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THE MECHANISM OF FLUID SECRETION IN THE RABBIT PANCREAS STUDIED BY MEANS OF VARIOUS INHIBITORS

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In order to increase our understanding of the mechanism of pancreatic fluid secretion we have studied the effects of various transport inhibitors on this process in the isolated rabbit pancreas. In this preparation, a high rate of unstimulated fluid secretion occurs, which probably originates from the ductular cells. Inhibitory are ouabain, furosemide, bumetanide, piretanide, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS) and acetazolamide, with their half-inhibitory concentrations: $2 \cdot 10^{-6}$ M (ouabain), $1.3 \cdot 10^{-3}$ M (furosemide), $2.2 \cdot 10^{-3}$ M (bumetanide and piretanide) and $1.4 \cdot 10^{-4}$ M (SITS). With acetazolamide a maximal inhibition of only 20% is found at 10^{-3} M. Amiloride (10^{-3} M) has no effect on pancreatic fluid secretion. The inhibitory effects on HCO_3^- output are always larger and those on Cl^- output lower than those on fluid secretion. The results suggest that the ouabain-sensitive $(Na^+ + K^+)$-ATPase system provides the energy for a Na^+-gradient-driven Cl^--HCO_3^--exchange transport system, sensitive to the loop diuretics furosemide, bumetanide and piretanide and to SITS. This system would drive the transcellular transport of HCO_3^- and secondarily that of cations, Cl^- and water.

Introduction

The exocrine pancreas secretes a fluid containing digestive enzymes, and inorganic ions. This secretory process is driven by the active transport of ions, with water following osmotically [1]. As in other fluid-secreting epithelia, the $(Na^+ + K^+)$-ATPase system has been shown to be the primary driving force for fluid secretion in the pancreas [2]. This system is located in the basolateral plasma membrane of the ductular and acinar cells [3], and pumps K^+ into the cells and extrudes Na^+ from the cells into the interstitial fluid space. However, the fluid is secreted into the lumen from the apical side of the cells and contains Na^+ as the main cation. This implies that there must be one or more other ion transport systems, driven by the $(Na^+ + K^+)$-ATPase system, which lead to the secretion of water and ions into the lumen.

Several studies have shown that in certain animal species there exist two separate, independent sources of fluid secretion in the pancreas. One is the ductular cell, which secretes a fluid containing a high HCO_3^- concentration, while the other is the acinar cell which secretes a fluid with a high Cl^- concentration. In the rat, the secretion of a Cl^--rich fluid is stimulated by acetylcholine and by cholecystokinin-pancreozymin, suggesting an acinar origin, and the secretion of a HCO_3^--rich fluid is stimulated by secretin, suggesting a ductular origin [4]. However, in the cat and probably also in the rabbit, the secretion of NaCl by the acinar cells is lacking or of minor importance [5,6]. Various models have been postulated to explain the secretion processes in these two cell types.

Abbreviation: SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid.

These models have in common that the secretion of the anions Cl^- and HCO_3^- is thought to be driven either by cotransport of Na^+ and Cl^- or by countertransport of Na^+ and H^+.

Previously we have suggested that in the rabbit pancreas the secretion of Na^+ and K^+ proceeds through a paracellular pathway, consisting of lateral intercellular spaces and leaky tight junctions [7]. In addition, we have shown that the unstimulated fluid secretion of the rabbit pancreas is primarily Na^+- and HCO_3^--dependent, and that Cl^- is secreted secondary to HCO_3^- [6,7]. From the results of these studies we have also concluded that the fluid secretion mechanism is probably present in the ductular cells [6,7]. In order to obtain more information about the involvement of ion transport systems other than $(Na^+ + K^+)$-ATPase, we have studied the effects of various inhibitors of ion transport systems on the secretory rate and the composition of the rabbit pancreas fluid.

Methods

Male and female New Zealand white rabbits of 3–4 kg are used. The animals are killed by a blow on the neck, immediately followed by carotic exsanguination. The pancreas is prepared essentially as described by Rothman [8] and modified by us [9,10]. The isolated pancreas is mounted on a frame and incubated in a bath containing 350 ml bathing medium. The main pancreatic duct is cannulated close to its junction with the duodenum, so the secreted fluid can be collected. The isolated pancreas is preincubated for 1 h after mounting in a balanced Krebs-Ringer bicarbonate medium in order to reach a steady-state condition. The composition of the normal Krebs-Ringer medium is (in mmol/l): Na^+ 143.5, K^+ 4.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 130.7, HCO_3^- 25, $H_2PO_4^-$ 1.2, glucose 5.5 (pH 7.4). The medium is continuously gassed with carbogene (95% O_2, 5% CO_2). After the preincubation period, the medium is replaced by fresh Krebs-Ringer medium and the experiment is started. The secreted fluid is collected in 10-min fractions in preweighed plastic tubes. The fractions, in which the HCO_3^- concentration is to be measured, are collected under paraffin oil [6]. From each fraction samples are taken for the appropriate assays.

The first hour of incubation (control period) is carried out in normal Krebs-Ringer medium. This is generally followed by two or three experimental periods of 60–90 min, and sometimes an additional 60-min control period. In the experimental periods an inhibitor is added, in a higher concentration in each successive experimental period. The secretory rate and composition of the secreted fluid reach a steady-state level within 30–60 min.

In some experiments, furosemide is added to a Cl^--free or HCO_3^--free incubation medium. In these cases, the normal Krebs-Ringer medium is replaced after the control period by a medium containing isethionate instead of Cl^- or acetate instead of HCO_3^-, and after 60 min in this medium the inhibitor is added.

The pH of the acetate-medium is brought to 7.4 by gassing with an appropriate mixture of carbogene and O_2, with fine adjustment, if necessary, with NaOH or HCl. A sample of the bathing medium is taken every 30 min for determination of the ion concentrations. For calculations the mean values are taken of the final 30 min of the control period and of each experimental period.

Assay methods. The volume of the secreted fluid fractions is determined by weighing the plastic tubes on a fully automatic Mettler electronic balance, assuming a fluid density of 1.0.

Na^+ and K^+ concentrations are measured in an Eppendorf flame photometer. Samples of 10 or 15 µl bathing medium or pancreatic fluid are diluted with distilled water to 3 ml. Standard solutions containing NaCl and KCl in the same concentration range are used for calibration curves, which are virtually linear.

Chloride is determined coulometrically. A 5- or 7.5-µl sample of bathing medium or secreted fluid is diluted with 3.5 ml of a solution containing 10% acetic acid and 0.1 M nitric acid. Three drops of a gelatin indicator solution are added and the chloride content of the sample is measured by titration in an Aminco-Cotlove chloride titrator. Standards and blanks are titrated prior to the samples. The Cl^- concentration of the samples is calculated from the titration time after correction for the blank.

The bicarbonate concentration in the secreted

fluid is calculated as the difference between the $(Na^+ + K^+)$ concentration and the Cl^- concentration.

Chemicals. Ouabain is obtained from Merck, furosemide (Lasix) is purchased from Hoechst AG, acetazolamide (Diamox) from Lederle Laboratories Division, SITS from I.C.N. Pharmaceuticals, Inc. and amiloride from Merck, Sharp and Dohme. Bumetamide and piretanide are a gift from Dr. C.P. Stewart, Abteilung Pharmakologie, Medizinischen Fakultät, Rhein.-Westf. Techn. Hochschule, Aachen. Both SITS, amiloride, bumetanide and piretanide were made up freshly for each experiment. All other chemicals used are commercial preparations of the highest obtainable purity.

Results

In normal Krebs-Ringer medium, the isolated rabbit pancreas secretes spontaneously for about 5–6 h after the isolation at a relatively constant secretory rate of approx. 600 µl/h. The secreted fluid contains (in mM): Na^+ 160, K^+ 6, HCO_3^- 90 and Cl^- 80, as the major ions. Upon addition of ouabain the fluid secretion rate decreases, while the Na^+ concentration of the secreted fluid remains constant and the K^+ concentration rises slightly (Fig. 1). The Cl^- concentration of the secreted fluid increases which means that the HCO_3^--concentration decreases simultaneously.

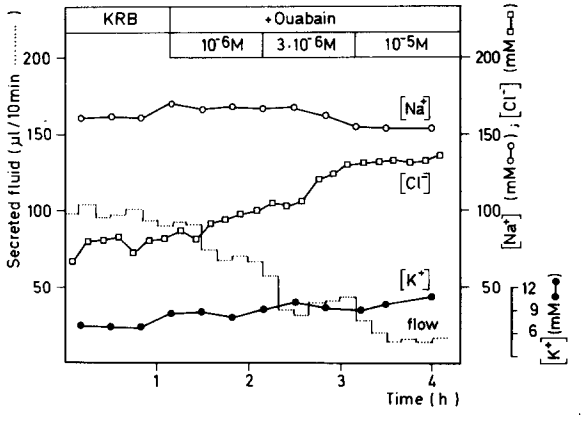

Fig. 1. Effect of ouabain on the rate of fluid secretion (·····) and on the concentrations of Na^+ (O——O), K^+ (●——●) and Cl^- (□——□) in the secreted fluid of the isolated rabbit pancreas.

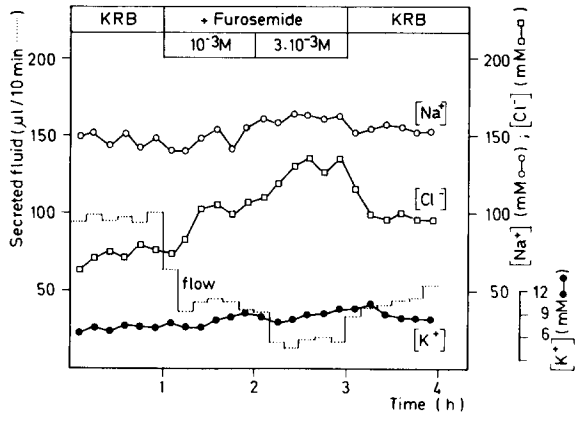

Fig. 2. Effect of furosemide on the rate of fluid secretion (·····) and on the concentrations of Na^+ (O——O), K^+ (●——●) and Cl^- (□——□) in the secreted fluid of the isolated rabbit pancreas.

Addition of furosemide decreases the fluid secretion rate and has virtually no effect on the cation concentrations of the fluid (Fig. 2). The Cl^- concentration of the secreted fluid increases and the (calculated) HCO_3^- concentration of the secreted fluid decreases. The effects of ouabain and furosemide are both dose-dependent (Table I). About 50% inhibition of fluid secretion is obtained with $2 \cdot 10^{-6}$ M ouabain or with $1.3 \cdot 10^{-3}$ M furosemide (Fig. 3).

In the Cl^--free isethionate medium, fluid secretion is inhibited by 50% as compared to normal Krebs-Ringer medium, and the secreted fluid contains mainly HCO_3^- (130 mM) and low concentrations of isethionate (15 mM) and Cl^- (15 mM) [6]. In the HCO_3^--free acetate medium, fluid secretion

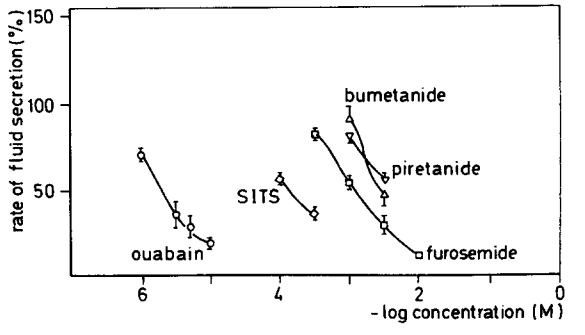

Fig. 3. Dose-response curves for the effects of ouabain (○), furosemide (□), bumetanide (△), piretanide (▽) and SITS (◇) on the rate of fluid secretion of the isolated rabbit pancreas. For the number of experiments see Table I.

is inhibited by 43%, and the concentrations of HCO_3^-, acetate and Cl^- are 27 mM, 33 mM and 92 mM [6]. When furosemide is added in isethionate or acetate medium, its effect (when expressed as percent inhibition of the fluid secretion rate in these media) is the same as in normal Krebs-Ringer medium (Table I). In isethionate medium, furosemide has virtually no effect on the HCO_3^-, Cl^- and isethionate concentrations, and since the secretion contains predominantly HCO_3^-, it inhibits mainly HCO_3^- secretion. In acetate medium, furosemide also has no effect on the anion concentrations in the secreted fluid, which means that it equally inhibits the secretion of Cl^-, HCO_3^- and acetate (Table I). Bumetanide and piretanide qualitatively have the same effect as furosemide, but they inhibit fluid secretion to a lesser extent (Fig. 3, Table I).

Acetazolamide inhibits fluid secretion only slightly, increases the Cl^- concentration, decreases the HCO_3^- concentration and has no effect on the Na^+ and K^+ concentrations in the secreted fluid. The effect on fluid secretion is maximal at a concentration of about 10^{-3} M, at which concentration an inhibition of 20% is obtained (Table I).

SITS inhibits the fluid secretion rate in an apparently dose-dependent manner (50% at $1.4 \cdot 10^{-4}$ M, Fig. 3). It decreases the HCO_3^- concentration and increases the Cl^- concentration of the secreted fluid and again has no effect on the cation concentrations (Table I).

Amiloride, in concentrations up to 10^{-3} M, has no effect on the rate of fluid secretion or on the ion concentrations in the secreted fluid, either in normal Krebs-Ringer medium or in a low Na^+-medium in which the Na^+ concentration is reduced from 143.5 to 25 mM by replacement with K^+ (Table I).

In Fig. 4 the percentages inhibition of Cl^-- or

TABLE I

EFFECTS OF VARIOUS TRANSPORT INHIBITORS ON FLUID SECRETION, Cl^--SECRETION AND HCO_3^--SECRETION

Mean values ± S.E. are given. N = number of experiments.

Medium	Inhibitor		N	% inhibition of		
				Fluid secretion	Cl^- secretion	HCO_3^- secretion
Normal	Ouabain	(10^{-6} M)	6	29 ± 2	15 ± 3	41 ± 3
		($3 \cdot 10^{-6}$ M)	4	64 ± 7	42 ± 9	84 ± 3
		($5 \cdot 10^{-6}$ M)	4	71 ± 6	62 ± 8	81 ± 4
		(10^{-5} M)	7	80 ± 2	66 ± 4	92 ± 2
Normal	Furosemide	($3 \cdot 10^{-4}$ M)	3	18 ± 3	12 ± 3	25 ± 2
		(10^{-3} M)	5	45 ± 3	35 ± 2	54 ± 6
		($3 \cdot 10^{-3}$ M)	4	70 ± 5	56 ± 6	82 ± 6
Cl^--free [a]		(10^{-3} M)	5	53 ± 10	60 ± 12 [d]	53 ± 10
HCO_3^--free [b]		(10^{-3} M)	5	53 ± 9	47 ± 9	58 ± 10 [e]
Normal	Acetazolamide	(10^{-3} M)	6	20 ± 2	1 ± 1	36 ± 3
Normal	Bumetanide	(10^{-3} M)	5	9 ± 6	1 ± 5	15 ± 8
		($3 \cdot 10^{-3}$ M)	4	53 ± 6	44 ± 6	55 ± 7
Normal	Piretanide	(10^{-3} M)	3	20 ± 2	19 ± 2	19 ± 3
		($3 \cdot 10^{-3}$ M)	3	44 ± 1	43 ± 2	45 ± 3
Normal	SITS	(10^{-4} M)	6	44 ± 2	32 ± 1	54 ± 1
		($3 \cdot 10^{-4}$ M)	5	64 ± 3	50 ± 4	76 ± 3
Normal	Amiloride	(10^{-3} M)	3	−9 ± 8		
Low Na^+ [c]		(10^{-3} M)	6	14 ± 13		

[a] 131 mM Cl^- replaced by 131 mM isethionate.
[b] 25 mM HCO_3^--replaced by 25 mM acetate.
[c] 118.5 mM Na^+ replaced by 118.5 mM K^+.
[d] This value represents inhibition of (Cl^- + isethionate)-secretion.
[e] This value represents inhibition of (HCO_3^- + acetate)-secretion.

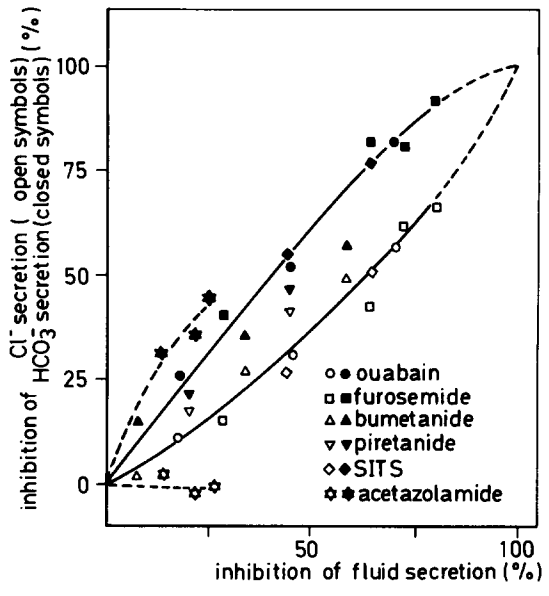

Fig. 4. Relationship between inhibition of fluid secretion and inhibition of HCO_3^- and Cl^- secretion. Data are taken from the experiments with ouabain (concentration range 10^{-6}–10^{-5} M) (○,●), furosemide (10^{-4}–10^{-3} M) (□, ■), bumetanide (10^{-3}–$3 \cdot 10^{-3}$ M) (△, ▲), piretanide (10^{-3}–$3 \cdot 10^{-3}$ M) (▽, ▼), SITS (10^{-4}–$3 \cdot 10^{-4}$ M) (◇, ◆), and acetazolamide (10^{-3} M). The results are expressed as percentages inhibition of the secretory rate in the control period, and represent mean values of 2–5 experiments.

HCO_3^--secretion rate (all data taken from Table I). This figure shows that the relation between these parameters is about the same for all inhibitors, except for acetazolamide, which does not inhibit Cl^- secretion.

Discussion

The Swanson-Solomon model

Several studies of pancreatic fluid secretion have shown that $(Na^+ + K^+)$-ATPase located in the basolateral membrane plays a primary and rate-limiting role in fluid secretion [2,3,11,12]. In addition, Swanson and Solomon [11] have suggested that a Na^+-dependent extrusion of protons via a $Na^+ - H^+$-exchange carrier is the mechanism responsible for transcellular transport of HCO_3^-. We have previously expanded this model to include the secretion of the monovalent cations Na^+ and K^+ via a paracellular route [7]. This model, with as key features the $(Na^+ + K^+)$-ATPase system and the Na^+-H^+-exchange carrier (Fig. 5a), explains that the inhibition of fluid secretion by ouabain is due to inhibition of the $(Na^+ + K^+)$-ATPase, resulting in a lowering of the Na^+ gradi-

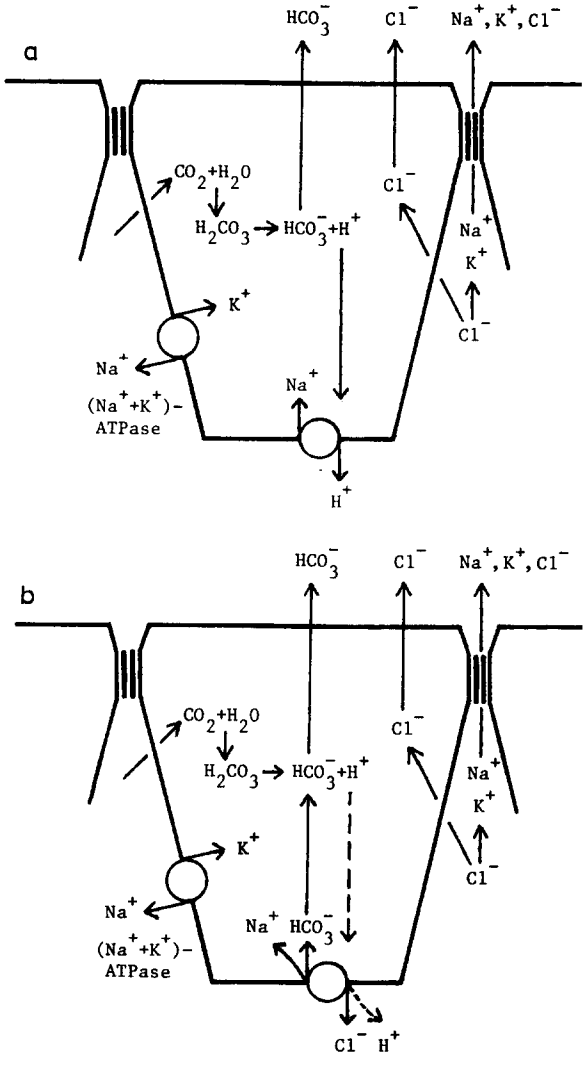

Fig. 5. Models for fluid secretion by the ductular cells of the rabbit pancreas. (a) The $(Na^+ + K^+)$-ATPase system maintains a Na^+-gradient across the basolateral cell membrane. The coupled Na^+-H^+ exchange is a secondary active process that serves to transport H^+ out of the cell and HCO_3^- into the cell and the secretory lumen. (b) The $(Na^+ + K^+)$-ATPase system drives via the Na^+-gradient a coupled Na^+-HCO_3^--Cl^- ($-H^+$) transport, which maintains the transcellular transport of HCO_3^-. In both models Na^+ and K^+ are secreted via the paracellular route. The pathway for Cl^- secretion is assumed to be paracellular, transcellular or both.

ent, a fall in intracellular pH and a decrease of the HCO_3^--concentration gradient across the apical membrane and consequently of the secretion rates for HCO_3^- and water. The inhibition of fluid secretion by acetazolamide would be the result of the inhibition of the enzyme carbonic anhydrase, which catalyzes the intracellular hydration of CO_2 leading to HCO_3^- and protons. However, 10^{-3} M acetazolamide, which should inhibit the carbonic anhydrase virtually completely, inhibits fluid secretion in the rabbit pancreas by only 20%. This suggests that either the rate of the uncatalyzed reaction is nearly sufficient to produce the HCO_3^- involved in the secretion process, or else that the reaction is only of minor importance in the fluid secretion process. In the perfused cat pancreas $2 \cdot 10^{-4}$ M acetazolamide inhibits fluid secretion by nearly 70% [13], so in this species intracellular CO_2 hydration appears to be a major factor in fluid secretion.

Arguments against the model

Although the model of Fig. 5a can explain the effects of ouabain and acetazolamide, it is unable to explain the effects of furosemide, piretanide, bumetanide and SITS and the lack of an effect of amiloride on pancreatic fluid secretion.

Amiloride has been studied in order to determine whether Na^+-H^+ exchange plays an essential role in fluid secretion. In concentrations above 1 μM it inhibits Na^+-H^+ exchange in various epithelial and non-epithelial systems [14]. This exchange has an important function in cellular pH regulation, volume regulation and growth processes [15,16], while a function in H^+ secretion has also been described [17]. Since amiloride competes with Na^+ for the exchange carrier, its effects are generally more pronounced in a low-Na^+ medium. However, we do not find any effect of 10^{-3} M amiloride on pancreatic fluid secretion, either in normal or low-Na^+ medium. This suggests that either the pancreas lacks such an amiloride-sensitive carrier, or that the carrier is not involved in fluid secretion, or that the low concentration of Na^+ (25 mM) is still too high to uncover an effect of amiloride. Thus, it remains unclear if the Na^+-H^+-exchange system postulated in the model of Fig. 5a is actually present.

The diuretics furosemide, bumetanide and piretanide have been used in order to determine whether a cation-coupled anion transport plays a role in pancreatic fluid secretion. In a number of epithelia, furosemide and other sulfonamide 'loop' diuretics have been shown to inhibit transepithelial Cl^- transport via inhibition of a Na^+-Cl^--cotransport system [18], which is energized by the Na^+-gradient. Recent evidence suggests that this system may actually be a Na^+-K^+-Cl^- cotransport carrier [19–21]. Palfrey et al. [22] have shown that the Na^+-K^+-Cl^--cotransport system in avian red cells is inhibited by furosemide in relatively high concentrations (10^{-4}–10^{-3} M) and that this system is 100-times more sensitive to bumetanide than to furosemide. However, these authors also find that furosemide is a rather aspecific inhibitor of the Na^+-K^+-Cl^- cotransport, since it inhibits also anion exchange in the erythrocytes in the same concentration as it inhibits the cotransport. Bumetanide can inhibit the anion exchange only at 1000-times the concentration in which it inhibits the Na^+-K^+-Cl^- cotransport (10^{-4} M). Thus, the relatively high concentration of furosemide and the high concentrations of bumetanide and piretanide (as compared to furosemide) needed to achieve an inhibitory effect on pancreatic fluid secretion, argue against a role of a Na^+-K^+-Cl^- cotransport and favour a role of a furosemide-sensitive anion-exchange carrier in pancreatic fluid secretion. It has been suggested that furosemide can inhibit Cl^- selfexchange in Ehrlich ascites cells [23] and in red blood cells [24], and Cl^--HCO_3^- exchange in the gallbladder [25].

The disulfonic stilbene derivative SITS, which is commonly assumed to be a highly specific inhibitor of anion exchange [26], inhibits pancreatic fluid secretion in concentrations similar to those required for inhibition of anion transport (10^{-4}–$5 \cdot 10^{-4}$ M). Inhibition of fluid secretion by SITS has previously been reported in the cat pancreas [27]. This also supports a role of an anion exchange system, probably a Cl^--HCO_3^--exchange system, in pancreatic fluid secretion. The finding that NO_3^- can substitute for Cl^- in fluid secretion by the cat pancreas [28] as well as in the anion-exchange system in Ehrlich ascites cells [23,29], but not in the Na^+-K^+-Cl^- cotransport in Ehrlich cells [20,30], is in accordance with this conclusion.

A revised model

The effects of various inhibitors on pancreatic fluid secretion suggest that the fluid secretion is dependent on the activity of the $(Na^+ + K^+)$-ATPase system, and that the involvement of the carbonic anhydrase system is at least questionable. In addition, an anion-exchange system, sensitive to the loop diuretics and SITS, appears to play an important role in the secretion process. Previous studies have shown that the entire fluid secretion is Na^+- and HCO_3^--dependent [6,7,11,13] and that HCO_3^- is the primary secreted anion [6,11]. In addition, the present study shows that ouabain, SITS and the loop diuretics have similar effects on the secretion of water, Cl^- and HCO_3^- and that these effects are comparable to those of omitting Na^+ or HCO_3^- from the bathing medium [6]. This suggests that these inhibitors eventually have a common mode of action, i.e. that they inhibit (different legs of) an ion transport system leading to secretion of HCO_3^- and water. This transport system appears to be a Na^+-gradient-dependent Cl^--HCO_3^--exchange mechanism.

According to these results we now postulate a revised model for pancreatic fluid secretion (Fig. 5b). The model includes a Na^+-dependent anion-exchange mechanism on the basolateral membrane that transports Na^+ and HCO_3^- into the cell and Cl^- out of the cell. Fluid secretion is based on the secondary active transport of HCO_3^- into the cell via this coupled transport with Na^+ and Cl^-. The involvement of a proton extrusion mechanism, whereby anions such as HCO_3^- and acetate may be accumulated into the cell via non-ionic diffusion of their protonated form and subsequent transport of the protons out of the cell, remains uncertain. Although the evidence for such a transport system is only indirect, it would account for the effects of the various inhibitors found in this study and for the effects of ion replacements found in previous work [6,11,12,28].

A coupled Na^+-Cl^--HCO_3^--transport system, as assumed here, appears to play a role in the regulation of the intracellular pH in squid axons, snail neurons and barnacle muscle [31]. This system is responsible for acid extrusion via influx of HCO_3^- and possibly efflux of H^+, and it is dependent on extracellular Na^+ and HCO_3^- and intracellular Cl^- [32]. The system can be inhibited by SITS and furosemide but not by amiloride, in contrast to the Na^+/H^+-Cl^-/HCO_3^- double ion exchanger which has been postulated to maintain NaCl transport in the brush border membrane of rat intestine [33,34] and which is sensitive to amiloride and SITS both. In addition, Guggino et al. [35] postulate a transport system in the basolateral cell membrane of the Necturus proximal tubule that can transport Cl^- out of the cell in exchange for both Na^+ and HCO_3^- and that is sensitive to SITS. In the pancreas, a similar coupled Na^+-Cl-HCO_3^- transport may thus function to transport HCO_3^- into the cell against its electrochemical gradient, with Na^+ entering and Cl^- leaving the cell simultaneously.

In conclusion, our findings support a model for fluid secretion by the rabbit pancreas, in which the $(Na^+ + K^+)$-ATPase system, located on the basolateral side of the ductular cell, plays a primary and rate-limiting role in fluid secretion and in which a Na^+-gradient-dependent Cl^--HCO_3^- exchange system serves to transport HCO_3^- through the cell (Fig. 5b). The latter system would be located on the basolateral membrane and may possibly involve a H^+ efflux component. Intracellular carbonic acid formation through the carbonic anhydrase would supply only a minor part of the secretory pool of HCO_3^-. Secretion of HCO_3^- on the apical side may proceed through a conductive pathway. The HCO_3^- secretion will induce the secretion of Na^+ and K^+ through the paracellular route and the secretion of Cl^- and water through trans- or paracellular pathways.

Acknowledgements

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