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Furosemide and Ca^{2+} affect $^{86}Rb^+$ efflux from pancreatic β-cells by different mechanisms

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The interaction between furosemide, calcium and D-glucose on the $^{86}Rb^+$ efflux from β-cell-rich mouse pancreatic islets was investigated in a perifusion system with high temporal resolution. Raising the glucose concentration from 4 to 20 mM induced an initial decrease in $^{86}Rb^+$ efflux, which was followed by a steep increase and then a secondary decrease. Removal of extracellular calcium increased the $^{86}Rb^+$ efflux at 4 mM D-glucose but reduced it at 20 mM. The initial biphasic changes in $^{86}Rb^+$ efflux induced by 20 mM D-glucose were inhibited by calcium deficiency. Furosemide (100 μM) reduced the $^{86}Rb^+$ efflux rate both at 4 and 20 mM D-glucose and the magnitudes appeared to be similar at either glucose concentration. Furosemide (100 μM) reduced the glucose-induced (10 mM) $^{45}Ca^+$ uptake but did not affect the basal (3 mM D-glucose) $^{45}Ca^+$ uptake. However, the ability of furosemide (100 μM) to reduce the $^{86}Rb^+$ efflux at a high glucose concentration (20 mM) was independent of extracellular calcium. The inhibitory effects of furosemide and calcium deficiency on the $^{86}Rb^+$ efflux rate appeared to be additive. It is concluded that the effect of furosemide on $^{86}Rb^+$ efflux is not secondary to reduced calcium uptake and that the effects of furosemide and calcium deficiency are mediated by different mechanisms. The effect of furosemide is compatible with inhibition of loop diuretic-sensitive co-transport of Na^+, K^+ and Cl^- and the effect of calcium deficiency with reduced activity of calcium-regulated potassium channels.

Introduction

Transmembrane fluxes of inorganic ions in the pancreatic β-cells are closely interrelated with the regulation of insulin release [1,2]. Recently, it was suggested that a system for coupled transport of Na^+, K^+, and Cl^- across the β-cell membrane may be involved in insulin secretion [3,4]. This transport system is similar to the electroneutral and bidirectional co-transport of Na^+, K^+, and Cl^-, that was first described in Ehrlich ascites cells [5] and by now has been demonstrated in a number of cell types [6–9]. Loop diuretics, such as furosemide, characteristically inhibit both the uptake [10–12] and efflux [10,13] of K^+ by this mechanism.

In the pancreatic β-cells, furosemide reduces the efflux as well as the ouabain-resistant uptake of $^{86}Rb^+$ [3,4]. However, preliminary data have shown that furosemide also reduces the glucose-induced uptake of calcium ions in the β-cells [14]. Other data have shown that the $^{86}Rb^+$ efflux can be stimulated by intracellular Ca^{2+} and that this efflux is decreased by blockers of calcium uptake [15,16]. Electrophysiological measurements indicate the presence of Ca^{2+}-stimulated K^+ channels in the β-cell plasma membrane [17–19]. Therefore, the possibility could not be excluded that the

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inhibitory effect of furosemide on $^{86}Rb^+$ fluxes might be secondary to reduced calcium uptake rather than being caused by a direct effect on NaCl/KCl co-transport. To further clarify the precise role of furosemide in K^+ transport in the β-cells, we have analysed the interaction between the effects of D-glucose, extracellular Ca^{2+}, and furosemide on $^{86}Rb^+$ (K^+ marker; [20–22]) efflux from isolated β-cell-rich pancreatic islets from mice.

Materials and Methods

Animals and isolation of islets. Adult non-inbred *ob/ob* mice from the Umeå colony (Umeå *ob/ob*) were used throughout. These animals are metabolically abnormal with mild hyperglycaemia and hyperinsulinaemia, which may be due to peripheral insulin resistance and hyperphagia [23,24]. However, their hyperplastic islets, which contain an unusually high proportion of β-cells (over 90%) [25]) respond adequately in vitro to various stimulators and inhibitors of insulin release [26–28]. After overnight starvation to normalise the blood sugar [29], pancreatic islets were isolated by microdissection under stereomicroscope without the use of enzymes [30].

Solutions. The basal medium used was a Hepes-buffered Krebs-Ringer solution. The composition of the 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, 3.0, with pH adjusted to 7.40 by addition of NaOH. Bovine serum albumin (1 mg/ml) was added and the gas phase was ambient air.

$^{86}Rb^+$ efflux. After preincubation for 30 min in 1 ml basal medium, groups of 15–20 islets were labelled during 120 min in 200 μl basal medium supplemented with 28 μM $^{86}RbCl$ (13.7 TBq/mol). The islets were washed for 5 min in non-radioactive basal medium, and then transferred to a flow-chamber and perifused as previously described [31,32]. This perifusion system has a small dead-space and after changing from control to test media about 90% change of effluent is effected within 45 s [32]. High temporal resolution and precision was obtained by manual collection of samples with short intervals (15–60 s). Radioactivity in the effluent samples and islets were measured in a liquid scintillation spectrometer and the fractional efflux rate was calculated as the amount of $^{86}Rb^+$ leaving the islets per time period in relation to the amount of isotope in the islets at the beginning of that period. The data are expressed as fractional efflux rate compared to the mean value of efflux (100%) during the 3 min directly preceding the test period.

$^{45}Ca^{2+}$ uptake. After a preliminary incubation for 30 min in 1 ml basal medium, groups of 4 or 5 islets were incubated for 10 min in 200 μl basal medium labelled with $^{45}Ca^{2+}$ (0.3 TBq/mol), and with or without test substance. Contaminating extracellular $^{45}Ca^{2+}$ was removed by washing for 60 min with 2 mM $LaCl_3$ before counting of radioactivity [33].

Statistics. Differences between groups were tested by using Student's two-tailed *t*-test on paired or unpaired data.

Chemicals. Furosemide (*N*-furfuryl-4-chlorosulfamoylantranilic acid) was a gift from Svenska Hoechst AB, Stockholm, Sweden. Hepes was from Boehringer-Mannheim, Mannheim, F.R.G. Bovine serum albumin (fraction V) was purchased from Miles Laboratories, Stoke Poges, U.K. $^{86}RbCl$ and $^{45}CaCl_2$ were from Amersham International, U.K. All inorganic chemicals were commercially available and reagents of analytical grade. Quartz-bidistilled water was used throughout.

Results

Effect of furosemide on $^{45}Ca^{2+}$ uptake

Elevating the concentration of D-glucose from 3 to 10 mM significantly increased the uptake of $^{45}Ca^{2+}$ in the control islets, but failed to increase it in the presence of 100 μM furosemide (3 mM: control, 3.72 ± 0.46 vs. furosemide, 3.45 ± 0.26; 10 mM: control, 5.10 ± 0.53 vs. furosemide, 3.98 ± 0.38 mmol/kg dry islet and 10 min (mean $\pm$ S.E. for 8 or 9 experiments, $P < 0.02$ for the effect of glucose alone)). Analysis of the effect of furosemide at the different glucose concentrations showed that the $^{45}Ca^{2+}$ uptake at 3 mM was unaffected, whereas the uptake at 10 mM was significantly reduced by 100 μM furosemide ($P < 0.05$ for the effect of furosemide).

Effect of furosemide on $^{86}Rb^+$ efflux at 4 or 20 mM glucose

Exposure to furosemide (100 μM) at a basal glucose concentration (4 mM), not inducing insulin secretion [34], resulted in a rapid reduction of the $^{86}Rb^+$ efflux rate (Fig. 1). This reduction was statistically significant within 45 s ($P < 0.005$) and persisted during the whole experimental period

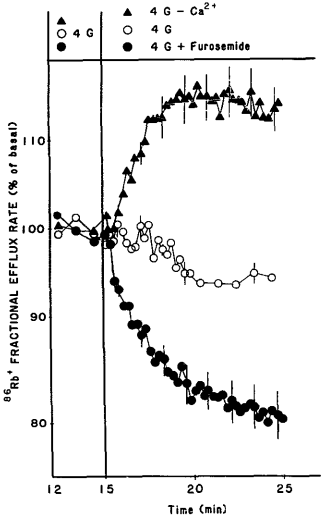

Fig. 1. Effect of furosemide or calcium deficiency on $^{86}Rb^+$ efflux at 4 mM D-glucose. After a preliminary incubation for 30 min in basal medium, groups of 15–20 islets were incubated for 120 min in basal medium labelled with 28 μM $^{86}RbCl$. Then, they were washed for 5 min in non-radioactive basal medium, moved to a flow chamber and perifused with control or test medium as indicated in the figure head. The data are expressed in relation to the mean efflux rate (100%) during the 3 min directly preceding the test period (100% corresponding to: 4 G, 3.09 ± 0.12 ($n = 4$); 4 G − Ca^{2+}, 3.34 ± 0.11 ($n = 7$); 4 G + furosemide, 3.61 ± 0.12 ($n = 7$). Results denote mean values ± S.E.

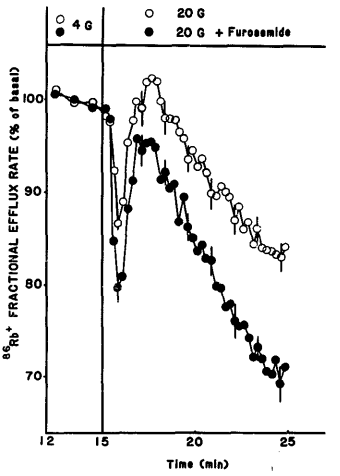

Fig. 2. Effect of furosemide on $^{86}Rb^+$ efflux at 20 mM D-glucose. The experimental design and handling of the data were as in Fig. 1, except for the different test substances (indicated in the figure head) (100% corresponding to: 20 G, 3.02 ± 0.06 ($n = 11$); 20 G + furosemide, 3.10 ± 0.09 ($n = 8$). Results denote mean values ± S.E.

of 10 min. The effect of furosemide on $^{86}Rb^+$ efflux was also tested at a high glucose concentration (20 mM), which gives a maximum stimulation of insulin release [35]. As shown in Fig. 2 (upper curve), the addition of 20 mM glucose alone resulted in a rapid and biphasic change in the $^{86}Rb^+$ efflux rate (cf. Ref. 32). First, a decrease of the efflux rate was observed, which reached a nadir within 60–75 s. This decrease was followed by a steep increase in efflux rate, with an apparent maximum within 165 s and then by a secondary gradual decrease, which persisted for the rest of the experimental period (Fig. 2). When 100 μM furosemide was added together with 20 mM glucose, the $^{86}Rb^+$ efflux was further reduced, al-

though the same biphasic pattern was retained. This furosemide effect was apparent from the third fraction (45 s; $P < 0.02$) after addition of the drug. The magnitudes of the furosemide effects at 4 mM (Fig. 1) and 20 mM glucose (Fig. 2) appeared to be similar.

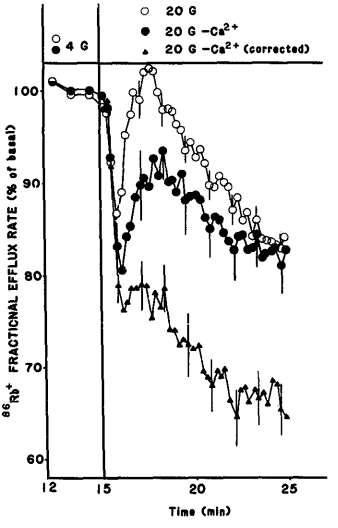

Fig. 3. Effect of calcium deficiency on $^{86}Rb^+$ efflux at 20 mM D-glucose. The experimental design and handling of the data were as in Fig. 1 except for the different test substances (indicated in the figure head) (100 % corresponds to: 20 G, 3.02 ± 0.06 ($n = 11$); 20 G−Ca^{2+}, 2.99 ± 0.15 ($n = 8$). The specific glucose effect in calcium deficiency (lower curve) was calculated by subtracting the elevation above baseline for each time point in the curve on 4 mM D-glucose in calcium deficiency (upper curve in Fig. 1) from the individual experiments on 20 mM D-glucose in calcium deficiency (middle curve in this figure). Results denote mean values ± S.E.

Effect of calcium deficiency on $^{86}Rb^+$ efflux at 4 mM and 20 mM glucose

At the lower glucose concentration (4 mM), removal of extracellular calcium resulted in an increase in the $^{86}Rb^+$ efflux rate (Fig. 1). This increase was established within the first minute of calcium deficiency and reached a steady state within approx. 5 min (Fig. 1).

Simultaneous removal of Ca^{2+} and increase in glucose concentration from 4 to 20 mM resulted in a further decrease in the $^{86}Rb^+$ efflux (Fig. 3, middle curve), as compared to the control with 20 mM glucose in the presence of calcium (Fig. 3, upper curve). The $^{86}Rb^+$ efflux still showed a biphasic pattern. However, the nadir of the initial fall as well as the maximum of the subsequent increase appeared later (Fig. 3, middle curve), and showed lower values than in the controls (Table I).

To further analyse the effects of glucose and Ca^{2+} on the secondary increase in $^{86}Rb^+$ efflux, the area under curves from fractions 5 to 20 (75–300 s) were calculated. As shown in Table II, the cumulated $^{86}Rb^+$ efflux during this period of time was significantly reduced by calcium deficiency. Taken together, these data show that the secondary increase in $^{86}Rb^+$ efflux in the presence of 20 mM D-glucose is markedly reduced by removal of extracellular calcium. Although there may be an interaction between the effects of Ca^{2+} deficiency and D-glucose, the shift in the biphasic efflux pattern (Fig. 3, middle curve) suggests that at least a portion of the increase in $^{86}Rb^+$ efflux induced by Ca^{2+} deficiency under basal conditions (4 mM D-glucose) is present also at the higher glucose concentration (20 mM). To try and estimate the specific effect of glucose in calcium deficiency, the stimulatory effect (elevation above baseline) of calcium deficiency at 4 mM D-glucose was therefore subtracted from the parallel data on 20 mM D-glucose (Fig. 3, middle curve). The resulting curve in the lower part of Fig. 3 shows that the biphasic pattern of $^{86}Rb^+$ efflux evoked by glucose (Fig. 3, lower curve) was completely lost by removal of extracellular calcium.

Interaction between furosemide and calcium deficiency on $^{86}Rb^+$ efflux

As shown in Fig. 4, furosemide (100 μM) reduced the $^{86}Rb^+$ efflux in the presence of 20 mM

TABLE I

NADIR AND PEAK VALUES OF BIPHASIC GLUCOSE-INDUCED $^{86}Rb^+$ EFFLUX: EFFECT OF FUROSEMIDE AND/OR CALCIUM-DEFICIENCY

The effects of furosemide and/or calcium deficiency on the level of the initial nadir (mean values of fractions 4–5) of $^{86}Rb^+$ efflux and the peak level (mean values of fractions 10–14) of the subsequent increase in efflux were calculated from the data in Figs. 2–4. Data are expressed as mean values ± S.E. for the number of experiments indicated in the parentheses. Differences from respective controls were tested by using Student's *t*-test for unpaired data.

Modification of medium	$^{86}Rb^+$ fractional efflux (% of basal)			
	nadir (fractions 4–5)		peak (fractions 10–14)	
+20 mM glucose (control, $n = 11$)	87.9 ± 1.2		100.9 ± 1.7	
+ Furosemide (100 μM, $n = 8$)	80.3 ± 1.7 **		94.4 ± 2.0 *	
Ca^{2+} deficiency ($n = 8$)	82.3 ± 2.2 *	$P < 0.02$	91.4 ± 3.3 *	$P < 0.02$
Ca^{2+} deficiency + Furosemide (100 μM, $n = 7$)	73.6 ± 1.9 ***		80.3 ± 2.1 ***	

* $P < 0.05$; ** $P < 0.02$; *** $P < 0.001$.

glucose also in a calcium-deficient medium. Both the nadir of the initial decrease and the maximum of the secondary increase were significantly reduced as compared to the relevant controls (Table I). Calculation of the area under curve from fractions 5 to 20 (75–300 s) showed that, within the limits for experimental variation, the effects of furosemide and calcium deficiency appeared to be additive (Table II).

TABLE II

EFFECT OF FUROSEMIDE AND/OR CALCIUM DEFICIENCY ON THE GLUCOSE-INDUCED SECONDARY INCREASE IN $^{86}Rb^+$ EFFLUX

The effects of furosemide and/or calcium deficiency on the glucose-induced secondary increase in $^{86}Rb^+$ efflux was estimated by calculating the area under curves from fraction 5–20 from the data in Figs. 2–4. Data are expressed as mean values ± S.E. for for the number of experiments indicated in the parentheses and differences from respective controls were tested by using Student's *t*-test on unpaired data.

Modification of medium	Area under curve (fractions 5–20)		Difference from control
+20 mM glucose (control, $n = 11$)	390.3 ± 6.6		–
+ Furosemide (100 μM, $n = 8$)	362.2 ± 6.3 **		28.1 ± 6.3
Ca^{2+} deficiency ($n = 8$)	356.2 ± 12.8 *	$P < 0.02$	34.1 ± 12.8
Ca^{2+} deficiency + Furosemide (100 μM, $n = 7$)	308.9 ± 9.1		81.2 ± 9.4

* $P < 0.05$; ** $P < 0.02$; *** $P < 0.001$.

Discussion

The temporal resolution of the perifusion system used here made it possible to study the biphasic pattern of $^{86}Rb^+$ efflux induced by 20 mM glucose during different test conditions. These initial biphasic changes are rapid and transient events that are most marked within the first 2–3 min and therefore have escaped discovery until recently. They were first clearly demonstrated by Sehlin and Freinkel [32] in studies on isolated islets from rats and lean mice. In the present work with islets from Umeå *ob/ob* mice. the early changes in $^{86}Rb^+$ efflux induced by 20 mM glucose were almost identical to those observed in the previous study [32]. This suggests that the biphasic, glucose-induced change in $^{86}Rb^+$ efflux indeed is representative of the β-cells, since the *ob/ob* mouse islets contain more than 90% of this cell type [25]. The results also add further support to the view that the β-cells of these *ob/ob* mice respond adequately to glucose stimulation in vitro.

Electrophysiological studies have suggested that the β-cells are equipped with calcium-stimulated

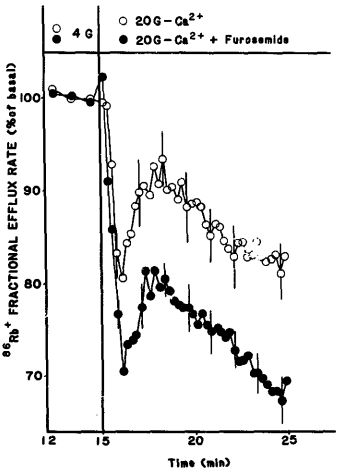

Fig. 4. Effect of furosemide and calcium deficiency on $^{86}Rb^+$ efflux. The experimental design and handling of the data were as in Fig. 1 except for the different test substances (indicated in the figure head) (100% corresponds to: 20 G−Ca^{2+}, 2.99± 0.15 (n = 8); 20 G−Ca^{2+} + furosemide, 3.37±0.12 (n = 7). Results denote mean values±S.E.

potassium channels that contribute to the regulation of the membrane potential [17–19]. Therefore, it could be expected that exposure of the β-cells to calcium deficiency would lead to reduced potassium permeability. However, previous results from such studies are contradictory. Boschero and Malaisse [22] concluded that the $^{86}Rb^+$ efflux was scarcely sensitive to changes in extracellular calcium and that the effect of glucose on the efflux was strictly unaffected by calcium deficiency. On the other hand, the $^{86}Rb^+$ efflux was reduced by inhibitors of calcium uptake [15,16]. Results from experiments with blockers of calcium uptake, such as cobalt and verapamil, appear difficult to interpret, since it was also shown that, in addition to blocking Ca^{2+} fluxes, these agents had direct effects on the potassium permeability [16].

In the present work we found that the $^{86}Rb^+$ efflux was indeed affected by extracellular calcium. In the presence of 4 mM glucose, calcium deficiency produced a clear increase in $^{86}Rb^+$ efflux. This finding is not easily explained by any known mechanism for regulation of K^+ permeability in the β-cells. However, it could be speculated that it is due to release of membrane-bound Ca^{2+}, leading to increased negative surface potential and changed K^+ permeability, as described for other cell types (cf. Ref. 36). After compensation for this increase in $^{86}Rb^+$ efflux, calcium deficiency was shown to abolish the glucose-induced secondary peak totally, whereas the glucose-induced inhibition of the efflux was retained. This leads us to suggest that the glucose-induced secondary increase in $^{86}Rb^+$ efflux could reflect opening of calcium-stimulated potassium channels.

In a previous study [4] we found only a marginal inhibition by furosemide of the $^{86}Rb^+$ efflux in the absence of glucose. In the present study, using a more sensitive experimental set-up, furosemide was found to clearly reduce the efflux of $^{86}Rb^+$ at a low glucose concentration that does not induce insulin release [34]. We could also confirm the previous result [4] that furosemide reduces the $^{86}Rb^+$ efflux at a glucose concentration stimulating secretion.

The present data show that furosemide inhibits $^{45}Ca^{2+}$ uptake by islet cells. The furosemide-induced inhibition of $^{86}Rb^+$ efflux could thus in principle be secondary to decreased calcium uptake. Yet, this is not likely, since the inhibitory effects of furosemide and calcium deficiency appeared to be additive and since furosemide reduced the $^{86}Rb^+$ efflux both at the low (4 mM) and high (20 mM) glucose concentrations, whereas it inhibited only the glucose-induced $^{45}Ca^{2+}$ uptake. The decrease in $^{45}Ca^{2+}$ uptake may be secondary to inhibition of more proximal steps in the stimulus-secretion coupling sequence in the β-cells [3].

In conclusion, the present study provides evidence that both furosemide and calcium deficiency reduce the efflux of $^{86}Rb^+$ from the β-cells and suggest that this is due to inhibition of two

separate mechanisms. The effect of furosemide is compatible with the inhibition of loop diuretic-sensitive co-transport of Na^+, K^+ and Cl^- [4,6–9], and the effect of calcium deficiency is best explained by inhibition of calcium-stimulated potassium channels.

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