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Biochimica et Biophysica Acta 1566 (2002) 162–168

BIOCHIMICA ET BIOPHYSICA ACTA
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Where have all the Na^+ channels gone? In search of functional ENaC in exocrine pancreas

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Received 30 May 2002; received in revised form 10 July 2002; accepted 3 September 2002

Abstract

Many epithelia express specific Na^+ channels (ENaC) together with the cystic fibrosis regulator (CFTR) Cl^- channels. Pancreatic ducts secrete HCO_3^--rich fluid and express CFTR. However, the question whether they possess ENaC has not been consistently addressed. The aim of the present study was to investigate if pancreatic ducts express functional ENaC. Membrane voltages (V) of ducts isolated from rat pancreas were measured with microelectrodes or whole-cell patch-clamp technique. Amiloride and benzamil given from bath or luminal sides did not hyperpolarize V. Lowering of extracellular Na^+ concentrations had effects that were not consistent with a simple Na^+ conductance, but rather with a Na^+/Ca^{2+} exchange. Acute or long-lasting treatment of pancreatic ducts with mineralocorticoids had no effect on V of unstimulated or secretin-stimulated preparations. Furthermore, pre-treatment of animals with glucocorticoids had no effect on pancreatic fluid secretion evoked from ducts, or from acini. Hence, our study shows that pancreas especially pancreatic ducts do not express functional ENaC.

Keywords: ENaC; Na^+ channel; Cystic fibrosis regulator; Pancreas; Pancreatic duct; Steroid hormone

1. Introduction

The first step in the transepithelial Na^+ transport is the passive electrodiffusion through the apical membrane and subsequent Na^+ extrusion by the basolateral pump. This concept, developed by Ussing et al. in the seminal studies of the frog skin laid grounds and impetus to the field of experimental epithelial physiology (see E.H. Larsen, this volume). Absorption of Na^+ against a large concentration gradient is a task of several epithelia, such as those lining the distal nephron, colon, ducts of salivary and sweat glands and toad bladder. These tight epithelia, responsive to mineralocorticoids, are involved in salt and water balance [1,2]. In other epithelia, for example those lining upper respiratory pathways and lung, Na^+ transport is not involved in overall salt balance, but in maintaining hydration of the mucosa and keeping the alveoli dry. The Na^+ channel responsible for mediation of electrodiffusion across the apical membranes of these epithelia is highly selective for Na^+ and sensitive to amiloride. This epithelial Na^+ channel (ENaC) has been molecularly characterized as a hetero-oligomeric protein consisting of subunits α-ENaC, β-ENaC and γ-ENaC, probably in a $\alpha_2\beta\gamma$ constellation [1,3,4].

In several epithelia (intestine, kidney, respiratory epithelia), ENaC is expressed along with the cystic fibrosis transmembrane regulator (CFTR) Cl^- channel, and in many cases, there seems to be a reciprocal control of these channels, such that an epithelium can switch between secretion and absorption. For example, when the CFTR-Cl^- channel is stimulated, ENaC is inhibited and thus Cl^- and fluid secretion prevails. In cystic fibrosis (CF), where CFTR is defective, Na^+ absorption dominates in respiratory epithelia, resulting in dehydration of the mucous surface [5,6]. However, the research field of CFTR–ENaC interactions is quite controversial, and in some co-expression studies, there are indications that ENaC can stimulate CFTR activity [7,8]. There are many studies addressing the question of these complex interactions between CFTR and ENaC. The proposals include protein–protein interactions, involvement of PDZ domain proteins, cytoskeletal proteins, control of exocytosis, G-proteins and other regulatory proteins, such as Nedd4 that are sensitive to intracellular Na^+ (and Cl^-) [9–12]. Notably in absorptive epithelia, such as sweat and salivary ducts that also express ENaC and CFTR,

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PII: S0005-2736(02)00598-9

Na^+ and Cl^- transport is electrically coupled and regulated in parallel [12–15]. This speaks for more flexible, perhaps tissue-specific control pathways for regulation of Na^+ and Cl^- permeabilities.

Pancreas is one of the main organs affected by CF, and the primary defect is in HCO_3^- secretion that is dependent on CFTR. Bicarbonate secretion originates in pancreatic ducts and the apical membrane of intralobular ducts is lined with CFTR that can function as a Cl^- channel [16–19]. Although the native ductal epithelium is not a tight epithelium [20], and thus would not fit with most steroid-regulated epithelia, the question of Na^+ channels is not settled. In our earlier electrophysiological work on rat pancreatic ducts, where we studied CFTR-Cl^- channels and K^+ channels, analysis of fractional conductances indicated that there was a small cation conductance, but it was not immediately recognizable as due to Na^+ channels [16,20]. Nevertheless, it was shown that pancreas, along with brain, testis, and ovary, contains transcripts for a δ-ENaC subunit [21]. In expression systems, δ-ENaC together with β and γ subunits can form Na^+ channels that have a slightly lower selectivity for Na^+ over Li^+, and 30× lower sensitivity to amiloride compared to the αβγ-ENaC [21]. Whether δβγ-ENaC occurs in vivo is not clear. Moreover, transcripts for human ENaC were detected in human pancreas [22], and recent immunohistochemical studies on rat pancreas show that there is expression of α-, β- and γ-ENaC subunits in the apical membrane of pancreatic acini and intercalated ducts (Jeppe Praetorius, personal communication).

Therefore, the aim of the present study was to investigate whether there are functional Na^+ channels in rat pancreatic ducts. For this purpose, we perfused intra- and interlobular pancreatic ducts and measured cell voltages with microelectrodes, and in smaller intercalated and intralobular ducts, cell voltages were measured with a whole-cell patch-clamp technique. In addition, we investigated effects of mineralocorticoids and glucocorticoids on membrane voltages and also monitored secretion from intact pancreas in in vivo preparations.

2. Materials and methods

2.1. In vitro preparations

Pancreatic tissue was obtained from female Wistar rats (100–300 g), which were kept on a standard laboratory diet. In some experiments, small intra- and interlobular ducts were isolated by microdissection and perfused in vitro, as described previously [23]. Dissected ducts (o.d. 20–60 μm) were held between two holding pipettes and placed in a perfusion chamber mounted on a stage of an inverted microscope (Axiovert 10, Zeiss, Germany). The ducts were bathed in a control bicarbonate containing solution of the following composition (in mmol/l): Na^+ 145, K^+ 3.6, Ca^{2+} 1.5, Mg^{2+} 1, Cl^- 125, HCO_3^- 25, phosphate 2 and glucose 5. The pH was equilibrated to 7.4 with 5% CO_2 in O_2. The lumen of the ducts was perfused through the double-barrelled pipette with a HCO_3^--free medium where HCO_3^- was replaced with gluconate. Individual cells were impaled with KCl-filled electrodes (100–200 MΩ) from the basolateral side of perfused ducts and the voltage across the basolateral membrane (V_{bl}) was measured with respect to the grounded bath as described previously [23].

For patch-clamp experiments, pancreatic ducts were obtained from collagenase digests of rat pancreas as described previously [24]. Briefly, pancreas cut into small pieces was incubated with collagenase V (1.3 mg/ml) in DMEM 1000/nutrient mixture F12 (HAM) medium mix containing trypsin inhibitor (0.17 mg/ml). Single intercalated and small intralobular duct fragments (o.d. 10–40 μm) were identified with the aid of a dissection microscope and later in an inverted microscope (Axiovert 100TV, Zeiss). Suction pipettes held ducts placed in a perfusion chamber such that both luminal and the basolateral sides of the epithelium were accessible to the bathing solutions. The cell responses were monitored in whole-cell nystatin patch-clamp recordings adopted for pancreatic ducts [24]. Patch pipettes had resistances of about 5 MΩ and the initial seal was at least 1 GΩ. The cell membrane under the pipette was permeabilized to make direct electrical access to duct cells. Nystatin (0.02–0.1 mmol/l) was dissolved in a pipette solution adjusted to pH 7.2 that had the following composition (mmol/l): Na^+ 11, K^+ 125, Cl^- 32, gluconate 96, Mg^{2+} 1, phosphate 6, glucose 5, adenosine 5′-triphospate (ATP) 1, and Ca^{2+} was adjusted to 0.1 μM with EGTA. A flowing 1-M KCl electrode was used as a reference. The membrane voltage (V_m) was continuously monitored during experiments in a current-clamp mode (zero current-clamp). Periodically, the whole-cell current (I) was measured in a voltage-clamp mode of the patch-clamp amplifier (EPC 9, Heka, Germany).

The chambers were continuously perfused at a rate of 10–15 ml/min with control solutions as given above. The temperature was kept constant at 37°C during all experiments. In some experiments, Cl^- concentrations were decreased from 125 to 32 or 5 mM and replaced with gluconate. In other experiments, Na^+ was decreased to 0 or 5 mM Na^+ and replaced with *N*-methyl-D-glucamine ($NMDG^+$) titrated with HCl, or in a few experiments with Li^+. Ca^{2+}-free solutions were made by adding 1 or 5 mM EGTA to solutions. The following transport inhibitors and agonists were used: amiloride (RBI, Research Chemicals), benzamil (Molecular Probes) and aldosterone, secretin, cholecystokinin octapeptide (CCK8) and ATP (Sigma).

2.2. In vivo preparations

For several experiments where pancreatic secretion was monitored, in vivo preparation of pancreas was

performed. The necessary permission was obtained from the Danish Animal Ethical Committee. Female Wistar rats weighing 100–300 g were used. In one series of experiments, animals were injected intramuscularly for 4–9 days with 50 mg/kg body weight of deoxycorticosterone pivolate (DOC) (Sigma). In another series of experiments, animals were injected intraperitoneally for 2 days with 10 mg/kg body weight of dexamethasone (Dexa) (Sigma). Control and treated animals were fasted 16–24 h before experiments. Rats were anesthetized with Mebumal (pentobarbital, 40 mg/kg i.p.; Nycomed-DAK) and the facial vein was cannulated for infusions. Anesthesia was maintained during the experiments by additional, intravenous injections of Mebumal. A thermostatically controlled heating table was used to maintain the animal's body temperature at 38 °C. The animals were tracheostomized and the abdomen was opened by a midline incision. The pylorus and the proximal end of the bile duct were ligated. A polyethylene tube was inserted into the common pancreatic bile duct. Collection of pancreatic juice was started with a control period of 30–60 min during which a mix of DMEM 1000/F12 (HAM) medium (Gibco) was infused. Secretion was then stimulated by infusion of secretin (10 pmol/min/animal) and subsequently, after a period without an agonist, by infusion of CCK8 (3 pmol/min/animal). The infusion rate (0.03 ml/min animal) was held constant with a syringe pump (Cole-Palmer). Pancreatic juice was collected in preweighed vials and the secretion rate was corrected for pancreas weight determined at the end of the experiments. Pancreatic juice was also analyzed for Na^+ concentrations by flame photometry.

The data are shown as original recordings, summaries and mean values ± S.E.M. To determine the statistical significance, we used Student's *t*-test, where *n* refers to the number of experiments from different ducts/animals and $P < 0.05$ was accepted as significant.

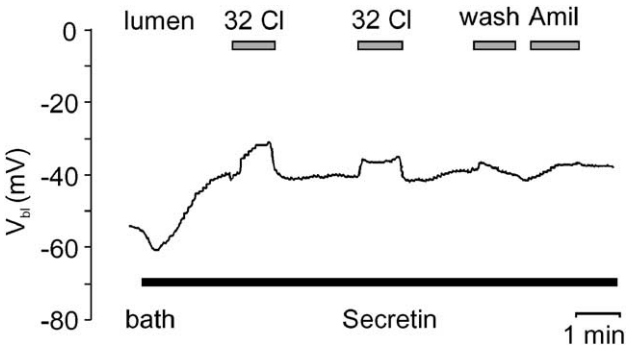

Fig. 1. Luminal amiloride has no effect on the basolateral membrane voltage, V_{bl}. The figure shows an original recording of V_{bl} from a perfused pancreatic duct that was stimulated with secretin (8×10^{-11} M). Following the stimulation, luminal Cl^- concentration was decreased from 125 to 32 mM, and then after a wash of the pipette barrel containing the test solutions, the duct was infused with 0.1 mM amiloride. Small deflections in V_{bl} are due to the current injection from the luminal pipette that can be used to monitor fractional resistance of the basolateral membrane.

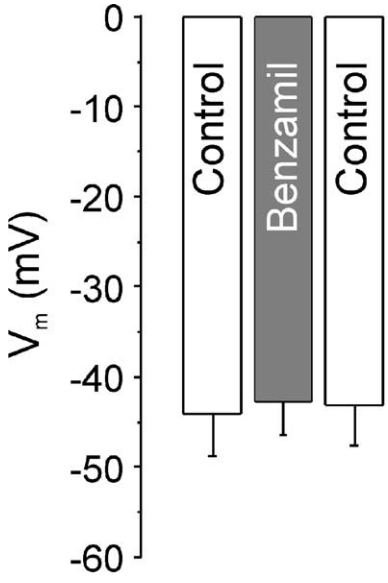

Fig. 2. Benzamil has no effect on the membrane voltage, V_m. Summary of data obtained on eight ducts where V_m was measured in whole-cell patch-clamp recordings. The first and second controls indicate V_m just immediately before and after benzamil additions (0.01 mM) in the same ducts.

3. Results

In the first series of experiments, we tested the effect of amiloride on V_{bl} of perfused ducts. In unstimulated ducts,

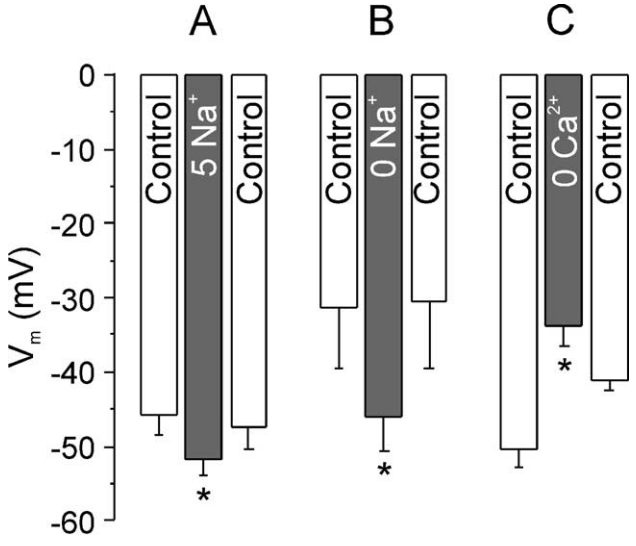

Fig. 3. Lowering of extracellular Na^+ hyperpolarizes, and lowering of extracellular Ca^{2+} depolarizes V_m. The extracellular Na^+ concentration was lowered from 145 to 5 mM or nominal 0 mM and the substituting cation was $NMDG^+$. The Ca^{2+}-free solutions were made with EGTA. There are 13, 4 and 7 experiments in the series shown, respectively, and the asterisks indicate that $P < 0.05$.

where V_{bl} was about −60 mV, amiloride applied from the basolateral side had very small effects on V_{bl}–depolarizing it by 2.1 ± 0.6 mV (amiloride 0.1 mM) and by 3.2 ± 0.4 mV (amiloride 1 mM), respectively ($n = 15$, 21). This is in agreement with our earlier study, where we proposed that this effect of amiloride is probably due to inhibition of a Na^+/H^+ exchanger (NHE) and intracellular pH effect on K^+ channels [23,25]. In several experiments, amiloride was tested from the luminal side and Fig. 1 shows an original recording of V_{bl} from such an experiment where the duct was stimulated with secretin. Initially, there was a short-lasting hyperpolarization, due to opening of basolateral K^+ channels, which was quickly taken over by depolarization of V_{bl} due to opening of CFTR-Cl^- channels, as described earlier [16,25]. The Cl^- conductance was demonstrated by a concentration step in the luminal Cl^- from 125 to 32 mM, which further depolarized V_{bl} in this leaky epithelium. Most importantly, after the low-Cl^- solution was washed out of the lumen, infusion of amiloride (0.1 mM) had no detectable effect on V_{bl}.

In another set of experiments, where V_m was measured in a whole-cell nystatin technique, we tested the effect of another Na^+ channel blocker–benzamil. In these experiments, duct fragments, usually from smaller ducts than those used for perfusion, were used and the inhibitor had access to both the luminal and basolateral sides of the epithelium (see Materials and Methods). Fig. 2 shows the summary of the data obtained. Benzamil (0.01 mM) had no significant effect on V_m in eight ducts, including three ducts that were stimulated with secretin. Thus, benzamil, like amiloride, could not hyperpolarize the membrane voltage of duct cells.

Fig. 3 shows the results of experiments where we investigated the effect of low-Na^+ solutions on V_m, again

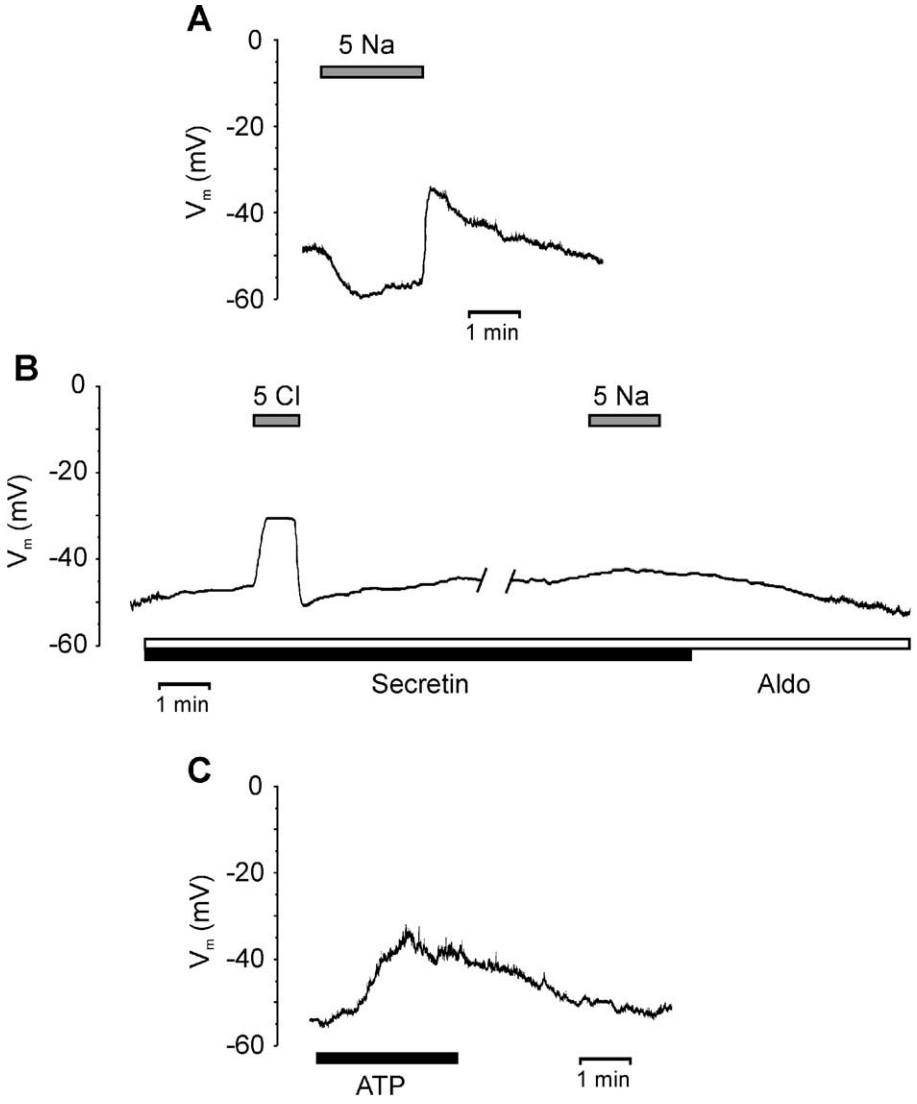

Fig. 4. Low Na^+ concentrations effect on V_m is inhibited in a duct stimulated with secretin. An original recording of V_m in one pancreatic duct: (A) first unstimulated and exposed to extracellualr solution with 5 mM Na^+; (B) then stimulated with secretin (10^{-9} M) and exposed to aldosterone (10^{-10} M) and exposed to low Cl^- (5 mM Cl^-) or low Na^+ (5 mM); and lastly, (C) stimulated with ATP (0.1 mM).

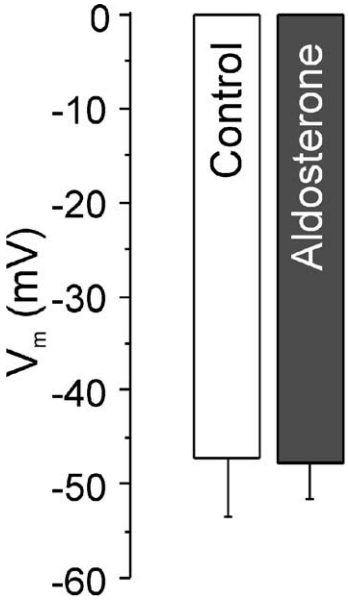

Fig. 5. Aldosterone has no effect on V_m. Summary of experiments where pancreatic ducts were exposed to aldosterone (10^{-10} M) for about 30 min ($n=3$).

measured in whole-cell nystatin recordings. A decrease in Na^+ concentrations from 145 to 5 mM hyperpolarized V_m from -46 ± 3 to -52 ± 2 mV (Fig. 3A). With 0 mM Na^+ solutions, the hyperpolarization was from -31 ± 8 to -46 ± 5 mV (Fig. 3B). This effect could have indicated presence of Na^+ channels. However, when Li^+ was used as a substituting cation instead of $NMDG^+$, responses were similar ($n=3$), indicating that Li^+ was rather impermeable–unlikely for a Na^+ channel [2]. Moreover, the time course of the V_m responses was somewhat unusual for a Na^+ channel response. Often the V_m hyperpolarization was transient, as already shown earlier [24], and there was a transient overshoot in V_m when the duct was returned to the control Na^+ solution (Fig. 4A). In an earlier study, we showed that such V_m transients and corresponding intracellular Ca^{2+} changes are compatible with an electrogenic $3Na^+/Ca^{2+}$ exchanger [24]. That is, when the extracellular Na^+ is lowered, the exchanger works in the Na^+ efflux mode, thus hyperpolarizing V_m. Present experiments in Fig. 3C show that a removal of extracellular Ca^{2+} led to depolarization of V_m from -50 ± 2 to -34 ± 3 mV, which could arise from influx of $3Na^+$ in exchange for Ca^{2+} exiting the cell down its chemical gradient. These Na^+ (and Ca^{2+}) substitution experiments are indicative of Na^+/Ca^{2+} exchange rather than a Na^+ channel.

Nevertheless, it was important to investigate whether Na^+ channels can be opened during secretin stimulation. Fig. 4 shows an original recording of V_m of a duct that was exposed to low Na^+, first in an unstimulated state (Fig. 4A) and then after being stimulated with secretin (Fig. 4B). Secretin stimulation opened CFTR-Cl^- channels, again as demonstrated by the depolarizing V_m response to low Cl^- in the extracellular medium. Notably, the low-Na^+ solution now had no effect on V_m. Also benzamil had no effect in this experiment (not shown). Later in the experiment (Fig. 4C), the duct was stimulated with ATP, which resulted in a depolarization of V_m. This is due to closing of K^+ channels and influx of Na^+ and Ca^{2+} through different P2 purinergic receptors [26]. Note that during secretin stimulation, V_m became steadier, as it was dominated by the activity of many channels (CFTR-Cl^- channels) and as shown earlier, the fractional conductance of the luminal membrane increased 20-fold [20]. In contrast, during the ATP stimulation, the recording became more "noisy", as the overall membrane conductance decreased due to P2Y2/P2Y4 purinergic-mediated closure of K^+ channels [26]. Notably, this duct was also exposed to aldosterone (10^{-10} M) together with secretin.

In some epithelia, aldosterone is able to induce acute non-genomic effects on Na^+ and other channels and NHE (see below). Yet, in pancreatic ducts, aldosterone even after about 30 min had no significant effect on V_m as summarized in Fig. 5. The classical effects of mineralocorticoids are genomic with early phase effects (0.5–1 h), and late phase effects apparent around 3 h. Hence, in one series of experiments, we compared V_{bl} of perfused pancreatic ducts from animals pre-treated with DOC to control animals. Fig. 6 shows that the resting V_{bl} was very similar on both series. In addition, the data show that secretin had a similar depolarizing effect on V_{bl} of both types of ducts. Thus, in ducts from control rats, secretin depolarized V_{bl} by 34 ± 1 mV ($n=41$), and in ducts from DOC rats, the resting V_{bl} was very similar and secretin depolarized V_{bl} by 37 ± 3 mV ($n=8$). These experiments show that DOC pre-treatment did not lead to synthesis and insertion of ENaC that could be detected in resting or stimulated ducts.

Since glucocorticoids can increase expression of ENaC in some epithelia, for example, lung [27,28], and glucocorticoids also have effects on pancreatic enzymes [29,30], we

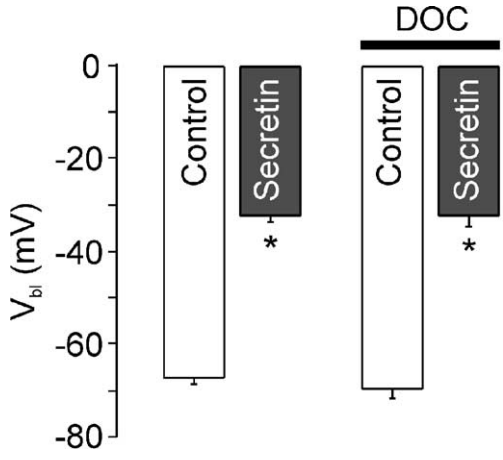

Fig. 6. DOC has no effect on V_{bl} of unstimulated or stimulated pancreatic ducts. V_{bl} was measured in perfused ducts dissected from untreated ($n=41$) and treated ($n=8$) animals. The resting V_{bl} and that of ducts stimulated with secretin (10^{-10}–10^{-9} M) were similar in the two series of experiments. The asterisks indicate that $P<0.05$ between unstimulated and the corresponding secretin-stimulated ducts.

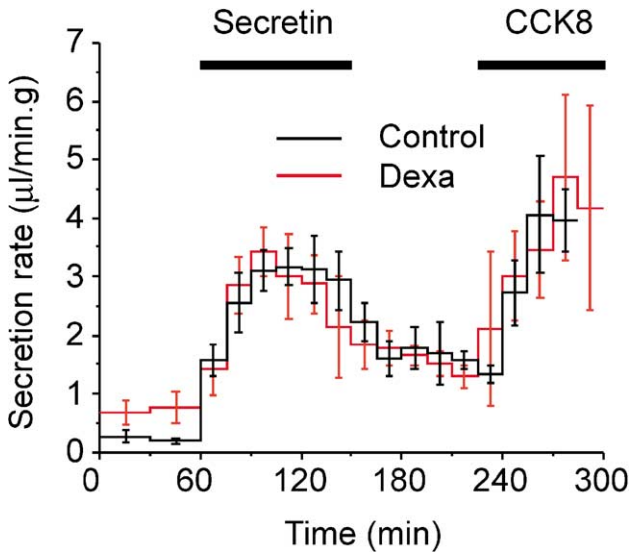

Fig. 7. Dexa has no effect on pancreatic secretion. Pancreatic secretion was evoked by secretin (10 pmol/min/animal) and later with CCK8 (3 pmol/min/animal) in pancreas from control and Dexa pre-treated rats ($n=3$ in each series).

tested the effect of Dexa treatment on pancreatic secretion. The total function of the pancreas was estimated by measuring the secretory capacity of the intact pancreas in anaesthetized animals as shown in Fig. 7. Secretion was elicited by secretin, the main agonist of pancreatic ducts, and subsequently by CCK8, the main agonist of pancreatic acini. The data show that the secretory response was very similar in both series of animals. In addition, Na^+ concentrations in the pancreatic juice were about 155 mM and did not change with the treatment or the secretory rate (data not shown).

4. Discussion

The present study shows that amiloride and benzamil did not hyperpolarize membrane voltages (V_{bl} or V_m) of unstimulated or stimulated pancreatic ducts (Figs. 1 and 2) as one would have expected upon inhibition of ENaC. The concentrations of the inhibitors used in the present study were sufficient to block even the less sensitive hetero-oligomers with the δ-ENaC subunit [21]. In fact, amiloride caused a small depolarization of V, which we ascribed to inhibition of NHE, followed by intracellular pH effects on K^+ channels. This effect on V was even more pronounced with another amiloride derivative EIPA [25].

Lowering of extracellular Na^+ did hyperpolarize V (Figs. 3 and 4), as would have been expected for a Na^+ channel. However, the effect as also observed earlier, showed unusual transients, also upon reintroduction of Na^+ back to the media, and also occurred with Li^+ as the substituting cation. These transients were accompanied by Ca^{2+} transients, as they were dependent on provision of extracellular Ca^{2+}, and we concluded that they were due to electrogenic Na^+/Ca^{2+} exchanger [24]. Our present data with low Na^+ and Ca^{2+} support these interpretations. Interestingly, the hyperpolarizing effect of low Na^+ is eliminated in ducts stimulated with secretin (Fig. 4). In a recent study, we showed that pancreatic ducts express two isoforms of the Na^+/Ca^{2+} exchanger, NXC1.3 and NCX1.7, and that secretin stimulation inhibits Ca^{2+} transients mediated by the exchanger [31].

Mineralocorticoid pre-treatment, either acute (Figs. 4 and 5) or longer lasting (Fig. 6), had no effect on V of resting or stimulated ducts. Clearly, if ENaC were upregulated by any signaling pathways including posttranslational control, or were newly synthesized and inserted in the apical membrane [32,33], resting and also stimulated V should have been more depolarized. This clearly was not the case. Even acutely (<1 min), there were no significant changes in V, which indicates that there was no non-genomic activation of Na^+ and K^+ channels or NHE, as seen in colon and kidney epithelia [34].

Most importantly, the treatment of rats with glucocorticoids had no effect on pancreatic secretion either stimulated with the predominantly ductal agonist secretin, or the acinar agonist CCK8 (Fig. 7). Thus, electrolyte and fluid secretion from the two epithelia is not affected, although glucocorticoids can stimulate synthesis and storage of proteins [29,30], and although human pancreas expresses the serum- and glucocorticoid-dependent serine/threonine kinase (sgk1) [35] that can be involved in ENaC regulation [33]. Regarding pancreatic acini, our results with CCK8 support a large number of electrophysiological studies, which so far have not come up with selective Na^+ channels, but rather non-selective cation channels of unknown functions [36].

Taken together, despite the fact that there might be one or more subunits of ENaC expressed in pancreas, the present study finds no evidence for functional Na^+ channels either in stimulated or unstimulated pancreatic ducts. Hence, possible ENaC subunits are not assembled into a functional channel, or the channel is down-regulated. In fact, this lack of functional ENaC makes sense, as native pancreatic ducts form a leaky and secretory epithelium. Moreover, it is well established that Na^+ concentrations in pancreatic juice are plasma-like and do not change with secretory rate.

Nevertheless, there are Na^+ conductive pathways in pancreatic ducts that are due to a regulated Na^+/Ca^{2+} exchanger [24,31], and due to P2X7/P2X4 purinergic receptors that are Ca^{2+}/Na^+ channels when stimulated with ATP [26]. The role of these two types of cation conductance is probably associated with Ca^{2+} signaling and/or Ca^{2+} transport. In addition, although rat pancreatic ducts do have a Na^+/HCO_3^- cotransporter (NBC) [37,38], the transporter is not electrogenic in these pancreatic ducts, as already addressed and shown in earlier electrophysiological studies [20,23].

In conclusion, the present study on electrophysiological responses of isolated pancreatic ducts, and secretory re-

sponses of intact pancreas shows that there are no functional ENaC, although there is functional CFTR. This is in accordance with the function of pancreatic ducts as a secretory epithelium.

Acknowledgements

The Novo-Nordisk Foundation, the Danish Natural Science Research Council, the Danish Medical Research Council and the Carlsberg Foundation supported this project. The authors acknowledge excellent technical assistance of Mr. A. Nielsen, Ms. A.V. Olsen and Ms. B. Petersen. Critical comments of N. Møbjerg, C.P. Hansen and E.H. Larsen are gratefully acknowledged, as well as the initial engagement of K. Sonne-Hansen in the in vivo studies.

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