Contribution of Na/Ca Exchange to Ca^{2+} Outflow and Entry in the Rat Pancreatic β-Cell

Studies With Antisense Oligonucleotides

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To characterize the role played by Na/Ca exchange in the pancreatic β-cell, phosphorothioated antisense oligonucleotides (AS-oligos) were used to knock down the exchanger in rat pancreatic β-cells. Na/Ca exchange activity was evaluated by measuring cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in single cells using fura-2. Exposure of β-cells to 500 nmol/l of the AS-oligos for 24 h inhibited Na/Ca exchange activity by ~77%. In contrast, control oligonucleotides (scrambled and mismatched) did not affect Na/Ca exchange activity. In AS-oligo–treated cells, the increase in $[Ca^{2+}]_i$ induced by membrane depolarization (K^+ or the hypoglycemic sulfonylurea, tolbutamide) was reduced by 28 or 40%, respectively. Likewise, the rate of $[Ca^{2+}]_i$ decrease after K^+ or tolbutamide removal was reduced by 72 or 40%, respectively. AS-oligos treatment also abolished the nifedipine-resistant increase in $[Ca^{2+}]_i$ induced by K^+ and profoundly altered the oscillatory or sustained increases in $[Ca^{2+}]_i$ induced by 11.1 mmol/l glucose. The present study shows that AS-oligos may specifically inhibit Na/Ca exchange in rat pancreatic β-cells. In those cells, Na/Ca exchange appears to mediate Ca^{2+} entry in response to membrane depolarization and to be responsible for up to 70% of Ca^{2+} removal from the cytoplasm upon membrane repolarization. ***Diabetes*** **47:1873–1880, 1998**

Calcium (Ca^{2+}) plays an important second messenger role in a large number of cells. To allow such a role, the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) must be tightly controlled. Two processes located at the plasma membrane appear to mediate Ca^{2+} extrusion from cells and participate in the control of $[Ca^{2+}]_i$: the Ca^{2+}-ATPase and Na/Ca exchange (1,2). While the Ca^{2+}-ATPase has a high affinity but low capacity for Ca^{2+}, the Na/Ca exchanger has a low affinity but high capacity for the divalent cation (1). The Na/Ca exchanger is an electrogenic transporter coupling Na^+ and Ca^{2+} countertransport with a stoechiometry of three Na^+ for one Ca^{2+} (3). The Na/Ca exchanger has been cloned from heart (4). Three genes coding for three different exchangers (NCX1, NCX2, and NCX3) have been identified in mammals (4–6). Further variability results from alternative splicing of NCX1, and tissue-specific variants have been identified and called NaCa1 to NaCan (7,8). While NCX1 appears to be widely distributed, NCX2 and NCX3 have been located only in the brain and the skeletal muscle (5,6).

In the heart, Na/Ca exchange appears to be the predominant mechanism for Ca^{2+} extrusion, being able to restore and control basal Ca^{2+} level on a beat-to-beat basis (9). Na/Ca exchange has also been proposed to reverse and mediate Ca^{2+} entry in cardiac cells during the action potential and to trigger Ca^{2+} release from the sarcoplasmic reticulum (10–13). However, the extent of the contribution of the exchanger to trigger Ca^{2+} release during the normal action potential and the physiological significance of the phenomenon remain controversial (14–16). Na/Ca exchange has been proposed to play key roles in Ca^{2+} homeostasis in noncardiac cells as well, such as neuronal, vascular (2), kidney (17), or pancreatic β-cells (18). In β-cells, however, the respective contribution of Ca^{2+}-ATP-ase and Na/Ca exchange to Ca^{2+} extrusion remains unknown. Likewise, the participation of the exchanger to Ca^{2+} entry during physiological cell signaling has not yet been demonstrated.

Na/Ca exchange remains difficult to investigate because of the lack of specific inhibitors of the exchanger. Several compounds are known to inhibit Na/Ca exchange but suffer from different weaknesses. Amiloride and its derivatives are not specific inhibitors of Na/Ca exchange (19). The exchange inhibitory peptide (XIP) is inactive in intact cells (20). Phe-Met-Arg-Phe-NH_2 (FMRFa)-related peptides and positively charged cyclic hexapeptides like Phe-Arg-Cys-Arg-Cys-Phe-NH_2 (FRCRCFa) (21,22) are, respectively, weak or nonspecific inhibitors of Na/Ca exchange in intact cells (23) (F.V.E., A.H., unpublished observations). The isothiourea derivative (no. 7943) inhibits the reverse mode of Na/Ca exchange but is much less potent in inhibiting forward Na/Ca exchange (24).

The aim of the present study was to determine the contribution of Na/Ca exchange to both Ca^{2+} outflow and entry in the pancreatic β-cell under physiological conditions. To do so, the new antisense oligonucleotides (AS-oligos) approach was used to repress the expression of Na/Ca exchange in rat pancreatic β-cells. The AS-oligos were designed to target the region encompassing the AUG start codon of the two NCX1 mRNA isoforms known to be present in the rat pancreatic

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Received for publication 29 January 1998 and accepted in revised form 11 August 1998.

AS, antisense; AUC, area under the curve; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; MM, mismatched; oligo, oligonucleotide; RT-PCR, reverse transcriptase–polymerase chain reaction; SCR, scrambled; XIP, exchange inhibitory peptide.

β-cell (25). AS-oligos may first act as a physical block to binding of the initiator complex to mRNA and second induce the degradation of the target transcript by RNase-H (26).

RESEARCH DESIGN AND METHODS

Phosphorothioated AS-oligos were designed to target the region encompassing the AUG start codon of the rat NCX1 mRNA. Two phosphorothioated controls were also designed to assess the specificity of the AS-oligos on Na/Ca exchange and evaluate the potential toxicity of phosphorothioate oligonucleotides: scrambled (SCR)-oligos, presenting the same base composition as AS-oligos but in disorder, and mismatched (MM)-oligos, containing 5 mismatches compared with AS-oligos. Nonsense oligonucleotides (reverse 5′ to 3′ orientation compared with antisense) were envisaged but not used because of 10- and 7-base common sequences with the AS-oligos. The sequences of the oligonucleotides follow, including the nonsense below the original nucleotide sequence of rat NCX1 cDNA (accession number x68191) running from base −7 to base 24:

NCX1 (rat)	5′-GTACAAC**ATG**CTTCGACTAAGTCTCCCACCC-3′
AS-oligo	5′-GGGTGGGAGACTTAGTCGAAG**CAT**GTTGTA*C*-3′
SCR-oligo	5′-GAACTTGGAGCGTAGGATGACGTGTGTTGCA-3′
MM-oligo	5′-GTGTGTGAGCCTTAGGCGAAG**AAT**GTTGTAC-3′
NS-oligo	5′-CATGTTG**TAC**GAAGCTGATTCAGAGGGTGGG-3′

The letters in bold are related to the start codon, the five mismatches introduced in the MM-oligos are underlined, as are the 17 common bases between the AS- and NS-oligos. Care was taken to verify that the sequences showed no homology with other known sequences in GenBank and the European Molecular Biology Laboratories. The phosphorothioated oligodeoxynucleotides were synthesized on an Applied Biosystems 394 Synthesizer (Perkin Elmer, Zaventem, Belgium) using the phosphoramidite method and the specific thiolating reagent.

To measure Na/Ca exchange mRNA levels, primers were designed to anneal to sequences flanking the hybridization region of AS-oligo with the AUG initiation codon. The sense primer 5′-GGTTGGAACAATTGGAAG-3′ and the antisense primer 5′-ACATGTAGACCATGGCCA-3′ correspond to nucleotides −32 to −15 and 239 to 256, respectively, based on rat heart sequence (accession no. X68191). Na/Ca exchange mRNA was isolated using the MicroFast Track kit (Invitrogen, Carlsbad, CA) from rat pancreatic islet cells incubated with AS-oligo for 0, 8, 16, 20, and 24 h and reverse-transcribed using the cDNA cycle kit (Invitrogen). The cDNA was then treated with RNase H for 20 min at 37°C, and the reaction was terminated by heating to 70°C for 15 min. To quantify and compare the amount of NCX1 transcripts in rat pancreatic islet cells incubated with AS-oligo for 0, 8, 16, 20, and 24 h, quantitative reverse transcription–polymerase chain reaction (RT-PCR) was used (25). The number of cycles corresponding to the linear part of the exponential phase of the PCR amplification was first determined. Single-strand cDNA (100 ng) was amplified by PCR in a volume of 20 µl using GoldStar DNA polymerase kit (Eurogentec, Ougrée, Belgium) with dATP, dCTP, dGTP, and dTTP (200 µmol/l each) (Boehringer Mannheim, Brussels, Belgium), 10 pmol of each primer, and 2.5 U GoldStr DNA polymerase. The amplification was conducted in a thermal cycler (GeneAmp PCR system 2400; Perkin Elmer) under the following conditions: initial denaturation at 94°C for 3 min; 28–36 cycles at 94°C, 52°C, and 72°C (1 min each); and a final extension at 72°C for 5 min. After amplification, the samples were analyzed on a 1.0% agarose gel stained with ethidium bromide, and the cDNA bands were quantified by scanning densitometry. The number of cycles chosen for further work was 34.

Pancreatic islets were isolated by the collagenase technique from the pancreas of fed albino (Wistar) rats. The method used to isolate pancreatic islet cells has been described elsewhere (27). After isolation, the cells were suspended in RPMI medium containing 10% newborn calf serum, 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 µg/ml gentamicine. A drop of the cell suspension (20 µl) containing 50,000 cells was placed on a round glass coverslip pretreated with poly-L-lysine, placed in a petri dish, and incubated at 37°C in a 5% CO_2, 95% O_2 incubator. After 2 h, the AS-oligos, the SCR-oligos, or the MM-oligos were added to a final concentration of 500 nmol/l in 180 µl. Control cells were grown in parallel without oligonucleotides. The cells were further incubated for ~20 h at 37°C before use.

The medium used to incubate or perfuse the islet cells consisted of Krebs-Ringer bicarbonate-buffered solution (pH 7.4, 37°C) having the following composition (in mmol/l): NaCl 115, KCl 5, $CaCl_2$ 2.6, $MgCl_2$ 1, $NaHCO_3$ 24, and glucose 2.8. The medium was equilibrated against a mixture of O_2 (95%) and CO_2 (5%) at pH 7.4. For Na^+-free solutions, NaCl was iso-osmotically replaced by choline chloride and $NaHCO_3$ by choline bicarbonate. To avoid cholinergic effects, the latter media contained atropine (10 µmol/l) that was also present in appropriate control solutions. The different media also contained, when required, glucose 11.1 mmol/l, tolbutamide 100 µg/ml, KCl 50 mmol/l, nifedipine 20 µmol/l, and ouabain 1 mmol/l. Stock solution containing fura-2 acetoxymethyl ester was prepared in dimethyl sulfoxide.

Cells were incubated with fura-2 acetoxymethyl ester (final concentration 2 µmol/l) during 60 min at 37°C in the Krebs-Ringer bicarbonate-buffered solution. The coverslips were then transferred to a tissue chamber mounted on an inverted fluorescence microscope (Diaphot TDM, Nikon, Tokyo, Japan) for epifluorescence. Fura-2 fluorescence of single cells was measured by dual-excitation fluorimetry using a camera-based image analysis system (Magical; Applied Imaging, Sunderland, U.K.). The excitation and emissions wavelengths were set at 340/380 and 510 nm, respectively, and a pair of ratioable images (at the excitations of 340 and 380 nm, 30 ms interval) were taken every 2.5 s. The $[Ca^{2+}]_i$ was calculated from the ratios of the 340- and 380-nm signals using the following:

$$[Ca^{2+}]_i = K_d \times \frac{(R - R_{min})}{(R_{max} - R)} \times \frac{Sf_2}{Sb_2}$$

where K_d is the dissociation constant for the fura-2–Ca^{2+} complex (224 nmol/l at 37°C), R is the ratio of fluorescence at 340 over 380 nm, Sf_2 is the fluorescence of free dye measured at 380 nm, and Sb_2 is the fluorescence of bound dye measured at 380 nm. R_{max}, R_{min}, and Sf_2/Sb_2 were determined in separate experiments by recording the fluorescence of fura-2 (free form) in the absence of extracellular Ca^{2+} or in the presence of a saturating Ca^{2+} concentration (28). In control experiments, the camera field encompassed ~40–50 cells, and the $[Ca^{2+}]_i$ in each individual cell (averaged over the cell surface) was measured. The tissue chamber (1 ml) was kept at a constant temperature of 37°C and perfused at a rate of 1.4 ml/min. The period of equilibration before starting fluorescence measurements was 10 min, so that the islet cells were exposed to 2.8 mmol/l glucose during ~75 min before being challenged with 11.1 mmol/l glucose.

To quantify the effect of the oligonucleotides on Na/Ca exchange and $[Ca^{2+}]_i$ changes induced by various secretagogues, the areas under the curves (AUCs) of the increases in $[Ca^{2+}]_i$ induced by extracellular Na^+ removal or the secretagogues were computed. The rates of $[Ca^{2+}]_i$ increases or decreases were computed by linear regression analysis of the $[Ca^{2+}]_i$ rising or decreasing phases in each individual cell. The results are expressed as means ± SE. The statistical significance of differences between data was assessed by using analysis of variance followed by a Bonferroni post-test. The difference in percent and type of responses to glucose was evaluated by using the χ^2 test.

RPMI, L-glutamine, penicillin, streptomycin, and gentamicine were from Gibco BRL (Merelbeke, Belgium); poly-L-lysine and ouabain from Sigma, St. Louis, MO; glucose from Merck, Darmstadt, Germany; atropine from Fluka Chemie, Buchs, Switzerland; tolbutamide from Hoechst, Frankfurt, Germany; nifedipine from Bayer, Leverkusen, Germany; and fura-2 from Molecular Probes, Eugene, OR.

RESULTS

Effect of AS-oligos on pancreatic islet cell attachment. Modified oligomers such as phosphorothioates were used because they display an increased resistance to cellular nucleases, an enhanced affinity for their target, and an improved uptake by intact cells (26). Exposure of the islet cells to AS-oligos during ~20 h was characterized by a reduction in the number of cells remaining attached to the coverslips. Indeed, the number of cells present in the camera field of preparations treated with 0.5 µmol/l AS-oligos (27 ± 4 cells) was reduced by ~46% compared with controls (50 ± 3 cells, $P < 0.001$). This reduction could not be attributed to any nonspecific effect of the oligonucleotides used (phosphorothioates), because the number of cells present in SCR- and MM-oligo–treated preparations did not differ from that of the nontreated preparations (46 ± 4 and 46 ± 3 cells, respectively). In AS-oligo–treated preparations, the cells remaining attached maintained a normal morphology and did not show any sign of apoptosis or necrosis as judged by morphology or trypan blue exclusion, respectively (data not shown). In view of the role played by Ca^{2+} in many physiological processes including cell adhesion (29), this may attest to the importance of the Na/Ca exchanger for Ca^{2+} homeostasis in the β-cell.

In preliminary experiments, various concentrations of the AS-oligos were tested, starting with a rather high concentration (3 µmol/l) (30). At concentrations higher than 0.5 µmol/l, very few cells remained attached, while concentrations lower than 0.5 µmol/l did not inhibit Na/Ca exchange to a significant extent. Therefore, all further experiments were carried out after 18–22 h (Fig. 2) of exposure to 0.5 µmol/l of the oligonucleotides.

Na/Ca exchange knockdown. Exposure of the islet cells to AS-oligos did not affect basal $[Ca^{2+}]_i$: the mean basal $[Ca^{2+}]_i$ was 193 ± 6 nmol/l in AS-oligo–treated cells compared with 190 ± 6 nmol/l in control cells ($P > 0.5$). To obtain functional evidence of Na/Ca exchange knockdown, the effect of AS-oligos treatment on $[Ca^{2+}]_i$ changes induced by extracellular Na^+ removal (stimulation of reversed Na/Ca exchange) was examined. In previous studies, we showed that this maneuver stimulated Ca^{2+} entry and $[Ca^{2+}]_i$ rise in pancreatic β-cells selectively by Na/Ca exchange (18,31). Figure 1 illustrates the

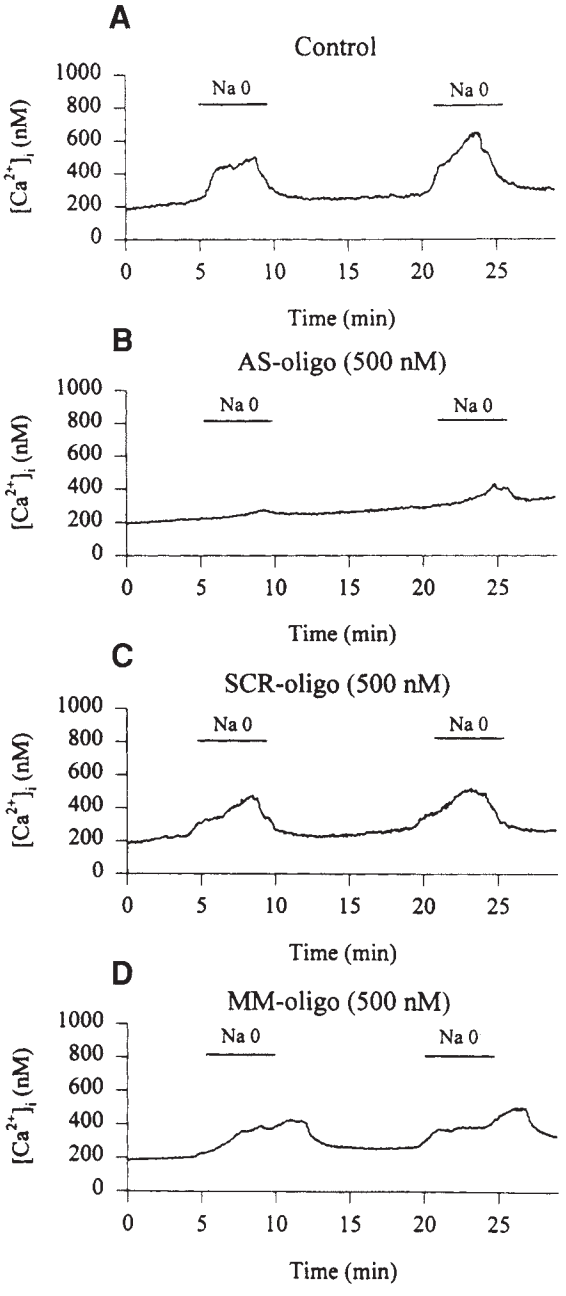

FIG. 1. $[Ca^{2+}]_i$ increases were induced by two successive reductions of the extracellular Na^+ concentration from 139 to 0 mmol/l in the presence of 2.8 mmol/l glucose in control cells (*A*), AS-oligo–treated cells (*B*), SCR-oligo–treated cells (*C*), and MM-oligo–treated cells (*D*). Periods of exposure to Na^+-free medium are indicated by bars above curves. The curves shown are the mean of more than 30 traces in each case, from two (AS, MM) or three (SCR) cell preparations.

effect of two successive removals of Na^+ from the extracellular milieu on $[Ca^{2+}]_i$ in control islet cells (*A*), AS-oligo–treated islet cells (*B*), SCR-oligo–treated islet cells (*C*), and MM-oligo–treated islet cells (*D*). In control cells, the two successive and transient Na^+ removals induced two successive, reversible, and reproducible $[Ca^{2+}]_i$ rises, a similar picture being observed in SCR- and MM-oligo–treated cells. In contrast, in AS-oligo–treated cells, the two increases in $[Ca^{2+}]_i$ were markedly reduced. Thus, in AS-oligo–treated cells, the increase in $[Ca^{2+}]_i$ induced by the absence of extracellular Na^+ (measured as the AUC during the two stimulations) was reduced by 77% compared with control cells (72.7 ± 7.6 μmol · l^{-1} · s^{-1}; $P < 0.05$). In MM- and SCR-oligo–treated cells, the mean increases in $[Ca^{2+}]_i$ did not differ from that of control cells ($P > 0.2$).

To confirm that AS-oligos acted by specifically knocking down Na/Ca exchange, the kinetics of the effect of the AS-oligos on Na/Ca exchange activity and mRNA levels was evaluated. AS-oligos induced a rapid and reversible decrease in Na/Ca exchange activity (Fig. 2*B*). Thus, after 4 h of exposure to AS-oligos, the increase in $[Ca^{2+}]_i$ induced by the absence of extracellular Na^+ (measured as in Fig. 1) only averaged 42% of control values ($P < 0.01$). At 8 and 20 h, the activity was minimal ($P < 0.001$), an increase in activity being observed at 24 h ($P < 0.05$), reflecting relief from inhibition by the AS-oligos. Figure 2*A* shows that Na/Ca exchange mRNA levels measured in the same cells showed a parallel and reversible decrease, a nadir being observed after ~16 h of exposure to the AS-oligos.

Effect of AS-oligos on $[Ca^{2+}]_i$ changes induced by various agents. To evaluate the role played by Na/Ca exchange in Ca^{2+} homeostasis, we examined the effects of Na/Ca exchange knockdown on the changes in $[Ca^{2+}]_i$ induced by different agents. Figure 3*A* illustrates the effect of membrane depolarization induced by K^+ (50 mmol/l) on $[Ca^{2+}]_i$. In control cells, K^+ induced a biphasic increase in $[Ca^{2+}]_i$ consisting of an initial peak followed by a plateau phase. The increase in $[Ca^{2+}]_i$ was rapidly reversible upon removal of K^+ from the solution. The increase in $[Ca^{2+}]_i$ recorded in AS-oligo–treated cells differed from that of control cells in several respects (Fig. 3*A* and Table 1): *1*) the increase in $[Ca^{2+}]_i$ observed during the initial peak was reduced by 28%; *2*) the rate of $[Ca^{2+}]_i$ rise during the initial peak was reduced by 53%; *3*) the rate of $[Ca^{2+}]_i$ decrease after K^+ removal from the medium was reduced by 72%; and *4*) the $[Ca^{2+}]_i$ at the end of the plateau phase was slightly lower than in controls. In contrast, none of these parameters were reduced in SCR-oligo–treated cells (Table 1). In MM-oligo–treated cells, three of the parameters were increased versus the decrease observed in AS-oligo–treated cells. The present study provides no further insight into the reason for these increases, which may represent some additional effects of the oligonucleotides (see DISCUSSION). Therefore, our data demonstrate that the changes observed in AS-oligo–treated cells are due to Na/Ca knockdown and not to any nonspecific effect of the phosphorothioates. Taken together, these data indicate that Na/Ca exchange plays a major role in Ca^{2+} extrusion from the β-cell and contributes modestly to Ca^{2+} entry in the secretory cell.

To confirm such a view, the same experiments were repeated using the hypoglycemic sulfonylurea tolbutamide to increase $[Ca^{2+}]_i$. Tolbutamide was chosen because the drug is known to increase the intracellular Na^+ content of the β-cell (32), in addition to exerting its well-known inhibitory effect

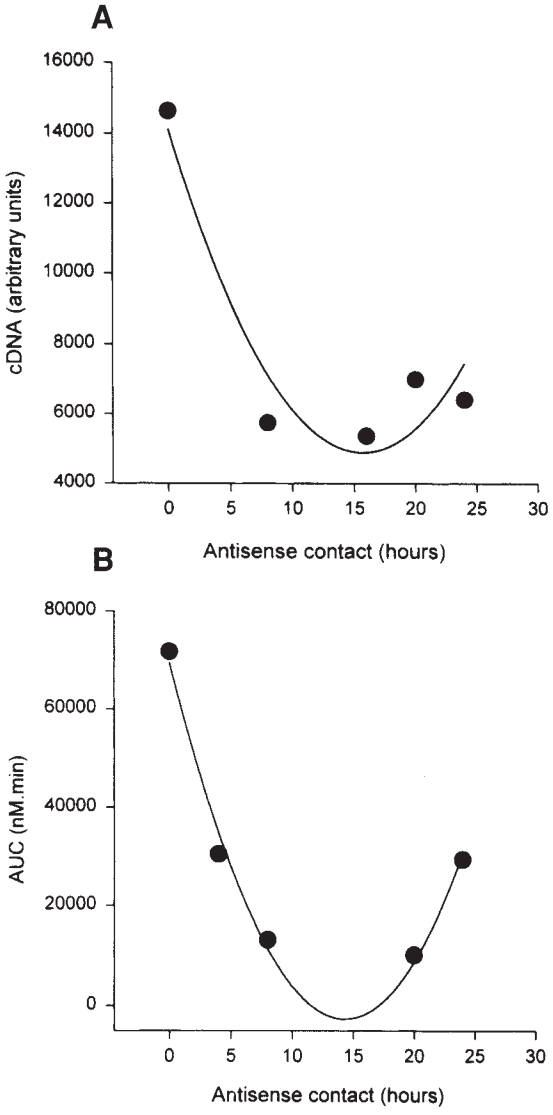

FIG. 2. Kinetics of the effect of AS-oligos on Na/Ca exchange mRNA levels (*A*) and Na/Ca exchange activity (*B*). *A*: mRNA levels were measured in batches of cells of the same cellular preparation as in *B*, exposed during various periods of time to the AS-oligos. mRNA levels were measured using the quantitative RT-PCR method (see METHODS). *B*: Same protocol as in Fig. 1, except that batches of cells from the same cellular preparation as in *A* were exposed to the AS-oligos for various periods of time. The data shown correspond to the AUC measured during the two periods of extracellular Na^+ removal and are the mean of more than 30 traces in each case.

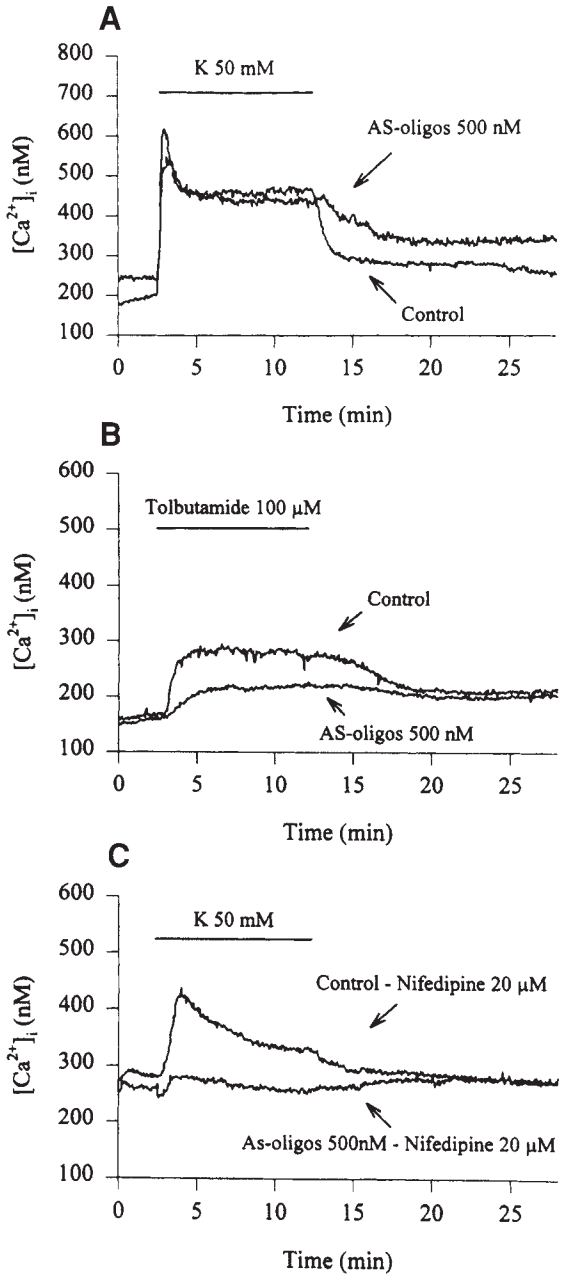

FIG. 3. Effect of KCl (*A*) or tolbutamide (*B*) on $[Ca^{2+}]_i$ in control and AS-oligo–treated pancreatic β-cells. *C*: Effect of KCl on $[Ca^{2+}]_i$ in control and AS-oligo–treated pancreatic β-cells perfused in the presence of nifedipine (20 µmol/l) throughout the experiment. Periods of exposure to KCl or tolbutamide are indicated by the bars above the curves. The curves shown are the mean of more than 50 traces in each case.

on ATP-dependent K^+ channels (33). The increase in intracellular Na^+ content should favor the reversal of Na/Ca exchange and hence Ca^{2+} entry by this mechanism. Figure 3*B* shows that tolbutamide, like K^+, rapidly and reversibly increased $[Ca^{2+}]_i$ but that the increase was of more modest magnitude and monophasic. As expected (see DISCUSSION), in AS-oligo–treated cells, the increase in $[Ca^{2+}]_i$ was more markedly inhibited than that induced by K^+. Indeed, in AS-oligo–treated cells, the increase in $[Ca^{2+}]_i$, as computed from the initial peak, was reduced by ~40% (Table 2). In agreement with this observation, there was a reduction (54%) in the rate of $[Ca^{2+}]_i$ increase, and the $[Ca^{2+}]_i$ reached at the steady state was lower in AS-oligo–treated cells (Table 2). In addition, $[Ca^{2+}]_i$ decrease after tolbutamide removal was reduced but to a lower extent (40%) than in the case of K^+. None of the changes observed with AS-oligos were seen in MM- or SCR-oligos, confirming again that the effects were not the result of any nonspecific effect of phosphorothioated oligonucleotides (Table 2).

If membrane depolarization stimulates Ca^{2+} entry into the β-cell by reverse Na/Ca exchange, the increase in $[Ca^{2+}]_i$ induced by membrane depolarization should not be completely inhibited by the Ca^{2+}-channel blocker nifedipine.

TABLE 1
Effect of AS-, MM-, and SCR-oligo treatment on $[Ca^{+2}]_i$ changes induced by 50 mmol/l KCl in the presence of 2.8 mmol/l glucose

	Control	AS	P_1*	MM	P_2	SCR	P_3
Δ peak (nmol/l)†	100 ± 3 (277)	72 ± 4 (54)	<0.001	123 ± 6 (69)	<0.001	102 ± 4 (74)	NS
Rate of initial peak increase (nmol · l^{-1} · min^{-1})	100 ± 6 (277)	47 ± 5 (54)	<0.001	134 ± 8 (69)	<0.01	110 ± 8 (74)	NS
Steady state (nmol/l)	100 ± 2 (277)	89 ± 1 (54)	<0.05	111 ± 1 (69)	<0.05	108 ± 3 (74)	NS
Rate of final decrease (nmol · l^{-1} · min^{-1})	100 ± 6 (277)	28 ± 3 (54)	<0.001	97 ± 9 (69)	NS	76 ± 6 (74)	NS

Data are means ± SE (number of experiments). Control experiments were pooled. Because there was a difference between controls, the data obtained after AS-, MM-, and SCR-oligo treatments are expressed in percent of their own controls. The mean ± SE data were 317 ± 10 nmol/l for Δ peak; 550 ± 32 nmol · l^{-1} · min^{-1} for the rate of initial peak increase; 397 ± 8 nmol/l for the steady state; and 67 ± 4 nmol · l^{-1} · min^{-1} for the rate of final decrease. *Significance of difference versus control values; †calculated as the difference between peak and basal $[Ca^{2+}]_i$ value.

Figure 3*C* shows that this is indeed the case. Thus, in the presence of a supramaximal concentration of nifedipine (13), the increase in $[Ca^{2+}]_i$ induced by K^+ (measured as the AUC during the stimulation) was inhibited by only 74% ($P < 0.025$). Figure 3*C* further shows that the residual increase in $[Ca^{2+}]_i$ seen in the presence of nifedipine was almost completely abolished (97%, $P < 0.01$) in AS-oligo–treated cells, confirming that it reflected Ca^{2+} entry through Na/Ca exchange.

Effect of AS-oligonucleotides on glucose-induced $[Ca^{2+}]_i$ increases and oscillations. Finally, to further examine the effect of antisense inhibition of Na/Ca exchange on Ca^{2+} handling in β-cells, Ca^{2+} responses evoked by 11.1 mmol/l glucose were examined. Glucose is the major physiological stimulus of insulin release, and, at 11.1 mmol/l, glucose induces cyclic $[Ca^{2+}]_i$ oscillations in a majority of cells. Figure 4 illustrates four typical individual $[Ca^{2+}]_i$ responses induced by the sugar in control cells. The cells displayed large oscillations with an almost complete return of the $[Ca^{2+}]_i$ to the basal level between the oscillations (*A*), less ample oscillations on a progressively increasing basal level (*B*), or a biphasic increase in $[Ca^{2+}]_i$ onto which no oscillations or small amplitude oscillations are superimposed (*C* and *D*, respectively). In the control situation, 82% of the cells responded to glucose by presenting one of these four profiles, and 55% of the responses were clearly oscillatory (*A* or *B* type). In contrast, only 31% of the AS-oligo–treated cells responded to glucose ($P < 0.001$), and only 33% of the responding cells displayed $[Ca^{2+}]_i$ oscillations ($P < 0.001$) that, in addition, were altered quantitatively and qualitatively as shown in Fig. 5. Thus, the oscillations were often delayed (Fig. 5*B*) or of lower magnitude (Fig. 5*A* and *C*). Again, none of these alterations were seen in SCR- and MM-oligo–treated cells ($P > 0.3$). The Ca^{2+} responses to glucose are known to display a high interindividual variability (27), so the curves were not averaged and the AUC not calculated.

DISCUSSION

When stimulated by glucose, the pancreatic β-cell displays bioelectrical activity consisting of slow waves of membrane depolarization on which action potential (spikes) are superimposed (34). In response to glucose, the β-cell also displays slow $[Ca^{2+}]_i$ oscillations mediated in part by Ca^{2+} entry through voltage-sensitive Ca^{2+} channels (27). In addition, in the pancreatic β-cell, Na/Ca exchange displays a quite high capacity (18). Therefore, the pancreatic β-cell may represent a good model for examining in depth, in a noncardiac cell, the role played by Na/Ca exchange as an extrusion or entry mechanism.

AS-oligos have been successfully used in cardiac and arterial myocytes to repress Na/Ca exchange expression and activity, and it has been proposed that AS-oligos may be useful to investigate the physiological role played by the exchanger (30,35,36). The view that the AS-oligos acted specifically by knocking down Na/Ca exchange was confirmed by the fact that none of the various effects of the AS-oligos on the changes in $[Ca^{2+}]_i$, induced by several distinct agents or experimental conditions, could be observed in MM- and SCR-oligo–treated cells. In two cases, the control oligonucleotides, instead of having no effect, exerted an effect opposite that of the AS-oligos. However, these differences only concerned some but not all parameters affected by the AS-oligos and were observed in each case with only one of the two control oligonucleotides at once. Hence, the differences may represent some sequence-selective instead of nonspecific effects of the oligonucleotides, a finding that does not challenge the view that the AS-oligos acted specifically by knocking down Na/Ca exchange. In agreement also with the specificity of the action of the AS-oli-

TABLE 2
Effect of AS-, MM-, and SCR-oligo treatment on $[Ca^{+2}]_i$ changes induced by 100 μmol/l tolbutamide in the presence of 2.8 mmol/l glucose

	Control	AS	P_1*	MM	P_2	SCR	P_3
Δ peak (nmol/l)	156.1 ± 5.2 (194)	90.6 ± 5.4 (87)	<0.001	140.5 ± 8.3 (70)	NS	147.7 ± 8.4 (55)	NS
Rate of initial peak increase (nmol · l^{-1} · min^{-1})	134.3 ± 7.8 (194)	61.7 ± 7.1 (87)	<0.001	151.5 ± 15.9 (70)	NS	190.8 ± 21.7 (55)	<0.01
Steady state (nmol/l)	308.3 ± 6.1 (194)	225.9 ± 5.5 (87)	<0.001	288.7 ± 9.6 (70)	NS	303.0 ± 8.5 (55)	NS
Rate of final decrease (nmol · l^{-1} · min^{-1})	35.5 ± 1.9 (194)	21.3 ± 1.8 (87)	<0.001	37.1 ± 3.8 (70)	NS	30.9 ± 3.0 (55)	NS

Data are means ± SE (number of experiments). *Significance of difference versus control values.

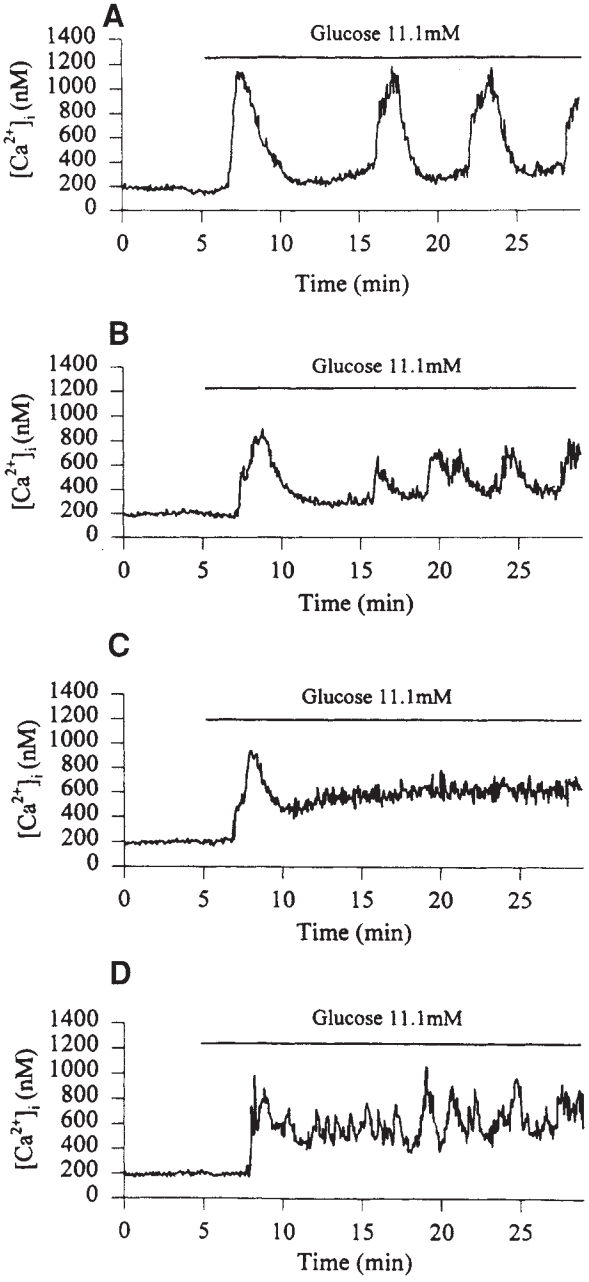

FIG. 4. Effect of glucose on $[Ca^{2+}]_i$ in control pancreatic β-cells. The curves shown are individual traces representative of the different types of response to glucose (number of cells, *A*, 21; *B*, 33; *C*, 30; *D*, 14). Periods of exposure to glucose are indicated by the bars above the curves.

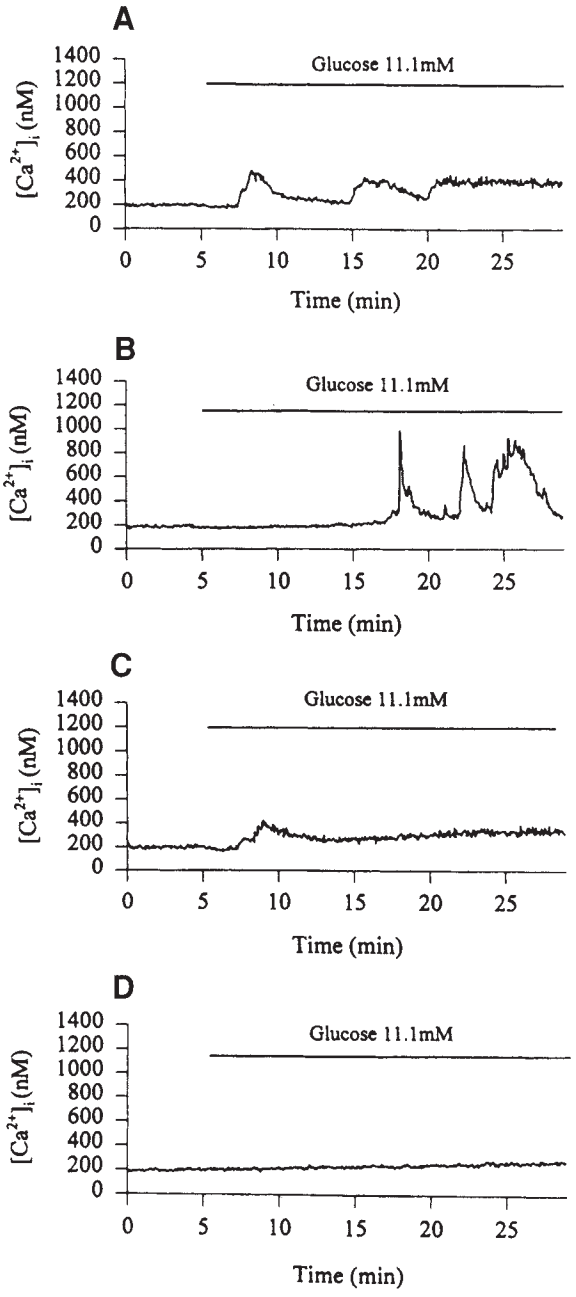

FIG. 5. Effect of glucose on $[Ca^{2+}]_i$ in AS-oligo–pretreated pancreatic β-cells. The curves shown are individual traces representative of the different types of response to glucose (number of cells, *A*, 27; *B*, 12; *C*, 28; *D*, 50). Periods of exposure to glucose are indicated by the bars above the curves.

gos, was the fall in Na/Ca exchange mRNA levels that attended the fall in Na/Ca exchange activity. Moreover, the effect on both parameters was transient and reversible, indicating that it was not of toxic or nonspecific nature. The reversible fall in both mRNA levels and Na/Ca exchange activity indicates that the hybridization of the AS-oligos with Na/Ca mRNA led to the degradation of the complex, explaining the dissipation of the effect of the AS-oligos. The short-term effect of the AS-oligos (~20 h) is in agreement with the knowledge that the exchanger is a high-turnover, low-abundance protein (37). Hence, the present study shows that AS-oligos may also specifically inhibit Na/Ca exchange expression and activity in the rat pancreatic β-cell. It further demonstrates that Na/Ca exchange knockdown profoundly alters both Ca^{2+} extrusion and Ca^{2+} entry in the β-cell in response to various agents.

Contribution of Na/Ca exchange to Ca^{2+} extrusion. The present data clearly show that Na/Ca exchange plays a predominant role in Ca^{2+} extrusion from the rat pancreatic β-cell. Indeed, within the range of the $[Ca^{2+}]_i$ reached after K^+ stimulation (200–500 nmol/l), the rate in $[Ca^{2+}]_i$ decrease seen after K^+ removal from the extracellular milieu was inhibited by ~70% in AS-oligo–treated cells, indicating that Na/Ca exchange accounts for up to 70% of the return of the $[Ca^{2+}]_i$ to its basal level upon repolarization. This view is compatible with previous indirect measurements showing

that removal of extracellular Na^+ reduced ^{45}Ca outflow from rat pancreatic islets by ~70% (38). In the heart, Na/Ca exchange plays a major role in Ca^{2+} homeostasis (9), and in the rabbit, ferret, cat, and guinea pig ventricular muscle, Ca^{2+} extrusion mechanisms are thought to be responsible for ~20–30% of Ca^{2+} removal from the cytoplasm, the extra 80–70% being removed by the sarcoplasmic reticulum (9). Therefore, in the rat β-cell, Na/Ca exchange appears to play an even more important role than in the heart, the process accounting for ~70% of the return of $[Ca^{2+}]_i$ to the basal level. This finding is consistent with the modest role played by intracellular Ca^{2+} release and hence reuptake mechanisms in β-cell stimulation, with the exception of responses evoked by agents acting on a receptor coupled to phospholipase C (39). The contribution of Na/Ca exchange to cytosolic Ca^{2+} removal was less important when the cells were stimulated by tolbutamide than by K^+. This probably results from the smaller increase in $[Ca^{2+}]_i$ induced by tolbutamide compared with K^+, Na/Ca exchange being known to display a low affinity for Ca^{2+} (1).

Contribution of Na/Ca exchange to Ca^{2+} entry. The second major finding of this study was that Na/Ca exchange also contributed to Ca^{2+} entry in the β-cell in response to membrane depolarization. In the case of K^+ stimulation, the contribution of Na/Ca exchange to Ca^{2+} entry was rather modest (28% of the initial peak). This is consistent with the situation seen in the heart, where the voltage-dependent Na/Ca exchange is suggested to generate an initial "spike" of Ca^{2+} entry, which is sufficient to trigger a fraction of the normal SR Ca^{2+} release, via Ca^{2+}-induced Ca^{2+} release (13). During the upstroke of the action potential, the membrane potential transiently becomes more positive than Na/Ca exchange equilibrium potential, so Na/Ca exchange current becomes briefly outward (40). It is interesting to notice that this reduction of 28% was equivalent to the nifedipine-resistant portion (26%) of the increase in $[Ca^{2+}]_i$ induced by K^+. In the case of tolbutamide stimulation, the contribution of the exchanger was more substantial (42% of the peak). This was expected, because tolbutamide is known to increase intracellular Na^+ content of the pancreatic β-cell (32), a phenomenon that should favor reversal of Na/Ca exchange. In the heart also, it has been proposed that Na^+ accumulation due to the opening of voltage-sensitive Na^+ channels could mediate Ca^{2+} entry through Na/Ca exchange (10,11). The contribution of the exchanger to Ca^{2+} entry in response to glucose was more difficult to quantify because the Ca^{2+} responses to glucose display a high heterogeneity (27). Nevertheless, it is clear from our data that Na/Ca exchange knockdown had a definite effect on Ca^{2+} entry in response to glucose. Indeed the $[Ca^{2+}]_i$ responses to glucose were reduced instead of increased in magnitude, as one would expect from the knockdown of a mechanism responsible only for Ca^{2+} extrusion.

Cells are known to be equipped with complex regulatory systems, many of them competitive or redundant. For instance, in the case of Ca^{2+} removal from the cytoplasm upon membrane repolarization, three systems could be in competition: the ATPases of the plasma membrane and the endoplasmic reticulum and Na/Ca exchange. Hence, the knockdown of one system could be compensated for by an increase in the activity of the other Ca^{2+}-handling mechanisms (41). Therefore, the exact contribution of Na/Ca exchange activity to Ca^{2+} entry and outflow as proposed in the present study could in fact be underestimated. Likewise, one may wonder whether the changes in $[Ca^{2+}]_i$ observed in our study may not result, at least in part, from changes in internal Ca^{2+} stores. Indeed, interventions promoting Ca^{2+} store refilling have been proposed to produce β-cell hyperpolarization and inhibit Ca^{2+} influx (42). Although such a view cannot be completely excluded, the present study did not explore the possibility that Na/Ca exchange knockdown may indeed fill or refill intracellular stores.

Incidentally, the conclusions reached in the present study concerning the role played by the exchanger in Ca^{2+} outflow and entry in the rat pancreatic β-cell may not necessarily be valid in the β-cell of other species. In the heart, species differences in Na/Ca exchange activity have been demonstrated (9). For example, whereas the exchanger accounts for 20–30% of the total Ca^{2+} removal in rabbit, ferret, cat, and guinea pig myocytes, the corresponding value in the rat myocyte is 7% (9). Such differences may also exist between β-cells from various species. In fact, we recently observed that Na/Ca exchange activity and mRNA level of transcription were 50% lower in mouse compared with rat pancreatic islets. In addition, the isoforms expressed in rat and mouse islets are not the same, leaving the possibility of differential regulation of the exchanger in different species (F.V.E., V. Duquesne, A. Bollen, A.H., unpublished observations). In agreement with the view of an interspecies difference, Nadal et al. (43) observed that Na/Ca exchange was less effective than Ca^{2+} pumps in the handling of Ca^{2+} in mouse β-cells. However, it must be emphasized that this view was based on studies in which extracellular Na^+ was reduced to 20 mmol/l instead of 0 mmol/l. In previous studies, we showed that reduction of extracellular Na^+ to 0 mmol/l inhibited Na/Ca exchange (forward mode) and stimulated reverse Na/Ca exchange more markedly than reduction of Na^+ to 24 mmol/l (18,38). In addition, the use of AS-oligos has the advantage over extracellular Na^+ removal in that it does not perturb various intracellular processes such as Na^+/H^+ exchange, Na^+-K^+-ATPase, glucose metabolism, etc., which may confuse the situation. Nadal et al. also neglected the fact that Na/Ca exchange is ATP regulated (44). Also, Garcia-Barrado et al. (45) suggested that Ca^{2+} influx by reverse Na/Ca exchange may not occur in the mouse pancreatic β-cell.

In conclusion, our study shows that AS-oligos may specifically inhibit Na/Ca exchange expression and activity in the rat pancreatic β-cell. In the latter cell, Na/Ca exchange appears to mediate Ca^{2+} entry in response to membrane depolarization and to be responsible for up to 70% of Ca^{2+} removal from the cytoplasm upon membrane repolarization. The present data underline the importance of the exchanger in the regulation of Ca^{2+} homeostasis in rat pancreatic β-cells.

ACKNOWLEDGMENTS

This work was supported by the Belgian Fund for Scientific Research (FRSM Nr 3.4545.96, LN 9.4514.93, and LN 9.4510.95), of which F.V.E. has been a research assistant (1993–1997).

We thank P. Galand and R. Pochet (Laboratoire de Cytologie Experimentale and Laboratoire d'Histologie, respectively) for their help in analyzing cell morphology.We are indebted to C. Pastiels for technical help and to A. Van Elsen for oligonucleotide synthesis.

REFERENCES

1. Carafoli E: Membrane transport of calcium: an overview. *Method Enzymol* 157:3–11, 1988
2. Blaustein MP, Goldman WF, Fontana G, Krueger BK, Santiago EM, Steele TD, Weiss DN, Yarowsky PJ: Physiological roles of the sodium-calcium exchanger in nerve and muscle. *Ann N Y Acad Sci* 639:254–274, 1991
3. Eisner DA, Lederer WJ: Na-Ca exchange: stoichiometry and electrogenicity. *Am J Physiol* 248:C189–C202, 1985
4. Nicoll DA, Longoni S, Philipson KD: Molecular cloning and functional expression of the cardiac sarcolemmal Na^+-Ca^{2+} exchanger. *Science* 250:562–565, 1990
5. Li Z, Matsuoka S, Hryshko LV, Nicoll, DA, Bersohn MM, Burke EP, Lifton RP, Philipson KD: Cloning of the NCX2 isoform of the plasma membrane Na^+-Ca^{2+} exchanger. *J Biol Chem* 269:17434–17439, 1994
6. Nicoll DA, Quednau BD, Qui Z, Xia Y-R, Lusis AJ, Philipson KD: Cloning of the third mammalian Na^+-Ca^{2+} exchanger, NCX3. *J Biol Chem* 271:24914–24921, 1996
7. Kofuji P, Lederer WJ, Schulze DH: Mutually exclusive and cassette exons underlie alternatively spliced isoforms of the Na/Ca exchanger. *J Biol Chem* 269:5145–5149, 1994
8. Lee S-L, Yu ALS, Lytton J: Tissue-specific expression of Na^+-Ca^{2+} exchanger isoforms. *J Biol Chem* 269:14849–14852, 1994
9. Bers DM, Bassani JWM, Bassani RA: Na-Ca exchange and Ca fluxes during contraction and relaxation in mammalian ventricular muscle. *Ann N Y Acad Sci* 779:430–442, 1996
10. Leblanc N, Hume JR: Sodium current-induced release of calcium from cardiac sarcoplasmic reticulum. *Science* 248:372–376, 1990
11. Lipp P, Niggli E: Sodium current-induced calcium signals in isolated guinea-pig ventricular myocytes. *J Physiol* 474:439–446, 1994
12. Kohmoto O, Levi AJ, Bridge HB: Relation between reverse sodium-calcium exchange and sarcoplasmic reticulum calcium release in guinea pig ventricular cells. *Circ Res* 74:550–554, 1994
13. Levi AJ, Spitzer KW, Kohmoto O, Bridge JHB: Depolarization-induced Ca entry via Na-Ca exchange triggers SR release in guinea pig cardiac myocytes. *Am J Physiol* 266:H1422–H1433, 1994
14. Sham JSK, Cleemann L, Morad M: Gating of the cardiac Ca^{2+} release channel: the role of Na^+ current and Na^+-Ca^{2+} exchange. *Science* 255:850–853, 1992
15. Bouchard RA, Clark RB, Giles WR: Role of sodium-calcium exchange in activation of contraction in rat ventricle. *J Physiol* 472:391–413, 1993
16. Cannell MB, Grantham CJ, Main MJ, Evans AM: The roles of the sodium and calcium current in triggering calcium release from the sarcoplasmic reticulum. *Ann N Y Acad Sci* 779:443–450, 1996
17. Windhager EE, Frindt G, Milovanovic S: The role of the Na-Ca exchanger in renal epithelia. *Ann N Y Acad Sci* 639:577–591, 1991
18. Plasman P-O, Lebrun P, Herchuelz A: Characterization of the process of sodium-calcium exchange in pancreatic islet cells. *Am J Physiol* 259:E844–E850, 1990
19. Garcia ML, King VF, Shevell JL, Slaughter RS, Suarez-Kurtz G, Winquist RJ, Kaczorowski GJ: Amiloride analogs inhibit L-type calcium channels and display calcium entry blocker activity. *J Biol Chem* 265:3763–3771, 1990
20. Li Z, Nicoll DA, Collins A, Hilgemann DW, Filoteo AG, Penniston JT, Weiss JN, Tomich JM, Philipson KD: Identification of a peptide inhibitor of the cardiac sarcolemmal Na^+-Ca^{2+} exchanger. *J Biol Chem* 266:1014–1020, 1991
21. Khananshvili D, Price DC, Greenberg MJ, Sarne Y: Phe-Met-Arg-Phe-NH_2 (FMRFa)-related peptides inhibit Na^+-Ca^{2+} exchange in cardiac sarcolemma vesicles. *J Biol Chem* 268:200–205, 1993
22. Khananshvili D, Shaulov G, Weil-Maslansky E, Baazov D: Positively charged cyclic hexapeptides, novel blockers for the cardiac sarcolemma Na^+-Ca^{2+} exchanger. *J Biol Chem* 270:16182–16188, 1995
23. Van Eylen F, Gourlet P, Vandermeers A, Lebrun P, Herchuelz A: Inhibition of Na/Ca exchange by Phe-Met-Arg-Phe-NH_2 (FMRFa)-related peptides in intact rat pancreatic B-cells. *Mol Cell Endocrinol* 106:R1–R5, 1994
24. Iwamoto T, Watano T, Shigekawa M: A novel isothiourea derivative selectively inhibits the reverse mode of Na^+/Ca^{2+} exchange in cells expressing NCX1. *J Biol Chem* 271:22391–22397, 1996
25. Van Eylen F, Svoboda M, Herchuelz A: Identification, expression pattern and potential activity of Na/Ca exchanger isoforms in rat pancreatic B-cells. *Cell Calcium* 21:185–193, 1997
26. Hélène C, Toulmé JJ: Specific-regulation of gene expression by antisense, sense and antigene nucleic acids. *Biochem Biophys Acta* 1049:99–125, 1990
27. Gobbe P, Herchuelz A: Does glucose decrease cytosolic free calcium in normal pancreatic islet cells? *Res Commun Chem Pathol Pharmacol* 63:231–247, 1989
28. Grynkiewicz G, Poenie M, Tsien RY: A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 260:3440–3450, 1985
29. Heimark RL: Calcium-dependent and calcium-independent cell adhesion molecules in endothelium. *Ann N Y Acad Sci* 639:229–239, 1991
30. Lipp P, Schwaller B, Niggli E: Specific inhibition of Na-Ca exchange function by antisense oligodeoxynucleotides. *FEBS Lett* 364:198–202, 1995
31. Herchuelz A, Lebrun P: A role for Na/Ca exchange in the pancreatic B cell: studies with thapsigargin and caffeine. *Biochem Pharmacol* 45:7–11, 1993
32. Ali L, Wesslén N, Hellman B: Sulphonamide modulation of sodium content in rat pancreatic islets. *Eur J Pharmacol* 158:257–262, 1988
33. Sturgess NC, Ashford MLJ, Cook DL, Hales CN: The sulphonylurea receptor may be an ATP-sensitive potassium channel. *Lancet* 8453:474–475, 1985
34. Meissner HP, Schmelz H: Membrane potential of beta-cells in pancreatic islets. *Pflügers Arch* 351:195–206, 1974
35. Slodzinski MK, Juhaszova M, Blaustein MP: Antisense inhibition of Na^+/Ca^{2+} exchange in primary cultured arterial myocytes. *Am J Physiol* 269:C1340–C1345, 1995
36. Takahashi K, Bland KS, Islam S, Michaelis ML: Effects of antisense oligonucleotides to the cardiac Na^+/Ca^{2+} exchanger on cultured cardiac myocytes. *Biochem Biophys Res Commun* 212:524–530, 1995
37. Cheon J, Reeves JP: Site density of the sodium-calcium exchange carrier in reconstituted vesicles from bovine cardiac sarcolemma. *J Biol Chem* 263:2309–2315, 1988
38. Herchuelz A, Sener A, Malaisse WJ: Regulation of calcium fluxes in rat pancreatic islets: calcium extrusion by sodium-calcium countertransport. *J Membr Biol* 57:1–12, 1980
39. Prentki M, Matschinsky FM: Ca^{2+}, cAMP, and phospholipid-derived messengers in coupling mechanisms of insulin secretion. *Physiol Rev* 67:1185–1248, 1987
40. Janvier NC, Boyett MR: The role of Na-Ca exchange current in the cardiac action potential. *Cardiovasc Res* 32:69–84, 1996
41. Liu B-F, Xu Xin, Fridman R, Muallem S, Kuo TH: Consequences of functional expression of the plasma membrane Ca^{2+} pump isoform 1a. *J Biol Chem* 271:5536–5544, 1996
42. Worley JF III, McIntyre MS, Spencer B, Dukes ID: Depletion of intracellular Ca^{2+} stores activates a maitotoxin-sensitive nonselective cationic current in β-cells. *J Biol Chem* 269:32055–32058, 1994
43. Nadal A, Valdeolmillos M, Soria B: Metabolic regulation of intracellular calcium concentration in mouse pancreatic islets of Langerhans. *Am J Physiol* 267:E769–E774, 1994
44. DiPolo R, Beaugé L: Ca^{2+} transport in nerve fibers. *Biochim Biophys Acta* 947:549–569, 1988
45. Garcia-Barrado M-J, Gilon P, Sato Y, Nenquin M, Henquin J-C: No evidence for a role of reverse Na^+-Ca^{2+} exchange in insulin release from mouse pancreatic islets. *Am J Physiol* 271:E426–E433, 1996