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Evidence for a Na^+-Ca^{2+} exchanger in rat pancreatic ducts

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Abstract Only recently has it been recognized that intracellular Ca^{2+} is an important cellular mediator in pancreatic ducts. The aim of the present study was to characterize the Ca^{2+} efflux pathway in ducts freshly prepared from rat pancreas. Lowering of extracellular Na^+ concentration resulted in a significant increase in intracellular Ca^{2+}. This effect was fast, reversible, dependent on the extracellular Na^+ concentration and did not correlate with intracellular pH changes. It was abolished in Ca^{2+}-free solutions, indicating that the outwardly directed Na^+ gradient was directly coupled to a flufenamate insensitive Ca^{2+} influx. Removal and reintroduction of extracellular Na^+ induced transient hyperpolarization and depolarization of V_m, respectively. Taken together, our data indicate that pancreatic ducts possess an electrogenic Na^+-Ca^{2+} exchanger, which under control conditions is responsible for transporting Ca^{2+} out of resting duct cells.

Key words: Pancreatic duct; Na^+-Ca^{2+} exchanger; Ca^{2+} efflux; Intracellular Ca^{2+}; Intracellular pH; Membrane voltage

1. Introduction

Pancreatic bicarbonate secretion is thought to originate in the ductal epithelium in response to secretin and vasoactive intestinal polypeptide, well-established agonists that act via the cAMP transduction pathway. In the past few years, the number of ductal agonists has grown to include those that act via the Ca^{2+} transduction pathway, such as acetylcholine and ATP. Indeed, these agonists evoke marked Ca^{2+} transients in duct cells of rat pancreas [2,12,13,32]. Furthermore, pancreatic ducts have a rather high resting level of intracellular Ca^{2+} activity ($[Ca^{2+}]_i$), possibly correlating with the resting Cl^- conductance [21], and spontaneous secretion [1,18]. Some properties of the Ca^{2+} stores and influx pathways in pancreatic ducts have been characterized recently [13]. The aim of our present study was to investigate how Ca^{2+} is transported out of pancreatic duct cells. In general, the two major pathways for cellular Ca^{2+} export are the Na^+-Ca^{2+} exchanger and the plasma membrane Ca^{2+}-ATPase. The exchanger operates in excitable cells [3,5,6,11,27]. The ATPase seems to be the dominant transport mechanism for Ca^{2+} export in epithelial cells, including exocrine gland cells [19,35,36,38], although two short studies indicated the presence of the exchanger [17,31]. A notable and well-established exception in epithelia is the distal part of the nephron, which possesses the Na^+-Ca^{2+} exchanger [4,10,28,34,37]. Since our preliminary studies showed that low Na^+ concentrations had marked effects on intracellular Ca^{2+} activity [26], we began our investigations on Ca^{2+} efflux mechanisms by testing the possibility whether pancreatic ducts possess the exchanger. These studies were carried out on unstimulated ducts, which nevertheless show a substantial Ca^{2+} influx sensitive to extracellular Ca^{2+}, pH, micromolar concentrations of La^{3+} and flufenamate [13].

2. Materials and methods

Pancreas were obtained from female Wistar rats (100–200 g), which were kept on a standard laboratory diet. Ducts were isolated by microdissection and perfused in vitro, as already described [20]; or they were obtained from collagenase-digested pancreas, our new preparation for ducts [24]. Isolated ducts were held by means of two pipettes in a chamber mounted on a stage of an inverted microscope (Axiovert, Zeiss, Oberkochen, Germany). The control bath solution had the following composition (in mmol/l): Na^+ 145, K^+ 4, Ca^{2+} 1.5, Mg^{2+} 1, Cl^- 125, HCO_3^- 25, phosphate 2, glucose 5. The solutions were equilibrated with 5% CO_2 in O_2; the pH was 7.4 and all experiments were performed at 37°C. In some experiments the Na^+ concentration was reduced from 145 mmol/l to 5 mmol/l, whereby Na^+ was replaced with *N*-methyl-D-glucamine ($NMDG^+$) titrated with HCl. This solution was HCO_3^--free, but it contained phosphate and was adjusted to pH 7.4. In another series of experiments the Na^+ concentration was 25 mmol/l, and HCO_3^- was retained in the solution. In several experiments 20 out of 145 mmol/l NaCl were replaced with an equimolar concentration of Na acetate. Ca^{2+}-free solutions were made by adding 1 or 5 mmol/l EGTA.

Intracellular Ca^{2+} activity, $[Ca^{2+}]_i$, was estimated using the fura-2 method according to the procedure of Grynkiewicz, as described for pancreatic ducts earlier [12]. Duct fragments were loaded with 0.5–1 μmol/l fura-2/AM for 30–45 min at room temperature. After excitation at 340, 360 and 380 nm, the emission intensity, collected from about 10 cells, was measured with a photomultiplier at 510 nm. The fluorescence ratio 340/380 nm was an estimate of $[Ca^{2+}]_i$. At the end of the experiments the ducts were permeabilized with ionomycin (1–5 μmol/l), and the signal was calibrated. In separate experiments ducts were incubated for 20–30 min with 1 μmol/l BCECF-AM (2′,7′-bis carboxyethyl 25(6)-carboxyfluorescein). The emission intensity was measured at 530 nm after excitation at 436 and 488 nm. The 488/436 ratio was an estimate of intracellular pH, pH_i. In situ calibrations were performed on ducts with 1 μmol/l of the protonophore CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), or with the K^+/H^+ antiporter nigericin (10 μmol/l) given in a bath solution of high K^+ concentration (145 mmol/l). As in the cortical collecting ducts [30], calibration with either substance leads to similar results.

In one separate series of experiments the cell membrane voltage (V_m) was measured by a whole-cell-nystatin method, as recently applied to duct cells [24]. Briefly, 20–100 μg/ml nystatin (20–100 μmol/l) was included in a pipette solution containing cell-like solution of the following composition (mmol/l): Na^+ 11, K^+ 127, Mg^{2+} 1, Cl^- 32, gluconate 96, phosphate 6, glucose 5, ATP 1; Ca^{2+} was adjusted with EGTA to 10^{-7} mol/l and the pH was 7.2. After formation of a seal, the patch clamp amplifier, EPC-9 (HEKA electronik, Lambrecht, Germany), was run in a current clamp mode and V_m was recorded.

All chemicals used to make the standard salt solutions were of the purest analytical grade. Other special chemicals used were as follows: ATP, ionomycin, CCCP, HEPES, EGTA, bepridil (Sigma), fura-2/AM and BCECF/AM (Molecular Probes, Eugene, OR, USA). S933240 was a kind gift from Dr. H.J. Lang (Hoechst, Frankfurt, Germany). The data are shown as original recordings, summaries and mean values ± S.E.M. Most data are presented as changes in

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the fluorescence ratios, as we are interested in the relative changes in $[Ca^{2+}]_i$ and pH_i. Control and test measurements were made within one duct, thus n refers to the number of measurements made in different ducts. Paired Student's t-tests were applied to all data and $P < 0.05$ was accepted as significant.

3. Results

3.1. Effect of low extracellular Na^+ concentrations on $[Ca^{2+}]_i$

Fig. 1 shows the effect of lowering the extracellular Na^+ concentration from 145 mmol/l to 5 and 25 mmol/l on the fura-2 ratio in unstimulated pancreatic ducts. There is a significant and reversible increase in $[Ca^{2+}]_i$, which is dependent on the Na^+ concentration. With 5 mmol/l Na^+ the 340/380 ratio increased from 2.53 ± 0.10, to a peak value of 3.34 ± 0.12 ($n = 76$). This corresponds to an increase in $[Ca^{2+}]_i$ from 134 ± 27 to 294 ± 46 nmol/l ($n = 25$). With 25 mmol/l Na^+ solutions the fura-2 ratio increased from 2.59 ± 0.20 to 3.00 ± 0.26 ($n = 12$). One of the simplest theories, which could explain this increase in $[Ca^{2+}]_i$, is that pancreatic ducts have a Na^+-Ca^{2+} exchanger. Under normal conditions, where there is a steady and small influx of Ca^{2+} via a separate Ca^{2+} influx pathway [13], the exchanger would be responsible for taking Ca^{2+} out of the cell. In a situation where the extracellular Na^+ is lowered, the inwardly directed Na^+ gradient would be reduced, or even reversed, leading to accumulation of Ca^{2+} in the cell, or even bringing Ca^{2+} into the cell. If this were the case, one would postulate that the Ca^{2+} transient

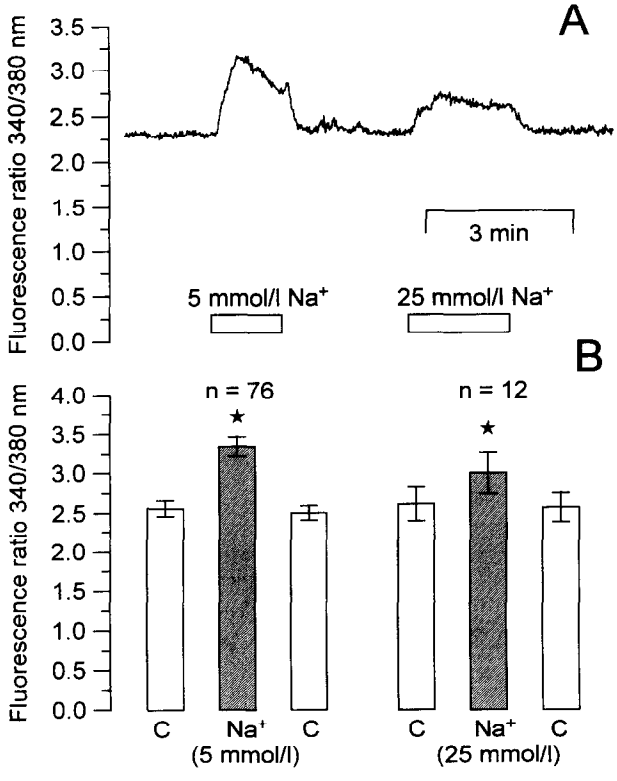

Fig. 1. (A,B) Effect of low extracellular Na^+ concentrations on fura-2 fluorescence ratio in pancreatic ducts. (A) Original recording of the 340/380 nm ratio in a single experiment, where extracellular Na^+ was lowered from 145 mmol/l to 5 or 25 mmol/l as indicated by the bars. (B) Summary of data for similar experiments. (A) statistically significant difference ($P < 0.05$) between the control (C) and the low Na^+ solution (Na^+) is indicated by an asterisk.

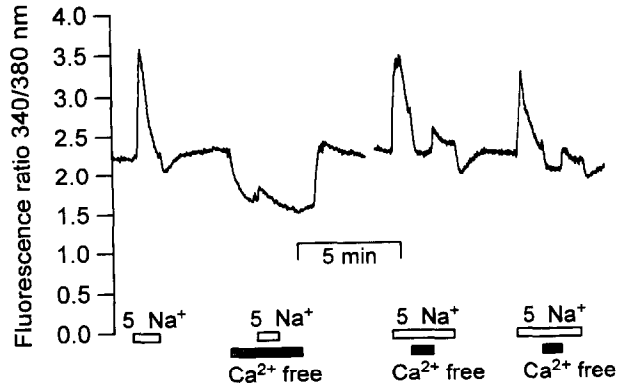

Fig. 2. Effect of a Ca^{2+}-free solution on the Ca^{2+}-transient evoked by a low Na^+ solution (5 mmol/l Na^+) in a pancreatic duct.

evoked by low Na^+ would depend on extracellular Ca^{2+}. Fig. 2 shows an experiment where the Na^+ step was repeated in Ca^{2+}-free solutions. Here, removal of extracellular Ca^{2+} prevented or interrupted the Ca^{2+} transient induced by low Na^+. Similar results were obtained in 19 experiments. These data indicate that influx of Ca^{2+} from the extracellular medium is necessary for the Ca^{2+} transient.

3.2. Effect of various inhibitors of Ca^{2+} transport

Flufenamate is an inhibitor of non-selective cation channels [9,13,14], through which Ca^{2+} might be able to 'slip in' if the concentration of the competing cation, Na^+, is lowered. Verapamil and nifedipine, on the other hand, are not effective in blocking Ca^{2+} influx in pancreatic ducts [13]. Therefore, we assessed the effect of flufenamate (10^{-4} mol/l) on the Na^+-induced Ca^{2+} transient. In 5 experiments we found that flufenamate had no inhibitory effect; in contrast, in 4 experiments it seems that it even increased the effect of low Na^+ on the peak $[Ca^{2+}]_i$ by about 15%. Thus, these results indicate that when extracellular Na^+ is lowered, Ca^{2+} does not enter cells via Ca^{2+} influx channels, but rather in exchange for Na^+.

In excitable tissues bepridil has been described as a blocker of the Na^+-Ca^{2+} exchanger [8]. We tested this inhibitor at a concentration of 10^{-6} mol/l in 10 experiments and at 10^{-5} mol/l in 4 experiments. On its own bepridil caused a slow, but significant increase in the fura-2 ratio in a concentration-dependent manner. In summary, 10^{-6} mol/l bepridil increased the fura-2 ratio from 2.74 ± 0.21 to 3.04 ± 0.31 (i.e. by 11%, $P = 0.03$); 10^{-5} mol/l bepridil increased the ratio from 3.60 ± 0.87 to 4.88 ± 0.88. (i.e. by 36%, $P = 0.01$). Indeed, one would expect an increase in $[Ca^{2+}]_i$, if the compound were to block the Ca^{2+} efflux via the Na^+-Ca^{2+} exchanger. However, in the presence of bepridil, the low Na^+ step still had more or less the same effect as in the preceding control ($n = 4$). In a further 2 experiments, however, the effect of low Na^+ was not detected after bepridil, but neither were effects of agonists that usually evoke increase in $[Ca^{2+}]_i$.

The biguanidylhydrazone, S933240, is a putative blocker of the Na^+-Ca^{2+} exchanger in cardiac cells [29]. Similar to bepridil, addition of this substance to the extracellular solution (10^{-6}–10^{-5} mol/l) caused an increase in the fura-2 ratio, possibly because the Ca^{2+} efflux was inhibited. In altogether 6 ducts, S933240 increased the fura-2 ratio from 2.94 ± 0.27 to 3.97 ± 0.76 (i.e. by 35%, $P = 0.05$). However, lowering extracellular Na^+ to 5 mmol/l still caused an increase in $[Ca^{2+}]_i$

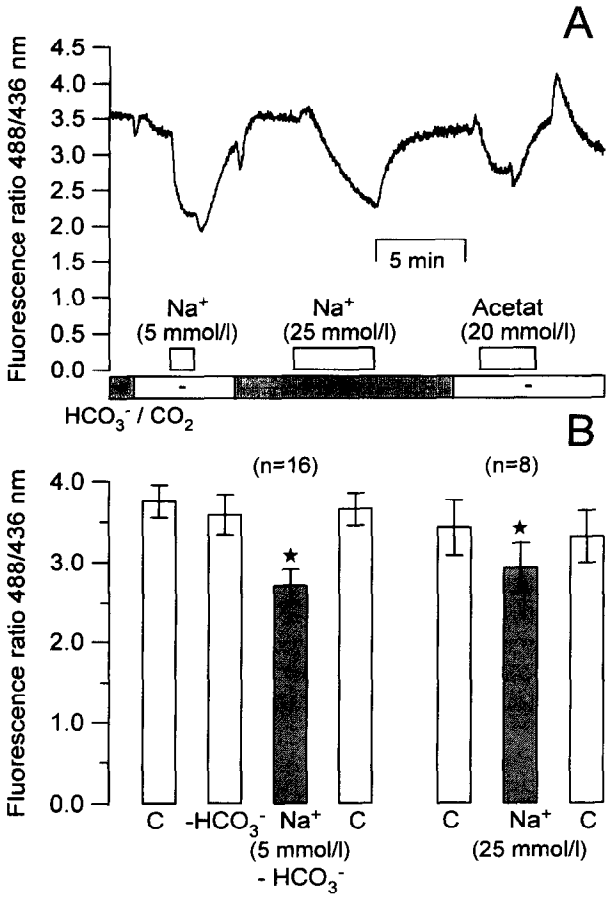

Fig. 3. (A,B) Effect of low Na^+ solutions and acetate/acetic acid on intracellular pH as estimated from the 488/436 nm ratio of ducts loaded with BCECF. (A) The extracellular Na^+ concentration was decreased to 5 mmol/l (in HCO_3^-/CO_2-free solution), and to 25 mmol/l (in HCO_3^-/CO_2-containing solutions). As last acetate/acetic acid (20 mmol/l) was given in the control solution with normal Na^+ concentrations. (B) Summary of similar experiments. A statistically significant difference ($P < 0.05$) between the control (C) and the low Na^+ solution (Na^+) is indicated by an asterisk; $-HCO_3^-$ indicates the solutions free of HCO_3^-.

(ratio increased by 0.84 ± 0.04), which appeared to be about 10% lower than in the corresponding control (ratio increased by 0.97 ± 0.15), but the significance was borderline ($P = 0.18$).

3.3. Effects of low Na^+ and acetate on intracellular pH

Fig. 3A shows the effect of Na^+ concentration steps on pH_i as estimated from the BCECF fluorescence ratio. The data show that lowering of extracellular Na^+ led to a significant decrease in pH_i, which depended on the concentration of Na^+. In summary, as shown in Fig. 3B, the BCECF ratio decreased by 1.00 ± 0.07 with 5 mM Na^+ in 16 experiments. In 8 experiments, where 25 mM Na^+ was also tested, the ratio decreased by 0.51 ± 0.11. Since pancreatic ducts are rather sensitive to the presence of HCO_3^-/CO_2 [22,23], it would have been preferable to retain the buffer as in 25 mmol/l Na^+ solutions. This was not possible, however, with solutions containing only 5 mM Na^+. Nevertheless, any effects due to removal of HCO_3^-/CO_2 were insignificant and negligible compared to those caused by lowering of the extracellular Na^+ concentration. For comparison, Fig. 3A also demonstrates the effect of acetate/acetic acid (normal Na^+ concentrations), which transiently acidified pancreatic ducts. Fig. 4 shows the effect of acetate and low Na^+ on pH_i, as measured in a fura-2 loaded duct, and on $[Ca^{2+}]_i$, as measured in a BCECF loaded duct. Clearly, acidification induced directly by the addition of acetate/acetic acid leads to a decrease in $[Ca^{2+}]_i$. Recently, we have also shown that lowering of pH_i by means of HEPES or HCO_3^-/CO_2 buffers also decreased $[Ca^{2+}]_i$ [13]. In contrast, acidification caused by low Na^+ solutions is associated with an increase in $[Ca^{2+}]_i$ (Fig. 4). Hence, most likely, the effect of low Na^+ on $[Ca^{2+}]_i$ is direct and not mediated via changes in pH. It is even conceivable that the fall in pH induced by low Na^+ actually diminishes the Ca^{2+} transient.

3.4. Effects of low Na^+ on membrane voltage

In a series of experiments we investigated the effect of low Na^+ solutions on membrane voltage, V_m, measured in whole-cell nystatin recordings. Fig. 5 (top) shows the effect of low extracellular Na^+ on V_m of a duct cell. With 5 mmol/l Na^+ solution V_m hyperpolarized, and in many cells V_m returned towards the control value within 3–5 min. Fig. 5 (bottom) is a summary of 14 such experiments. Immediately after lowering the Na^+ concentration, V_m hyperpolarized from -44 ± 3 to -51 ± 2 mV ($n = 14$). Within a few minutes V_m was recovering towards its control value. Upon return to the control Na^+ concentrations, there was a significant transient overshoot in the voltage to -29 ± 2 mV ($n = 14$), before V_m returned to the control value. Such transient voltage changes were also present with solutions containing Li^+ as the substituting cation, and they were more pronounced in 0 mmol/l Na^+ solutions ($n = 4$).

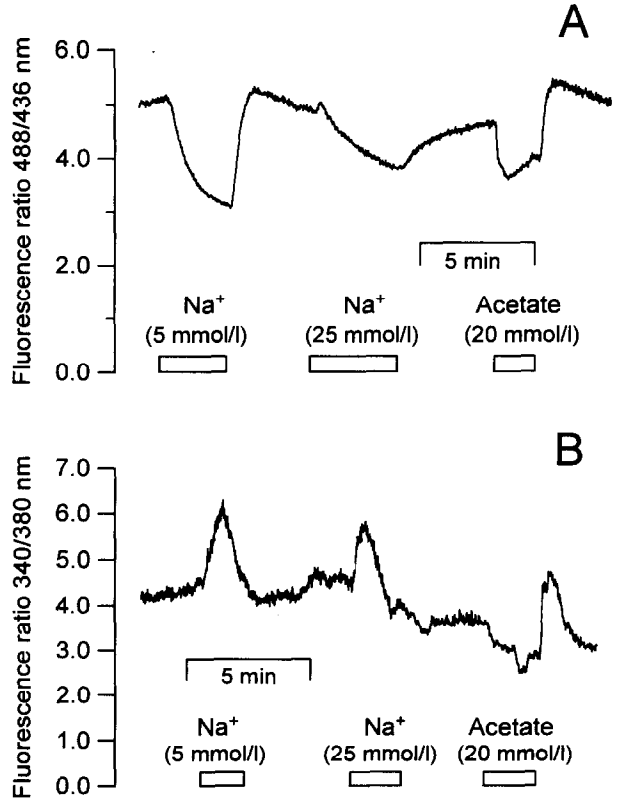

Fig. 4. (A,B) Effect of low Na^+ concentrations and of acetate/acetic acid (in control Na^+ concentrations) on: (A) pH_i of a pancreatic duct loaded with BCECF; and (B) $[Ca^{2+}]_i$ of a duct loaded with fura-2.

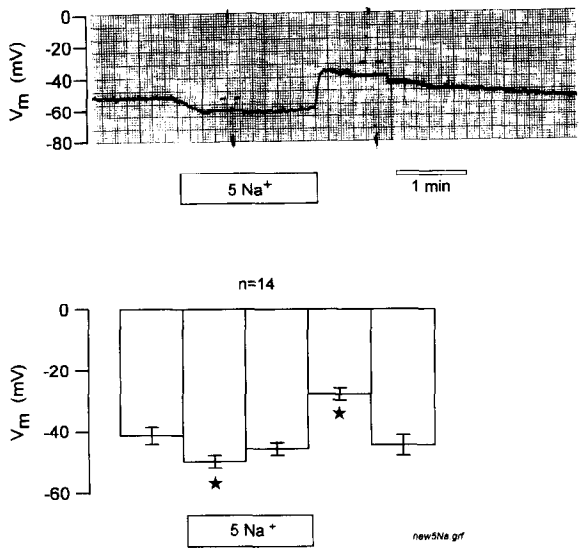

Fig. 5. Effect of low Na^+ solution on membrane voltage, V_m, measured in whole-cell-nystatin recording. The upper trace shows the original voltage recording from a single cell in a duct cell cluster. The lower part shows the summary of data from 14 experiments, and the bars correspond to the control, hyperpolarization and recovery during the low Na^+, depolarization and recovery during the control. An asterisk indicates a statistically significant difference ($P < 0.05$) between the steady-state control voltage and transient effects of the low Na^+ and its removal.

4. Discussion

The key finding in our study is that the reduction in extracellular Na^+ leads to a significant, relatively fast and spontaneously reversible increase in $[Ca^{2+}]_i$. The simplest interpretation is that the fluxes of Ca^{2+} and Na^+ are directly coupled. In fact, similar experiments and results from excitable cells and distal parts of kidney nephrons were interpreted as a part of the evidence for the Na^+-Ca^{2+} exchanger [4,6,10,34]. In contrast, in pancreatic acini and other epithelial cells, the Na^+-Ca^{2+} exchanger does not participate in the Ca^{2+} efflux significantly, since this efflux is independent of the Na^+ gradient [19,35,36]. In the following paragraphs we will put forward arguments in favour of the Na^+-Ca^{2+} exchanger in pancreatic ducts.

If it were that ducts had such an exchanger, lowering of extracellular Na^+ would decrease, or even reverse the gradient, such that the exchanger would run in the reverse mode. That is, Na^+ would come out of the cell and Ca^{2+} would be brought into the cell in exchange. Since removal of extracellular Ca^{2+} abolishes the low Na^+ effect (Fig. 2), it is clear that the increase in Ca^{2+} we observe is indeed due to influx of Ca^{2+} from outside. The effect of low Na^+ is transient (Fig. 2), while the fall in pH_i and hyperpolarization of V_m seemed more sustained (Figs. 4 and 5). The most likely explanation is that duct cells do not run out of the Na^+ gradient, but that Ca^{2+} becomes sequestered into intracellular stores. Cells are still producing H^+ (which cannot be taken out by the Na^+/H^+ exchanger) and thus pH_i remains low; and as some Na^+-Ca^{2+} still occurs, V_m is hyperpolarized.

In view of the fact that non-selective cation channels can mediate a measurable Ca^{2+} influx (in low Na^+) [14], it was important to check that this did not happen in our experiments with low Na^+ concentration. In fact, our recent study shows that pancreatic ducts have Ca^{2+} influx pathways that have some characteristics of the non-specific cation channels, e.g. sensitivity to flufenamate [13]. However, since flufenamate did not decrease the Ca^{2+} transient induced by low Na^+, Ca^{2+} did not enter via such channels, but rather in exchange for Na^+. In our quest to provide evidence for the exchanger, we have also tried two putative blockers of the antiport – bepridil and S933240 [8,29]. However, our experience with these inhibitors is inconclusive. Applied alone they increased $[Ca^{2+}]_i$, which would be consistent with the inhibition of the antiport, but they were not able to abolish the low Na^+ effect successfully.

Pancreatic ducts possess a Na^+-H^+ exchanger [20,23,25,33,39], and hence changes in the Na^+ gradient might affect intracellular pH. Since pH is known to affect $[Ca^{2+}]_i$ in many cells, including pancreatic ducts [13], it was imperative to test whether the low Na^+ effect on $[Ca^{2+}]_i$ is indirectly due to its effect on pH. Lowering of extracellular Na^+, which decreases the rate of Na^+-H^+ exchange and thus leads to cell acidosis, is associated with an increase in $[Ca^{2+}]_i$. If, on the other hand, one induces cell acidosis directly by, for example, acetate/acetic acid (Figs. 3 and 4), or by exposure to HCO_3^-/CO_2 or HEPES solutions of low pH_e [13], $[Ca^{2+}]_i$ decreases, not increases as with low Na^+. Hence, the low Na^+ effect cannot be due to the pH effect. In contrast, the pH effect might even reduce the height of the Ca^{2+} transient induced by low Na^+. In fact, in pancreatic β-cells the Na^+-Ca^{2+} exchanger is markedly pH sensitive [27].

Let us now consider our voltage data, which show that low Na^+ leads to hyperpolarization of V_m (Fig. 5). One immediate possibility that comes to mind is that this low Na^+ effect could be explained in terms of a Na^+ conductance. However, amiloride at a concentration of 1–10 μmol/l has no effect on the membrane voltage of normal pancreatic ducts. In the millimolar range, amiloride has a depolarizing effect on V_m, which is due to inhibition of the Na^+-H^+ exchanger and the resulting pH effects on the K^+ conductance [20,25]. Another possibility which one should consider is that the changes in membrane voltage we observe are due to an increase in the K^+ conductance followed by an increase in the Cl^- conductance, both initiated by a high $[Ca^{2+}]_i$. However, this could possibly explain the hyperpolarization of V_m, but not the depolarizing overshoot after readmission of Na^+. Furthermore, preliminary data indicate that Ba^{2+} does not inhibit the effect of low Na^+. Thus, the most direct explanation of our data is that there is an electrogenic Na^+-Ca^{2+} exchanger, presumably carrying 3 Na^+:1 Ca^{2+} (see below), which switches from depolarizing the cell membrane voltage under normal conditions, to hyperpolarizing it, as it reverses in low Na^+ conditions. Return to high Na^+ causes transient and large overshoot in the depolarizing direction.

One important question we need to consider is, whether there is enough driving force for this exchanger to function in pancreatic ducts. Strictly speaking, we do not know what the stoichiometry of the transporter is. However, except for the outer rod segments, where $4Na^+:(1Ca^{2+}+1K^+)$ stoichiometry is accepted [5], excitable cells have the $3Na^+:1Ca^{2+}$ exchanger, which is encoded by a different gene [6,7,11,15,16]. A number of tissues, including the distal convoluted tubule and pancreas, express the cardiac Na^+-Ca^{2-} exchanger [16,28,37,40]. Hence, one might assume that the epithelial ex-

changer is also functionally related, and if we take $3Na^+:1Ca^{2+}$ for pancreatic ducts, the reversal potential for the exchanger is given by [7]:

$$E_{NaCa} = 3E_{Na} - 2E_{Ca}$$

and the net driving force is $\Delta V = V_m - E_{NaCa}$, where V_m is the membrane potential. Therefore, if $\Delta V < 0$, the Na^+-Ca^{2+} exchanger will operate in the forward mode and Ca^{2+} will be transported out of the cell. If, on the other hand, $\Delta V > 0$; the Na^+-Ca^{2+} exchanger will run in the reverse mode and bring Ca^{2+} into the cell. Taking the resting $[Ca^{2+}]_i$ as 150–200 nmol/l [12,13], $[Ca^{2+}]_e$ as 1.5 mmol/l, $[Na^+]_e$ as 145 mmol/l and assuming that $[Na^+]_i$ is about 10 mmol/l, and the resting V_m as about −60 mV, as estimated from many recordings [20,24], it can be calculated the that ΔV is about −30 mV. Hence, the exchanger could take Ca^{2+} out of the cell, i.e it could serve as a Ca^{2+}-efflux pathway in non-stimulated ducts. As we have recently shown, even in a resting pancreatic duct there is a significant Ca^{2+} influx, which keeps $[Ca^{2+}]_i$ high [13]. The function of this exchanger might be to take some of this Ca^{2+} back out of the cell. Whether the exchanger is important in ducts undergoing stimulation with acetylcholine or ATP, or whether the plasma membrane Ca^{2+}-ATPase is also involved under those conditions, remains to be determined.

In conclusion, the present study provides the first evidence for the Na^+-Ca^{2+} exchanger in rat pancreatic ducts. It seems that in resting ducts it provides an efflux pathway for Ca^{2+}. This finding of the exchanger in pancreatic ducts seems to be unique, as most other epithelia, except for the distal parts of nephron, utilize the plasma membrane Ca^{2+}-ATPase to transport Ca^{2+} out of the cell.

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