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Na^+ participates in loop diuretic-sensitive Cl^--cation co-transport in the pancreatic β-cells

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In order to investigate whether Na^+ participates in loop diuretic-sensitive Cl^--cation co-transport in the β-cells, we tested the interaction between the effects of Na^+ deficiency, furosemide and D-glucose on $^{86}Rb^+$ fluxes in β-cell-rich mouse pancreatic islets. Removal of extracellular Na^+ slightly reduced the ouabain-resistant $^{86}Rb^+$ influx and the specific effect of 1 mM furosemide on this influx was significantly smaller in Na^+-deficient medium. The capacity of 20 mM D-glucose to reduce the ouabain-resistant $^{86}Rb^+$ influx was not changed by removal of extracellular Na^+. The $^{86}Rb^+$ efflux from preloaded islets was rapidly and reversibly reduced by Na^+ deficiency. Furosemide (1 mM) reduced the $^{86}Rb^+$ efflux and the effect of the combination of Na^+ deficiency and 1 mM furosemide was not stronger than the effect of furosemide alone. $^{22}Na^+$ efflux was reduced by both ouabain and furosemide and the effects appeared to be additive. The data suggest that Na^+ participates in loop diuretic-sensitive Cl^--cation co-transport in the pancreatic β-cells. This adds further support to the idea that β-cells exhibit a Na^+,K^+,Cl^- co-transport system. Since some of the furosemide effect on $^{86}Rb^+$ efflux persisted in the Na^+-deficient medium, it is likely that also loop diuretic-sensitive K^+,Cl^- co-transport exists in this cell type.

Introduction

Recently, we described a system for loop diuretic-sensitive co-transport of chloride and cations in the pancreatic β-cells [1,2]. The experimental evidence were obtained from studies of the effect of furosemide on $^{36}Cl^-$ uptake and $^{86}Rb^+$ (K^+ analogue) fluxes in β-cell-rich mouse pancreatic islets [1,2]. That this system may have a role in normal β-cell function was suggested by the observation that furosemide inhibits glucose-induced insulin release from isolated pancreatic islets in vitro [2,3].

From studies in other cell types it has become evident that more than one mode of loop diuretic-sensitive co-transport of chloride and cations may exist. A loop diuretic-sensitive Na^+,K^+,Cl^- co-transport system was first demonstrated in Ehrlich ascites cells [4] and has since then been described in a number of cell types [5–7]. An important role for this system in ion transport across epithelia and in regulatory volume increase has been suggested [7–9]. A system for loop diuretic-sensitive K^+, Cl^- co-transport has been suggested in red blood cells and is belived to mediate efflux of K^+ and Cl^- during regulatory volume decrease [10]. Coupled fluxes of Na^+ and Cl^- may also exist but it has been argued that such transport is only a separate mode of action of the more extensively characterized Na^+,K^+Cl^- co-transport system [11].

The loop diuretic-sensitive co-transport systems outlined above have been identified by investigating their sensitivity to loop diuretics and the interdependency of ion fluxes. Thus, these transport systems are inhibited by loop diuretics, such as furosemide and bumetanide and transport of one ion requires the presence of the co-transported ion(s). At present the role of Na^+ in loop diuretic-sensitive co-transport in the β-cells is not clear. Therefore, in the present study we have investigated the interaction between furosemide and Na^+ on $^{86}Rb^+$ (K^+ analogue) fluxes and measured the direct effect of furosemide on $^{22}Na^+$ fluxes in isolated β-cell-rich mouse pancreatic islets.

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Materials and Methods

Animals and isolation of islets

Adult non-inbred ob/ob mice from the Umeå colony (Umeå ob/ob) were used as source of pancreatic islets. The hyperplastic islets of these mice contain an unusually high proportion (> 90%) [12] of β-cells that respond adequately to various stimulators and inhibitors of insulin release [13,14]. Therefore, the results are probably representative of this endocrine cell type. The animals were starved overnight to normalize their blood-sugar and their pancreatic islets were dissected out under a stereomicroscope without the use of enzymes [15]. Islets for $^{22}Na^+$ efflux experiments were isolated by collagenase digestion essentially as previously described [16].

Solutions

Hepes-buffered Krebs-Ringer solution was used as basal medium and the composition of this medium was (in mM): NaCl, 130; KCl, 4.7; $CaCl_2$, 2.6; KH_2PO_4, 1.2; $MgSO_4$, 1.2; Hepes, 20 and D-glucose 3. The medium was supplemented with 1 mg/ml bovine serum albumin and the pH was adjusted to 7.4 by addition of NaOH. The gase phase was that of ambient air. In experiments with Na^+ deficiency, 130 mM NaCl was replaced by equimolar amounts of choline chloride. In order to avoid any possible cholinergic effects of choline, 10 μM atropine was added to all media containing choline. To make sure that differences in $^{86}Rb^+$ fluxes observed with these media were not due to the presence of 10 μM atropine, this drug was also added to the respective control media.

$^{86}Rb^+$ uptake

After an initial incubation for 30 min at 37°C in 1 ml basal medium, groups of 4 or 5 islets were incubated for 5 min at 37°C in 200 μl basal medium supplemented with 28 μM ^{86}RbCl and with 8 μM [6′,6′-^{3}H]sucrose as an extracellular space marker [17]. After the incubations the islets were freeze-dried overnight (−40°C, 0.1 Pa) and weighed on a quartz-fibre balance. Their radioactivity was measured by liquid scintillation counting and the islet content of $^{86}Rb^+$ in excess of the extracellular (sucrose) space was calculated.

$^{86}Rb^+$ and $^{22}Na^+$ efflux

After an initial incubation for 30 min at 37°C in basal medium as above, groups of 15–30 islets were labelled with $^{22}Na^+$ or $^{86}Rb^+$ for 120 min at 37°C in 200 μl medium. The islets were then washed for 2 min ($^{22}Na^+$ efflux) or 5 min ($^{86}Rb^+$ efflux) with non-radioactive basal medium and were then placed in a flow chamber and perifused with non-radioactive medium essentially as previously described [18]. High precision and temporal resolution was obtained by manual collection of effluent samples with short intervals (30 or 60 s). Radioactivity of effluent samples and islets was measured by liquid scintillation counting and the fractional efflux rate was calculated as the amount of radioacitvity leaving the islets per minute in relation to the amount of radioactivity in the islets in the beginning of that minute. Data are expressed in relation to the mean of the three last samples (100%) directly preceding the test period.

Statistics

Differences between control and test groups were analysed by using Student's two-tailed *t*-test for paired or unpaired data.

Chemicals

Furosemide (*N*-furfuryl-4-chlorosulfamoylanthranilic acid) was a gift from Svenska Hoechst AB. Hepes

TABLE I

Interaction between Na^+ deficiency, furosemide and D-glucose on ouabain resistant $^{86}Rb^+$ influx

After preincubation for 30 min in basal medium with 1 mM ouabain, groups of 4 or 5 islets were incubated for 5 min in glucose-free basal medium supplemented with 28 μM ^{86}RbCl and 8μM [6′,6′-^{3}H]sucrose, 1 mM ouabain and the test substances indicated in the table. Addition of furosemide and removal of Na^+ also applied to the preincubation period. Data are expressed as mean values ± S.E. for the numbers of experiments indicated. Note that the groups 'control' and '$-Na^+$' ($n = 16$) represent the pooled data from two separate sets of experiments ($n = 8$) testing the interaction with furosemide and D-glucose, respectively. Statistical differences between groups were evaluated by using Student's two tailed *t*-test for paired data. $^*P < 0.05$, $^{**}P < 0.01$.

Modification of medium	$^{86}Rb^+$ uptake (mmol/kg dry islets and 5 min)	Specific effect of furosemide	Specific effect of D-glucose
None (control)	0.124 ± 0.016 (16)		
$-Na^+$	0.106 ± 0.014 (16)		
+1 mM furosemide	0.080 ± 0.014 * (8)	0.048 ± 0.015 (8)	
$-Na^+$ +1 mM furosemide	0.084 ± 0.017 (8)	0.014 ± 0.007 * (8)	
+20 mM D-glucose	0.084 ± 0.010 ** (8)		0.038 ± 0.008 (8)
$-Na^+$ +20 mM D-glucose	0.079 ± 0.011 * (8)		0.046 ± 0.008 (8)

was purchased from Boehringer Mannheim, Mannheim, F.R.G. and bovine serum albumin (fraction V) was from Miles Laboratories, Stokes Poges, U.K. ^{86}RbCl and ^{22}NaCl were from Amersham International, U.K. All inorganic chemical were commercially available and of analytical grade. Quartz bidistilled water was used throughout.

Results

Interaction between the effects of Na^+ deficiency, furosemide and D-glucose on ouabain-resistant $^{86}Rb^+$ influx

$^{86}Rb^+$ influx in β-cell-rich mouse pancreatic islets measured in the presence of 1 mM ouabain (ouabain-resistant influx) is likely to represent influx by other pathways than the Na^+/K^+ pump, since this transport systems in the β-cells is almost completely inhibited by 1 mM ouabain (Ref. 19, and Sandström, Klærke, Sehlin and Jørgensen, unpublished data).

Table I shows that removal of Na^+ induced a small reduction in the $^{86}Rb^+$ influx, although the difference failed to reach a high level of statistical significance ($0.05 < P < 0.10$). The effect of furosemide (1 mM) on $^{86}Rb^+$ influx was tested both in Na^+-containing and in Na^+-deficient medium. This concentration of furosemide was chosen because previous studies on $^{86}Rb^+$ [1] and $^{36}Cl^-$ [2] transport in islets have shown that furosemide acts as a dose-dependent inhibitor up to at least 1 mM, although its effects on insulin secretion may be more complex [2]. An analysis of the specific effect of furosemide showed that the furosemide-sensitive $^{86}Rb^+$ uptake was significantly smaller in the absense of Na^+ (Table I). Also 20 mM D-glucose reduced the ouabain-resistant $^{86}Rb^+$ influx (Table I). The effect was present both in the Na^+-containing and in the Na^+-deficient medium and the specific effect of D-glucose was similar in either medium (Table I).

Interaction between the effects of Na^+ deficiency and furosemide on $^{86}Rb^+$ efflux

The dynamics and reversibility of the effects of Na^+ deficiency and furosemide on K^+ transport was studied by means of $^{86}Rb^+$ efflux. As shown in Fig. 1A, removal of Na^+ resulted in a rapid reduction in $^{86}Rb^+$ efflux that was fully established within 2 min. The effect of Na^+ deficiency was readily reversible and the $^{86}Rb^+$ efflux rate was back to control level within the first minute after reintroduction of Na^+ (Fig. 1A). In studies on rat islets, extracellular Na^+ deficiency indeed leads to a rapid fall in total intracellular Na^+ content. The decrease from starting level was about 40% after 5 min and 60% after 20 min of treatment (Hellman, B., personal communication).

Also furosemide reduced $^{86}Rb^+$ efflux from preloaded islets (Fig. 1B). Maximum effect was reached within approx. 5 min. After removal of furosemide, the

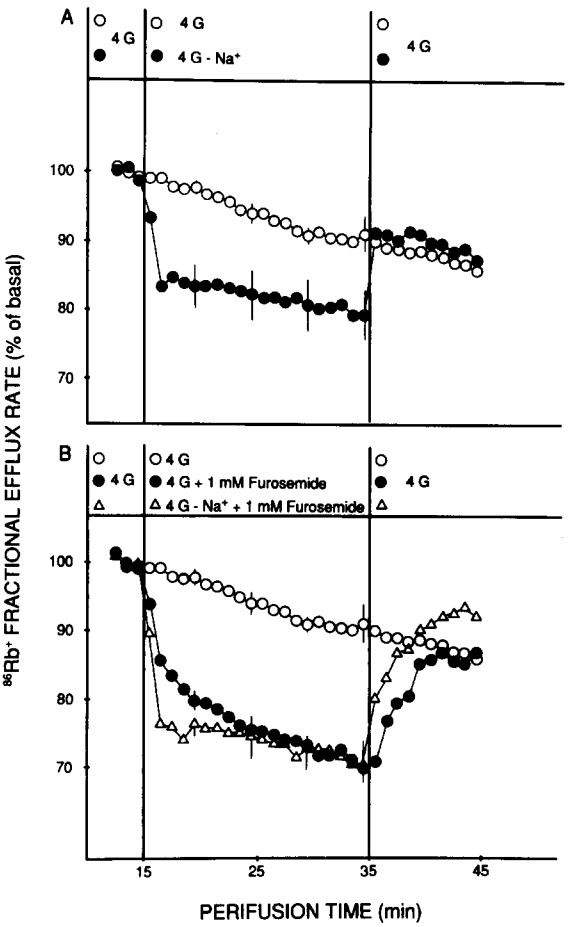

Fig. 1. Interaction between furosemide and Na^+ deficiency on $^{86}Rb^+$ efflux. After an initial incubation for 30 min in basal medium the islets were labelled for 120 min in basal medium supplemented with ^{86}RbCl. The islets were then washed for 5 min in non-radioactive basal medium and perifused with control or test medium as indicated in the figure head. Samples of the effluent were collected every 60 s. 4 G means that the perifusion medium contained 4 mM D-glucose. Data are expressed as mean values ± S.E. for 5–7 experiments.

$^{86}Rb^+$ efflux rate returned to control level within a similar period of time (Fig. 1B). The bottom curve in Fig. 1B (Δ) shows the effect of the combination of Na^+ deficiency and 1 mM furosemide on the $^{86}Rb^+$ efflux rate. During the first minutes of the test period the combination of Na^+ deficiency and furosemide reduced the $^{86}Rb^+$ rate to lower values than furosemide alone (min 17–18: 1 mM furosemide, 85.9 ± 1.0% vs. Na^+ + 1 mM furosemide, 76.5 ± 1.6%, $P < 0.005$) but no difference between curves was detected from approx. 5 min and on (Fig. 1B).

The interaction between Na^+ deficiency and furosemide was further analysed by calculating the specific effect of furosemide in the presence or absence of Na^+. This was done by subtracting the individual values for $^{86}Rb^+$ efflux rate in the presence of furosemide and with or without Na^+ (two lower curves in Fig. 1B) from the values in the corresponding control curves in the absence of furosemide (lower curve in Fig. 1A and

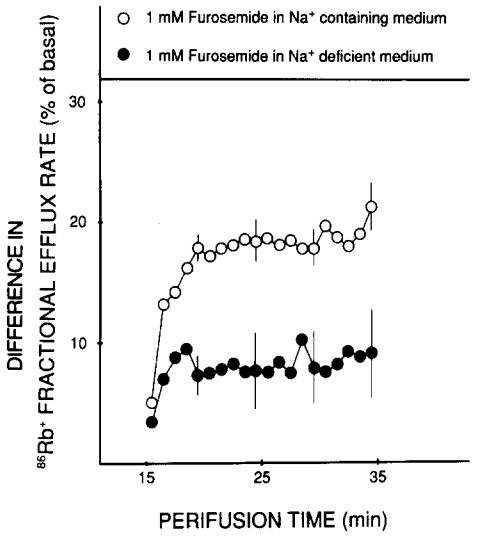

Fig. 2. Specific effect of furosemide on $^{86}Rb^+$ efflux. The specific effects of furosemide on $^{86}Rb^+$ efflux under control or Na^+ deficient conditions were calculated by subtracting the value for each individual experiment with furosemide (shown in Fig. 1B) from the mean value for the corresponding control curve in the absence of furosemide (shown in Figs. 1A and B). Data are expressed as mean values ± S.E. for five experiments.

upper curve in Fig. 1B). The resulting data depicted in Fig. 2 show that the specific effect of furosemide was stronger in the presence of Na^+. This suggests that furosemide-sensitive $^{86}Rb^+$ efflux is mediated by a Na^+-dependent mechanism(s). However, in 4 out of 5 experiments the specific effect of furosemide was above zero also in Na^+ deficiency (lower curve in Fig. 2). This suggests that furosemide-sensitive $^{86}Rb^+$ efflux may to some extent be mediated by Na^+-independent mechanism(s).

Effect of furosemide on $^{22}Na^+$ efflux

To more directly investigate whether Na^+ participates in loop diuretic-sensitive ion fluxes in the β-cells we tested the effect of furosemide on $^{22}Na^+$ efflux from preloaded β-cell-rich mouse pancreatic islets. There was no statistically significant lowering effect of 1 mM furosemide (Fig. 3). However, withdrawal of furosemide resulted in a significant increase in $^{22}Na^+$ efflux rate (min 13–15: 69.5 ± 3.7% vs. min 18–20: 73.8 ± 4.4%, $P < 0.02$ with unpaired Student's *t*-test),

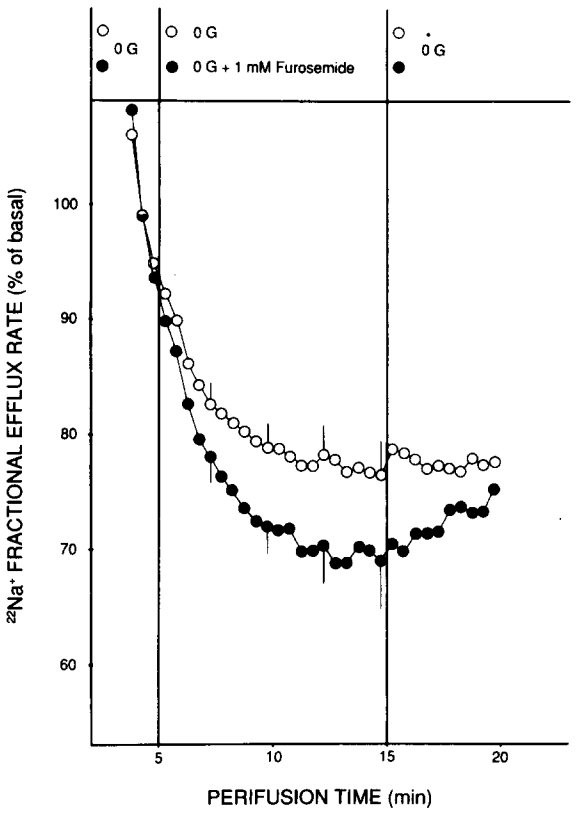

Fig. 3. Effect of furosemide on $^{22}Na^+$ efflux. After an initial incubation for 30 min in basal medium islets were labelled for 120 min in basal medium supplemented with $^{22}NaCl$. The islets were then washed for 2 min in non-radioactive basal medium and perifused with glucose-free ('0 G') control or test media as indicated in the figure head. Samples of the effluent were collected every 30 s. Data are expressed as mean values ± S.E. for 6–8 experiments.

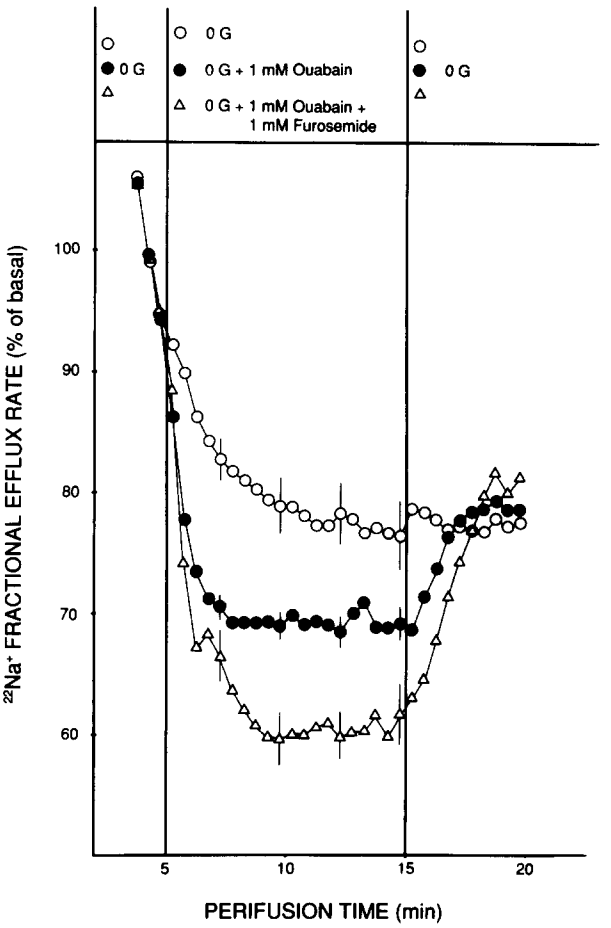

Fig. 4. Interaction between ouabain and furosemide on $^{22}Na^+$ efflux. The experimental design was as in Fig. 3. except for the different test substances (indicated in the figure head). Data are expressed as mean values ± S.E. for 5–7 experiments.

which supports the idea that $^{22}Na^+$ efflux is reduced by furosemide.

Interaction between the effects of ouabain and furosemide on $^{22}Na^+$ efflux

Fig. 4 shows the interaction between the effects of ouabain and furosemide on $^{22}Na^+$ efflux. As shown by the middle curve, 1 mM ouabain rapidly and reversibly reduced the $^{22}Na^+$ efflux rate. The combination of ouabain and furosemide led to a further reduction of $^{22}Na^+$ efflux as compared to ouabain alone (min 13–15: ouabain, 69.4 ± 1.5% vs. ouabain + furosemide, 60.9 ± 2.2%, $P < 0.05$). This observation strengthens the idea that furosemide reduces $^{22}Na^+$ efflux and suggests that this is not mediated by an effect on the Na^+/K^+ pump. The effect of ouabain alone was fully established within 2 min (Fig. 4) and the effect of ouabain + furosemide reached a maximum after approx. 5 min. This may suggest that 5 min is needed for maximum effect of furosemide on $^{22}Na^+$ efflux (Fig. 4).

Discussion

In previous work we showed that the loop diuretic furosemide reduces ouabain-resistant $^{86}Rb^+$ influx and efflux as well as $^{36}Cl^-$ influx and accumulation in the pancreatic β-cells [1,2]. These data in combination with the observation that the β-cell chloride content is higher than would be expected from a strictly Nernstian distribution [20] led to the suggestion that the β-cells accumulate chloride by secondary active, loop diuretic-sensitive chloride-cation co-transport [1]. The idea that Na^+ participates in this co-transport system was based on the observation that removal of extracellular Na^+ slightly reduced the islet accumulation of $^{36}Cl^-$ [1] as well as $^{36}Cl^-$ influx (Sandström and Sehlin, unpublished data). To obtain more final evidence that Na^+,K^+,Cl^- co-transport exists in the β-cells, coupling between the fluxes of Na^+ and K^+ as well as loop diuretic-sensitivity of Na^+ fluxes must be shown. Such data are provided in the present investigation.

Coupling between the fluxes of Na^+ and K^+ was suggested by the observation that ouabain-resistant $^{86}Rb^+$ influx as well as $^{86}Rb^+$ efflux from preloaded islets were reduced by removal of extracellular Na^+. Recently, Lebrun and coworkers [21] suggested that Na^+ deficiency reduces $^{86}Rb^+$ efflux by inhibiting K^+ channel activity. Our present data, showing that Na^+ deficiency failed to reduce $^{86}Rb^+$ efflux in the presence of 1 mM furosemide, rather suggest that reduced $^{86}Rb^+$ efflux is mediated by lowered Na^+,K^+, Cl^- co-transport. This idea is further strenghtened by the observation that the specific effect of furosemide on ouabain-resistant $^{86}Rb^+$ influx as well as $^{86}Rb^+$ efflux was lowered in a Na^+-deficient medium. In the previous study [21] it was also shown that the effect of D-glucose on β-cell $^{86}Rb^+$ permeability was inhibited in a Na^+ free medium, which led to the suggestion that Na^+ deficiency mimics the effect of D-glucose on β-cell $^{86}Rb^+$ permeability. We could not detect any effect of Na^+ deficiency on the capacity of D-glucose to reduce ouabain-resistant $^{86}Rb^+$ uptake. We have no explanation for this apparent discrepancy but the present data suggest that Na^+,K^+,Cl^- co-transport rather than glucose-regulated K^+ channels are inhibited by Na^+ deficiency.

The observation that a small specific effect of furosemide on the $^{86}Rb^+$ efflux was detected also in the Na^+-deficient medium suggests that part of the loop diuretic-sensitive K^+ efflux is mediated by a Na^+-independent mechanism. In other cell types, the cellular contents of K^+ and Cl^- during regulatory volume decrease (RVD) are reduced by loop diuretic-sensitive K^+, Cl^- co-transport and/or conductive K^+ and Cl^- pathways (for review, see Ref. 9). It has previously been shown that the β-cell K^+ and Cl^- contents are reduced in hypotonic medium [22], and recently we found that loop diuretic-sensitive $^{86}Rb^+$ efflux is stimulated during RVD in the β-cells (Engström, Sandström and Sehlin, unpublished data). Taken together, these data suggest that Na^+-independent, loop diuretic-senstive K^+,Cl^- co-transport may exist also in the β-cells.

Previous studies on $^{22}Na^+$ efflux from pancreatic β-cells [23,24] have met with considerable difficulties because of the the high fractional turnover rate and the low specific radioactivity of the available isotope. In spite of these difficulties, we were able to detect an inhibitory effect of furosemide on $^{22}Na^+$ efflux. An inhibitory effect of the Na^+/K^+, pump inhibitor ouabain was also observed. The effect of ouabain is in agreement with previous results [24] but adds further information about the time course. In the previous study ouabain was present during the whole experiments and its effect was calulated from the slope of the $^{22}Na^+$ efflux. By adding ouabain only during part of the efflux period we found that the onset of the inhibitory effect as well as its reversal were very rapid (approx. 2 min).

Furosemide reduced the $^{22}Na^+$ efflux rate also in the presence of ouabain which makes it is likley that the effect is not mediated by inhibition of the Na^+/K^+ pump but rather due to inhibition of loop diuretic-sensitive Na^+,K^+,Cl^- co-transport. From comparisons of the effects of furosemide in the presence or absence of 1 mM ouabain it appeared as if the effect of furosemide was more stable and easily detectable in the presence of 1 mM ouabain. An explanation for the unstable effect in the absence of ouabain could be that inhibition of $^{22}Na^+$ efflux by furosemide leads to increased Na^+/K^+ pump activity, which hides the inhibitory effect of furosemide.

Further support for coupled, loop diuretic-senstive Na^+ and K^+ transport is obtained from the time dependence of the furosemide effect on $^{86}Rb^+$ and $^{22}Na^+$ efflux. Thus, the dynamics of the furosemide inhibition of these ion fluxes were almost identical.

Taken together our present data provide strong evidence for loop diuretic-sensitive Na^+,K^+,Cl^- co-transport in the β-cells. Since part of the loop diuretic-senstive $^{86}Rb^+$ efflux was Na^+-independent, K^+,Cl^- co-transport is also likely to exist in this cell type.

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