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THE PRESENCE OF A HCO_3^--ATPase IN PANCREATIC TISSUE

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SUMMARY

An ATPase was isolated and partially purified from mammalian pancreas. It was shown to be stimulated several fold by HCO_3^- and other anions, and to be completely inhibited by SCN^- and CNO^-. In many of its properties it resembles gastric ATPase and a model is suggested to account for pancreatic HCO_3^- secretion.

INTRODUCTION

Recently it has proved possible to further characterize an ATPase from gastric mucosa[1,2], stimulated by HCO_3^- and inhibited by SCN^- (refs 3, 5). Summarizing our findings: (a) This enzyme is localized in the acid secreting cell of the mucosa. (b) It is localized in a smooth membrane vesicular fraction of that cell banding between 1.09 and 1.12 density in a sucrose gradient. (c) It is solubilized by Triton X-100, and can be further purified on a Sephadex G-200 column and 2 to 22 % sucrose density gradient. (d) A variety of bases can stimulate the enzyme, such as arsenate, sulfite, *etc.* as a function of the p*K* of the base. (e) The enzyme can be distinguished from mitochondrial ATPase because of location of this enzyme and mitochondrial markers such as succinate dehydrogenase and monamine oxidase on the density gradient. (f) A variety of inhibitors has been found for this enzyme such as SCN^-, CNO^-, NO_2^-, *etc.* which all inhibit acid secretion (G. Sachs, unpublished results). In short, the various properties have been interpreted to mean that this enzyme is involved in some critical phase of acid secretion and two types of model have been suggested, based on Mitchell's[6] ATPase I model or on a coupled redox-ATPase model where the ATPase provides the necessary vectorialization.

Since the pancreas is known to secrete HCO_3^- actively[7], it seemed likely that a similar type of enzyme might be involved in this process, and with some differences in detail, this has been shown for pancreas from dog or cat. Hence a model is proposed for active HCO_3^- secretion by this organ.

METHODS AND MATERIALS

Preparation of membrane-bound HCO_3^--ATPase

Dog or cat pancreatic tissue was freed of fat and adjacent connective tissue,

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rinsed to remove blood, weighed, chilled, and maintained in a homogenizing medium containing 0.25 M sucrose and triethanolamine-HCl (10 mM, pH 7.6). After mincing to very small pieces with scissors, tissue was homogenized with 5 parts (w/w) of the above. The homogenate was layered on a discontinuous gradient which consisted of 7 ml 15, 25 and 35 % (w/w) sucrose in 10 mM triethanolamine (pH 7.6) respectively and centrifuged for 2 h at 105000 × *g*. After the centrifugation 7 fractions were collected, Fractions 1–3 corresponding to 15 % (w/w) sucrose concentration, Fraction 5 to 25 % and Fraction 7 to 35 % sucrose concentrations; the parts between 15 and 25 %—and between 25 and 35 %—sucrose concentration were named Fraction 4 and Fraction 6. The sediment was discarded. Alternatively, the homogenate was divided into $10^4 \times g \cdot$min, $10^5 \times g \cdot$min, $4 \cdot 10^5 \times g \cdot$min and $6 \cdot 10^6 \times g \cdot$min fractions by differential centrifugation. All fractions obtained by these methods described above were stored overnight at —40 °C.

Gradient centrifugation was carried out either for 16 h in a Beckman SW25 rotor at 23000 rev./min on a linear sucrose gradient from 20–43 % (w/w) or in an Omega II ultracentrifuge (Heräus–Christ) in a 20–30 % linear sucrose gradient. Sucrose concentration was measured in an Abbé refractometer.

Preparation of the solubilized HCO_3^--ATPase

(*A*) *Solubilization*

Solubilization of the enzyme preparation was carried out using Triton X-100 with a Triton/protein ratio of 3:1 in a final volume of 1.0 ml as previously described[3]. Triton X-100 was dissolved in 25 mM imidazole–HCl buffer, pH 7.0 and 20 mM NaCl. The reaction mixture was incubated for 1 h at 4 °C with stirring; the suspension was centrifuged at 105000 × *g* for 1 h, and the supernatant concentrated by ultrafiltration in a Diaflo ultrafiltration cell (Model 10) equipped with XM-50 membrane (Amicon-Corp.) and stored at 4 °C.

(*B*) *Purification steps*

(1) *Gel chromatography on Sephadex G-200.* 1 ml of the solubilized enzyme solution was applied to Sephadex G-200 column (0.9 cm × 34 cm) equilibrated with the elution buffer and the column was operated at a flow rate of 5 ml/h. Elution buffer was 20 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES), pH 7.4. Absorbance in the elution fluid was measured at 380 nm. 1-ml fractions were collected and concentrated by ultrafiltration according to (A).

(2) *Continuous sucrose gradient.* 0.6 ml of the concentrated enzyme solution was placed on top of a continuous sucrose gradient. This gradient had been formed from 5 ml of 2 % and from 5 ml of 22 % sucrose–triethanolamine (10 mM, pH 7.6). Centrifugation time was 90 min at 105000 × *g* at +2 °C. The use of Strohmaier cells made it possible to separate 11 fractions, No. 1 corresponding to the top of the gradient.

Enzyme assays

(A) Alkaline phosphatase (EC 3.1.3.1) was determined by the Mercko-test method in the presence of 0.5 % bovine albumin (A. G. Merck, Darmstadt, Germany).

(B) The determination of acid phosphatase (EC 3.1.3.2) was carried out according to Lansing[8].

(C) The assay for aminopeptidase (EC 3.4.1.2) contained leucine-β-naphthylamide as substrate and was performed with a micromodification of the method described by Goldberg and Rutenburg[9].

(D) The activity of lactate dehydrogenase was measured according to Bücher *et al.*[10].

(E) The activity of Mg^{2+}-ATPase and (Na^+–K^+)-ATPase was measured in 75 mM Tris buffer, pH 7.6, using three different assays: (1) In the presence of 6 mM $MgSO_4$; (2) with $MgSO_4$, 100 mM NaCl and 20 mM KCl; and (3) with $MgSO_4$, NaCl, KCl and 2 mM ouabain in the incubation media. 3 mM Tris–ATP was used as substrate. After 30 min of incubation at 37 °C, the samples were heated for 2 min in boiling water, chilled and centrifuged (2 min at 14000 units/min, microsystem Eppendorf). The amount of P_i liberated was determined in aliquots of the supernatant using the method of Yoda and Hokin[11] or Bartlett[12]. Prior to the measurement of the enzyme activity the fractions of the discontinuous sucrose gradient were treated with deoxycholate and EDTA for 30 min at 4 °C.

(F) The activity of Mg^{2+}-ATPase and HCO_3^--ATPase was assayed in a medium containing 3 mM Na^+-ATP, 3 mM $MgCl_2$, 2 mM ouabain, 75 mM Tris–HCl buffer (pH 7.6) with or without 25 mM $NaHCO_3$. Following a 15-min incubation at 37 °C the samples were heated for 1–2 min in boiling water, chilled and centrifuged (2 min at 14000 units/min, microsystem Eppendorf). The amount of P_i liberated was determined in aliquots of the supernatant.

(G) Succinate dehydrogenase was determined according to ref. 13.

The protein concentrations of the fractions were measured after precipitation

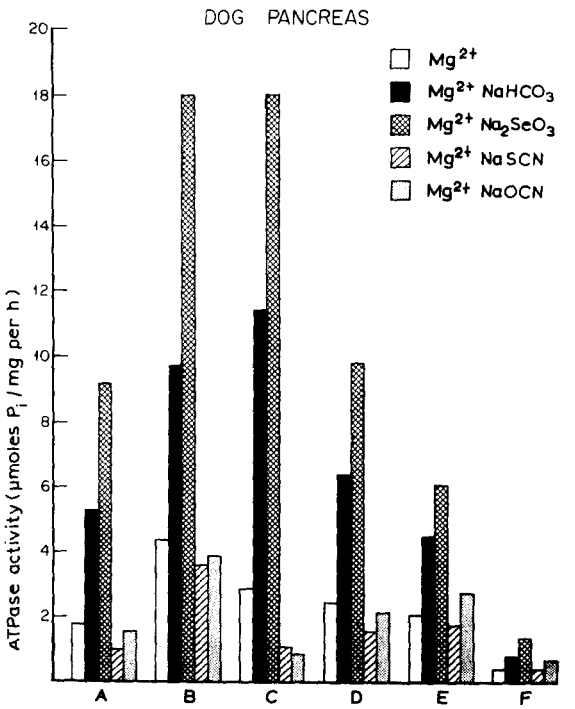

Fig. 1. Distribution of Mg^{2+}-, HCO_3^--ATPase in different subfractions of homogenate of dog pancreas, and effect of selenite (20 mM) and thiocyanate (10 mM) and cyanate (1 mM). A, total homogenate; B, $10^4 \times g \cdot min$; C, $10^5 \times g \cdot min$; D, $5 \cdot 10^5 \times g \cdot min$; E, $6 \cdot 10^6 \times g \cdot min$; F, supernatant.

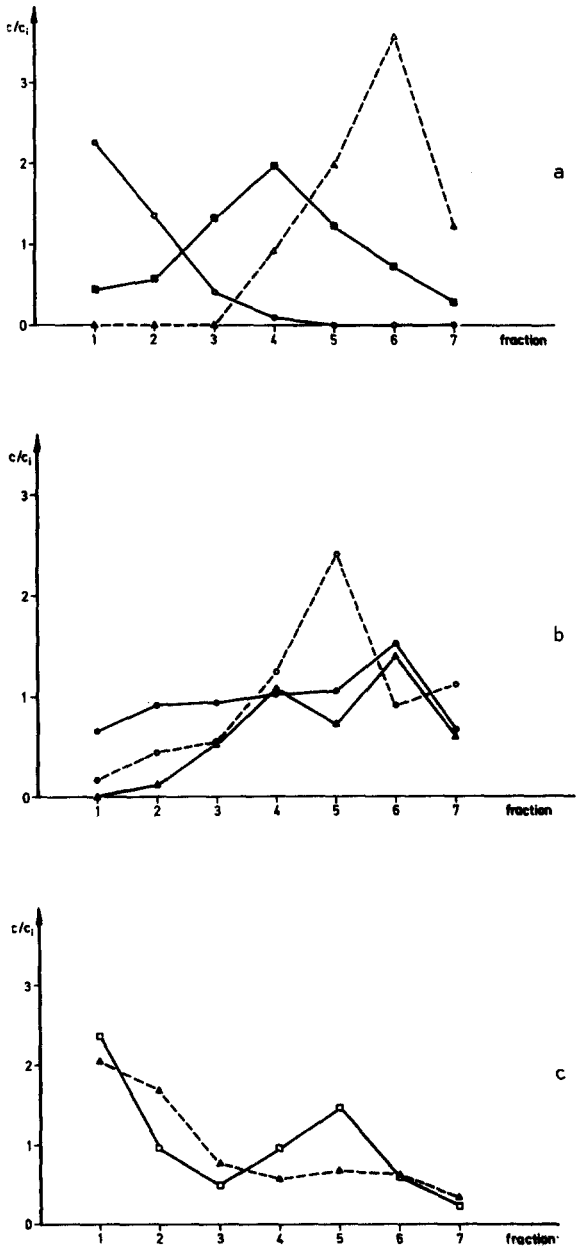

Fig. 2. (a) Distribution of acid phosphatase (■—■), lactate dehydrogenase (○—○) and succinate dehydrogenase (△---△) in the discontinuous sucrose gradient. The means of 5 experiments are given. (b) Distribution of Mg^{2+}-ATPase (●—●), (Na^+–K^+)-ATPase (▲—▲) and HCO_3^--ATPase (○---○) in the discontinuous sucrose gradient. The means of 5 experiments are given. (c) Distribution of alkaline phosphatase (□—□) and aminopeptidase (▲---▲) in the discontinuous sucrose gradient. The means of 4 experiments are given. c is the concentration measured in a single fraction; c_i is the concentration which would be present in the fractions if a substance is distributed homogeneously. A ratio of c/c_i greater than 1 indicates an enrichment of a substance in the fraction. For explanation of fraction number see Methods.

of the protein by 10 % trichloroacetic acid in the cold and dissolution of the precipitate in 1 M NaOH according to Lowry *et al.*[14].

RESULTS

Distribution of the HCO_3^--ATPase

Fig. 1 shows the distribution of the HCO_3^--ATPase in the different subcellular fractions. It can be seen that the highest activity was present mainly in the crude nuclear and mitochondrial (10^4 and $10^5 \times g \cdot$min) fractions, but was also distributed into the $5 \cdot 10^5 \times g \cdot$min and $6 \cdot 10^6 \times g \cdot$min fractions. Based on this, since stimulation of mitochondrial ATPase by HCO_3^- (ref. 15), and inhibition by SCN^- (ref. 2) has been described, it was necessary to establish the exact location of this enzyme.

Using discontinuous sucrose gradients, and enzyme markers, the results of Fig. 2 were obtained. Peak activity of the HCO_3^--ATPase was obtained at about 25 % (w/w) sucrose whereas succinate dehydrogenase showed a heavier distribution peaking at 35%. Of the other enzymes tested, only alkaline phosphatase activity peaked in a similar manner. This is of interest since such a phosphatase associated with the ATPase of gastric mucosa has been described[16]. Oligomycin did not inhibit the ATPase of Fraction 5, but inhibited the ATPase of Fraction 6. Where the high-activity fraction from the discontinuous gradient was layered on a continuous sucrose gradient of 20–30%, the results of Fig. 3 were obtained, showing a clear distinction between succinate dehydrogenase and the ATPase. Additional evidence was obtained using the 20–50 % gradient for the $10^5 \times g \cdot$min fraction where the HCO_3^--ATPase banded at a different density from the succinate dehydrogenase. Most of the activity of the $10^4 \times g \cdot$min fraction was also localized at about 26 % sucrose. It should be noted that in all cases, mitochondrial markers banded at sucrose values below that usually

Figure 3

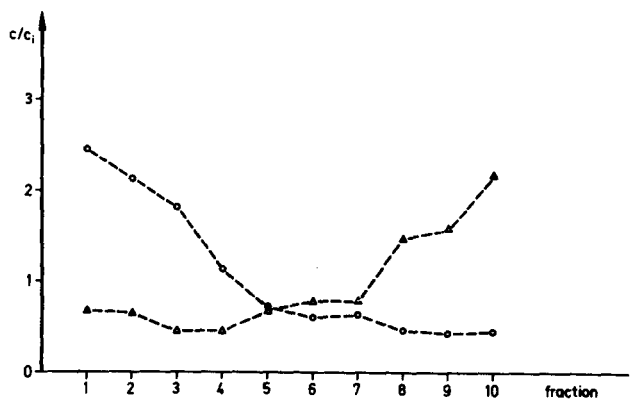

Fig. 3. Distribution of ("particulate") HCO_3^--ATPase (○---○) and succinate dehydrogenase (△---△) in the continuous sucrose gradient. 2 ml of Fraction 5 (discontinuous gradient) had been mixed with 3 ml 16.6% and with 3 ml 33.3% sucrose–triethanolamine (0.01 M, pH 7.6). Both parts had been combined by stirring to get a continuous gradient from 20–30% sucrose. For centrifugation (2 h and 105 000 × *g*, + 4 °C) Strohmaier cells were used which made it possible to separate 10 successive fractions, number 1 corresponding to the top of the gradient. The means of 4 experiments are shown. For c/c_1 see legend of Fig. 2.

found, for liver or gastric mitochondria which band isopycnically at about 41% sucrose.

Solubilization and partial purification

Applying the methods previously used for the gastric mucosal ATPase, the enzyme was solubilized with Triton X-100 and further purified by Sephadex G-200 chromatography and sucrose gradient centrifugation as shown in Table I. This procedure gives about 20-fold purification depending on species. Dog showed a greater degree of purification.

TABLE I

PURIFICATION OF HCO_3^--ATPase

	Purification ΔHCO_3^- *(-fold)*
I. Cat pancreas homogenate (0.788 mole/h per mg protein)	—
II. Discontinuous sucrose gradient (25–27% sucrose)	3.5
III. Supernatant after Triton X-100	10.0
IV. Sephadex G-200 filtrate	19.5
V. Continuous sucrose gradient (4–6% sucrose)	23.5

TABLE II

PROPERTIES OF PARTICULATE AND SOLUBILIZED ATPase FROM DOG AND CAT

	ATPase activity (μmoles P_i/mg per h)			
	Dog		*Cat*	
	Particulate ($10^5 \times g \cdot min$)	*Solubilized*	*Particulate (27% sucrose)*	*Solubilized*
Mg^{2+}	4.2	4.1	6.8	9.0
$Mg^{2+} + HCO_3^-$	11.4	22.0	9.7	18.8
$Mg^{2+} + HSeO_3^-$	18.0	29.8	—	—
$Mg^{2+} + SCN^-$	1.2	0.0	4.5	2.1
$Mg^{2+} + CNO^-$	0.8	0.0	—	—

Properties of enzyme

Table II shows the properties of the particulate and solubilized enzyme, showing that the HCO_3^- and $HSeO_3^-$ stimulation and SCN^- or CNO^- inhibition both significantly increase with solubilization. The enzyme prepared from dog is apparently more sensitive to the oxyanions tested than that from cat. It should be noted that there is a 3-fold stimulation of dog enzyme in the particulate form and 5-fold in the soluble form.

Effect of inhibitors

A variety of other compounds was tested for effect on the ATPase. 2,4-dinitrophenol (1 mM), valinomycin (1 μM), nigericin (1 μM) and diamox (0.1 mM) had no

effect on the ATPase. In contrast SH reagents such as 3,6-bio-acetomercurimethyl-dioxane or *p*-chloromercuribenzoate inhibited the enzyme by about 50 % at 1 mM.

DISCUSSION

The gastric mucosa secretes H^+ and the pancreas HCO_3^-. Secretion by both these tissues is inhibited by carbonic anhydrase inhibitors such as diamox[17,18] and apparently, from the data presented here, they both contain an ATPase which has some strikingly similar properties, with some differences.

The distribution of the enzyme in crude subcellular fractions from dog pancreas showed that the enzyme is concentrated in the low-speed fraction, in contrast to gastric mucosa, where it is mainly present in the crude microsomal or light mitochondrial fraction. This may relate to the difficulty of homogenization of the pancreas. However, it can be separated from a mitochondrial marker both on rate-isopycnic and isopycnic centrifugation. Moreover, the density corresponded to the peak density of the gastric ATPase particle[5].

Both enzymes were solubilized by Triton X-100 and showed similar purification on Sephadex and sucrose-gradient centrifugation.

Since it had previously been shown that stimulation of the enzyme was not confined to HCO_3^-, but other oxybases were also effective, $HSeO_3^-$ was used, and shown to stimulate dog pancreatic enzyme more than HCO_3^-, similar to the enzyme

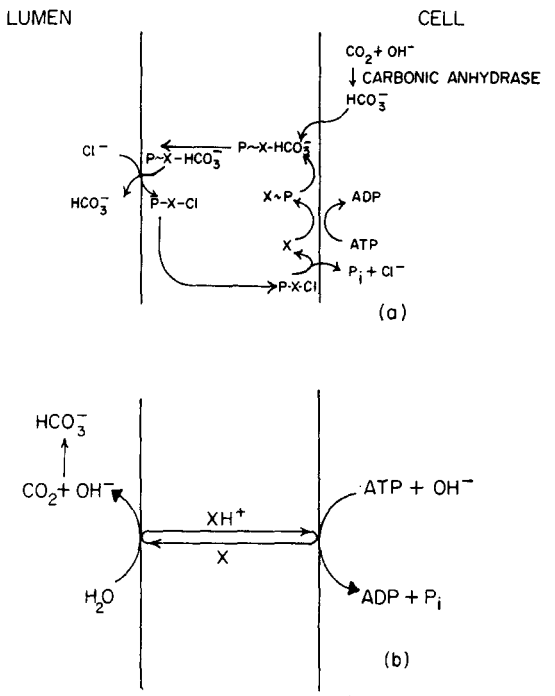

Fig. 4. Two hypothetical models for function of the HCO_3^--ATPase in the pancreas: (a) This is a HCO_3^-–Cl^- exchange model, with HCO_3^- as the primary transported ion. (b) This is a proton absorption model based on Mitchell's ATPase I, where HCO_3^- secretion is accounted for by inward H^+ transport.

obtained from Necturus gastric mucosa[4]. SCN^- and CNO^- also inhibited both enzymes, CNO^- being more potent than SCN^-. In fact in contrast to gastric ATPase, CNO^- completely inhibited the soluble pancreatic ATPase.

In considering the possible mechanisms whereby this enzyme could be responsible for HCO_3^- secretion by this organ, various models may be considered:

(*A*) *HCO_3^- is the primary ion transported.* In this case a carrier model similar to the (Na^+–K^+)-ATPase may be considered, for example a Cl^-–HCO_3^- coupling mechanism. For this we may then postulate an intermediate phosphoprotein, varying the affinity for HCO_3^- or Cl^-. $HSeO_3^-$ could then also be transported.

(*B*) *HCO_3^- secretion is due to proton transport.* In this case, CO_2 diffuses into the lumen, and reacts with OH^- resulting from inward proton transport. This model would not easily account for the HCO_3^- stimulation of the ATPase, nor the effect of diamox in inhibiting HCO_3^- secretion, but essentially is an ATPase I mechanism of Mitchell[6]. The two models discussed are shown in Fig. 4.

Thus the finding that an enzyme is present in pancreas analogous to an enzyme in gastric mucosa is a challenging finding and warrants further exploration.

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