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Regulation of the Na^{+}/Ca^{2+} Exchanger in Rat Pancreatic Ducts

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Abstract. The Ca^{2+} content of pancreatic juice is closely regulated by yet unknown mechanisms. One aim of the present study was to find whether rat pancreatic ducts have a Na^{+}/Ca^{2+} exchanger, as found in some Ca^{2+} transporting epithelia. Another aim was to establish whether the exchanger is regulated by hormones/agonists affecting pancreatic secretion. Whole pancreas, pure pancreatic acini and ducts were obtained from rats and used for RT-PCR and Western blot analysis, immunohistochemistry and intracellular Ca^{2+} measurements using Fura-2. RT-PCR analysis indicated Na^{+}/Ca^{2+}-exchanger isoforms NCX1.3 and NCX1.7 in acini and pancreas. Western blot with NCX1 antibody identified bands of 70, 120 and 150 kDa in isolated ducts, acini and pancreas. Immunofluorescence experiments showed the Na^{+}/Ca^{2+} exchanger on the basolateral membrane of acini and small intercalated/intralobular ducts, but in larger intralobular/extralobular ducts the exchanger was predominantly on the luminal membrane. Na^{+}/Ca^{2+} exchange in ducts was monitored by changes in intracellular Ca^{2+} activity upon reversal of the Na^{+} gradient. Secretin (1 nM) and carbachol (1 μM) reduced Na^{+}/Ca^{2+} exchange by 40% and 51%, respectively. Insulin (1 nM) increased Na^{+}/Ca^{2+} exchange by 230% within 5 min. The present study shows that pancreatic ducts express the Na^{+}/Ca^{2+} exchanger. Its distinct localization along the ductal tree and regulation by secretin, carbachol and insulin indicate that ducts might be involved in regulation of Ca^{2+} concentrations in pancreatic juice.

Key words: Na^{+}/Ca^{2+} exchanger — NCX — Pancreatic duct — Ca^{2+}transport — Pancreas

Introduction

Bicarbonate and digestive enzymes are the main components of pancreatic juice important in digestive processes. Calcium is the minor component of the juice, but it is of critical importance in the formation of Ca^{2+}-containing stones. The origin of secreted Ca^{2+} is unclear. Some Ca^{2+} may be secreted together with digestive enzymes from zymogen granules; some may be accounted for by spillover from acinar cell signaling, which seems to be most prominent at the apical pole of acinar cells [2]. In addition, the lumen-negative transepithelial voltage in pancreatic ducts secreting bicarbonate [28] might provide the driving force for paracellular or transcellular Ca^{2+} transport. Total and free Ca^{2+} concentrations in the juice depend on secretory rates, and are notably lower after secretin stimulation [21]. Increased risk for Ca^{2+} stone formation and trypsinogen activation in hypercalcemia and pancreatitis would indicate that pancreatic Ca^{2+} homeostasis was disturbed in these conditions [21, 23]. It is thus very likely that the Ca^{2+} content of pancreatic juice is closely regulated, as also indicated by recent findings of Ca^{2+} sensing receptors on the luminal membrane of pancreatic ducts [4]. However, the nature of Ca^{2+} transport processes in pancreas and the regulation of these are largely unknown.

The distal part of kidney tubuli and intestine absorb Ca^{2+}, and one of the transporters involved in this process is the Na^{+}/Ca^{2+} exchanger [15, 38, 39]. Although the Na^{+}/Ca^{2+} exchanger is probably present in many cells, it is most extensively studied in excitable cells. The Na^{+}/Ca^{2+} exchanger (NCX) expressed by heart, kidney and intestine is the cardiac type of exchanger, NCX1 [38, 39]. NCX2 and NCX3 isoforms, which are products of distinct genes, are primarily expressed in brain and skeletal muscle, although one study shows mRNA for NCX2 in a variety of tissues [31]. NCX1 occurs in various splice forms, arising from alternative splicing of the variable

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region within the large intracellular loop, while no splice variants are detected in NCX2 [16, 31]. It is becoming apparent that the Na^+/Ca^{2+} exchanger, which transports $3Na^+$ for $1Ca^{2+}$, depends not only on the electrochemical gradients and modulation by transported ions, but also on intracellular signaling pathways and on the NCX isoforms expressed by a particular tissue [3, 30].

In our earlier studies on unstimulated rat pancreatic ducts we obtained preliminary evidence for the Na^+/Ca^{2+} exchanger using intracellular Ca^{2+} measurements and electrophysiological recordings [8]. That study was performed on unstimulated ducts and we have no information whether pancreatic secretagogues that elicit HCO_3^- and fluid secretion also influence Na^+/Ca^{2+} exchange and thereby the amount of Ca^{2+} secreted in the juice. Furthermore, we need to establish the presence of the Na^+/Ca^{2+} exchanger in pancreatic ducts by other methods. Thus, the aim of the present study was to demonstrate the Na^+/Ca^{2+} exchanger in native pancreatic ducts and acini using molecular biological techniques and immunohistochemistry. Furthermore, we studied regulation of the exchanger by the common pancreatic secretagogues secretin and acetylcholine, as well as by endocrine hormone insulin. This study shows that pancreatic exocrine tissue expresses NCX1 isoforms. The exchanger has a distinct pattern of distribution in pancreatic ducts depending on their size and proximity to acini. Secretin, acetylcholine and insulin regulate Na^+/Ca^{2+} exchange, indicating that the exchanger might be of relevance in pancreatic Ca^{2+} homeostasis.

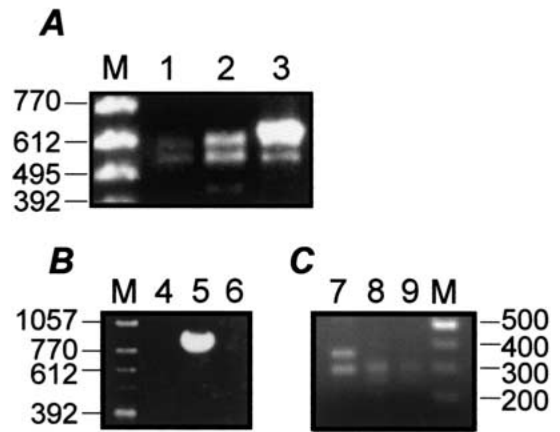

Fig. 1. RT-PCR determination of NCX expression in rat pancreas, acini and heart. (*A*) Total RNA from pure acini (lane *1*), whole pancreas (lane *2*), and heart (lane *3*) was reverse-transcribed, and the resulting cDNA was PCR-amplified using NCX1 primers. (*B*) Lanes *4*, *5* and *6* contained primers for carbonic anhydrase II, amylase and thrombin, respectively, and were used to check purity of the acinar preparation, (*C*) Restriction enzyme digestion of the PCR products from heart (lane *7*), acini (lane *8*) and pancreas (lane *9*). Lanes M_w contain molecular weight markers. The gel shown is representative of four independent experiments.

Materials and Methods

All standard chemicals were obtained from Sigma (Copenhagen, Denmark). Tissue culture media were purchased from Life Technologies Ltd. (UK). Fura-2/AM and Alexa 488-coupled goat anti-rabbit IgG were obtained from Molecular Probes (Leiden, The Netherlands). Polyclonal antibody against purified canine heart Na^+/Ca^{2+} exchanger (NCX1), described in detail in [29], was a generous gift of Prof. K. D. Philipson (UCLA School of Medicine, Los Angeles, CA). The same antibody was also later obtained from SWant (Bellinzona, Switzerland). Primers for RT-PCR were provided by MWG-Biotech, Germany. Protease inhibitor cocktail tablet was from Boehringer Mannheim (Hoersholm, Denmark).

Tissue Preparation

Pancreas was obtained from female Wistar rats (100–200 g) kept on standard laboratory diet. Rats were killed by cervical dislocation. Pancreatic ducts comprise 2–4% of the total tissue. Intralobular ducts were obtained from collagenase-digested pancreas as described previously [27], and single ducts were identified with the aid of a dissection microscope. Any remaining loose connective tissue and small blood vessels were removed with sharpened forceps. Subsequently, each duct was transferred to an inverted microscope (Axiovert 100TV, Zeiss, Oberkochen, Germany) and checked for "cleanliness" with a high-power objective, *see* Fig. 1 in [7]. Each preparation of rat pancreas yielded only a few "clean" ducts that were not damaged by exogenous and endogenous enzymes. These ducts were used for fluorescence measurements, as well as for molecular biology, where the purity of duct preparation was further checked (*see* below). For pancreatic acinar preparation we modified our standard procedure slightly. Pancreatic tissue was incubated with collagenase for a longer period (40–45 min) and the cell suspension was filtered through nylon filters with a pore size of 100 μm (Millipore, Denmark). Single acini were inspected for "cleanliness" in an inverted microscope and a few individual "clean" acini were used for molecular biology. Each sample of pancreatic ducts and acini that were further used for molecular biology were first checked for purity/contamination by RT-PCR amplification of amylase, anhydrase II and thrombin using specific primers as described recently [7]. Pancreas and heart were excised and washed several times in ice-cold phosphate buffer saline (PBS). For immunohistochemistry, organs were cut in small pieces and immediately put into fixative. For molecular-biology assays, pancreas and heart were minced with scissors and placed directly either into lysis buffer or total RNA isolation reagent (*see* below).

For fluorescence experiments single isolated pancreatic duct fragments were transferred into a chamber mounted on the stage of an inverted microscope. These ducts were usually intercalated and small intralobular ducts, which are too narrow, short and fragile for perfusion, as compared to ducts used for perfusion in our other studies [9, 28]. The outside diameter of duct fragments ranged from 17 to 50 μm, the majority being between 20 and 40 μm and the mean size of 31 ± 2 μm (n = 32). Holding pipettes were placed on a sidewall of the ducts, such that both luminal and basolateral membranes were accessible to bath solutions. The control bath solution containing HCO_3^- (+BIC) 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; it was equilibrated with 5% CO_2 in O_2 and pH was 7.4. In HCO_3^- free solutions (−BIC), $NaHCO_3$ was replaced with NaCl or Na gluconate and 5 mmol/l HEPES. All experiments were performed at 37°C. N-methyl-D-glucamine ($NMDG^+$), titrated with HCl, was used for a substitution of

extracellular Na^+ in the solutions where Na^+ was decreased from 145 mM to 0 mM or 5 mM. These solutions were HCO_3^--free. In several experiments, K^+ concentration was increased from 4 to 30 mM by substituting NaCl with KC1. Intracellular Ca^{2+} activity ($[Ca^{2+}]_i$) was measured using the fura-2 method as described for pancreatic ducts earlier [9]. Ca^{2+} measurements are presented as original recordings or summaries with the mean values ±SEM; *n* refers to the number of measurements carried out in different ducts. All effects were compared with control measurements done before and after maneuvers in the same preparation. The paired Student's *t*-test was applied and $p < 0.05$ was accepted as significant.

Immunohistochemistry

A polyclonal antibody raised against canine cardiac Na^+/Ca^{2+} exchanger (NCX1) [29] was used for localization of the Na^+/Ca^{2+} exchanger in pancreas and other control tissue. Pancreatic tissue was prepared using several different techniques.

Isolated Acini and Ducts

Pancreatic tissue (acini and ducts) was obtained from enzymatically digested pancreas (*see* above) and fixed for 1 hr in 4% paraformaldehyde buffered in PBS. After fixation, tissue was washed with PBS 3× for 5 min prior to staining. After staining (*see* below) suspensions were washed and then transferred to coverslips treated with poly-L-Lysine or Cell-TAK. Tissue was allowed to settle on coverslips for some hours. Subsequently, coverslips were mounted on microscope slides using DACO fluorescence-mounting media.

Paraffin Sections

Fixed pancreatic tissue was dehydrated with ascending ethanol series (50%, 80%, 95% and 100%) and xylene before embedding in paraffin. Paraffin-embedded pancreas was cut in 5–6 μm thick slices, and mounted on microscope slides. The slides were deparaffinated in xylene and rehydrated in a series of ethanol solutions (100%, 95%, 80% and 50%). After rinsing in PBS, pH 7.4, tissue was stained as described below.

Cryosections

Pancreas was perfused with fixative (4% paraformaldehyde with 75 mM lysine and 10 mM periodate buffered with phospate buffer) via blood supply, or pancreas was dissected and cut into small pieces and fixed for 24 hr. Prior to freezing, tissues were protected by incubating in 30% sucrose phosphate buffer for 24 hr at 4°C. Tissues were then mounted on metal rings by embedding in Cryo-Embed (Ax-Lab, Denmark) and frozen in liquid nitrogen. Frozen blocks were cut in 5–6 μm thick slices and these were re-hydrated in PBS twice for 5 min before staining. Tissues obtained from one of the above described preparations were incubated in PBS containing 10–20% normal goat serum (NGS) for 45 min at room temperature in order to block nonspecific binding. Primary antibody (diluted 1:100) was added in PBS containing 0.25% BSA (bovine serum albumin) and 0.1% Triton X-100 for 1 hr. After incubation with the primary antibody, tissue was washed 3× with PBS and Alexa-488-coupled secondary antibody (diluted 1:100) was added in PBS containing 10% NGS. Tissues were incubated for 45 min in the dark followed by wash with PBS. Control experiments were performed with primary or secondary antibody alone and the same procedures as above. Tissues were mounted and fluorescence signals were monitored on a confocal laser scanning microscope (CLSM) (Leica TCS SP) equipped with an ArKr laser. Fluorophore was excited with the 488 nm line and emission at 520 ± 20 nm was selected with the CLSM spectrophotometer unit and collected with a photomultiplier. For most measurements a 63× 1.20 NA w PL APO objective was used and 8–12 images at 512 × 512 pixels were accumulated. Corresponding transmitted light images were collected with another photomultiplier.

Immunoprecipitation and Western Blotting

For protein extraction, immunoprecipitation and immunoblotting, whole pancreas was excised and immediately frozen on a dry ice/ethanol mixture before homogenization in NP-40 lysis buffer (10 mM Tris-HCl, pH 7.4, 0.25 M Sucrose, 1 mM EDTA, 1 mM EGTA, 2% NP-40 and a protease inhibitor cocktail tablet). Pancreatic ducts and acini were isolated as described above. While the acini were lysed in NP-40 lysis buffer, due to the limited amount of ducts, these were lysed directly in boiling 2× Laemmli buffer and subjected to immunoprecipitation after dilution in NP-40 lysis buffer. Cellular debris from lysed heart, pancreas and acini were removed by centrifugation at 10,000 × g for 10 min, after which the protein content in each sample was measured using the Bio-Rad protein assay kit according to the manufacture's instructions (Bio-Rad, Hercules, CA). 2× Laemmli buffer was added to equal amounts of cell lysates and samples were subjected to SDS-PAGE followed by Western blotting (*see* below). In the absence of the primary antibody, no signal was detected (*not shown*). Pancreatic ducts were immunoprecipitated with NCX1 antibody overnight at 4°C. Immunoprecipitates were collected by adding 100 μl of a 10% Protein A-sepharose 4B slurry (Pharmacia, Upsala, Sweden) to protein extracts for 1 hr, followed by brief centrifugation. The precipitates were washed three times in (mM) 10 Tris-HCl, pH 7.4, 62.5 sucrose, 0.25 EDTA, 0.25 EGTA, 0.5% NP-40 containing a protease inhibitor cocktail tablet, 2× Laemmli buffer was added, the samples were boiled and proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Immunoreactive proteins were made visible using horseradish peroxidase-coupled secondary antibodies and enhanced chemiluminescence reagents (ECL) according to the manufacturer's instructions (Biological Industries, CO, Israel). Blots were visualized in a Kodak ImageStation. In order to check for nonspecific staining of the immunoprecipitated primary antibody, two tissue-free controls were carried out. In both samples the primary antibody was immunoprecipitated and subjected to 2× Laemmli buffer. The first sample was subjected directly to SDS-PAGE, while the second sample in addition was boiled prior to SDS-PAGE (reduced and denatured).

Molecular Biology

Total RNA was isolated from whole pancreas, acini and heart using total RNA isolation reagent (Advanced Biotechnologies Ltd.) according to the manufacturer's instructions. First-strand cDNA was synthesized utilizing an anchored oligo(dT) primer using Reverse-iT (Advanced Biotechnologies Ltd.). Polymerase chain reaction (PCR) conditions were identical for all primers. 2 μl of reverse-transcribed RNA was added to a solution of 10 μM each forward and reverse primers, 1.5 mM $MgCl_2$, PCR buffer (7.5 mM Tris HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, and 0.01% (v/v) Tween 20), 0.5 mM dNTPs, 1.25 units of Red Hot DNA polymerase (all from Advanced Biotechnologies Ltd.), and distilled H_2O in a total volume of 50 μl. We used primers described by K. D. Philipson for canine heart NCX1 [31]. The forward primer was 5′TCTTCAGAAGTCTCGGAAGAT-3′ and the reverse primer was 5′CACTTCCAGCTTGGTGTGT-3′ spanning the variable region within the large intracellular loop [16,31]. To confirm the identity of the PCR products, these were subjected to restriction-enzyme digestion with *Eco*RI. This should lead to two fragments for each of the following isoforms: NCX1.7 (286 and 320 bp),

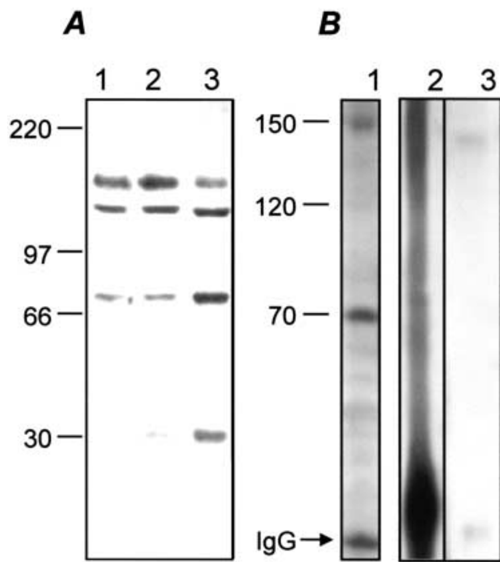

Fig. 2. Western blot analysis of NCX expression in whole pancreas, acini, and ducts. (*A*) Whole-cell lysates of heart (lane *1*), pancreatic acini (lane *2*) and whole pancreas (lane *3*) were separated by SDS-PAGE and Western blot analysis was performed using the antibody raised against the cardiac NCX1. (*B*) Cell lysates from pancreatic duct cells (lane *1*) were immunoprecipitated with an anti-NCX antibody and analyzed as above. To check for nonspecific crossreactivity between the antibodies, two control experiments were performed on tissue-free samples. One sample was untreated (lane *2*) and another was reduced and denatured (lane *3*), as in all the tissue preparations. One of five independent experiments is shown. Positions of molecular weight markers in kD and of IgG are indicated.

NCX1.3 (286 and 251 bp), NCX1.1 (286 and 359 bp) and NCX1.8 (286 and 290 bp). All digestions were performed directly on PCR products with appropriate buffers. Primers for amylase (acini), carbonic anhydrase II (ducts) and thrombin (blood vessels) were used as controls as described previously [7]. Temperature cycling proceeded as follows: 1 cycle at 94°C for 5 min and 35 cycles at 94°C for 30 sec, 55°C for 60 sec, and 72°C for 90 sec, followed by 72°C for 10 min. PCR products were then subjected to gel electrophoresis on a 1% agarose gel containing ethidium bromide.

Results

Expression of NCX in Rat Pancreas, Acini and Heart

In order to determine whether pancreas expresses NCX1, we performed RT-PCR analysis with the NCX1 primers for the rat pancreatic acini and whole pancreas and compared them to the heart (Fig. 1). In pancreas acini two major weak bands were detected at 605 base pairs (bp) and 536–539 bp. The same transcripts were detected in the whole pancreas. The band at 605 bp corresponds to the isoform NCX1.7. The band at 536–539 bp corresponds most likely to isoform NCX1.3. In heart controls we detected 644 bp and 575 bp bands, which reflect the established NCX1.1 and NCX1.8 isoforms [31]. A weak band at 450 bp was detected in pancreas and heart. Fig. 1 also shows controls run on the acinar preparation to check the purity of the preparation, as described earlier [7]. Only the transcript for amylase was detected, indicating that the preparation was devoid of ducts and blood vessels. The identity of the PCR products was confirmed using an enzyme digestion with *Eco*RI as described in the Material and Methods and the results are shown in Fig. 1*C*.

To confirm the expression of NCX at the protein level we performed Western blot analysis using an antibody raised against the full-length canine cardiac NCX1 [29]. Fig 2*A* shows Western blot analysis on total cell lysates from the rat heart, isolated acini and total pancreas. The tissues gave three bands at 70, 120 and about 150 kDa. Under nonreducing conditions the same bands were obtained (*not shown*). In Western blot analysis on total cell lysates from pancreatic ducts NCX could not be detected (*not shown*), probably due to the limited amount of the material. Therefore, we performed immunoprecipitation of NCX from pancreatic ducts and the Western blot analysis revealed strong bands with sizes at 70 and 150 kDa, and a weaker band at 120 kDa (Fig. 2*B*). Two controls on tissue-free sample were performed to check for nonspecific cross-reaction between antibodies (Fig. 2*B*, lanes 3, 4). None of these gave rise to bands seen with NCX obtained from the immunoprecipitated cell lysates.

Immunohistochemical Studies

NCX antibody was used on various preparations of pancreas and heart, and the fluorescence signal from the Alexa-488-coupled secondary antibody was detected in a confocal laser scanning microscope (Fig. 3). Fig. 3*A* and *B* shows NCX immunofluorescence in cryosections of the rat pancreas, and the corresponding transmission images. The two sections show NCX distribution in small intercalated/intralobular ducts (outer diameters of about 12 and 8 μm, respectively). The staining was localized on the basolateral membranes, whereas no staining was observed on the luminal membranes. Pancreatic acini were also labeled with the NCX antibody on the basolateral membranes. Fig. 3*C* shows a cross-section through a larger intralobular/extralobular duct (outer diameter about 20 μm) labeled with the NCX antibody. This duct exhibits most prominent staining on the luminal membranes. We repeated staining on different preparations using cryosections of fixed pancreatic tissue, snap freeze sections, paraffin-embedded tissue and paraformaldehyde-fixed cell suspensions — altogether 15 different preparations. In all tissues, irrespective of the preparation methods used, we observed distinct polarized labeling in ducts. Closer analysis showed

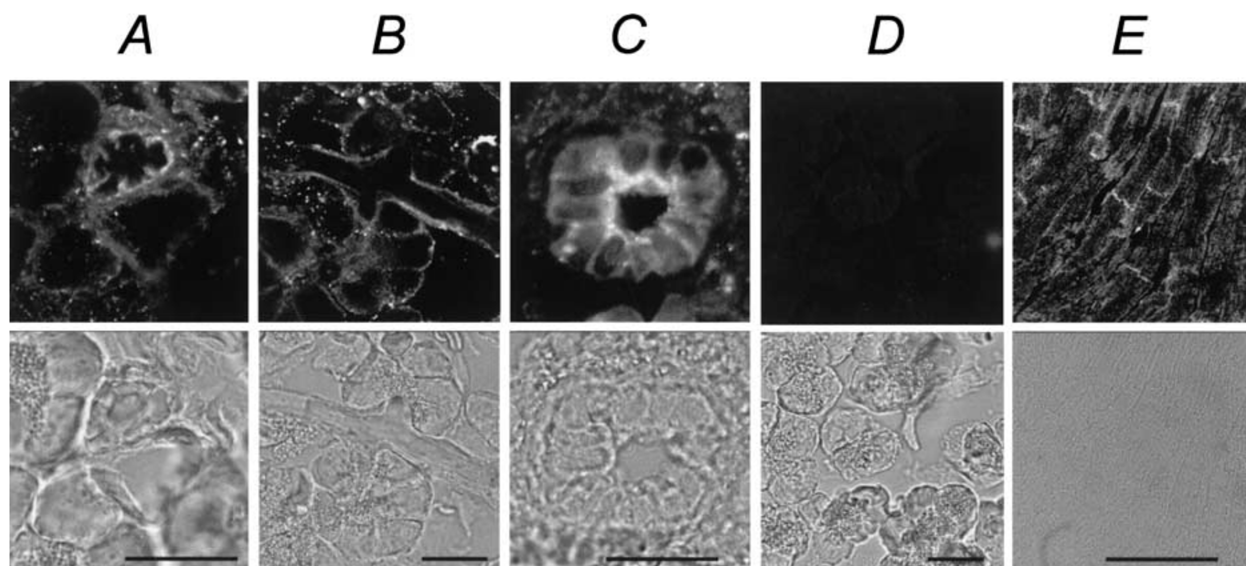

Fig. 3. Immunostainings with Na^+/Ca^{2+} exchanger antibody. Cryosections of the rat pancreas and heart were incubated with anti-NCXl antibody. Alexa 488-coupled goat anti-rabbit immunoglubulin (IgG) was used as a secondary antibody. (*A–C*) immunofluorescence images of the Na^+/Ca^{2+}-exchanger distribution in rat pancreas with corresponding transmission images. Labeling of the Na^+/Ca^{2+} exchanger is very intensive on the basolateral membrane of the smaller ducts, *A* and *B*. Larger ducts show strong labeling on the luminal membrane, *C*. (*D*) An example of a control experiment, where pancreatic tissue was treated only with the secondary antibody (Alexa 488). (*E*) Labeling of the Na^+/Ca^{2+} exchanger in the rat heart. In all images the bar is 20 μm.

that smaller ducts of 8.8 ± 0.8 μm in outside diameter expressed NCX on the basolateral membranes (n = 6). In contrast, larger ducts of 30 ± 6 μm in outside diameter, show the predominant expression of the exchanger on the luminal membranes (n = 8). In all preparations the pancreatic acini showed labeling for NCX on the basolateral membranes. Fig. 3*D* shows no significant staining in sections incubated with fluorophore-coupled secondary antibody alone. Also the autofluorescence of pancreas at the same settings used for detection of fluorescence signals was negligible (*not shown*). The rat heart, which possesses the Na^+/Ca^{2+} exchanger (*see* Introduction), was used as positive control. Fig. 3*E* shows that, as expected, the heart myocytes exhibited strong staining with the NCX antibody.

Effects of Carbachol and Secretin on Na^+/Ca^{2+} Exchange

The above results show that there is a Na^+/Ca^{2+} exchanger in pancreatic ducts. The present experiments addressed the issue whether the transporter is regulated by common pancreatic secretagogues — acetylcholine and secretin. One way to detect the Na^+/Ca^{2+} exchanger activity is by measurements of intracellular Ca^{2+} activity ($[Ca^{2+}]_i$) following the reversal of gradients for Na^+ and Ca^{2+}. Reduction of extracellular Na^+ concentrations (from 145 to 0 mM Na^+) caused the exchanger to run in the Ca^{2+} entry mode, so that it imports Ca^{2+} into the cell and Na^+ out (Fig. 4*A*, first response). In our earlier study, we have already established that such Ca^{2+} responses were due to the Na^+/Ca^{2+} exchanger, dependent on extracellular Ca^{2+}, and not to release of Ca^{2+} from intracellular pools or influx of Ca^{2+} through cation channels [8]. Assuming that the extracellular Na^+ concentration (nominally 0 mM) is about 1 mM, due to contamination, we can calculate that the driving force for Ca^{2+} entry is more than 300 mV (*see* Discussion). Acetylcholine is a regulator of secretion in pancreatic ducts and the PKC transduction pathway induces large biphasic Ca^{2+} transients with an initial peak and a secondary plateau phase, as shown earlier and also *see* below, Fig. 9, for a typical response [9, 10]. We tested if carbachol, a synthetic acetylcholine analogue, could influence Na^+/Ca^{2+} exchange. Carbachol induced an increase in $[Ca^{2+}]_i$ due to the release from intracellular Ca^{2+} stores and Ca^{2+} influx through plasma membrane channels as shown in the second part of Fig. 4*A* and Fig. 9. During the plateau phase of the carbachol response, solution was changed to a Na^+-free medium containing carbachol. The usual big increase in $[Ca^{2+}]_i$ following removal of extracellular Na^+ was reduced. Subsequent $[Ca^{2+}]_i$ response to zero Na^+ in the unstimulated ducts was restored. Fig. 4*A* also shows that HCO_3^-/CO_2 buffer can influence $[Ca^{2+}]_i$ indirectly due to changes in intracellular pH, as shown earlier [8, 10]. Fig. 4*B* shows the changes in $[Ca^{2+}]_i$ response to zero Na^+ summarized for 7 experiments. On average, carbachol (1 μM) diminished the zero-Na^+ effect by 51 ± 12%.

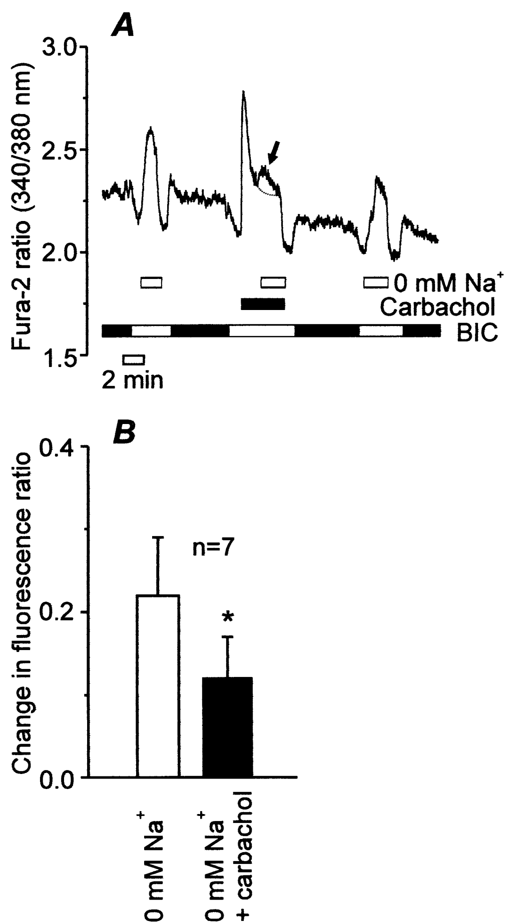

Fig. 4. Effect of carbachol on Na^+/Ca^{2+} exchange. (*A*) Original recording of the fura-2 fluorescence ratio in one pancreatic duct where Ca^{2+} influx was induced by 0 mM Na^+ in extracellular solution. The duct was stimulated by carbachol (1 μM). Note that the initial peak during carbachol stimulation is due to release of Ca^{2+} from intracellular Ca^{2+} stores. During the plateau phase (dotted line) Ca^{2+} influx was induced by 0 mM Na^+—the second small increase in Ca^{2+} (*arrow*). The duct was bathed in +BIC solution (*black bars*) or −BIC solutions (*white bars*). (*B*) Summary of data for 7 experiments shown as the mean ± SEM peak fluorescence changes just after the addition of 0 mM Na^+. *Asterisk* indicates the 0 mM Na^+ effect upon addition of carbachol was significantly lower compared to 0 mM Na^+ effect alone, taken as the average response before and after stimulation in each experiment ($p < 0.05$).

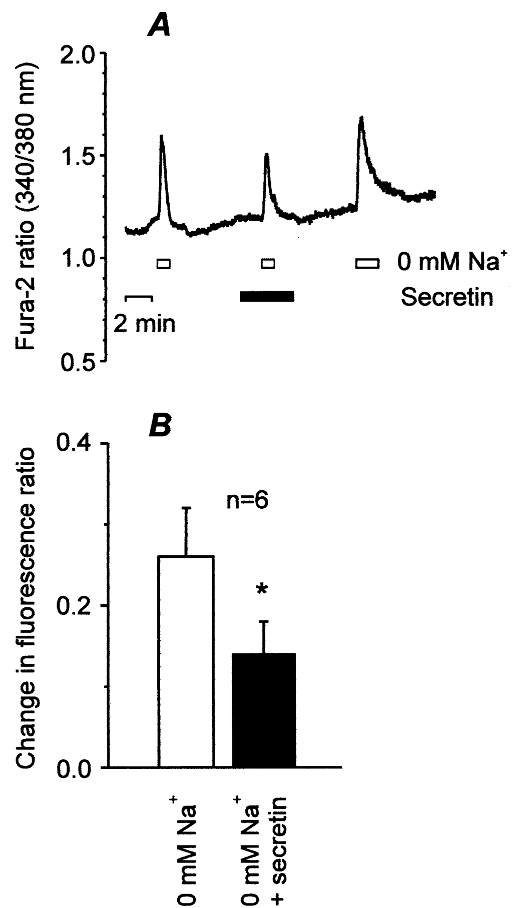

Fig. 5. Effect of secretin on Na^+/Ca^{2+} exchange. (*A*) Original recording of the fura- 2 fluorescence ratio in one duct exposed to 0 mM Na^+ three times and stimulated with secretin (1 nM). Bathing solutions just prior to 0-mM Na^+ steps were changed to -BIC (*not indicated*) as in Fig. 4. (*B*) Summary of data shown as means ± SEM from 6 experiments. *Asterisk* indicates the zero-Na^+ effect upon addition of secretin was significantly lower compared to zero-Na^+ effect alone ($p < 0.05$).

The most important regulator of pancreatic ducts is secretin. Secretin increases cAMP production and stimulates the PKA-transduction pathway, which activates cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels, as well as other transport pathways [28]. However, the action of secretin on the Ca^{2+} signaling in native pancreatic tissue is unclear. Figure 5*A* shows the effect of zero Na^+ on Ca^{2+} influx and the effect of secretin (1 nM) on this event. As the middle part of the figure shows, secretin reduced the $[Ca^{2+}]_i$ increase induced by zero Na^+. Fig. 5*B* shows a summary of six such experiments, where secretin decreased Na^+/Ca^{2+} exchange by 40 ± 10%. In these experiments secretin itself did not induce any significant changes in $[Ca^{2+}]_i$. Nevertheless, as shown in Fig. 6, sometimes secretin caused small increases in cellular Ca^{2+}. These secretin-evoked Ca^{2+} transients were small and sluggish, unlike the Ca^{2+} transients caused by carbachol and ATP. In a large series of experiments, about half of the ducts responded to secretin in the physiological range of concentrations (10 pM–10 nM), while the other half did not respond, although carbachol and ATP evoked usual increases in intracellular Ca^{2+} (n = 56)

Effect of Membrane Depolarization on $[Ca^{2+}]_i$

Although secretin and acetylcholine have such different effects on $[Ca^{2+}]_i$, they have similar effects on the membrane voltage (V_m) of pancreatic ducts, as

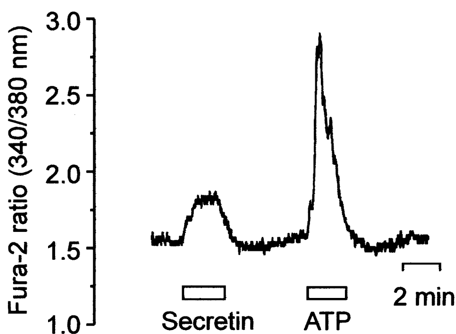

Fig. 6. Effect of secretin and ATP on intracellular Ca^{2+}. Original recording of fura-2 fluorescence ratio in one pancreatic duct. Note that secretin (10 nM) induced a moderate increase in $[Ca^{2+}]_i$ compared with ATP (0.1 mM).

shown by a number of our earlier studies [9, 27, 28]. Both secretin and acetylcholine depolarize V_m from about −60 mV to −30 mV. We performed experiments where extracellular K^+ concentration was increased from 3.6 to 30 mM, which depolarizes V_m to about −30 mV [28]. We predicted that the V_m depolarization would reverse the exchanger, and a possible small increase in $[Ca^{2+}]_i$ would be detected. However, in 16 experiments with a 30 mM K^+ step we could not detect significant effects on $[Ca^{2+}]_i$ Removal of extracellular Na^+ during the 30-mM K^+ step should accentuate the Ca^{2+} entry mode of exchange. Figure 7*A* shows that this was the case in the depicted experiment. In a large series of experiments shown in Fig. 7*B*, it can be seen that this effect was seen in 6 experiments, however, in 10 other experiments V_m depolarization decreased $[Ca^{2+}]_i$. The cause of this variable response is so far unclear and may possibly be related to the type of the duct.

Effect of Insulin on Na^+/Ca^{2+} Exchange

It is known that insulin potentiates effects of secretogogues on pancreatic secretion [18]. In cardiac myocytes insulin is also described as a stimulator of the Na^+/Ca^{2+} exchanger. In the present study, application of insulin (1 nM) itself did not change the basal cell Ca^{2+} significantly ($n = 6$). Fig. 8 shows the effect of insulin on the zero Na^+ induced changes in $[Ca^{2+}]_i$. Notably, the zero Na^+ effect was increased markedly after the application of insulin (Fig. 8*A*). Interestingly, about 5 min after removal of insulin the zero Na^+ effect became even larger. Such time-dependent and potentiating effect of insulin was observed in 6 experiments as shown in Fig. 8*B*. About 5 min after the removal of insulin, Na^+/Ca^{2+} exchange increased by 230 ± 59% above the control level. In one series of experiments we tested the theory whether insulin effects on Na^+/Ca^{2+} exchange could be due to stimulation of Ca^{2+} signaling. Hence, we tested if carbachol-induced increases in intracellular

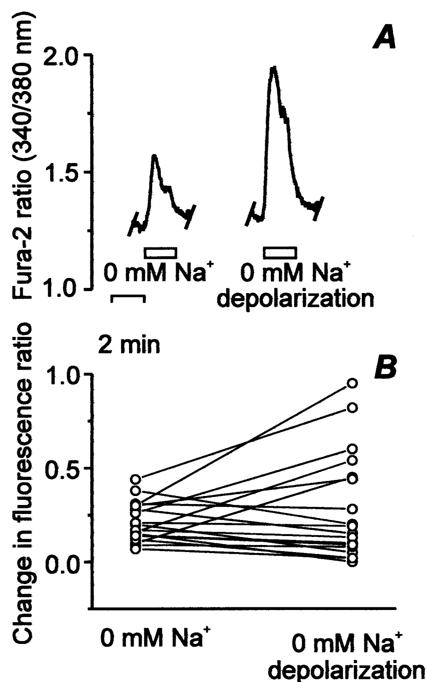

Fig. 7. Effects of V_m depolarization on Na^+/Ca^{2+} exchange. (*A*) Original recording of the fura-2 fluorescence ratio in one experiment. Depolarization of V_m induced by elevated 30 mM extracellular K^+ markedly increased zero Na^+ effect. (*B*) Summary of 17 such experiments. Paired experiments are connected with lines.

Ca^{2+} could be modulated by insulin. Fig. 9 shows that, as usual, carbachol induced biphasic Ca^{2+} transients [9]. The second part of the figure shows response of the same duct exposed to insulin (1 nM) about 5 min prior to addition of carbachol. The Ca^{2+} transient did not differ from pre-control. Similar results were obtained in altogether 5 experiments.

Discussion

The most important and new finding reported in this study is that exocrine pancreas expresses the Na^+/Ca^{2+} exchanger as demonstrated by RT-PCR and Western blot analysis. Furthermore, immunofluorescence studies show focal distribution of the Na^+/Ca^{2+} exchanger in different generations of pancreatic ducts. In functional studies on pancreatic ducts we show that pancreatic secretagogues, secretin and acetylcholine, and pancreatic hormone, insulin, influence Na^+/Ca^{2+} exchange independently of their effects on intracellular Ca^{2+} signals. Consequently, as discussed below, this exchanger might be poised for transmembrane or even transcellular Ca^{2+} transport by this tissue.

NCX1 in Pancreas

RT-PCR analysis on pancreas and pancreatic acini (Fig. 1) revealed transcripts, which most likely cor-

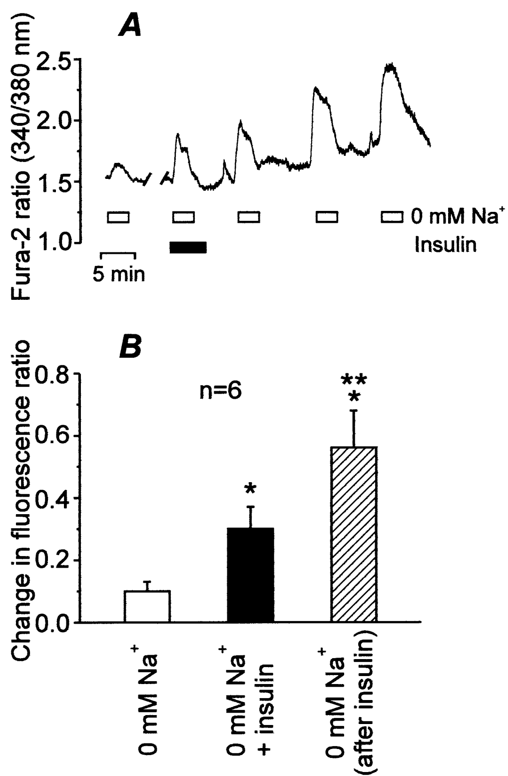

Fig. 8. Insulin increases activity of the Na^+/Ca^{2+} exchanger. (*A*) Original recording of the fura-2 fluorescence ratio in one duct repeatedly exposed to 0 mM Na^+. The zero-Na^+ effect in this experiment was relatively low (first peak), but during stimulation with insulin this effect was markedly elevated (other peaks). After washout of insulin, the zero Na^+-effect on Ca^+ increased during the rest of the experiment. Such upregulation of the response was not seen without insulin; on the contrary there was a rundown in $[Ca^{2+}]_i$ responses. Bathing solutions were +BIC or −BIC, similar to protocols shown in Fig. 4. (*B*) Summary of 6 such experiments. *Asterisk* indicates that increases in fura-2 fluorescence ratio are significantly bigger upon and about 5 min after stimulation with insulin compared with pre-control ($p < 0.05$). *Two asterisks* indicate that the $[Ca^{2+}]_i$ response after insulin was removed was significantly higher than during the preceding period ($p < 0.05$).

respond to the isoform NCX1.3, also found in other epithelial tissues [5, 19] and transcripts corresponding to isoform NCX1.7, which is also found in pancreatic B-cells [36, 37], Since the isoforms in the whole pancreas and acini were the same, one could infer that ducts do not express additional NCX1 isoforms. The heart tissue, used as the control for our primers, showed bands corresponding to the recognized isoforms NCX1.1 and NCX1.8 [31]. For the Western blot analysis (Fig. 2) it was necessary to immunoprecipitate NCX from pancreatic ducts, since they form only 2–4% of total tissue volume. By this approach we found that NCX1 antibody immunoreacted with proteins of 70, 120 and 150 kDa, although the 120-kDa band was weak. The same bands were detected in isolated acini and whole pancreas. These

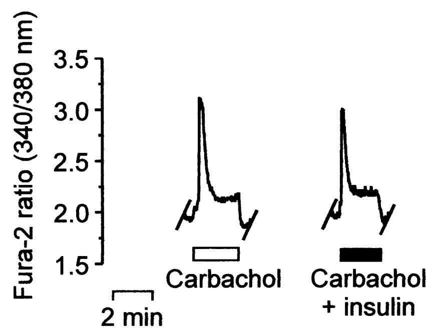

Fig. 9. Insulin does not modulate carbachol-induced increase in intracellular Ca^{2+}. Carbachol (1 μM) induces biphasic increase in intracellular $[Ca^{2+}]_i$, which is not significantly affected by insulin (1 nM). Example of one of five experiments.

results agree with studies on heart and pancreatic islet cells, indicating that they are due to the separate entities of NCX1, where the 120- and 150-kDa bands could be due to a different extent of glycosylation and/or phosphorylation of NCX1, while the origin of the 70-kDa band is still unclear [32, 37, 40].

The Na^+/Ca^+ Exchanger in Pancreatic Acini

The RT-PCR, Western blot and immunofluorescence data show localization of the Na^+/Ca^{2+} exchanger in pancreatic acini (Figs. 1–3). There are already several functional studies exploring the possibility whether the Na^+/Ca^{2+} exchanger is involved in agonist-mediated Ca^{2+} extrusion in pancreatic acini. In one study of plasma membrane vesicles there is a good indication for the Na^+/Ca^{2+} exchanger [1], which our present studies would support. Yet in studies of intact pancreatic acini, it has not been possible to unmask the exchanger, as it seems unimportant in comparison to the plasma membrane Ca^{2+}-ATPase [24, 35]. Our present studies cannot resolve this issue for pancreatic acini, but let us point out that there are other examples of epithelial cells, which probably have the Na^+/Ca^{2+} exchanger, but it is not active or important in the normal physiological conditions [12]. In contrast to pancreatic acini, pancreatic ducts have a high resting $[Ca^{2+}]_i$. The ductal Na^+/Ca^{2+} exchanger is active even in unstimulated tissue [8], and as we show in the present study (Figs. 4–9), it seems to be regulated by agonists (*see* below).

Regulation of Na^+/Ca^{2+} Exchange in Pancreatic Ducts

The present functional studies on pancreatic ducts show that carbachol and secretin inhibit about 40–50% of Na^+/Ca^{2+} exchange evoked by outwardly directed Na^+ gradient (0 mM Na^+ in extracellular medium). In contrast, insulin increased this exchange

by more than 200%. Let us first consider whether we can explain our data taking into account altered electrochemical gradients (Ca^{2+}, Na^+, pH and V_m). Regarding the Na^+ gradient, secretagogues stimulate the basolateral Na^+/H^+ exchanger and Na^+-HCO_3^- cotransporter [13], which would increase $[Na^+]_i$. This should have increased Na^+/Ca^{2+} exchange, which is not what we observe with secretin and carbachol. Regarding the intracellular pH, secretagogues alone have no detectable effects [26]. We have shown earlier that lowering of extracellular Na^+ acidified duct cells, but this did not correlate with Na^+/Ca^{2+} exchange [8]. Thus, pH variations are also unlikely to explain our results. Regarding V_m, measured in our preparation of pancreatic ducts [9, 27, 28], we can predict that secretin and acetylcholine could reverse Na^+/Ca^{2+} exchange to a Ca^{2+} entry mode even in normal extracellular Na^+ concentrations. Let us assume the stoichiometry of exchange is 3 Na^+ for 1 Ca^{2+} and the reversal potential for the exchanger is given as [3]:

$$E_{NaCa} = 3E_{Na} - 2E_{Ca}. \quad (1)$$

At the resting $[Ca^{2+}]_i$ of about 200 nM [9], $[Ca^{2+}]_e$ of 1.5 mM, $[Na^+]_e$ of 145 mM and $[Na^+]_i$ of 15 mM, E_{NaCa} would be −55 mV. Thus, at resting V_m of −60 mV, there is a small driving force on the exchanger to extrude Ca^{2+} out of the cell, as in fact observed earlier [8]. In ducts stimulated with acetylcholine, the peak $[Ca^{2+}]_i$ increases to 300 to 400 nM [9]. In normal extracellular Na^+ concentrations, E_{NaCa} would be −37 to −44 mV, and given that V_m is −30 mV, there is 7 to 14 mV of net driving force allowing Ca^{2+} entry. During the plateau phase of acetylcholine response where $[Ca^{2+}]_i$ is 250–300 nM, or in case of secretin, where a change in $[Ca^{2+}]_i$ is not detectable or at least low (*see* Results), the driving force for Ca^{2+} entry would be higher than at the peak of the acetylcholine reponse. However, experiments with simple depolarization of V_m to about −30 mV in the absence of agonists were not definitive (Fig. 7). The probable complicating factor is that V_m depolarization may also inhibit Ca^{2+} influx through Ca^{2+} permeable channels in pancreatic ducts [10]. With respect to Ca^{2+}, secretin, acetylcholine (and also insulin) have widely different effects on intracellular Ca^{2+} per se, which makes it difficult to argue that these would outweigh the large inwardly directed Ca^{2+} gradient set up by low extracellular Na^+. That is, lowering of $[Na^+]_e^+$ from 145 to about 1 mM (nominal 0 mM) would increase the driving force for Ca^{2+} entry to more than 300 mV with acetylcholine, and even more so with secretin and insulin. Yet, acetylcholine and secretin inhibited Na^+/Ca^{2+} exchange, while insulin stimulated it. Therefore, these effects on Na^+/Ca^{2+} exchange are most likely not due to increases in cellular Ca^{2+}, rather, they may be related to the regulation of the exchanger.

From many studies performed mostly on non-epithelial cells, there seems to be no common pattern of effects on protein kinase-mediated regulation of the Na^+/Ca^{2+} exchanger [3]. Stimulation of PKC pathways either upregulates, has no effect or even downregulates Na^+/Ca^{2+} exchange [3, 12]. Interestingly, phospholipase C activation may, via PIP_2, inhibit the exchanger, possibly by interacting with the endogenous XIP regulatory region on NCX [30]. PKA pathway stimulation most often downregulates, or in some cases upregulates, activity of the Na^+/Ca^{2+} exchanger [3, 22]. Considering the complexity of the protein kinase activation pathways and that there are so many isoforms of NCX, perhaps it is of no surprise that modulatory effects are so variable. It seems that the type of regulation depends on the isoforms of the NCX expressed by the given tissue. For example, PKA activation upregulates NCX1 isoforms from neurons and astrocytes that contain AD exons, but has no effect on isoforms that contain BD exons [6]. The answer to this question for pancreatic ducts and also for other cells (containing several NCX isoforms), must await further experiments on simple expression systems.

In contrast to acetylcholine and secretin, insulin had a striking stimulatory effect on the Na^+/Ca^{2+} exchanger, which persisted even after the hormone was withdrawn and was unrelated to $[Ca^{2+}]_i$ (Fig. 8). Interestingly, insulin upregulates the cardiac Na^+/Ca^{2+} exchanger, and correspondingly in diabetes Na^+/Ca^{2+} exchange is affected [34]. In some studies it seems that the activation is not only via the tyrosine kinase receptor, but may involve activation of G-proteins and PKC pathways [34], modulation of intracellular Ca^{2+} activity [14], or modulation of Na^+ activity [20]. As discussed above, it does not seem that insulin affects the Ca^{2+}/ PKC signaling pathways (Figs. 8 and 9). At this stage we cannot resolve the action of insulin on pancreatic ducts. Given the fact that there are insulin receptors on pancreatic ducts [33], that insulin released by B-cells enters directly to circulation to exocrine pancreas [25], and that insulin potentiates ductal secretion [18], makes this an important issue.

Distribution of the Na^+/Ca^{2+} Exchanger and Possible Function in Regulation of Juice ca^{2+}

One of the most interesting results of our study is the distinct immunolocalization pattern of the Na^+/Ca^{2+} exchanger in pancreatic ducts seen in pancreas prepared by different techniques. The smallest ducts, < 10 μm in outside diameter, showed basolateral localization of the exchanger, similar to pancreatic acini (Fig. 3). These ducts belong to the first generation of ducts, lying most proximal to acini, and are intercalated ducts and small intralobular ducts. Larger ducts, > 10 μm in diameter, showed the most

prominent immunolocalization of Na^+/Ca^{2+} exchanger on the luminal membranes. These larger ducts were of intralobular and extralobular origin. Histological and immunohistochemical studies of other transporters indicate differential distribution within the ductal tree (Sørensen, Amstrup and Novak, in preparation). The published data already show that it is the smallest (and most numerous) intralobular ducts that are richest in CFTR and carbonic anhydrase II expression [11, 17], and are probably the main site of secretion. In the present functional studies we could not detect any differences in the Na^+/Ca^{2+} exchanger. We used a spectrum of intralobular ducts of various sizes (*see* above), but the majority was between 20 and 40 μm in outside diameter. These ducts were similar to those used in our other studies and they have similar electrophysiological responses to acetylcholine and secretin [8, 9, 27, 28].

The differential localization of the Na^+/Ca^{2+} exchanger in ducts might reflect differences in handling of Ca^{2+}. As described in the Introduction, the origin of Ca^{2+} in pancreatic juice is unclear. In acini, Ca^{2+} would be spilled over or extruded into the lumen following Ca^{2+} signaling (*see* Introduction). In the smallest ducts, which have similar localization of the exchanger as acini, Ca^{2+} signaling and downregulation of the basolateral Na^+/Ca^{2+} exchanger with secretin or acetylcholine would lead to high intracellular Ca^{2+} that might also result in Ca^{2+} leak into the lumen if an appropriate Ca^{2+} extrusion pathway were present. It would be the job of the larger ducts to fine-regulate Ca^{2+} in the juice. One can postulate from our studies that agonists could directly, or indirectly via V_m depolarization, slow down or even reverse the luminal exchanger, so that Ca^{2+} would increase intracellularly, and in effect be available for transport across the basolateral membrane into interstitium. One set of experiments in our study indicates this might be the case: secretin inhibits the Na^+/Ca^{2+} exchanger in the Ca^{2+} exit mode and thus gives rise to small and relatively slow Ca^{2+} transients (Fig. 6). In parallel to the Ca^{2+} effect, intracellular Na^+ would decrease, thereby creating favorable gradients for other transporters involved in HCO_3^- and fluid secretion.In that respect Na^+/Ca^{2+} transport might be closely correlated with HCO_3^-/Cl^- transport and secretion. Returning back to Ca^{2+}, one study on intact dog pancreas shows that, indeed, secretin decreases the Ca^{2+} content in the pancreatic juice [21]. In support of our argument that pancreatic ducts are important in regulation of juice Ca^{2+} content are the observations that the Ca^{2+} sensing receptor is present on the luminal membrane of larger pancreatic ducts [4], and that pancreatic ducts also express calbindins thought to be associated with transepihelial Ca^{2+} transport (I. Ankorina-Stark, J. Amstrup & I. Novak, in preparation). In these respects the pancreatic ducts exhibit some parallels with the distal kidney tubules and small intestine [15, 38, 39]. Thus, future studies should be directed to elucidate the cellular regulation of the individual Na^+/Ca^{2+} exchanger isoforms, and the postulated Ca^{2+} transport pathway in ducts. This might enable us to start getting insights into the pathological conditions where increased Ca^{2+} in pancreatic juice can be one of the factors in the development of pancreatitis.

In conclusion, the present study shows that pancreatic ducts express the Na^+/Ca^{2+} exchanger. In native pancreatic ducts Na^+/Ca^{2+} exchange is downregulated by acetylcholine and secretin, but upregulated by insulin. Immunofluorescence localization of Na^+/Ca^{2+} exchanger shows distinct localization patterns within the ductal tree, which might be associated with different roles in Ca^{2+} transport.

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