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INSULIN ACTION ON CARDIAC GLUCOSE TRANSPORT

STUDIES ON THE ROLE OF THE Na^+/K^+ PUMP

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Isolated muscle cells from adult rat heart have been used to study the relationship between myocardial glucose transport and the activity of the Na^+/K^+ pump. $^{86}Rb^+$-uptake by cardiac cells was found to be linear up to 2 min with a steady-state reached by 40–60 min, and was used to monitor the activity of the Na^+/K^+ pump. Ouabain (10^{-3} mol/l) inhibited the steady-state uptake of $^{86}Rb^+$ by more than 90%. Both, the ouabain-sensitive and ouabain-insensitive $^{86}Rb^+$-uptake by cardiac cells were found to be unaffected by insulin treatment under conditions where a significant stimulation of 3-*O*-methylglucose transport occurred. $^{86}Rb^+$-uptake was markedly reduced by the presence of calcium and/or magnesium, but remained unresponsive towards insulin treatment. Inhibition of the Na^+/K^+ pump activity by ouabain and a concomitant shift in the intracellular $Na^+:K^+$ ratio did not affect basal or insulin stimulated rates of 3-*O*-methylglucose transport in cardiac myocytes. The data argue against a functional relationship between the myocardial Na^+/K^+ pump and the glucose transport system.

Introduction

One of the most prominent actions of insulin on its principal target tissues, which include liver, muscle and fat, is represented by the stimulation of glucose transport [1]. Despite extensive investigations, the exact mechanism of this stimulatory action remains presently unknown. Oxidation of key membrane sulfhydryl groups, alterations of ionic fluxes, especially of cellular calcium, phosphorylation-dephosphorylation reactions and the release of bioactive peptides from the plasma membrane have all to be considered as possible mechanisms of insulin receptor-effector coupling (for review, see Refs. 2,3).

Recently an alternative model, the 'insulin transduction system', which is energized by the Na^+/K^+ pump, has been suggested by Moore [4]. Moreover, as reported by Bihler [5], a negative feedback from the Na^+/K^+ pump to sugar transport appears to exist in the myocardium. In light of these and earlier observations on a possible regulatory effect of the Na^+/K^+ pump on glucose transport [6,7], the present study was initiated in order to reevaluate the role of this ion pump in regulating basal and insulin stimulated cardiac glucose transport. Isolated myocytes from the adult rat heart, which we have shown to be an excellent model for the study of insulin action at the molecular level [8–12], have been used to approach these problems. The data suggest that the Na^+/K^+ pump is not involved in the regulation of hexose transport in the heart.

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

Materials and Methods

Chemicals

3-*O*-[^{14}C]Methyl-D-glucose (spec. act. 57.2 mCi/mmol) and L-[1-^{14}C]glucose (spec. act. 58 mCi/mmol) were obtained from Amersham, Braunschweig, F.R.G. [^{86}Rb]RbCl (spec. act. 1.72–10.05 mCi/mg) was from New England Nuclear, Dreieich, F.R.G. Collagenase (EC 3.4.24.3) was supplied by Boehringer, Mannheim, F.R.G. Ouabain was obtained from Sigma, Munich, F.R.G. Hepes was purchased from Serva, Heidelberg, F.R.G., and bovine serum albumin (fraction V, reagent grade) from Miles, Frankfurt, F.R.G. All other chemicals were analytical grade and obtained from Merck, Darmstadt, F.R.G.

Isolation of heart cells

Male Wistar rats weighing 280–320 g were used in all experiments. The animals had free access to food and drinking water. Calcium tolerant myocytes were isolated by perfusion of the heart with collagenase as detailed previously [12]. The final cell suspension was washed three times with Hepes buffer (composition: 130 mmol/l NaCl, 4.8 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 25 mmol/l Hepes, 5 mmol/l glucose, 20 g/l bovine serum albumin, pH 7.4, adjusted with NaOH, equilibrated with oxygen) and incubated in siliconized erlenmeyer flasks in a rotating water bath shaker at 37°C until further use. Cell numbers were determined in a Fuchs-Rosenthal chamber; cell viability as checked by determination of the percentage of rod-shaped cells was found to be 85–95%.

Transport assays

All transport studies were performed at 37°C in Hepes buffer, pH 7.4, in the presence of D-glucose. Transport of 3-*O*-methylglucose was initiated by addition of 50 µl of the cell suspension ($2 \cdot 10^4$ cells) to 50 µl of Hepes buffer containing labelled 3-*O*-methylglucose (final concentration 100 µmol/l). Incubations were terminated by addition of 900 µl of cold stopping solution (composition: 38 µmol/l cytochalasin B, 0.1% ethanol, 150 mmol/l NaCl). Two 300 µl aliquots of the resultant suspension were immediately transferred to precooled microfuge tubes containing 100 µl of silicone oil (density 1.04) and centrifuged in a Beckman microfuge B for 40 s at $10000 \times g$. The tip of the tube was cut off and after solubilization of the pellet the radioactivity was determined by liquid scintillation counting. All data of 3-*O*-methylglucose uptake have been corrected for simple diffusion and extracellular trapping of radioactivity by subtracting the amount of L-[^{14}C]glucose uptake from the amount of 3-*O*-methylglucose uptake, and represent specific carrier mediated transport. All measurements of sugar uptake were carried out in triplicate, diplicate tubes containing L-[^{14}C]glucose instead of 3-*O*-[^{14}C]methylglucose were run in parallel to determine nonspecific uptake.

The above technique was also used for monitoring $^{86}Rb^+$-uptake, which substitutes for potassium in the active transport of K^+ into the cell [13]. Cardiac myocytes ($2 \cdot 10^4$ cells) were incubated in triplicate with ^{86}RbCl in a final concentration of 15 µmol/l. Termination of incubations using cold isotonic NaCl as a stopping solution and determination of radioactivity were performed as outlined above. All data of $^{86}Rb^+$-uptake were corrected for extracellular trapping of radioactivity by performing a 'zero-time' assay. This consisted of direct addition of cells to cold stopping solution containing labelled Rb^+, and immediately centrifuging aliquots of the resultant suspension as described above. Extracellular contamination never exceeded 3–5% of total $^{86}Rb^+$ uptake under all conditions.

Results

Effect of insulin on 3-O-methylglucose transport and $^{86}Rb^+$ uptake

We have recently reported on the kinetic characteristics of the glucose transport system in isolated cardiac myocytes and its modulation by insulin [12]. Stimulation of 3-*O*-methylglucose transport was found to be due to an increase in the maximal velocity of the carrier system, to be preceeded by a lag-phase of 20 s and to be dependent on intracellular magnesium and ATP [12]. As shown in Fig. 1, maximal stimulation of 3-*O*-methylglucose transport occurs at an insulin concentration of $3 \cdot 10^{-9}$ mol/l with a half-maximal effect at $3 \cdot 10^{-10}$ mol/l. Studies on insulin binding

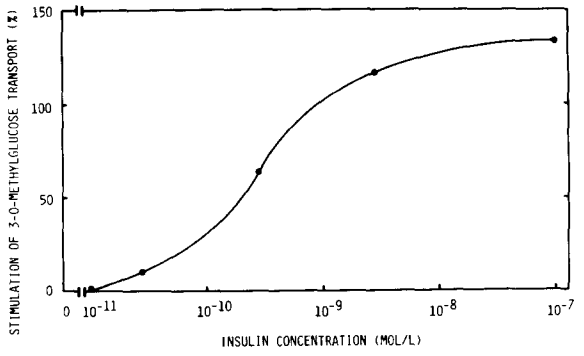

Fig. 1. Dose-response relationship for insulin stimulated transport of 3-*O*-methylglucose. Myocytes were incubated in Hepes buffer for 60 min at 37°C in the presence of increasing concentrations of insulin ($0.9 \cdot 10^{-11}$ mol/l to 10^{-7} mol/l). Transport of 3-*O*-[^{14}C]methylglucose (final concentration 100 μmol/l) was then determined after incubation of $2 \cdot 10^5$ cells/ml for 10 s as outlined in Methods. All data have been corrected for nonspecific uptake by use of L-[^{14}C]glucose. Data are a representative example of three separate experiments.

showed that the latter effect involves and occupancy of only 2% of total insulin receptors present on the myocytes (data not shown).

In order to directly correlate this stimulatory

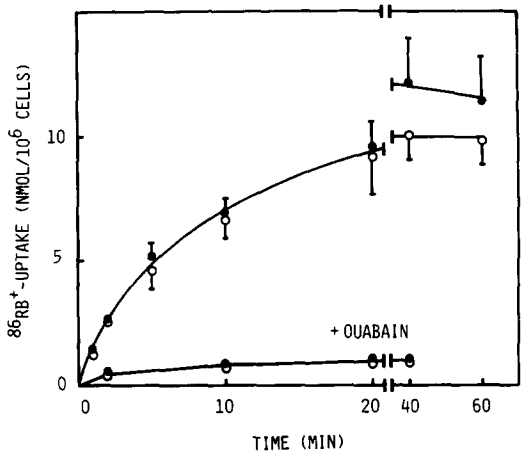

Fig. 2. Effect of insulin and ouabain on $^{86}Rb^+$-uptake by cardiac myocytes. Cells were incubated for 30 min at 37°C in the absence (●——●) or presence (○——○) of insulin ($3.5 \cdot 10^{-7}$ mol/l), both, in the absence or presence of ouabain (10^{-3} mol/l). Aliquots of the cell suspension (final cell concentration $2 \cdot 10^5$ cells/ml) were then incubated with ^{86}RbCl (final concentration 15 μmol/l) for the indicated times. Uptake was stopped and quantitated as outlined in the methods section. Data presented are mean values ± S.E. of 3–6 separate experiments.

action of the hormone to the activity of the myocardial ($Na^+ + K^+$)-ATPase, which is identical to the Na^+/K^+ pump [14], we have studied the effect of insulin on the uptake of the K^+ congener $^{86}Rb^+$ under incubation conditions identical to those of the hexose transport studies. $^{86}Rb^+$ uptake by control cells was found to be linear up to 2 min with a steady-state reached by 40–60 min (Fig. 2). Treatment of cardiac cells with insulin ($3.5 \cdot 10^{-7}$ mol/l) for 30 min did not significantly affect the rate of $^{86}Rb^+$-uptake. Identical results were obtained after longer treatment periods (up to 90 min, not shown in the figure). Preincubation of cardiac cells with ouabain (10^{-3} mol/l) for 30 min markedly reduced the rate of $^{86}Rb^+$ influx (Fig. 2). Under steady-state conditions more than 90% of $^{86}Rb^+$ uptake was found to be attributable to the function of the Na^+/K^+ pump. As shown in Fig. 2, insulin did not affect the ouabain-insensitive fraction of $^{86}Rb^+$ uptake. Thus, under conditions where the myocardial glucose transporter can be significantly stimulated by insulin, the Na^+/K^+ pump appeared to be unresponsive to this hormone.

In order to rule out the possibility that the Na^+/K^+ pump was already maximally stimulated under the above mentioned conditions, additional experiments were conducted using different con-

TABLE I

EFFECT OF CALCIUM AND MAGNESIUM ON $^{86}Rb^+$ UPTAKE BY ISOLATED CARDIAC MYOCYTES

Myocytes were incubated for 15 min at 37°C in the presence of calcium (1.25 mmol/l) and/or magnesium (1.2 mmol/l). Incubation was continued for 30 min in the absence or presence of insulin ($3.5 \cdot 10^{-7}$ mol/l). Uptake of $^{86}Rb^+$ was then determined using a 1 min assay period. Data are man values ± S.E. of three separate experiments.

Treatment	Insulin	$^{86}Rb^+$ uptake (nmol/10^6 cells per 1 min)	
		K^+, 1.2 mmol/l	K^+, 6 mmol/l
Control	–	2.05 ± 0.24	1.24 ± 0.12
	+	2.01 ± 0.18	1.28 ± 0.14
Ca^{2+}	–	0.90 ± 0.13	0.43 ± 0.05
	+	0.83 ± 0.08	0.40 ± 0.05
Mg^{2+}	–	1.30 ± 0.09	0.56 ± 0.06
	+	1.31 ± 0.11	0.58 ± 0.07
$Ca^{2+} + Mg^{2+}$	–	0.81 ± 0.08	0.37 ± 0.05
	+	0.77 ± 0.06	0.40 ± 0.06

centrations of extracellular ions. As presented in Table I, addition of calcium and, to a lesser extent, of magnesium resulted in a significant reduction of $^{86}Rb^+$ uptake both in the presence of high and low K^+ concentrations in the incubation medium, in agreement with recent observations of Liu and Onji [15] on K^+-activated *p*-nitrophenylphosphatase activity in dog heart myocytes. However, even a reduction of the pump activity by 60–70% by the presence of calcium and magnesium did not result in a stimulation by insulin (Table I). Moreover, insulin did not affect $^{86}Rb^+$ uptake by cardiac myocytes from 24 h fasted animals (data not shown).

Effect of ouabain on 3-O-methylglucose transport

Several studies on isolated perfused hearts suggested that drugs of the digitalis group at concentrations inhibitory to the Na^+/K^+ pump stimulate cardiac sugar transport [5,16]. This stimulatory action was found to be quantitatively correlated with the gain of Na^+ and loss of K^+ by the cells, rather than with the activity of the pump as such [6]. We have now studied the effect of the cardiac glycoside ouabain on initial rates of basal and insulin-stimulated transport of 3-*O*-methylglucose in isolated cardiac myocytes. Preincubation of cells with increasing concentrations of ouabain (10^{-6}–10^{-3} mol/l) for 30 min did not significantly affect the rate of 3-*O*-methylglucose transport (Fig. 3). In addition, insulin stimulated transport of 3-*O*-methylglucose remained unaffected by treatment of cells with ouabain. The activity of the Na^+/K^+ pump, however, as measured by Rb^+ uptake, was found to be significantly reduced using concentrations of ouabain in the range of 10^{-5}–10^{-3} mol/l (Fig. 3). These data argue against the suggested feedback control [5] of sugar transport by the Na^+/K^+ pump in the myocardium. It is noteworthy, that contractile activity of the myocytes was markedly increased by the presence of ouabain (10^{-4} mol/l, and more pronounced at 10^{-3} mol/l). However, both viability and ATP-

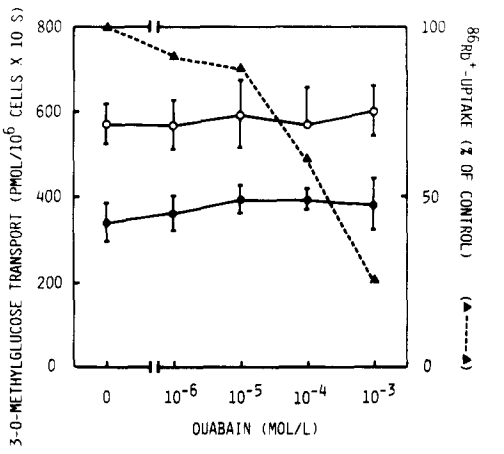

Fig. 3. Dose-response relationship for the effect of ouabain on 3-*O*-methylglucose transport and on $^{86}Rb^+$ uptake. Myocytes were incubated for 30 min at 37°C with the indicated concentrations of ouabain in the absence (●———●), (▲- -▲) or presence (○———○) of insulin. $2 \cdot 10^5$ cells/ml were then incubated with 3-*O*-[^{14}C]methylglucose (100 μmol/l) for 10 s for the determination of hexose transport, or with ^{86}RbCl (15 μmol/l) for 1 min for the determination of $^{86}Rb^+$ uptake. All samples were processed as outlined in the methods section. All data are mean values ± S.E. of three or four different experiments.

TABLE II

EFFECTS OF INSULIN AND OUABAIN ON 3-*O*-METHYLGLUCOSE TRANSPORT, Rb^+ UPTAKE, AND ON Na^+ AND K^+ CONTENT IN ISOLATED CARDIAC MYOCYTES

Cells were incubated for 30 min with insulin or ouabain at the indicated concentrations. Transport of 3-*O*-methylglucose and Rb^+ uptake were measured as described above. Intracellular sodium and potassium was determined by flame photometry as reported earlier [8]. The data presented are mean values ± S.E. with the number of experiments given in parentheses.

Treatment	3-*O*-Methylglucose transport (pmol/ 10^6 cells per 10 s)	Rb^+ uptake (nmol/10^6 cells per 1 min)	Na^+ (nmol/ 10^6 cells)	K^+ (nmol/ 10^6 cells)
Control	347 ± 52 (7)	1.52 ± 0.18 (5)	697 ± 38 (3)	2015 ± 267 (3)
Insulin ($3.5 \cdot 10^{-7}$ M)	573 ± 52 (4)	1.28 ± 0.19 (3)	680 ± 25 (3)	2044 ± 257 (3)
Ouabain (10^{-3} M)	387 ± 57 (4)	0.38 ± 0.06 (4)	2617 ± 217 (3)	530 ± 111 (3)

content remained unaltered even after incubation up to 90 min. Table II compares the effects of insulin and ouabain on 3-*O*-methylglucose transport, Rb^+ uptake, and on the Na^+- and K^+-content in isolated cardiac myocytes. The data clearly show that stimulation of the cardiac glucose transporter by insulin does not involve activation of the Na^+/K^+ pump, nor changes in the intracellular concentrations of Na^+ and K^+. On the other hand, inhibition of the pump activity by ouabain and changes in the $Na^+ : K^+$ ratio appear to be unrelated to the activity of the glucose carrier in myocardial cell.

Discussion

A variety of earlier studies on different preparations of muscle tissue suggested that the Na^+/K^+ pump, through its effect on internal levels of Na^+ and K^+, may exert a regulatory effect on sugar transport [5–7,16,17]. The present study was designed in order to detect such a possible relationship between the myocardial Na^+/K^+ pump and the glucose carrier using freshly isolated myocytes from the adult rat heart. These cells possess some major advantages when compared to other heart tissue preparations, as discussed in detail by Dow et al. [18]. Moreover, our laboratory has recently reported on the presence of specific insulin receptors and insulin responsiveness in this cell preparation [8–12].

The first major finding of the present investigation is represented by the lack of an effect of insulin on Na^+/K^+ pump activity in cardiac myocytes. In contrast, such an effect of insulin has been observed in a variety of noncardiac cell preparations including adipocytes [19], 3T3 cells [20], isolated hepatocytes [21] and various other tissues (for review, see Ref. 22). These studies have provided a molecular basis for the long known effect of insulin of lowering serum K^+ levels [23]. On the other hand, detailed informations on the modulation of myocardial Na^+/K^+ pump activity by insulin are lacking. A stimulatory action of insulin has been observed using a membrane-bound ($Na^+ + K^+$)-ATPase preparation from dog heart [24]. Werdan et al. [25] reported on a stimulation of Rb^+ uptake by insulin in cultures of neonatal rat heart cells. However, the known differences in pump activity between newborn and adult tissue [26] have to be taken into account. Recent studies by Ku and Sellers [27] on adult cardiac tissue demonstrated a reduction of myocardial Na^+/K^+ pump activity after induction of streptozotocin diabetes, in agreement with an earlier report by Onji and Liu on alloxan-induced diabetes in dog heart myocytes [28]. Addition of insulin either in vitro or in vivo completely reversed the pump depression, however, insulin failed to increase the Na^+/K^+ pump activity of the normal rat heart [27]. The results of the present investigation confirm the latter finding, suggesting that control of myocardial Na^+/K^+ pump activity by insulin may be related to the long term effects of this hormone. It may be argued, that perturbations of the cell membrane due to the collagenase isolation procedure used in the present study may have resulted in the apparent loss of insulin sensitivity of the Na^+/K^+ pump of cardiac myocytes. This, however, seems most unlikely, since retainement of specific insulin receptors [8–11] and receptor-effector coupling [12] in this cell preparation has been demonstrated by us.

Incubation of cardiac cells in the presence of calcium and/or magnesium resulted in a significant depression of Na^+/K^+ pump activity, in agreement with studies on dog heart myocytes [15] and on ($Na^+ + K^+$)-ATPase isolated from guinea-pig heart [29]. As discussed in detail by Liu and Onji [15], the nature of this inhibition is rather complex, involving competition at different activation sites and effects on the affinity for K^+ at two different sites. Another explanation stems from the studies of Hohl et al. [30], who observed a strong depression of passive sodium uptake by incubating cardiac myocytes in the presence of calcium. This ionic shift might result in a decrease in the activity of the Na^+/K^+ pump, as observed in the present investigation. Despite a marked reduction of the pump activity, the lack of an effect of insulin on the sodium pump persisted under all conditions. It is noteworthy, that basal activity of the glucose transport system is not decreased by the presence of calcium and magnesium, as recently shown by us [12].

An insulin-mimetic action of ouabain on hexose transport has been observed in adipose tissue [31], skeletal [17] and heart muscle [16]. This effect of

ouabain has been correlated with shifts in intracellular Na^+ or K^+ concentrations [5]. Based on these findings, the activity of the Na^+/K^+ pump has been suggested to operate as a negative feedback system controlling sugar transport in the myocardium [5]. Our data argue against this hypothesis. Both, a significant inhibition of Na^+/K^+ pump activity by ouabain and changes in intracellular $Na^+ : K^+$ ratio were found to be without effect on basal and insulin-stimulated hexose transport in isolated cardiac myocytes. The reason for this discrepancy is presently unclear. It may be related, however, to secondary actions of ouabain in the perfused tissue preparations used in the earlier studies [5,16].

In summary, our data suggest that myocardial glucose transport is independent of the activity of the Na^+/K^+ pump and of changes in intracellular Na^+ and K^+ concentrations. It appears that the pump activity is not acutely regulated by insulin in the normal heart.

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