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INHIBITION OF Na/Ca EXCHANGE IN PANCREATIC ISLET CELLS BY 3',4'-DICHLOROBENZAMIL

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Abstract—Na/Ca exchange may play a role in Ca^{2+} extrusion from the pancreatic B cell. The role played by the exchanger was examined by characterizing the effects of 3'-4'-dichlorobenzamil on ionic fluxes and insulin release in normal rat pancreatic islet cells. 3',4'-Dichlorobenzamil potently inhibited ^{45}Ca uptake mediated by reverse Na/Ca exchange (IC_{50}: 18 μM) in islet cells. The drug failed to decrease intracellular pH but reduced ^{86}Rb outflow from perifused islets. The effects of glucose and 3',4'-dichlorobenzamil on ^{86}Rb outflow were not additive. The drug potently blocked ^{45}Ca uptake through voltage-sensitive Ca^{2+} channels (IC_{50}: 7.5 μM).

In the presence of extracellular Ca^{2+} and 3',4'-dichlorobenzamil, glucose lost part of its ability to reduce ^{45}Ca outflow. The drug failed to affect the secondary rise in ^{45}Ca outflow induced by the sugar. In the absence of extracellular Ca^{2+}, 3',4'-dichlorobenzamil induced a delayed inhibition of ^{45}Ca outflow, the effect of the sugar and the drug being not additive. This effect of 3',4'-dichlorobenzamil and its ability to impair the inhibitory effect of glucose were reproduced by the removal of extracellular Na^+ and disappeared under the latter experimental condition. 3',4'-Dichlorobenzamil did not affect insulin release in the absence of glucose but significantly increased glucose-induced insulin release when used at a high concentration. It is concluded that 3',4'-dichlorobenzamil is a potent inhibitor of the process of Na/Ca exchange in the pancreatic B cell. Unfortunately, the drug is of poor specificity and blocks, in the same range of concentrations, both K^+ channels and voltage-sensitive Ca^{2+} channels. The data also indicate that glucose inhibits ^{45}Ca outflow from pancreatic islets to a great extent (at least 75%) by inhibiting Na/Ca exchange. The type of Na/Ca exchange that is inhibited by glucose, remains to be elucidated.

A rise in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) represents a major step in the process of glucose-induced insulin release from the pancreatic B cell [1–4]. Although it is well established that an increase in Ca^{2+} inflow is a major modulator of $[Ca^{2+}]_i$ in B cells, the mechanisms of Ca^{2+} extrusion from these cells remain poorly characterized [5].

Na/Ca exchange plays an important role in Ca^{2+} outflow from many types of cells [6, 7]. This process of exchange is powered by the Na^+ electrochemical gradient and couples the transport of Na^+ and Ca^{2+} in opposite directions. Since Na/Ca exchange is thought to be an electrogenic and completely reversible transport system, it may mediate either Ca^{2+} efflux or influx depending on the prevailing Na^+ electrochemical gradient and the membrane potential [7].

The existence of a process of Na/Ca exchange in pancreatic B cells has been suggested about 10 years ago [8]. However, the process was only recently shown to display properties similar to those observed in other cells: complete reversibility and sensitivity to membrane potential [5]. Initially, glucose, the main physiological stimulant of insulin release, was proposed to inhibit Na/Ca exchange, working in the Ca^{2+} efflux mode, of the pancreatic B cell [8, 9]. This view, however, has been challenged [10, 11]. Therefore, the aim of the present study was to further characterize the process of Na/Ca exchange in the pancreatic B cell and to investigate its role in the process of glucose-induced insulin release.

For this purpose, we have examined the secretory and ionic actions of 3',4'-dichlorobenzamil (DCB) in pancreatic islet cells. DCB is one of the most potent organic inhibitor of the process of Na/Ca exchange [12].

MATERIALS AND METHODS

Cell preparations. Perifusion experiments were carried out using pancreatic islets removed from fed albino rats. ^{45}Ca uptake and intracellular pH measurement were performed with dissociated islet cells prepared from the latter islets. The method used to isolate pancreatic islet cells has been described elsewhere [13]. In brief, after exposure to a Ca^{2+} deprived medium, the islets were disrupted using dispase and centrifuged through an albumin solution to remove cell debris and dead cells. Cell viability as assessed by trypan blue exclusion was about 97%. The responsiveness to glucose of the cell preparation has been previously assessed [13]. After isolation, the cells were incubated at 37° during 1 hr in 0.5 mL of RPMI 1640 culture medium (Gibco Europe, Gent, Belgium) containing 0.5% albumin, glutamine 2.3 mM and glucose 11.1 mM under a 95% O_2 and 5% CO_2 gas phase.

Media. The media used for incubating, washing of perifusing the islets consisted of a Krebs–Ringer

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bicarbonate-buffered solution containing (in mM) 115 NaCl, 5 KCl, 2.56 $CaCl_2$, 1 $MgCl_2$ and 24 $NaHCO_3$. The medium was supplemented with 0.5% (w/v) dialyzed albumin (fraction V; Sigma Chemical Co., St Louis, MO, U.S.A.) and equilibrated against a mixture of O_2 (95%) and CO_2 (5%).

When complete suppression of sodium was required, NaCl (115 mM) was replaced by sucrose (200 mM) (Merck, Darmstadt, F.R.G.), while $NaHCO_3$ (24 mM) was replaced by choline bicarbonate (24 mM) (Sigma Chemical Co.). In all experiments performed in the presence of choline salts, the media also contained atropine sulfate (3.6 μM) in order to prevent cholinergic side effects [8]. Some media contained no $CaCl_2$ and were enriched with 0.5 mM EGTA (ethyleneglycolbis (β-aminoethylether)*N*,*N*'-tetra-acetic acid).

Experiments conducted on incubated islet cells for the determination of intracellular pH (pH_i) and ^{45}Ca uptake were performed using a Krebs–Ringer solution containing (in mM) 115 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$ buffered with *N*-2-hydroxyethylpiperazine-*N*'-2 ethanesulfonic acid-sodium hydroxide (Hepes–NaOH, 10 mM; pH 7.4) and gassed with ambiant air. In some experiments NaCl was isoosmotically replaced by sucrose (241 mM) and Hepes–NaOH by Hepes–KOH. The different media also contained, as required, glucose (Merck, Darmstadt, F.R.G.), nifedipine (Bayer, Belgium), 3',4'-dichlorobenzamil (DCB) prepared as described by Cragoe *et al.* [14]. Nifedipine and DCB were dissolved in dimethylsulfoxide, which was added to both control and test media at final concentrations not exceeding 0.1% (v/v).

^{45}Ca efflux, ^{86}Rb efflux and insulin release. The method used for the measurement of ^{45}Ca efflux, ^{86}Rb efflux and insulin release from perifused islets has been described elsewhere [8, 15]. Briefly, groups of 100 islets each were incubated for 60 min in the presence of 16.7 mM glucose and either ^{86}Rb (0.15–0.25 mM; 50 μCi/mL) or ^{45}Ca (0.02–0.04 mM, 100 μCi/mL). After incubation, the islets were washed three times and then placed in a perifusion chamber. The perifusate was delivered at a constant rate (1.0 mL/min). From the 31st to the 90th min, the effluent was collected continuously over successive periods of 1 min each. An aliquot of the effluent (0.6 mL) was used for scintillation counting while the remainder was stored at −20° for insulin assay. At the end of the perifusion, the radioactive content of the islets was also determined. The efflux of ^{86}Rb and ^{45}Ca (cpm/min) was expressed as a fractional outflow rate (% of instantaneous islet content per min (FOR, see Ref. 8)). The validity of ^{86}Rb as a tracer for the study of K^+ handling in the islets has been previously assessed [16].

^{45}Ca uptake. The method used for the measurement of ^{45}Ca uptake has been previously described [5]. In brief, after incubation in culture medium, the cells were centrifuged (500 *g*, 3 min) to remove the supernatant, and preincubated at 37° in 1 mL of a non radioactive modified Krebs–Ringer solution buffered with Hepes. After 30 min, the cells were again centrifuged and incubated at 37° for 5 min in 1 mL of the same medium containing in addition ^{45}Ca (10 μCi/mL). When required, KCl (20 mM) was added to the incubation medium. 3'.4'-dichlorobenzamil was added to both preincubation and incubation media.

At the end of the incubation, the short term ^{45}Ca uptake was stopped by the addition of 5 mL of an ice-cold "stop-solution". The "stop-solution" consisted of a Krebs–Ringer solution containing $LaCl_3$ (2 mM) and albumin (20 mg/mL), and that had been adjusted at pH 7.1 to avoid any precipitation of the lanthanum ion. After 20 min, the cells were centrifuged, the radioactive supernatant was discarded and the cells resuspended in 1 mL of the same ice-cold "stop-solution". Aliquots of 0.1 mL of this suspension were then placed in polyethylene microcentrifuge tubes. A first centrifugation (30 sec; Beckman Microfuge) was performed to deposit the cells in the tip of the tube. Di-*n*-butylphthalate (0.1 mL, Sigma Chemical Co.) was layered on the top of the solution and a second centrifugation (30 sec) performed to separate the islet cells from the medium. The bottom of the tube was cut and transfered to a counting vial to which 5 mL of scintillation fluid was added (Lumagel, Lumac, The Netherlands). The uptake of ^{45}Ca was expressed as femtomoles of Ca^{2+} with the same specific activity as that of the incubation medium.

Intracellular pH. Intracellular pH measurements were performed using the pH-sensitive fluorescent dye BCECF according to Rink *et al.* [17]. After preincubation in culture medium for 1 hr, the cells were incubated during 30 min in the same medium containing, in addition, BCECF-AM (2',7'-bis-(2-carboxyethyl)-5(and-6)-carboxy fluorescein, tetra-acetoxymethylester; Molecular Probes, Eugene, OR, U.S.A.), to reach a final concentration of 1 μM. After loading, the cells were washed twice and placed in a quartz cuvette (1.5 mL) containing 0.75 mL of Hepes–NaOH buffer. BCECF fluorescence of loaded cells was measured using a Perkin-Elmer spectrofluorometer (LS 5). The fluorometric cuvette holder was thermostated at 37° and the cell suspension continuously stirred. Excitation and emission wave lengths were 500 and 530 nm, respectively. After 15–20 min equilibration, fluorescence measurement was started and the islet cells challenged about 10 min later. Tests substances were added to the cuvette in small aliquots to avoid dilution to the medium. Calibration of BCECF fluorescence was carried out by adding successively to the cuvette digitonin (final concentration: 50 μM) and aliquots of 1 μL of HCl 1 N to measure fluorescence at various values of extracellular pH. The pH of the incubation medium was measured using a pH-sensitive electrode. pH_i was estimated from the traces by reference to a standard curve obtained at the end of each individual experiment.

Calculations and statistics. The basal value for ^{86}Rb and ^{45}Ca outflow, and for insulin release was computed from min 40 to min 44 of perifusion (Figs 3 and 5). The inhibitory effect of DCB on either ^{86}Rb outflow or ^{45}Ca outflow (Fig. 3, Fig. 5 upper panel and Fig. 6) was taken as the difference between the mean values for outflow recorded during min 40–44 in test and control experiments. The inhibitory effect of glucose on ^{86}Rb outflow (Fig. 3), was taken

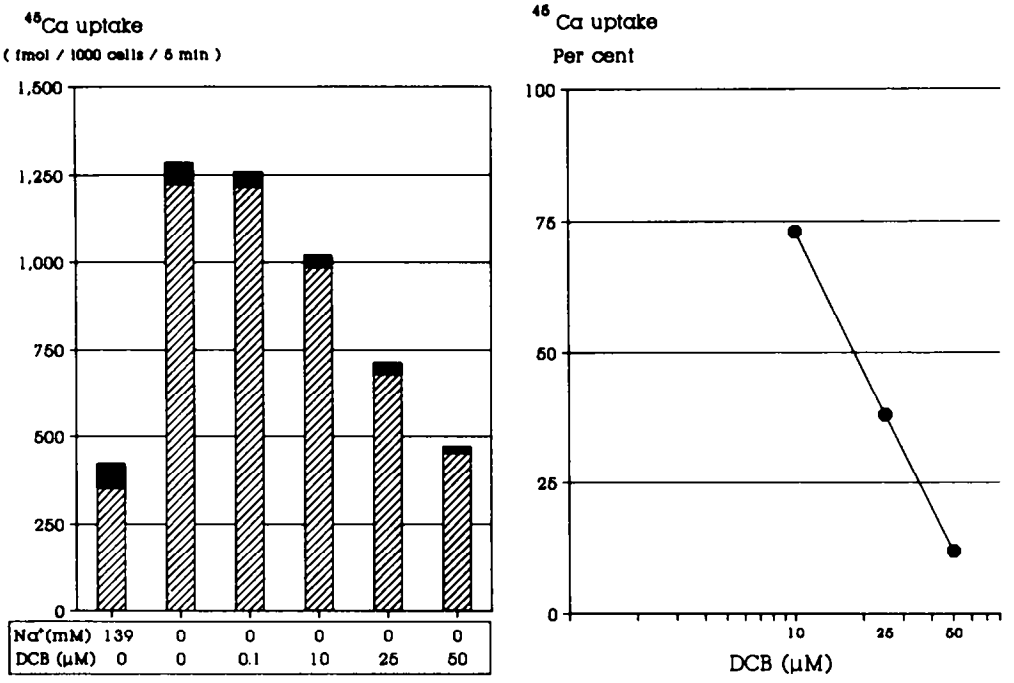

Fig. 1. Left panel: Effect of DCB on ^{45}Ca uptake induced by the absence of Na^+ in isolated islet cells. Extracellular Na^+ was isoosmotically replaced by sucrose. Mean values + SEM (black box at top of the bars) are expressed in fmol/1000 cells/5 min and refer to at least 14 individual samples in each case. Right panel: Semi-logarithmic plot of the same data presented in per cent of the control value found within the same experiment in the absence of DCB and after substraction of basal uptake seen at Na^+ 139 mM.

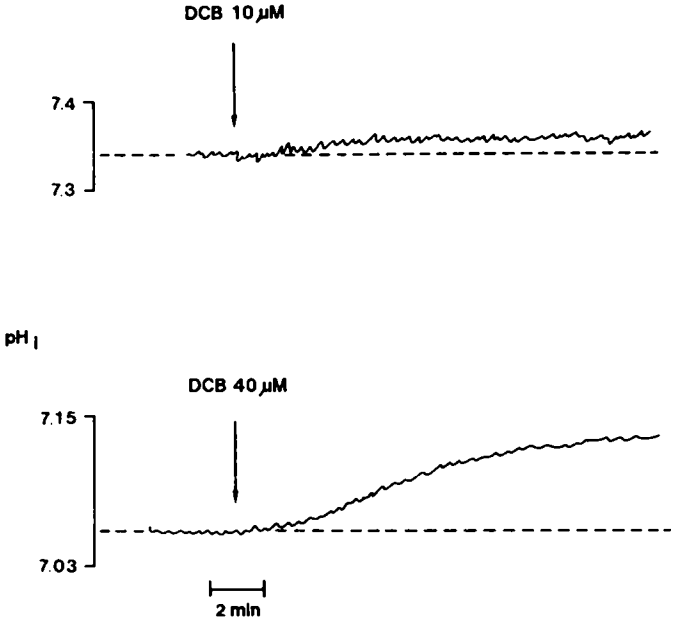

Fig. 2. Effect of DCB 10 and 40 μM on intracellular pH of isolated islet cells, measured using the fluorescent indicator BCECF. Basal medium contained glucose 2.8 mM and no HCO_3^-. Each trace is representative of at least four individual experiments.

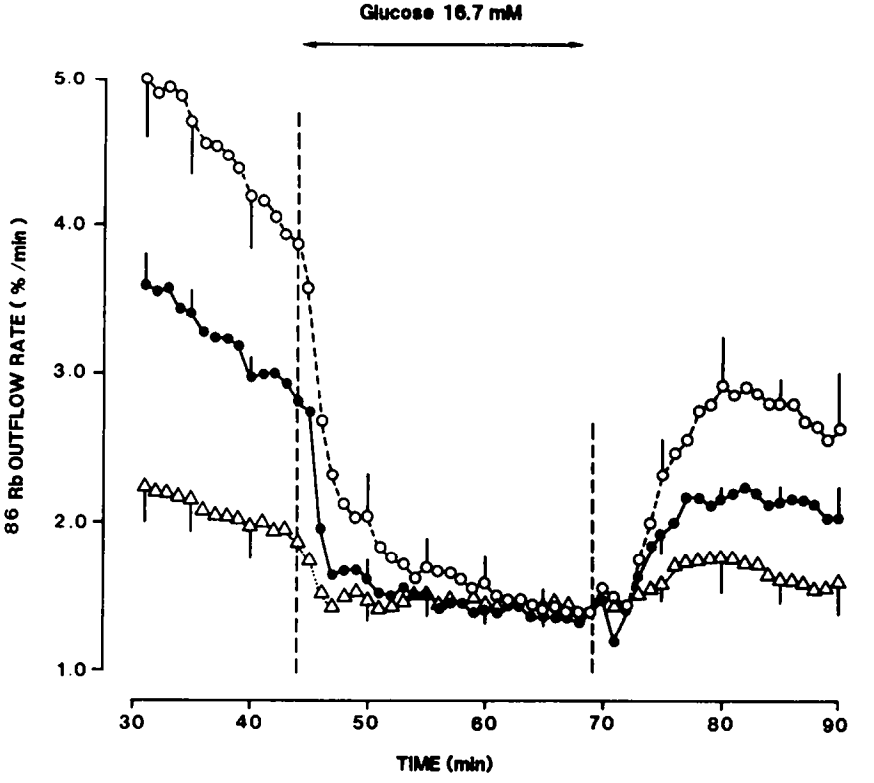

Fig. 3. Effect of glucose 16.7 mM on ^{86}Rb efflux from islets perifused in the absence (○——○) or the presence of 10 μM (●——●) and 40 μM (△ · · · △) DCB. Basal media contained no glucose. Mean ± SEM refer to at least four experiments in each case.

as the difference between the mean values for ^{86}Rb outflow recorded in each individual experiment between min 40 and 44 and min 64 and 68 of the perifusion. The inhibitory effect of glucose on ^{45}Ca outflow was taken as the difference between the mean values for ^{45}Ca outflow recorded in each individual experiment between the 40th and 44th min and either the lowest value seen immediately after glucose administration (experiments carried out in the presence of extracellular Ca^{2+}, e.g. Fig. 5) or the 64th and 68th min of perifusion (experiments carried out in the absence of extracellular Ca^{2+}, e.g. Fig. 6 and Table 1). The magnitude of the increase in ^{45}Ca outflow was estimated in each individual experiment from the integrated outflow of ^{45}Ca observed during stimulation after correction for basal value (taken as the lowest value seen before ^{45}Ca efflux reascension). The effect of DCB on glucose induced-insulin release was computed from the difference in insulin release recorded between min 45 and 69 in test and control experiments after correction for basal value (taken as the mean value seen in the absence of glucose, min 40–44).

All the results are expressed at the mean (± SEM) together with the number of individual experiments (N). The statistical significance of differences between mean data was evaluated by use of Student's *t*-test for two samples and variance analysis for multisample comparison followed by Fischer's Least Significant Difference test.

RESULTS

Effect of DCB on Na/Ca *exchange*

Isoosmotical replacement of extracellular Na^+ by sucrose provoked a 3–4-fold increase in ^{45}Ca uptake by isolated islet cells (Fig. 1, left panel). DCB provoked a marked and dose-related inhibition of this uptake (Fig. 1, left panel). The IC_{50} of the drug as determined from the semi-logarithmic plot of the data, was 18 μM (Fig. 1, right panel).

Effect of DCB on intracellular pH

DCB did not decrease and on the contrary increased intracellular pH of islet cells incubated in the presence of 2.8 mM glucose (Fig. 2). The effect of the drug was rather modest at 10 μM but clearly evident at 40 μM.

Effect of DCB on ^{86}Rb *outflow*

DCB did not produce any rapid decrease in ^{86}Rb outflow from islets perifused in the absence of glucose (data not shown). However, a late inhibitory effect (min 64–69) could be detected at high concentrations of the drug (40 μM).

Figure 3 illustrates the effect of a rise in glucose concentration from 0 to 16.7 mM on ^{86}Rb outflow from islets perifused throughout (since 45 min at the time of glucose addition) in the presence of increasing concentrations of DCB. In the absence of DCB, 16.7 mM glucose induced a rapid, sustained and

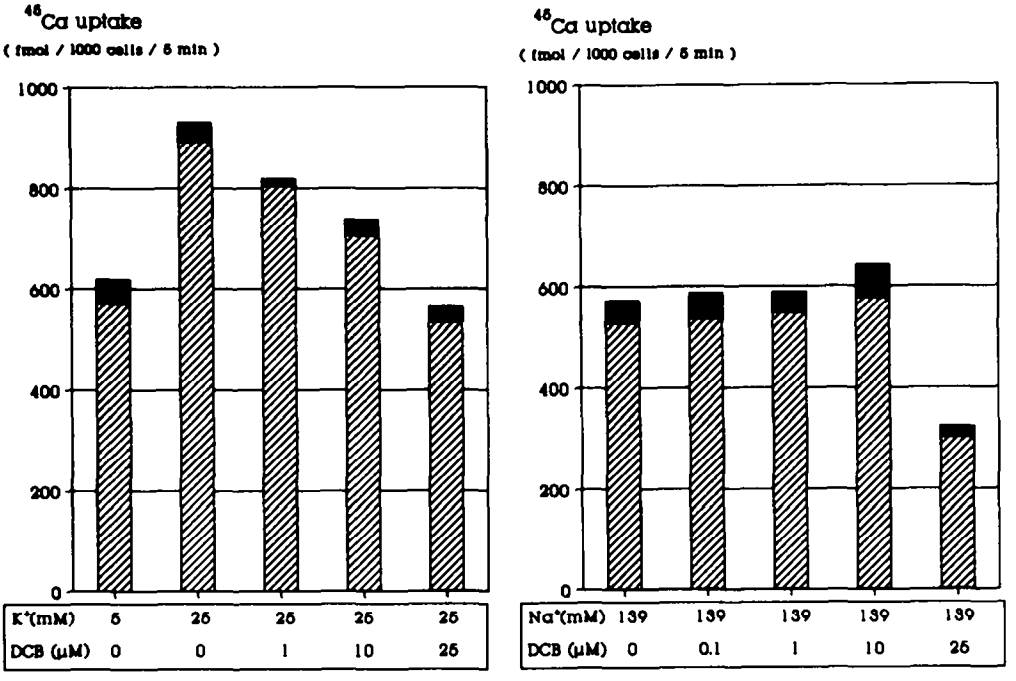

Fig. 4. Left panel: Effect of DCB on ^{45}Ca uptake induced by 25 mM K^+ in isolated islet cells. Mean values + SEM (black box at top of the bars) are expressed in fmol/1000 cells/5 min and refer to 24 individual samples in each case. Right panel: Effect of DCB on ^{45}Ca uptake observed under basal conditions (Na^+ 139 mM). Mean + SEM (black box at top of the bars) are expressed in fmol/1000 cells/5 min and refer to at least 13 individual samples in each case.

rapidly reversible decrease in ^{86}Rb outflow from perifused islets. DCB provoked a dose-related decrease in basal ^{86}Rb outflow ($P < 0.001$). In the presence of DCB, glucose lost, in a dose-related manner, its ability to reduce ^{86}Rb outflow, the effect of the sugar and the drug being not additive. Since, at the concentration used (16.7 mM), glucose does not maximally inhibit ^{86}Rb outflow [18], the data suggest that DCB and glucose act on the same target site to inhibit ^{86}Rb outflow. In the presence of 10 and 40 μM DCB, the inhibitory effect of the sugar only averaged 61% and 21%, respectively, of that seen in its absence ($P < 0.001$ in both cases).

Effect of DCB on ^{45}Ca uptake induced by membrane depolarization

A rise in the extracellular K^+ concentration from 5 to 25 mM increased the uptake of ^{45}Ca in isolated islet cells by about 57% ($P < 0.001$; Fig. 4, left panel). DCB potently inhibited this uptake, the half-maximal effect of the drug averaging 7.5 μM. Figure 4, right panel shows that DCB did not affect basal ^{45}Ca uptake except at a high concentration (25 μM) where the drug reduced the uptake.

Effect of DCB on ^{45}Ca outflow

DCB did not produce any immediate effect on ^{45}Ca outflow from islets perifused in the absence of glucose but the presence of extracellular Ca^{2+} (data not shown). However, similarly to what was found for ^{86}Rb outflow, a tiny and delayed inhibitory effect could be detected ($P < 0.025$ at 10 μM). A similar picture was observed in the absence of extracellular Ca^{2+} (data not shown) except that the effect was slightly more pronounced ($P < 0.001$).

In a next series of experiments, we examined whether DCB altered the effects of glucose on ^{45}Ca outflow. In the presence of extracellular Ca^{2+}, a rise in the glucose concentration from 0 to 16.7 mM has been shown [19] to induce a dual modification in ^{45}Ca outflow consisting in an initial fall rapidly followed by a secondary rise in ^{45}Ca outflow (Fig. 5, upper panel). In the presence of extracellular Ca^{2+}, DCB up to a concentration of 40 μM did not reduce basal ^{45}Ca outflow (the effect of 10 μM is illustrated in Fig. 5, upper panel). However, in the presence of the drug, the sugar lost part of its ability to reduce ^{45}Ca outflow. The effect was already present, though not significantly, at 1 μM (−30%, $P > 0.10$) and plateaued a 10 μM (−49% at 10 μM and −48% at 40 μM) ($P < 0.025$ in both cases). Last, DCB barely affected the secondary rise in ^{45}Ca outflow (−12% at 10 μM, $P > 0.5$ and −17% at 40 μM, $P > 0.4$). In Fig. 5, the secondary rise in ^{45}Ca outflow (min 55–70) appeared to be more sustained and ^{45}Ca outflow remained at a higher level after glucose removal (min 70–90) in the presence than in the absence of 10 μM DCB. However, such a behaviour was not observed at the two other concentrations of DCB tested (1 and 40 μM; data not shown).

In the absence of extracellular Ca^{2+} glucose has been shown to only induce a monophasic decrease in ^{45}Ca outflow [19] (Fig. 6, upper panel). In the absence of extracellular Ca^{2+}, DCB produced a

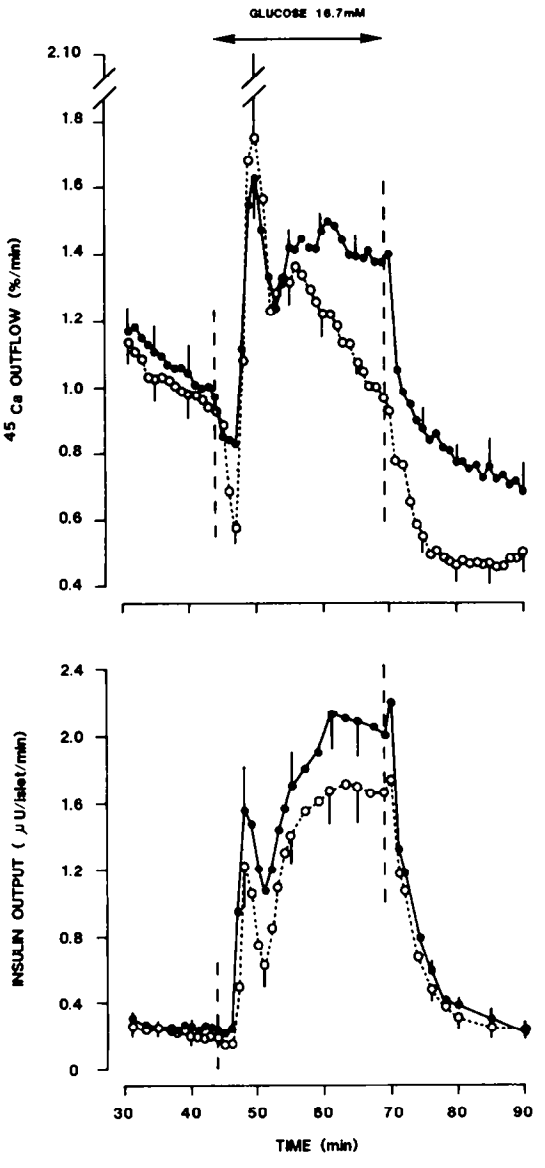

Fig. 5. Effect of 16.7 mM glucose on ^{45}Ca outflow (upper panel) and insulin release (lower panel) from islets perifused in the absence (○——○) or the presence (●——●) of DCB 10 μM. Basal media contained $CaCl_2$ but no glucose. Mean ± SEM refer to four individual experiments in each case.

dose-related decrease of basal ^{45}Ca outflow (Fig. 6, upper panel and Table 1, $P < 0.05$). In the presence of DCB, the sugar lost, in a dose-related manner, its ability to reduce ^{45}Ca outflow, the effects of the sugar and the drug being not additive (Fig. 6, upper panel and Table 1, $P < 0.001$). Since at the concentration used (16.7 mM), glucose does not maximally inhibit ^{45}Ca outflow (e.g. see Fig. 6) our data indicate that DCB and glucose act on the same target site to inhibit ^{45}Ca outflow. In the presence of DCB 1, 10 and 40 μM, the inhibitory effect of glucose only averaged 72%, 61% and 37%, respectively, of that seen in its absence.

To investigate the mechanism of action of DCB on ^{45}Ca outflow, the same experiments were repeated in the absence of extracellular Na^+, the monovalent cation being isoosmotically replaced by sucrose. As previously observed [8], the absence of extracellular Na^+ markedly reduced basal ^{45}Ca outflow recorded in the absence of glucose (compare upper and lower panels of Fig. 6 and see Table 1). In the absence of extracellular Na^+, the inhibitory effect of glucose only averaged 26% of that seen in the presence of extracellular Na^+ ($P < 0.001$). Figure 6, lower panel further shows that in the absence of extracellular Na^+, DCB completely lost its ability both to reduce basal ^{45}Ca outflow and to impair the inhibitory effect of glucose on ^{45}Ca outflow (see also Table 1).

Effect of DCB on insulin release

The effect of DCB on glucose-induced insulin secretion was examined in dynamic perifusion experiments (Fig. 5, lower panel). At 40 μM, DCB significantly increased glucose-induced insulin release (1.65 ± 0.26 μUnits/islet/min, N = 8, compared to 0.96 ± 0.15 μUnits/islet/min, N = 8, in the absence of DCB, $P < 0.05$). At lower concentrations (1 μM and 10 μM), DCB respectively failed to affect (0.83 ± 0.24 μUnits/islet/min, N = 4, compared to 0.97 ± 0.28 μUnits/islets/min, N = 5) or only tended to increase glucose-induced insulin release (1.38 ± 0.19 μUnits/islet/min, N = 8, compared to 1.10 ± 0.14 μUnits/islet/min, N = 8; see Fig. 5, lower panel; $P > 0.2$). DCB did not affect insulin release in the absence of glucose (data not shown).

DISCUSSION

Amiloride, the potassium sparing diuretic, is a weak inhibitor of Na/Ca exchange [20]. Recently, analogs of amiloride substituted by an hydrophobic group on the terminal nitrogen of the acyl guanidinium group, were shown to display a markedly enhanced potency as inhibitors of the transporter [12]. DCB is one of the most potent of these derivatives and has been used in many tissues to investigate the process of Na/Ca exchange [12].

Effect of DCB on Na/Ca *exchange*

In the islet cells, as investigated in the present study, DCB behaved as a potent inhibitor of the process of Na/Ca exchange. It was about two orders of magnitude more potent than amiloride [5] and displayed an IC_{50} (18 μM) close to that found in many other tissues [12]. Therefore, in the next part of our study, the selectivity of the inhibitory actions of DCB was examined.

Effect of DCB on intracellular pH

Amiloride is known to reduce the intracellular pH of many different cells including the pancreatic B-cell [21, 22]. Indeed, at concentrations close to 100 μM, amiloride inhibits the process of Na/H exchange, a carrier thought to play a major role in H^+ extrusion from cells [20]. Some analogs of amiloride, bearing substituents on the 5-amino group of the pyrazine ring, have a greatly enhanced inhibitory action on Na/H exchange as compared to amiloride [12]. The finding that DCB did not reduce

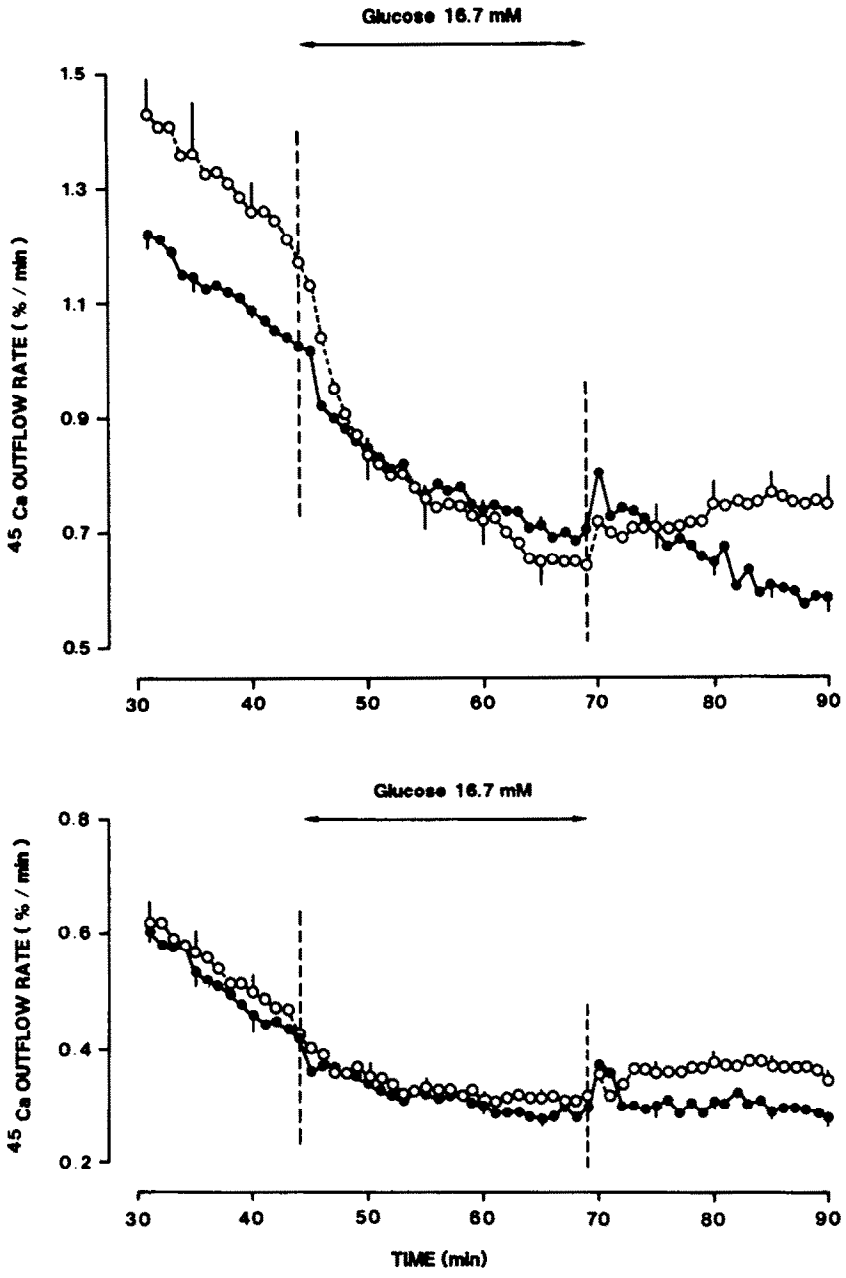

Fig. 6. Upper panel: Effect of 16.7 mM glucose on ^{45}Ca outflow from islets perifused either in the absence (○——○) or the presence of DCB 10 μM (●——●). Basal media contained no glucose, no Ca^{2+} and was enriched with EGTA 0.5 mM. Lower panel: Effect of 16.7 mM glucose on ^{45}Ca outflow from islets perifused either in the absence (○——○) or the presence of DCB 10 μM (●——●). Basal media contained no Na^+, no glucose, no Ca^{2+} and was enriched with EGTA 0.5 mM. Extracellular Na^+ was isoosmotically replaced by sucrose. Mean ± SEM refer to at least four individual experiments in each case.

the intracellular pH of our preparation clearly evidences that DCB does not inhibit Na/H exchange in the pancreatic B-cell. This is in agreement with the view that terminal guanidino-nitrogen derivatives of amiloride are devoid of inhibitory activity on Na/H exchange [23].

Effect of DCB on K^+ fluxes

DCB induced a potent, though delayed, inhibitory effect on ^{86}Rb outflow. Furthermore, the inhibitory effects of glucose and DCB were not additive. This is a good indication that DCB and glucose acted on the same K^+ permeability to reduce ^{86}Rb outflow from pancreatic islets. Since glucose is thought to reduce ^{86}Rb outflow from islet cells mainly by inhibiting ATP-sensitive K^+ channels, our data may indicate that DCB exerts an inhibitory action on these channels. The view that DCB may block K^+

Table 1. Effect of DCB on ^{45}Ca outflow and glucose-induced inhibition of ^{45}Ca outflow from islets perifused in the absence of extracellular Ca^{2+}

Experimental condition	3',4'-Dichlorobenzamil (μM)				P†
	0	1	10	40	
Na 139 mM					
Glucose 0 mM	1.24 ± 0.05 (16)*	1.13 ± 0.08 (9)	1.06 ± 0.01 (5)	0.90 ± 0.12 (4)	<0.05
Glucose 16.7 mM	0.66 ± 0.05 (16)	0.74 ± 0.07 (9)	0.70 ± 0.01 (5)	0.70 ± 0.07 (4)	>0.5
Difference	0.57 ± 0.03 (16)	0.41 ± 0.06 (9)	0.35 ± 0.02 (5)	0.21 ± 0.08 (4)	<0.001
Absence of Na^+					
Glucose 0 mM	0.47 ± 0.03 (4)	0.48 ± 0.05 (4)	0.44 ± 0.01 (4)		>0.5
Glucose 16.7 mM	0.31 ± 0.01 (4)	0.32 ± 0.04 (4)	0.28 ± 0.01 (4)		>0.5
Difference	0.15 ± 0.02 (4)	0.16 ± 0.01 (4)	0.16 ± 0.02 (4)		>0.5

* ^{45}Ca outflow is expressed as a fractional outflow rate (per cent/min).
† Probability of a difference between values of ^{45}Ca outflow seen at the different concentrations of DCB.

channels is not new [12]. Incidentally, amiloride also reduces ^{86}Rb outflow from perifused islets [22]. By blocking Na/H and/or Na/Ca exchange, DCB and amiloride are expected to reduce $[Na^+]_i$. Since the absence of extracellular Na^+, also expected to reduce $[Na^+]_i$, inhibits ^{86}Rb outflow like DCB and amiloride [24], it could be asked whether the two drugs may not reduce ^{86}Rb outflow by decreasing $[Na^+]_i$.

Effect of DCB on ^{45}Ca fluxes

The effects of DCB on ^{45}Ca uptake suggest that DCB also blocked voltage-sensitive Ca^{2+} channels in the pancreatic B-cells. The latter channels have been shown to equip the B-cell, and their opening is thought to mediate the increase in Ca^{2+} inflow mediated by a rise in the extracellular concentration of K^+ [1–4, 25]. In other tissues also, DCB has been shown to block voltage-dependent Ca^{2+} channels [12] more potently, as in the present study, than it blocks Na–Ca exchange activity.

At first glance, the two latter effects of DCB (block of K^+ and Ca^{2+} channels) could preclude the use of the drug in the study of the nature of the effects of glucose on ^{45}Ca fluxes under perifusion conditions. Although this view cannot be denied for experiments carried out in the presence of extracellular Ca^{2+}, the reverse holds true for experiments carried out in its absence.

Indeed, in the presence of extracellular Ca^{2+}, the effects of glucose on ^{45}Ca fluxes depend to a great extent on the ability of the sugar to reduce K^+ permeability and, as a consequence of this effect, to open voltage-sensitive Ca^{2+} channels [26]. Consistent with this view was the failure of DCB to affect the secondary rise in ^{45}Ca outflow induced by glucose (Fig. 7). This increase is thought to reflect an increase in Ca^{2+} inflow through voltage-sensitive Ca^{2+} channels and to represent a process of Ca–Ca exchange in which influent Ca^{2+} displaces ^{45}Ca from intracellular binding sites [19]. The absence of major effects of DCB on the secondary rise in ^{45}Ca outflow can be viewed as the balance between stimulatory effects of the drug on Ca^{2+} inflow resulting from the closure of K^+ channels and inhibitory effects resulting from a direct interference with the opening of Ca^{2+} channels. The rather modest actions of DCB on glucose induced-insulin release can be interpreted within the same frame.

On the other hand, in the absence of extracellular Ca^{2+}, none of the actions exerted by the sugar on ^{45}Ca fluxes are thought to result from changes in K^+ permeability and of course in Ca^{2+} inflow [2]. The decrease in ^{45}Ca outflow induced by glucose in the absence of extracellular Ca^{2+} (Fig. 8, upper panel) has been proposed to reflect an inhibition by glucose of Ca^{2+} outflow by Na–Ca exchange [8]. Indeed, the removal of extracellular Na^+ was initially observed to produce a marked decrease in ^{45}Ca outflow that did not cumulate with that of glucose. However, in a further study [11], an inhibitory effect of the sugar on ^{45}Ca outflow was shown to persist, to a limited extent, in the absence of extracellular Na^+. In a third study, it was even shown, that glucose completely retained its ability to reduce ^{45}Ca outflow from islets in a Na^+-free medium, provided K^+ salts are used as substitutes for Na^+ salts [10].

In the present study, the inhibitory effect of glucose was reduced by 75% in a medium where extracellular Na^+ was isoosmotically replaced by sucrose. This confirms that the effect of glucose is not completely suppressed in the absence of extracellular Na^+ [11]. In absolute value, however, it is reduced by 75%. The reason why the effect of glucose was reduced by 75% in the present study, compared to 50% in that of Henquin *et al.* [11] probably results from the use of different substitutes for NaCl (sucrose in the present study compared to choline salts). Isoosmotical replacement of Na^+ by sucrose instead of choline salts is 1.7-fold more potent in inhibiting Na/Ca exchange as evaluated by the ability of the two Na^+-free solutions to stimulate ^{45}Ca uptake in isolated islet cells [5]. Incidentally, in his study, Henquin *et al.* [11] observed also that the effect of glucose was more markedly reduced when extracellular Na^+ was replaced by sucrose instead of choline salts.

On the other hand, the claim by Hellman and Gylfe [10] of a maintained inhibitory effect of glucose on ^{45}Ca outflow when extracellular Na^+ is replaced by K^+ salts is of very limited value for two reasons.

First, in the latter study the data were expressed in per cent of the mean value found within the same experiment during the 10 min preceeding the introduction of glucose. This mode of expression allows to compare relative but not absolute effects (like in the present study and that of Henquin *et al.* Ref. 11) of experimental conditions on ^{45}Ca outflow. With such a mode of expression, an inhibition of the absolute effect of glucose in media where K^+ is used as a substitute for Na^+ would have been missed. Second, K^+ is probably the less appropriate substitute for Na^+, since K^+ may substitute for Na^+ in the exchange process [27], alter the kinetics of the exchanger [12] and even, as recently proposed, be cotransported with Ca^{2+} by the exchanger [28]. Thus, taken as a whole, the present data confirm that inhibition of Na/Ca exchange working in its Ca^{2+} efflux mode represents a significant, though not the sole, mechanism by which glucose inhibits ^{45}Ca outflow from pancreatic islet cells [8].

In this respect, it was interesting to see that DCB like glucose reduced basal ^{45}Ca outflow from Ca^{2+}-deprived islets, the effects of the sugar and the drug being not additive. Furthermore, this effect of DCB and its ability to impair the inhibitory effect of glucose were reproduced by the absence of extracellular Na^+ and disappeared under the latter experimental condition. Therefore, the present data reinforce the view that the inhibitory effect of glucose on ^{45}Ca outflow indeed results from an inhibition of Na/Ca exchange. Consistent also with this view, is the dose-related alteration by DCB of the initial inhibitory effect of glucose on ^{45}Ca outflow from islets exposed to extracellular Ca^{2+} (Fig. 7). This decrease represents the same phenomenon than that seen in the absence of extracellular Ca^{2+} but, in the presence of extracellular Ca^{2+}, it is masked by the secondary rise in ^{45}Ca outflow [19]. Incidentally, a distinct non-electrogenic Na/Ca exchange is present in mitochondria derived from some electrically excitable cells [12]. This distinct Na/Ca exchange is sensitive to DCB [12] and probably present in pancreatic islet cells as suggested by both direct [29] and indirect evidences [15]. Our data do not exclude that the inhibition of ^{45}Ca outflow induced by DCB and glucose results from an interference with this second type of Na/Ca exchange. Measurements of the effect of DCB on $[Ca^{2+}]_i$ could help in choosing between these two hypotheses. Unfortunately, measurements of the $[Ca^{2+}]_i$ using the fluorescent indicator fura 2 could not be done since DCB strongly fluoresces at the excitations wave-lengths of fura 2.

CONCLUSIONS

The present study shows that DCB is a potent inhibitor of the process of Na/Ca exchange in the pancreatic B-cell. Unfortunately, the drug is of poor selectivity and blocks, in the same range of concentrations, both K^+ channels and voltage-sensitive Ca^{2+} channels. Hence, caution must be observed when interpreting the effects of DCB on intact cells. However, when used under appropriate experimental conditions, the drug may be of value in elucidating the role played by Na/Ca exchange [12]. In the present study, the use of DCB allowed the confirmation of the existence in the pancreatic B-cell of a process of Na–Ca exchange and that glucose inhibits ^{45}Ca outflow to a great extent (at least 75%) by inhibiting Na–Ca exchange. The type of Na–Ca exchange (electrogenic and localized at the plasma membrane or nonelectrogenic and localized at the level of the mitochondria) that is inhibited by glucose, remains to be elucidated.

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