	38 ± 6 (3)
P3	1.14 ± 0.03 (3)	0.19 ± 0.04 (2)	0.23 ± 0.05 (6)	6.46 ± 1.41 (3)	1.78 ± 0.12 (3)	3.37 ± 0.22 (3)	25 ± 7 (3)
B3	7.86 ± 1.42 (5)	1.63 ± 0.14 (3)	1.78 ± 0.44 (4)	1.54 ± 0.31 (4)	0.29 ± 0.14 (4)	2.96 ± 0.45 (4)	78 ± 3 (4)
F1	12.8 ± 1.33 (5)	2.28 ± 0.28 (3)	3.12 ± 0.52 (4)	1.33 ± 0.79 (4)	0.10 ± 0.02 (4)	1.88 ± 0.12 (4)	
F2	2.50 ± 1.06 (3)	1.74 ± 0.19 (2)	0.85 ± 0.07 (3)	0.27 ± 0.02 (2)	0.05 ± 0.01 (2)	1.45 ± 0.40 (3)	
F3	1.74 ± 0.30 (3)	1.20 ± 0.11 (2)	0.84 ± 0.08 (2)		0.01 ± 0.01 (2)	1.65 ± 0.67 (2)	
F4	2.30 ± 0.35 (3)	0.84 ± 0.10 (2)	0.48 ± 0.12 (3)	0.45 ± 0.01 (2)	0.09 ± 0.04 (2)	2.30 ± 0.11 (3)	
PM	23.9 ± 3.32 (6)	2.37 ± 0.80 (3)	3.12 ± 0.62 (6)	0.50 ± 0.01 (4)	0.10 ± 0.04 (4)	1.77 ± 0.37 (4)	2 ± 0.5 (5)

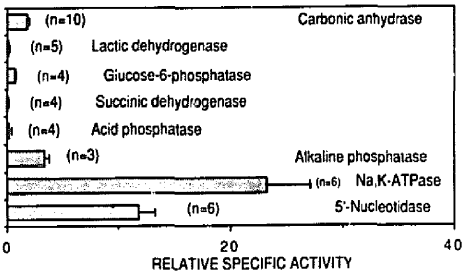

Fig. 1. Relative specific activity (the ratio of marker enzyme activity in the fraction to activity in the homogenate) in the PM fraction. Values are means ± S.E.. The number of experiments is given in parentheses.

membranes with a very low degree of contamination by other cellular materials.

An important carbonic anhydrase activity was found in the PM fraction (1.20 units per mg protein, Fig. 2), with a 1.79-fold enrichment (± S.E. 0.16, $n = 10$). This activity was sensitive to acetazolamide inhibition (Fig. 3); it was significantly enhanced by Triton X-100 (approx. 50%), but was unaffected by anions such as 200 mM Cl^- (data not shown) or 10 μM CNO^-.

Although hepatic carbonic anhydrase has been classically thought of as a cytoplasmic enzyme, with a small amount distributed in the intracellular compartments, the present result, as well as those of preliminary reports [12,13], suggest the presence of this enzyme in the plasma membrane of rat hepatocytes. The possibility of plasma membrane-bound carbonic anhydrase being an artifact

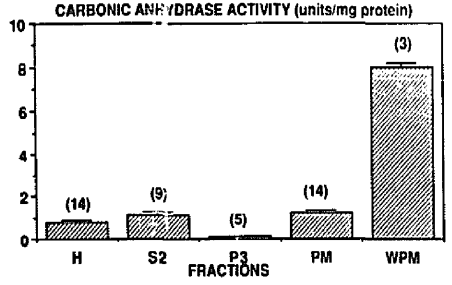

Fig. 2. Specific carbonic anhydrase activity in homogenate (H), and S2, P3, PM and washed PM (WPM), which are fractions with enrichment of cytoplasmic, intracellular membranes, and plasma membrane marker enzymes, respectively. The values are means ± S.E.. The number of experiments is given in parentheses.

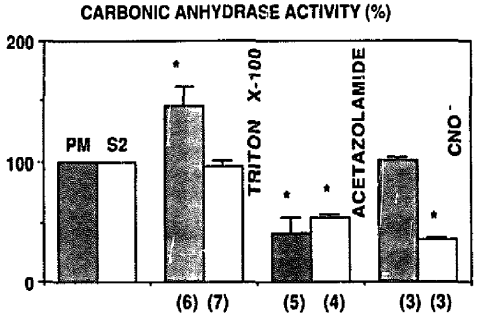

Fig. 3. Percent inhibition or activation of carbonic anhydrase in rat liver plasma membrane fraction by $2 \cdot 10^{-8}$ M acetazolamide or 5% Triton X-100 (which were incubated with the membranes at the indicated concentration at 4°C for 10 min) or 10 μM CNO^- added to the reaction chamber before carrying out carbonic anhydrase measurement. Values are means ± S.E.. The number of experiments is given in parentheses. *, $P < 0.05$, as compared with values obtained in samples with no addition of acetazolamide, Triton X-100 or CNO^-.

due to the adsorption of cytoplasmic enzyme onto the plasma membrane or due to enclosure of the cytoplasm into membrane vesicles can reasonably be ruled out. Firstly, this is because resuspension/washing in ionic media of the PM fraction, resulted in the WPM fraction, where carbonic anhydrase activity was not lower, but higher. Secondly, because lactic dehydrogenase, a cytoplasmic enzyme, showed considerably less enrichment than did carbonic anhydrase. Other authors have also reported experimental evidence to exclude the adsorption of cytoplasmic carbonic anhydrase onto isolated membranes in other cell types [16].

It is very unlikely that the carbonic anhydrase activity found in the PM fraction could be due to contamination by intracellular membranes containing this enzyme. Fig. 2 shows a comparison of carbonic anhydrase activity in PM and in three other characteristic fractions obtained during the isolation process, i.e., H, S2 and P3. S2 is a fraction containing abundant soluble cytoplasmic material (lactic dehydrogenase was enriched 1.75-times). P3 is a fraction with a high enrichment of intracellular membrane marker enzymes (acid phosphatase, glucose-6-phosphatase and succinic dehydrogenase were enriched 3.7, 1.9- and 2.3-times, respectively), but with a low enrichment of plasma membrane (0.47, 0.88- and 0.61-times,

(Na^+/K^+)-ATPase, 5′-nucleotidase, and alkaline phosphatase, respectively) or cytoplasmic (0.14-times, lactic dehydrogenase) marker enzymes. Fig. 2 shows that both the S2 and PM fractions contained an important carbonic anhydrase activity (higher than that found in H, $P < 0.05$). However, very low activity values were measured in the P3 fraction. The contamination of PM with intracellular membranes does not seem to be the cause of the high carbonic anhydrase activity found in the PM fraction. This is so because intracellular membrane marker enzymes in the PM fraction were much less enriched than carbonic anhydrase. Another reason would be because the low specific activity of carbonic anhydrase associated with intracellular membranes might not be responsible for the carbonic anhydrase activity measured in the PM fraction.

The recovery of carbonic anhydrase activity in the PM fraction was 0.3% of the total activity determined in the homogenate (H). According to the recovery of the activity of the plasma membrane marker enzymes and assuming that the plasma membrane fraction is highly purified, it may be estimated that the carbonic anhydrase bound to the plasma membrane of rat hepatocytes would be 2.2, 17.3 or 5.7% of total hepatic activity, considering a similar distribution for membrane-bound carbonic anhydrase and (Na^+/K^+)-ATPase, alkaline phosphatase, or 5′-nucleotidase, respectively.

Due to its strategic location, this low carbonic anhydrase activity presumably plays an important role in cellular functions, such as control of cell volume, of intracellular pH, anion transport and bile formation. On considering the role of carbonic anhydrase in secretion, it seems clear that such activity must reside in the membranes, since cytoplasmic enzyme, despite its abundance, would not confer the essential vectorial properties [27]. Although our results do not permit us to state whether membrane-bound carbonic anhydrase is located at the basolateral plasma membrane or the canalicular plasma membrane, or both, in several types of secretory cell this enzyme has been found at both the luminal and anti-luminal surface [27], and this is probably also true for hepatocyte. Further investigations on the differences and similarities between hepatic membrane-bound carbonic anhydrase and other forms of this membrane-bound enzyme (tentatively termed CA IV) are needed. Awareness of the presence of carbonic anhydrase in the plasma membrane of rat hepatocytes will be the starting point for further studies on the physiological role of this enzyme in liver.

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