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CHANGES IN RAT AND MOUSE SALIVARY GLANDS AND PANCREAS AFTER CHRONIC TREATMENT WITH DIURETICS: A POTENTIAL ANIMAL MODEL FOR CYSTIC FIBROSIS

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Abstract

Defective transepithelial chloride and water transport is thought to be the cellular basis of the disease cystic fibrosis (CF). Therefore, it was attempted to develop an animal model for this disease by chronically inhibiting transepithelial chloride transport in experimental animals by long term treatment with high doses of diuretics. In the present study, changes in the salivary glands and pancreas after such treatment were investigated by X-ray microanalysis and electron microscopy.

Treatment of rats for one month with diuretics caused a significant decrease in chloride and an increase in calcium in the acinar cells of the submandibular gland. This increase was due to accumulation of mucus in the cells. The strongest effect was obtained after combined treatment with furosemide and acetazolamide. Only minor changes were noted in the parotid gland and the pancreas. Treatment of mice for three months with diuretics caused similar changes in the submandibular glands. In addition, marked changes in the pancreas were observed. The chloride content of the pancreatic acinar cells was decreased. In many acinar cells, only very few zymogen granules were present. The morphological and microanalytical results point to severe dysfunction of the exocrine pancreas. These changes parallel those found in patients with CF, and the chronically furosemide-treated mouse thus could serve as an animal model for this disease.

Key Words: cystic fibrosis, epithelium, chloride transport, water transport, pancreas, salivary glands, diuretics, furosemide, animal model.

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Introduction

In recent years, evidence has accumulated that a defective transepithelial chloride and water transport is a central factor in the pathogenesis of the congenital, hereditary, lethal disease cystic fibrosis (CF) (Quinton, 1983; Knowles *et al.*, 1983 a,b; Boucher *et al.*, 1983; Bijman, 1984; Bijman and Frömter, 1986; Widdicombe *et al.*, 1985; Goldstein *et al.*, 1988; Orlando *et al.*, 1989). In many epithelia, e.g., in the epithelia lining the respiratory and digestive tract, as well as in salivary glands and pancreas, chloride is taken up across the basolateral membrane via a $Na^+/K^+/2Cl^-$ cotransport system, and secreted across the apical membrane via a Cl^- channel (Liedtke, 1989). With the exception of salivary gland acinar cells, where it is primarily regulated by intracellular Ca^{2+} ions, this Cl^- channel is opened by cyclic AMP (cAMP). Water follows passively across the epithelium with the flux of Cl^- ions. In CF patients it appears that the Cl^- channel cannot be opened by cAMP (Welsh and Liedtke, 1986; Frizzell *et al.*, 1986; Schoumacher *et al.*, 1987; Chen *et al.*, 1989; Hwang *et al.*, 1989; Li *et al.*, 1989).

A model for CF could be constructed (Boucher, 1984) in which the reduced transepithelial chloride and water transport would result in an abnormally low water content of the secretory products at the luminal side of the affected epithelia. In the respiratory tract, the inhibition would be responsible for the formation of viscous mucus blocking the airways. Similarly, in the exocrine pancreas, the ducts would be obstructed by viscous enzymatic secretions, which would then digest the pancreatic tissue. This course of events would explain the two major clinical symptoms of CF, namely chronic obstructive lung disease and pancreatic insufficiency. A model for CF should ideally explain all the different symptoms and pathological changes observed in connection with the disease. Among those changes is an increase in calcium in a

number of tissues and secretions in CF patients. This abnormality in calcium handling has given rise to the "calcium theory" of CF (stating that the basic cellular defect in CF was linked to an error in the intracellular calcium homeostasis), which for a long time has been the dominating theory on the pathogenesis of CF (Katz *et al.*, 1984).

For the study of the chain of events leading from the primary cellular defect underlying CF to the clinical symptoms, an animal model of the disease would be very valuable. Martinez *et al.* (1975a,b) proposed the chronically reserpinized rat as animal model for CF. At that time it was thought that an abnormality in the nervous regulation of epithelial transport was of importance in the pathogenesis of CF, and chronic treatment with the noradrenaline depleter reserpine disturbs the nervous regulation of exocrine gland function. This animal model indeed displays a number of parallels with the human disease (Martinez, 1985), although it is unclear which of the many possible effects of reserpine at the cellular level is responsible for the observed effects.

It therefore appeared logical to attempt to develop an animal model based on chronic inhibition of transepithelial chloride and water transport by treatment with known inhibitors of these processes. The best parallel with CF would be to block chloride efflux via the apical Cl^- channel, but at present this is difficult because of lack of suitable inhibitors. However, blocking chloride entry across the basolateral membrane would have a similar effect on transepithelial chloride and water transport. In a previous study (Scarlett *et al.*, 1988) rats were treated for one month with the diuretic furosemide. Inhibition of fluid transport in the submandibular gland was demonstrated, and morphological changes in salivary glands and pancreas indicative of duct obstruction were observed. However, the dramatic changes in the structure of the pancreas found in CF patients were not observed in this animal model. It was considered that this could be due to an incomplete inhibition of fluid transport by furosemide. In particular in the pancreas, water transport may be mainly coupled to the bicarbonate flux, rather than to the chloride flux (Kuijpers and De Pont, 1987). For the present study, therefore, experiments were carried out in which animals were chronically treated with a combination of inhibitors of anion and fluid transport. In addition, in a separate set of experiments, the treatment was continued over a period of three months.

Materials and Methods

In one set of experiments, male Sprague-Dawley rats (4 to 5 weeks old at the start of the experiment) were used. The animals were divided into groups of six animals each that during one month received food mixed with diuretics as follows: [1] furosemide + acetazolamide to give a daily dose of 40 mg furosemide and 8 mg acetazolamide per animal, [2] furosemide + amiloride to give a daily dose of 40 mg furosemide and 4 mg amiloride, and [3] bumetanide, to give a daily dose of 8 mg bumetanide. The doses were calculated based on average food intake by the animals. The drugs were mixed with the standard rat chow as described previously (Scarlett et al. 1988). The animals had access to food and water *ad libitum*. A control group of eight animals was kept for the same period under identical housing conditions and received standard rat chow. For the results on animals treated with furosemide alone (40 mg daily) the data of a previous study (Scarlett et al. 1988) were used.

In the second set of experiments, female mice (5 weeks old at the start of the experiment) were used. The animals were divided into groups of six animals that during three months received food mixed with diuretics as follows: [1] furosemide, to give a daily dose of 4 mg, [2] amiloride, to give a daily dose of 1 mg, [3] acetazolamide, to give a daily dose of 4 mg, [4] furosemide + amiloride, to give a daily dose of 4 mg furosemide and 0.5 mg amiloride, and [5] furosemide + acetazolamide, to give a daily dose of 4 mg furosemide and 4 mg acetazolamide. The doses were calculated based on average food intake by the animals. The drugs were mixed with the standard chow and the animals had access to food and water *ad libitum*. A control group of nine animals was kept for the same period under identical housing conditions and received standard chow and water *ad libitum*.

The animals were deprived of food 16 h prior to sacrifice, but had access to water *ad libitum*. Tissues of interest were removed from animals heavily anesthetized with sodium pentobarbital, to minimize the period of anoxia. Small pieces (about 1 mm^3) of submandibular gland, parotid gland and pancreas were excised and rapidly frozen in liquid nitrogen. Thick (16 μm) cryosections were cut on a conventional cryostat at -30°C, mounted on a carbon specimen holder, freeze-dried overnight in the cryostat, and coated with a conductive carbon layer (Wróblewski *et al.*, 1987). The specimens were viewed in the scanning mode (in either a JEOL 1200EX TEMSCAN or a Philips 525 SEM) and analyzed at an accelerating voltage of 20 kV with either a Tracor TN 5500 or a Link AN 10000 energy-dispersive X-ray microanalysis system. Quantitative analysis was carried out by determining the ratio of characteristic counts to the background under the peak (P/B-ratio), and comparing P/B-ratios obtained in the specimens with P/B-ratios obtained from standards, consisting

of known concentrations of mineral salts in a 20% gelatin/5% glycerol matrix, that were frozen and sectioned in the same way as the specimen (Roomans and Sevéus 1977, Roomans 1988).

The statistical significance of concentration differences was determined by one-way analysis of variance (ANOVA) followed by calculation of the minimal critical difference between means.

For light and transmission electron microscopy (TEM), small pieces of tissue were fixed by immersion in phosphate-buffered glutaraldehyde, postfixed with osmium tetroxide, dehydrated and embedded in epoxy resin. Semi-thin sections for light microscopy were stained with toluidine blue, ultrathin sections for TEM were contrasted with uranyl acetate and lead citrate.

Results

Chronic treatment of rats with diuretics for one month resulted in a number of changes in the elemental composition of the mucous acinar cells of the submandibular gland. A significant decrease of the intracellular Na, K, and Cl concentrations was noted with most treatments (Figs 1-3), whereas the intracellular Ca concentration was significantly increased (Fig 4), in particular after combined treatment with furosemide and acetazolamide. The intracellular Mg concentrations followed the Ca concentrations, but the changes were less pronounced (Fig 5). Observations by light and electron microscopy showed an increase in acinar cell size and in the intracellular mucus content of the cells, similar to what was found previously after treatment with furosemide alone (Scarlett *et al.*, 1988). Changes in elemental concentrations in the parotid gland and the pancreas were much smaller than in the submandibular gland and generally not significant (data not shown). Marked changes in the morphology of the pancreas were not observed.

Chronic treatment of mice with diuretics for three months resulted in marked changes in elemental composition and morphology. In the submandibular gland, most treatments (except with amiloride alone) resulted in a significant decrease of the Cl and K content of the acinar cells (Figs 6 and 7) and increase of the Mg concentration (Fig 8). Treatment with furosemide (alone or combined with acetazolamide) as well as with amiloride caused a significant increase in cellular Ca content (Fig 9). The Na content of the cells was not significantly changed by any of the treatments (data not shown). In those treatments that resulted in a significant increase of the intracellular Ca content, morphological studies showed an increase in intracellular mucus content (Figs 10 and 11).

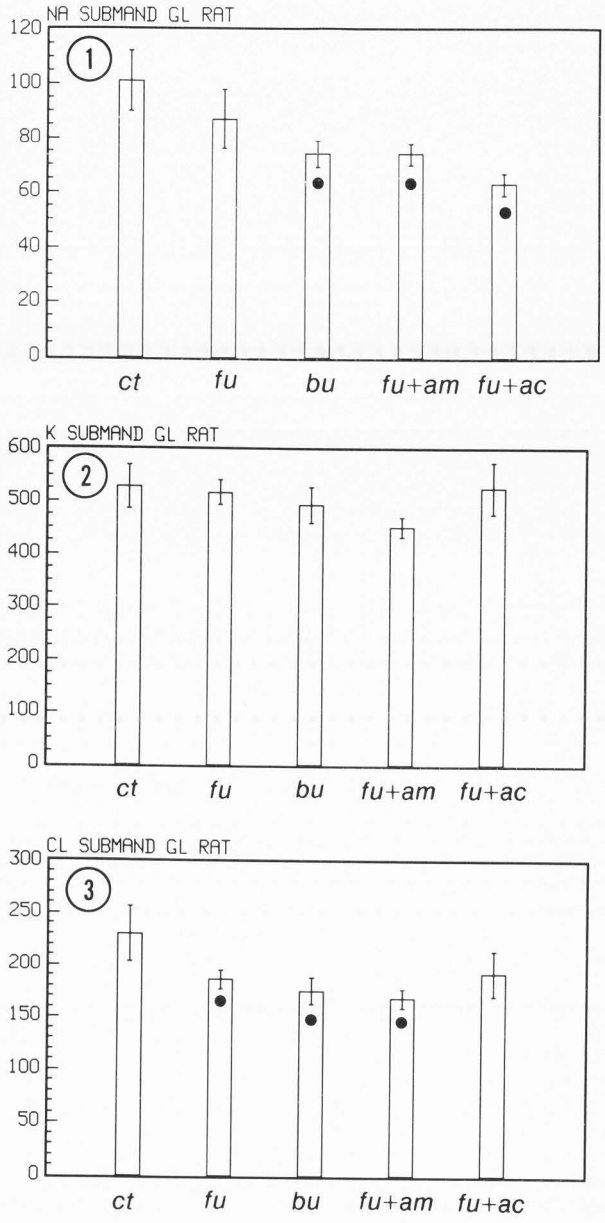
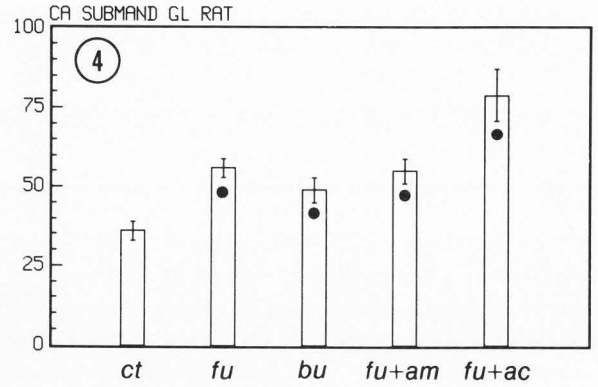


Fig 1: Na concentrations (in mmol/kg dry weight) in rat submandibular gland acinar cells: ct = control, fu = furosemide only (data from Scarlett *et al.*, 1988), bu = bumetanide, fu+am = furosemide + amiloride, fu+ac = furosemide + acetazolamide. The vertical bar denotes the standard error. The dot denotes a significant difference from the control value at $p < 0.05$.

Fig 2: K concentrations (in mmol/kg dry weight) in rat submandibular gland. Other legends as in Fig 1.

Fig 3: Cl concentrations (in mmol/kg dry weight) in rat submandibular gland. Other legends as in Fig 1.



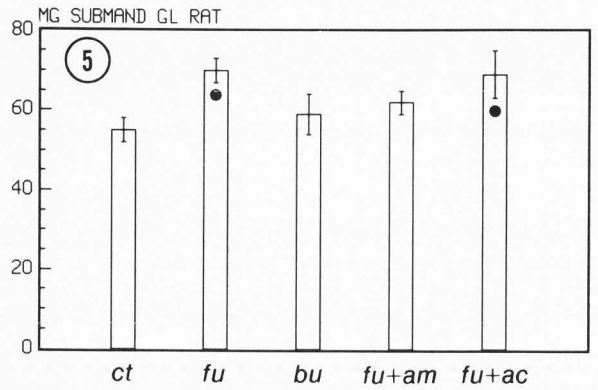


Fig 4: Ca concentrations (in mmol/kg dry weight) in rat submandibular gland. Other legends as in Fig 1.

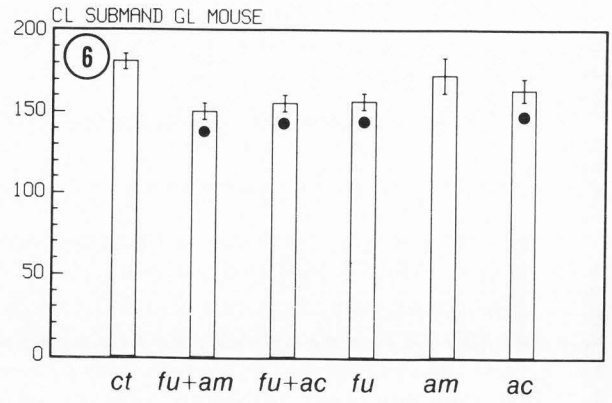
Fig 5: Mg concentrations (in mmol/kg dry weight) in rat submandibular gland. Other legends as in Fig 1.

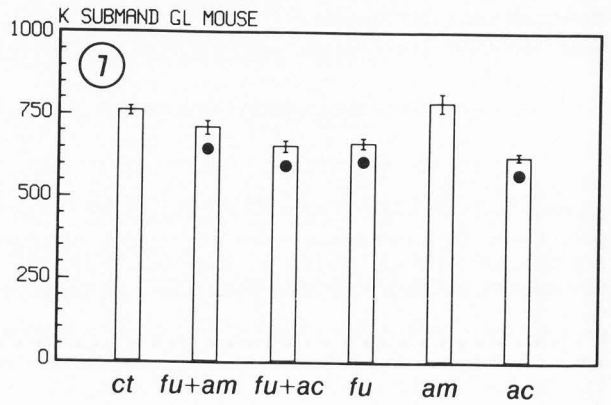
In the pancreas, the pattern of changes is in some respects different from that observed in the salivary glands. Diuretic treatment results in a significant decrease in Cl (Fig 12) and S (Fig 13) content of the cells, whereas Mg, P, and K show a virtually parallel increase (Figs 14-16). No increase in Ca was noted; instead, Ca was decreased or not significantly changed (Fig 17). Morphological studies showed a dramatic reduction of the number of zymogen granules in a large number of pancreatic acinar cells (Figs 18-21).

In particular treatment with diuretics for three months, as in the experiment with mice, causes some weight loss in the animals (Fig 22). Weight loss was less pronounced in rats after a one month treatment.

Discussion

In an earlier study it was shown that chronic treatment with furosemide caused some changes in the elemental composition of salivary gland and pancreas cells,





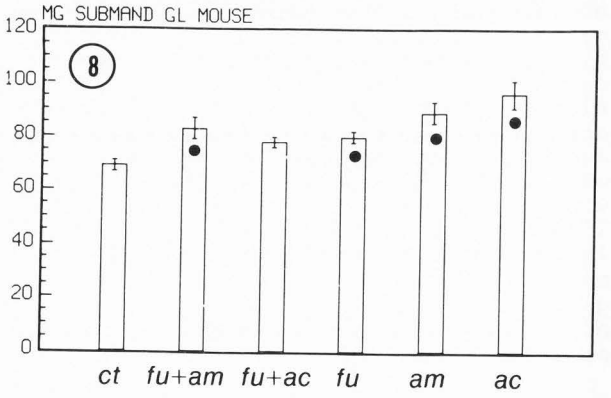


Fig 6: Cl concentrations (in mmol/kg dry weight) in mouse submandibular gland acinar cells. ct = control, fu+am = furosemide + amiloride, fu+ac = furosemide + acetazolamide, fu = furosemide only, am = amiloride only, ac = acetazolamide only. The vertical bar denotes standard error. The dot indicates a significant difference from the control (at $p < 0.05$).

Fig 7: K concentrations (in mmol/kg dry weight) in mouse submandibular gland acinar cells. Other legends as in Fig 6.

Fig 8: Mg concentrations (in mmol/kg dry weight) in mouse submandibular gland acinar cells. Other legends as in Fig 6.

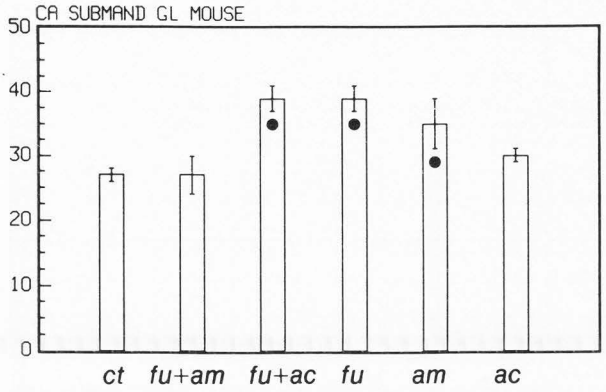
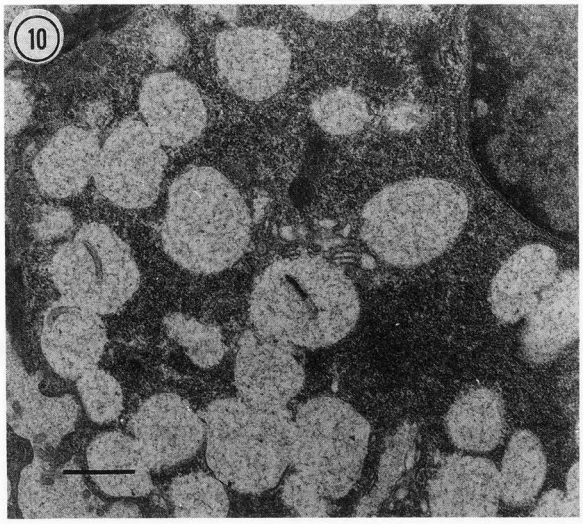


Fig 9: Ca concentrations (in mmol/kg dry weight) in mouse submandibular gland acinar cells. Other legends as in Fig 6.

changes in the flow rate and composition of submandibular saliva, and relatively minor changes in the morphology of salivary gland and pancreas. Although the changes generally parallelled those found in other animal models of CF (in particular, the chronically reserpinized rat) and in CF patients, major pathological changes, such as degeneration of the exocrine pancreas, were not observed. It was considered that this could be due to either the relatively short duration of the experiment or to the fact that furosemide alone would not be sufficiently effective to inhibit transepithelial chloride and water transport. The latter consideration was especially valid for the pancreas, where a large part of the water transport appears to be coupled to bicarbonate, rather than chloride transport (Kuijpers and De Pont, 1987).

In the one-month experiments on rat, furosemide (alone, or in combination with other diuretics) caused a decrease in the Na, Cl and K content of the cells, which is in accordance with inhibition of the basolateral $Na^+/K^+/Cl^-$ cotransporter. Bumetanide, a more specific inhibitor of this cotransporter, has similar effects. The Ca content of the cells is increased. In earlier studies (Müller and Roomans, 1985; Roomans, 1986; Roomans *et al.*, 1989) we have shown that most of the calcium in submandibular gland acinar cells is localized in the mucus granules, and that the Ca content of the cells directly depends on the mucus content of the cells. Chronic treatment with diuretics appears to cause an accumulation of intracellular mucus, possibly by inhibiting the response to low-intensity β-adrenergic stimulation (Scarlett *et al.*, 1988). The changes in Mg content parallel the changes in Ca content, but are less pronounced, because the difference in Mg content between mucus granules and cytoplasm is less than the difference in Ca content (Roomans, 1986). The effect of furosemide on mucus and Ca



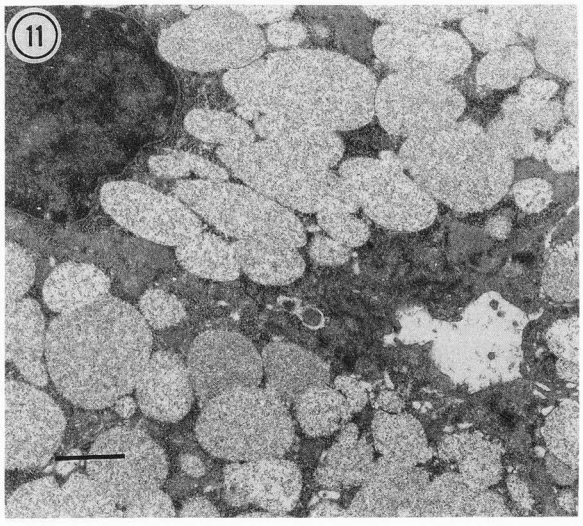
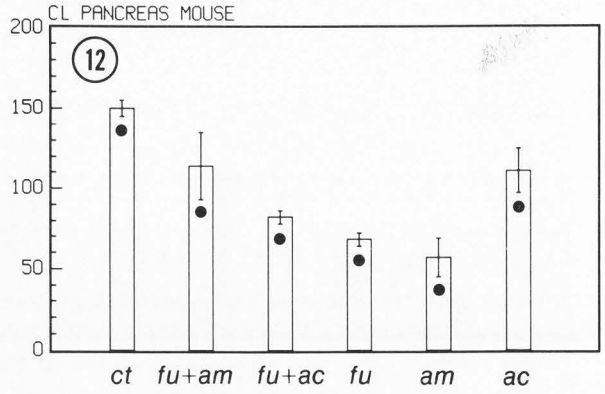


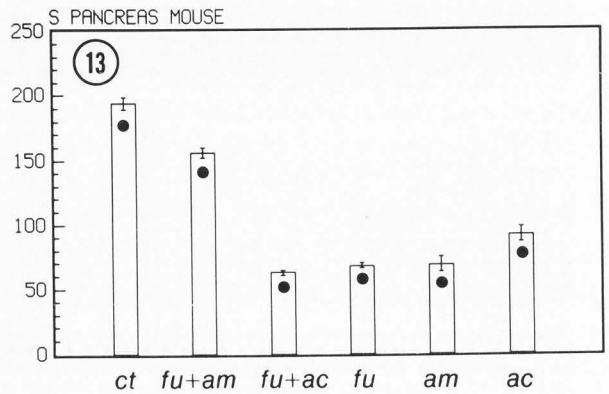
Fig 10: Transmission electron micrograph of a submandibular gland acinar cell of a control rat. Bar = 1 μm.

Fig 11: Transmission electron micrograph of a submandibular gland acinar cell of a rat treated for one month with furosemide and acetazolamide. Note the increase in number of mucus granules. Bar = 1 μm.

content of the acinar cells is potentiated by acetazolamide. A tentative explanation is that acetazolamide causes changes in cell pH by decreasing the intracellular bicarbonate concentration. Previous studies have shown that changes in cell pH cause accumulation of calcium-rich mucus (Roomans and Bardon, 1984; Roomans, 1986). Apparently the effect of acetazolamide in this case is separate from and additive to the effect of inhibition of the $Na^+/K^+/Cl^-$ cotransporter.

However, even combined treatment with furosemide and acetazolamide for one





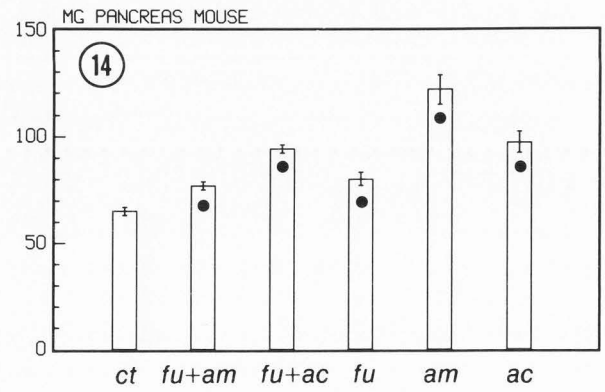
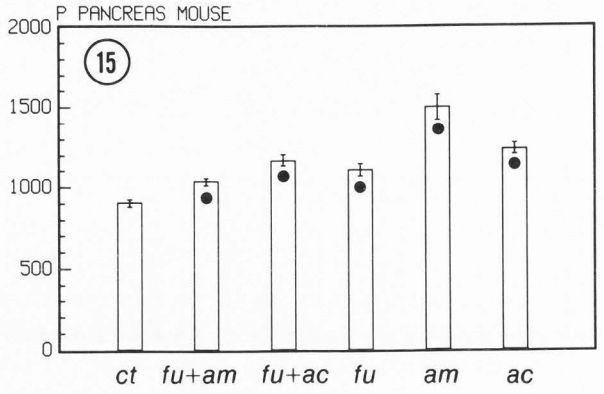
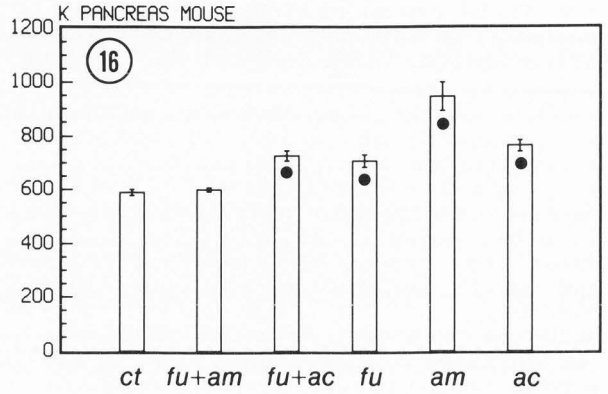


Fig 12: Cl concentrations (in mmol/kg dry weight) in mouse pancreatic acinar cells. Other legends as in Fig 6.

Fig 13: S concentrations (in mmol/kg dry weight) in mouse pancreatic acinar cells. Other legends as in Fig 6.

Fig 14: Mg concentrations (in mmol/kg dry weight) in mouse pancreatic acinar cells. Other legends as in Fig 6.





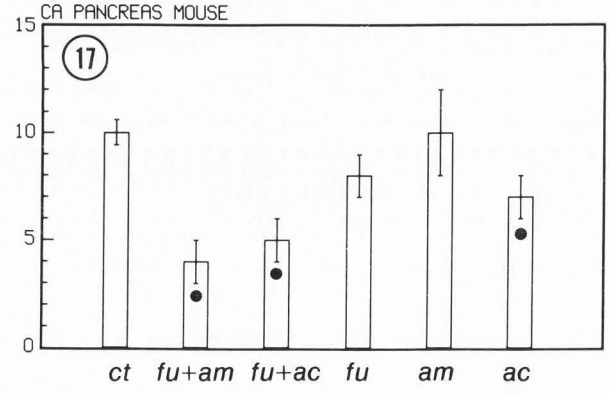


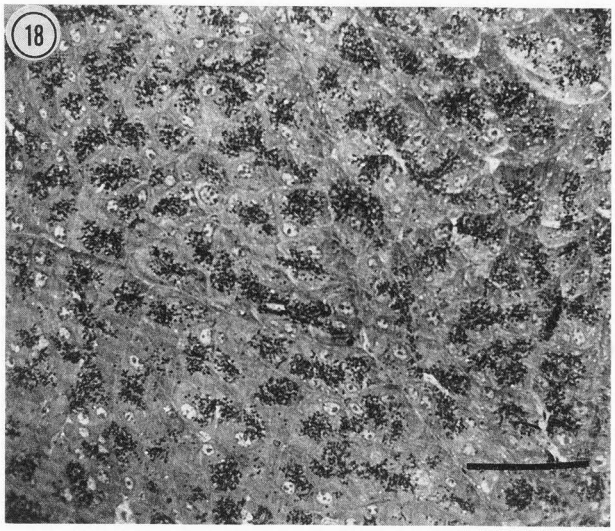
Fig 15: P concentrations (in mmol/kg dry weight) in mouse pancreatic acinar cells. Other legends as in Fig 6.

Fig 16: K concentrations (in mmol/kg dry weight) in mouse pancreatic acinar cells. Other legends as in Fig 6.

Fig 17: Ca concentrations (in mmol/kg dry weight) in mouse pancreatic acinar cells. Other legends as in Fig 6.

month, which would be expected to virtually completely inhibit fluid transport in the pancreas (Kuijpers *et al.*, 1984) did not cause significant changes in morphology and elemental content.

On the other hand, treatment of mice for three months with diuretics caused dramatic changes in the pancreatic acinar cells. A significant decrease in Cl levels is observed. Other changes in elemental



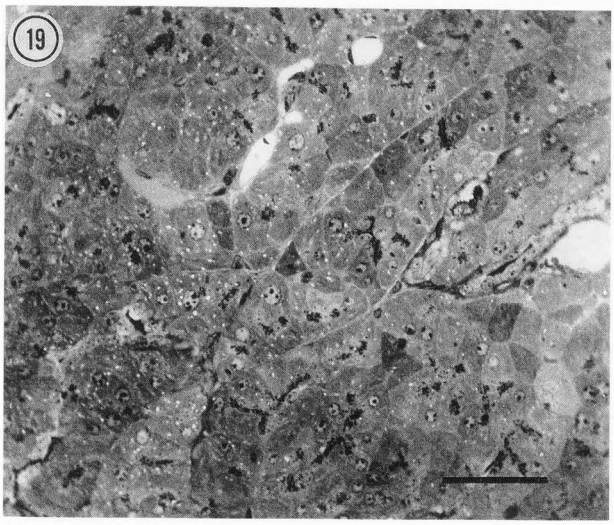
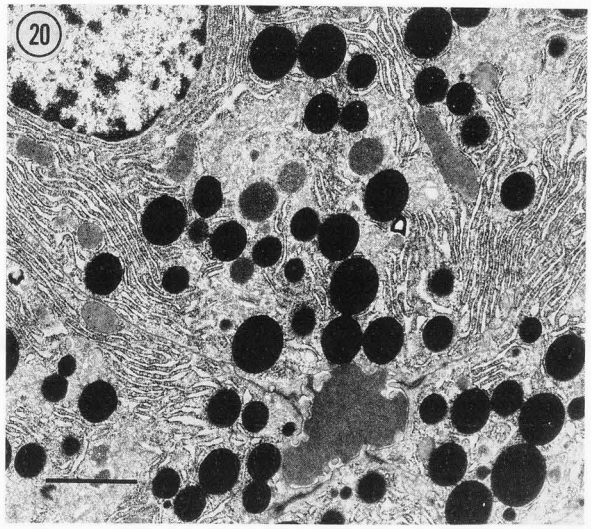


Fig 18: Light micrograph of the pancreas of a control mouse. The bar denotes 50 μm.

Fig 19: Light micrograph of the pancreas of a mouse treated with furosemide for 3 months. Note the absence of zymogen granules in many cells. The bar denotes 50 μm.

composition are correlated with the morphological changes. The zymogen granules of the pancreatic acinar cells are rich in sulfur and relatively poor in phosphorus (Roomans and Wei, 1985; Roos, 1988) and the decrease in sulfur concentration is paralleled by a decrease in the number of zymogen granules. Changes in Mg and K appear to parallel the changes in P. As a consequence of the disappearance of the zymogen granules the relative volume of nucleus and endoplasmic reticulum-containing cytoplasm are increased. Since these structures are rich in nucleic acids (that contain phosphorus and preferentially bind K^+ and Mg^{2+} ions) the cellular content of



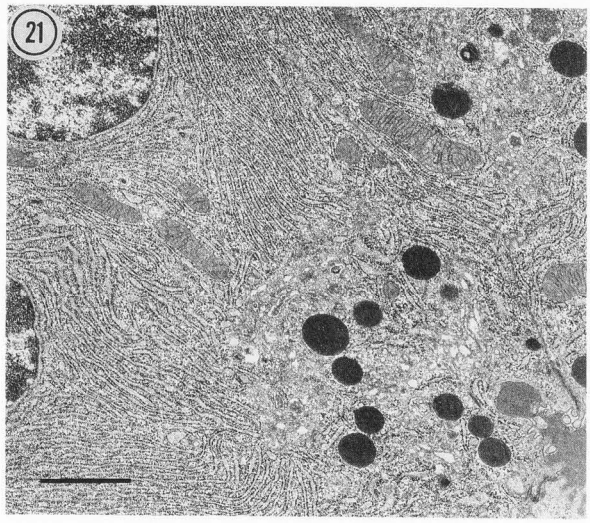


Fig 20: Transmission electron micrograph of pancreatic acinar cells of a control mouse. The bar denotes 1 μm.

Fig 21: Transmission electron micrograph of the pancreas of a mouse treated with furosemide for 3 months. Note the reduced number of zymogen granules. The bar denotes 1 μm.

these elements increases. Since the zymogen granules contain higher concentrations of calcium than do other parts of the pancreatic acinar cell (Roomans and Wei, 1985; Roos, 1988) their disappearance also causes a decrease of the cellular calcium content. The effects of diuretics on the acinar cells need not be direct. Indeed, *in vitro* experiments on the effect of diuretics on pancreatic acinar cells (McMillan *et al.*, unpublished results) showed that these substances do not directly affect the ionic composition of the

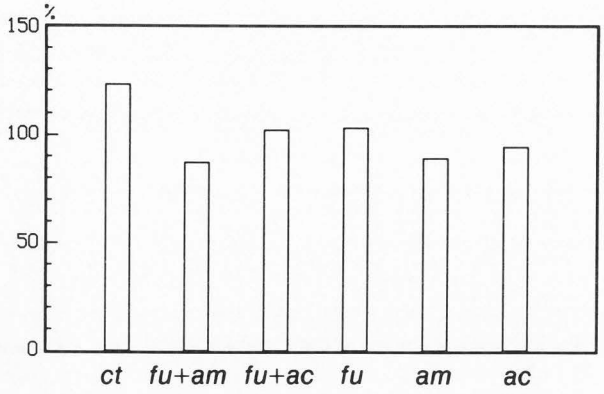


Fig 22: Relative weight changes of the mice during a three month treatment with diuretics (data show weight in % of initial weight). ct = control, fu+am = furosemide + amiloride, fu+ac = furosemide + acetazolamide, fu = fursoemide only, am = amiloride only, ac = acetazolamide only.

cells. Fluid transport in the pancreas is thought to be mainly located in the duct cells. Obstruction of the duct by released secretory material that cannot be transported to the intestinal lumen because of lack of solvent water could result in secondary effects on the acinar cells.

The main changes observed after three months in mouse submandibular gland, after chronic treatment with furosemide alone or in combination with other diuretics, are similar to those found after one month in rats: a decrease in the cellular chloride content and an increase in calcium. As in the rat, the increase in cellular calcium is paralleled by an increase in cellular mucus content, and the explanation for this finding is likely to be the same as discussed above for the rat. We have previously shown (Scarlett *et al.*, 1988) that the calcium and protein content of stimulated saliva is increased already after one month treatment with furosemide. This could be explained by assuming that the inhibition of water transport is more severe than the inhibition of mucus secretion, so that the relative amount of calcium-rich mucus in the saliva is increased. In addition, the cells have accumulated more mucus than normal and strong stimuli appear to be able to overcome the inhibition of mucus secretion found in this and other animal models of CF (Müller and Roomans, 1985).

In the three-month treatment, combination of furosemide with other diuretics does not appear to enhance the effect of furosemide alone. Hence, there appears to be no advantage in animal models involving more than one diuretic. Rather, given the possibility of side effects and indirect systemic effects, as is also evident from the weight loss in, e.g., the combined treatment with furosemide and amiloride, an animal model based on treatment with furosemide alone would be preferable.

The changes in salivary gland structure and function found in the previous (Scarlett *et al.*, 1988) and the present study parallel those found in CF patients. However, the changes in the salivary glands are not a clinical problem in CF. Together with chronic obstructive lung disease, pancreatic insufficiency is the main clinical sign of CF. In most studies of the reserpinized rat, which at present is considered the most reliable animal model for the study of CF, there are relatively minor changes in the structure of the pancreas (Setser *et al.*, 1979; Roomans *et al.*, 1982; Spicer *et al.*, 1985) and no more than a 30% functional impairment of the exocrine pancreas (Hazlett *et al.*, 1986); only one study shows more extensive changes (Grondin *et al., 1989*). The chronically furosemide-treated mouse, on the other hand, shows dramatic changes in the structure of the pancreas that are indicative of severe dysfunctioning. In this respect, this animal model for CF appears to be the best so far developed. Further studies with chronically furosemide-treated animals, also involving other relevant organ systems, are therefore indicated.

Acknowledgements

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Discussion with Reviewers

M.B. Engel: The elemental analyses are expressed in terms of dry weight. Changes in intracellular water might be anticipated in cystic fibrosis and in these experimental studies. Would the results be significantly different if the values were expressed in relation to cell water ?
Authors: You are correct in pointing out that changes in dry weight concentrations need not be the same as changes in concentrations related to wet weight or cell water volume. The intracellular water content cannot be determined by analysis of freeze-dried tissue, and it is certainly conceivable that treatment with diuretics causes changes in water content. We therefore plan to extend this work to analysis of frozen-hydrated specimens so that we can measure wet weight concentrations.

M.B. Engel: The diuretic-treated animals show a consistent decrease in intracellular Cl concentrations especially in acinar cells of the mouse pancreas. Could this effect reflect a change in cellular macromolecules including an increase in their negative charge density ?
Authors: In the pancreas the intracellular P concentration is increased after most treatments, indicating an increase in phosphate groups. However, this increase in negative charge appears to be paralleled and neutralized by changes in K and Mg. X-ray microanalysis does not give information on other negative groups such as carboxyl groups. Hence, your question cannot be answered with certainty.

M.B. Engel: Are there significant changes in electrolyte concentrations in blood ?
Authors: Serum electrolyte concentrations were not measured in the present study.

K. Izutsu: How do the doses of diuretics used in these studies compare with the clinical doses used in the chronic treatment of patients? Is there any evidence for iatrogenic exocrine gland changes in these patients?
Authors: The furosemide dose used in the experiments is the equivalent of about 13 g per day for an adult, which is about 300 times the normal clinical daily dose for long-term treatment. For amiloride the dose used is about 250 times the normal clinical dose for long-term treatment, and for acetazolamide 15-30 times. No specific side effects of these drugs on exocrine glands are given in the pharmaceutical literature, but it appears unlikely that long-term treatment with such excessive doses would ever take place.

G.A.J. Kuijpers: Do you have an explanation for the fact that treatment of the mouse with furosemide or amiloride alone largely decreases the Cl and S concentration in the pancreas (Figs 12 and 13), while treatment with the two drugs simultaneously affects the ion concentrations to a much lesser degree? The same question applies to the data presented on Ca in the submandibular gland (Fig 9).

Could you conclude from the data presented in Figs 14-16 that treatment with furosemide in combination with amiloride prevents the effects of amiloride (alone) on Mg, P and K concentration?
Authors: Combined treatment with furosemide and amiloride appeared to severely affect the animals. This was the only group in which the death of an animal during the experiment occurred. We would speculate that secondary changes could occur in tissues in these animals, e.g., increase of Cl and decrease of K and Mg due to suboptimal energy metabolism. We have not investigated the problem further because at this stage we felt that it did not contribute to the goal of our research.

G.A.J. Kuijpers: The reduction in the number of pancreatic zymogen granules in furosemide-treated animals (Fig. 19) may well be correlated to the decrease of the S concentration in the pancreas (Fig 13). The data on Ca, however, show that Ca is not significantly decreased upon furosemide treatment. Nevertheless, in the Discussion you suggest that the decrease in granule number causes the decrease of the cellular Ca content. Can you comment on this?
Authors: Although even in the case of furosemide there is a small decrease of the Ca concentration (Fig 17) it is true that there is no straight correlation between changes in Ca and changes in S. It is likely that in the zymogen granules the Ca is bound both to the sulfated polyanion that is responsible for the condensation of the granules and to the secretory proteins. Changes in the calcium binding properties of the secretory proteins may occur in addition to other changes and complicate the interpretation of the resulting change in cellular Ca concentrations.