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Insulin action on the glucose transport system in isolated cardiocytes from adult rat*

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Calcium-tolerant myocytes from the adult rat heart were used to study the effects of insulin on the kinetics of myocardial 3-*O*-methylglucose transport at 37°C. Insulin increased the initial velocity of sugar influx without affecting the equilibrium uptake values. Maximal stimulation averaged 50–80%, with a half-maximal response at an insulin concentration of 0.1 nM and maximal stimulation occurring at 1 nM. The onset of insulin action was preceded by a lag-phase of 20 s, reaching maximal action by 60 s. The $V_{max.}$ of the glucose transport system was increased from 160 to 287 nmol/min per 10^6 cells with an unaltered affinity. Neither extracellular nor intracellular calcium was found to be involved in the stimulatory action of insulin. Removal of intracellular magnesium resulted in a loss of insulin action. This study demonstrates that activation of the cardiac glucose transporter by insulin is due exclusively to an increase in the maximal velocity representing one of the very early effects of insulin on myocardial metabolism. The data suggest involvement of magnesium in the transmission of the insulin signal.

Stimulation of overall glucose metabolism is known to be one of the major biological actions of insulin in a variety of target tissues (Levine & Goldstein, 1955; Park *et al.*, 1959). The major rate-limiting step in the consumption of glucose is represented by its transport across the plasma membrane of insulin-sensitive cells like muscle and fat (Levine *et al.*, 1950; Morgan *et al.*, 1961) and this has been recognized to be the principal site of insulin action (Randle & Morgan, 1962; Crofford & Renold, 1965). Glucose transport is mediated by a carrier system exhibiting saturation kinetics, stereospecificity, competitive inhibition and counter-transport (for review, see Regen & Morgan, 1964). Insulin increases the maximal velocity of the glucose carrier with little or no change in the apparent affinity (Czech, 1980). This increase has been suggested to be due to a recruitment of carrier molecules from intracellular sites (Cushman & Wardzala, 1980; Susuki & Kono, 1980); alternatively, covalent modification or phosphorylation of the carrier may be involved (Czech, 1977). The early events, however, occurring in response to insulin–receptor interaction and finally leading to glucose transport stimulation remain completely unknown.

Cardiac glucose uptake and its regulation by insulin has been extensively studied using the isolated perfused rat heart (Morgan *et al.*, 1959, 1961; Fischer, 1971; Cheung *et al.*, 1978). However, information on the initial mechanism of insulin action is lacking, since modulation of the glucose transport system by insulin cannot be studied at the cellular and molecular level with this preparation. The aim of the present investigation was to approach these problems by using freshly isolated cardiocytes from adult rat, which we have shown to be an excellent model for the study of cardiac insulin receptors (Eckel & Reinauer, 1980*a*,*b*; Eckel *et al.*, 1982; Eckel & Reinauer, 1982). The data show that insulin increases the maximal velocity without affecting the affinity of the glucose transport system; the effect of insulin appears to be very rapid, with a lag-phase of about 20 s. Magnesium but not calcium may be involved in the transmission of the insulin signal in the cardiac cell.

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

* This paper is dedicated to Professor Karl Oberdisse on the occasion of his 80th birthday.

Materials and methods

Chemicals

3-*O*-[^{14}C]Methyl-D-glucose (sp. radioactivity 57.2 Ci/mol) and L-[1-^{14}C]glucose (sp. radioactivity

58 Ci/mol) were purchased from Amersham, Braunschweig, Germany. Porcine monocomponent insulin was from Novo, Bagsvaerd, Denmark. Collagenase (EC 3.4.24.3) and ionophore A23187 were obtained from Boehringer, Mannheim, Germany. 3-*O*-Methylglucose, cytochalasin B, EDTA, EGTA and hyaluronidase (EC 3.2.1.35) were supplied by Sigma, München, Germany. Trypsin (EC 3.4.21.4) and soya-bean trypsin inhibitor were from Worthington, Freehold, NJ, U.S.A. Hepes was purchased from Serva, Heidelberg, Germany. Purified bovine serum albumin was obtained from Behringwerke, Marburg, Germany. Bovine serum albumin (fraction V, reagent grade) was supplied by Miles, Frankfurt, Germany. ATP-monitoring-kit was from LKB, Gräfelfing, Germany. TS-1 and Quickszint 212 were from Zinsser, Frankfurt, Germany. Silicone oil (AR 200) (density 1.04 g/ml) was purchased from Wacker Chemie, München, Germany. All other chemicals were analytical grade and obtained from Merck, Darmstadt, Germany.

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. Myocytes were isolated by perfusion of the heart with collagenase as previously described (Eckel & Reinauer, 1980*a*) with the following modifications. The heart was preperfused *in situ* through the aorta for 3 min at 37°C with a nominally calcium-free Krebs–Ringer bicarbonate buffer, pH 7.4 (composition: NaCl, 35 mM; KCl, 4.75 mM; KH_2PO_4, 1.19 mM; Na_2HPO_4, 16 mM; $NaHCO_3$, 25 mM; sucrose 134 mM; Hepes 10 mM; glucose, 10 mM). Perfusion was continued for about 40 min with the same buffer supplemented with purified bovine serum albumin (5 g/l), collagenase (1 g/l) and hyaluronidase (1.5 g/l). The perfusion medium was gassed with O_2/CO_2 (19:1). At the end of the perfusion period the softened heart was removed, minced and incubated for 15 min at 37°C in 15 ml of the perfusion medium. The resultant dispersion was filtered through a nylon mesh and centrifuged for 5 min at 50 *g*. The supernatant was discarded and the cells were washed once with Hepes buffer (composition: NaCl, 130 mM; KCl, 4.8 mM; KH_2PO_4, 1.2 mM; Hepes, 25 mM; glucose, 5 mM; bovine serum albumin, 20 g/l; pH 7.4, equilibrated with O_2). The concentration of free calcium in this buffer was found to be less than 10 μM, as measured with an ion-selective electrode (Orion Research, Cambridge, MA, U.S.A). After washing, the cells were suspended in Hepes buffer and incubated for 30 min at 37°C in the presence of trypsin (1.9 units/ml). At the end of the incubation period soya-bean trypsin inhibitor was added and after filtration and centrifugation the cells were washed three times with Hepes buffer. Cell viability was judged by determination of the percentage of rod-shaped cells. Determination of the cell number was carried out in a Fuchs–Rosenthal chamber. Monitoring of cellular ATP content was performed on trichloroacetic acid extracts using a Luminometer 1250 (LKB) and the firefly luciferase reaction (Lundin *et al.*, 1976).

3-O-Methylglucose transport assay

All transport studies were performed in Hepes buffer, pH 7.4, at 37°C, containing D-glucose unless otherwise indicated. The reaction was started by addition of 50 μl of the cell suspension (2×10^4 cells) to 50 μl of Hepes buffer containing labelled 3-*O*-methylglucose and all other additions as indicated. Incubations were terminated by addition of 900 μl of cold (4°C) stopping solution, containing cytochalasin B (38 μM), 0.1% ethanol and 150 mM-NaCl. Control experiments demonstrated that this stopping solution completely inhibits sugar transport for at least 2 min. Two 300 μl portions of the resultant suspension were immediately transferred to precooled microfuge tubes containing 100 μl of silicone oil and centrifuged in a Beckman microfuge B for 40 s at 10000 *g*. The tip of the tube was cut off, added to a counting vial and treated with 1 ml of TS-1. After solubilization of the pellet, 3 ml of scintillation fluid was added and the radioactivity was determined by liquid-scintillation counting.

Expression of results

All data for sugar uptake reported in the present paper 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 experiments were carried out in triplicate; duplicate tubes containing L-[^{14}C]glucose instead of 3-*O*-[^{14}C]methylglucose were run in parallel to determine non-specific uptake. Results are expressed as means of at least three different experiments. Data were analysed by Student's *t* test; a *P* value less than 0.05 was considered to be statistically significant.

Results

Cell preparations

The use of adult cardiac myocytes has been limited by their hypersensitivity to calcium, which is expressed as a rapid irreversible decline in viability on exposure of the cells to physiological concentrations of calcium (Farmer *et al.*, 1977; for review, see Dow *et al.*, 1981). On the other hand, calcium has been suggested to be directly involved in the mechanism of insulin action on heart and skeletal muscle (Bihler, 1974; Clausen, 1975). Therefore, the

use of calcium-tolerant myocytes seems to be an important prerequisite for studies of insulin action.

To meet these requirements we have modified our previously published procedures (Eckel & Reinauer, 1980*a*) by performing an additional trypsin treatment of the cell suspension after the collagenase digestion. As shown in Fig. 1, freshly prepared cells exhibit an initial viability of 90%, which slightly decreases on incubation at 37°C. In the presence of calcium (2.5 mM) only an insignificant decrease in viability has been observed even after incubation for 3 h, demonstrating the calcium tolerance of this cell preparation. Moreover, electron-microscopic studies showed a well preserved fine structure of the cell. Myofibrils, mitochondria and nuclei appeared normal, as previously reported for calcium-tolerant myocytes (Kao *et al.*, 1980). It should be noted that insulin binding to calcium-tolerant cardiocytes is comparable with our previous observations on calcium-sensitive cells (Eckel & Reinauer, 1980*a*) (results not shown).

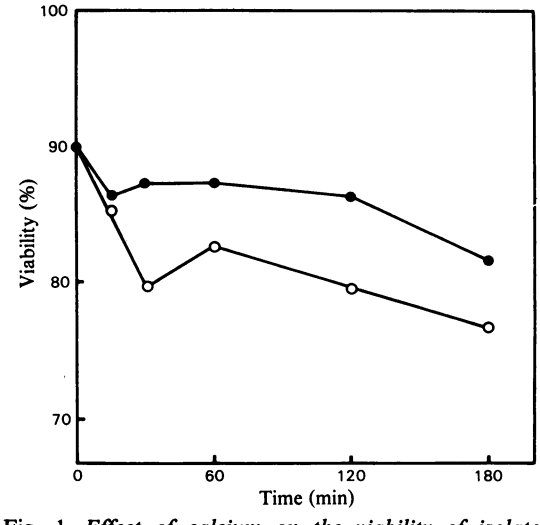

Fig. 1. *Effect of calcium on the viability of isolated cardiac myocytes*

Heart cells (1×10^5 cells/ml) were incubated at 37°C in the absence (●) or in the presence (○) of calcium (2.5 mM). At the indicated times, portions of the cell suspension were removed and the viability was determined from the percentage of rod-shaped cells. Data are means of three separate experiments.

Insulin action has been found to be dependent on a minimal amount of cellular ATP (Siegel & Olefsky, 1980). We have therefore monitored the ATP content of cardiac myocytes under various incubation conditions. In the presence of glucose the initial amount of ATP (190 nmol/10^6 cells) decreased by about 30% after 3 h at 37°C (Fig. 2*a*). This decrease could be prevented by the addition of calcium (2.5 mM). In the absence of exogenous substrates the initial ATP content fell to 130 nmol/10^6 cells and, upon incubation at 37°C, again decreased by about 30% after 3 h. This decrease, however, was not significantly affected by the addition of calcium. Insulin was without effect on the ATP content of cardiac cells under these conditions (Fig. 2*b*).

Time course of 3-O-methylglucose transport and the effect of insulin

Characterization of the cardiac glucose carrier was performed by measuring the transport of the

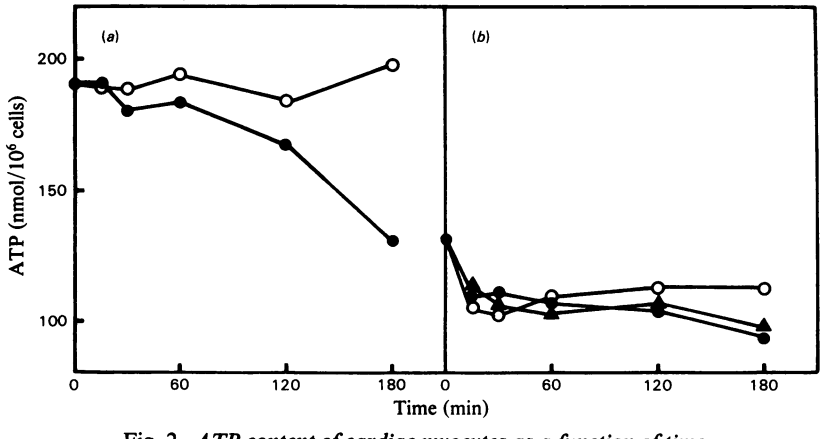

Fig. 2. *ATP content of cardiac myocytes as a function of time*

(*a*) 1×10^5 cells/ml were incubated in Hepes buffer in the absence (●) or in the presence (○) of calcium (2.5 mM). Determination of cellular ATP was performed at the indicated times on portions of the incubation mixture as outlined in the Materials and methods section. (*b*) Conditions were as for (*a*) with the exception that glucose was omitted. Incubations were performed in the absence (●) or in the presence of calcium (○) or insulin (0.2 μM) (▲). All data are means of three different experiments.

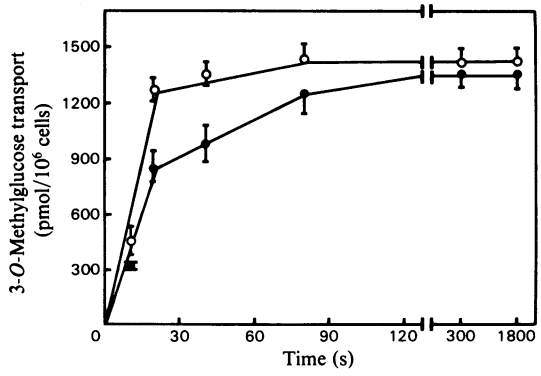

Fig. 3. *Effect of insulin on the time course of 3-O-methylglucose transport in cardiac myocytes*
Cardiocytes were incubated for 30 min at 37°C in Hepes buffer (without D-glucose) in the absence (●) or in the presence (○) of insulin (0.27 μM). Portions of the cell suspension (final cell concentration 2×10^5 cells/ml) were then incubated with 3-*O*-[^{14}C]-methylglucose (final concentration 50 μM) for the indicated times. Uptake was stopped and quantified as described in the Materials and methods section. All data have been corrected for non-specific uptake by the use of L-[^{14}C]glucose. Data are means ± S.E.M. of three separate experiments.

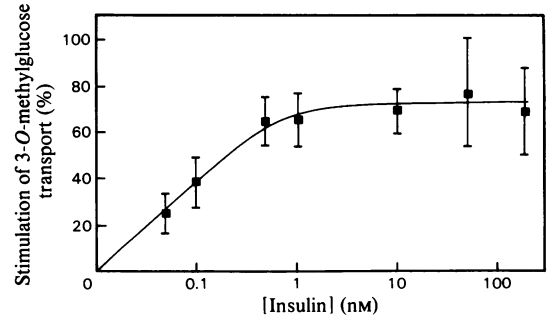

Fig. 4. *Dose–response relationship for insulin-stimulated transport of 3-O-methylglucose*
Myocytes were incubated for 60 min with increasing concentrations of insulin (50 pM to 0.27 μM). Transport of 3-*O*-[^{14}C]methylglucose (100 μM) was then determined after incubation of 2×10^5 cells/ml for 20 s. Data are means ± S.E.M. taken from three to nine different experiments.

non-metabolizable glucose analogue 3-*O*-methylglucose, which has been shown to enter the cell by the same transport system as glucose in this tissue (Morgan *et al.*, 1964). The transport process exhibited saturation kinetics with a K_m of 6.8 mM, stereospecificity, counter-transport and temperature-dependence with an activation energy of 17.1 kcal/mol (results not shown). These observations confirm a very recent report by Gerards *et al.* (1982) on the uptake of glucose and 3-*O*-methylglucose by adult cardiac myocytes.

The time course of 3-*O*-methylglucose transport in cardiac cells at 37°C is presented in Fig. 3. After an initial influx phase of up to 20 s the transport velocity declines, reaching a plateau by 5 min, which can be maintained up to at least 30 min. Treatment of cells with insulin (270 nM, 30 min) resulted in an acceleration of the initial sugar influx without affecting the equilibrium uptake values. The time for half-maximal uptake decreased from 17 s to 12 s in basal and insulin-treated cells respectively. It is noteworthy that the uptake of L-glucose (representing diffusion plus extracellular trapping of radioactivity in the cell pellet) never exceeded 20% of total 3-*O*-methylglucose uptake.

The sensitivity of cardiocytes towards glucose transport stimulation by insulin was assessed by incubating the cells with increasing concentrations of insulin for 60 min in the presence of D-glucose after which 3-*O*-methylglucose transport was determined. As shown in Fig. 4, a half-maximal response was achieved at 0.1 nM, with maximal stimulation occurring at 1 nM. Studies on insulin binding showed that half-maximal stimulation occurs with an occupancy of only 2% of total insulin receptors present on the myocytes (results not shown). Maximal stimulation of glucose transport averaged 50–80%, depending on the estimation of the basal transport rate. It is noteworthy that activation of the glucose carrier by insulin has been found to be an energy-requiring process and that at least an amount of 60 nmol of ATP/10^6 cells has been found to be necessary for insulin to activate the cardiac glucose transport system (results not shown).

The coupling of insulin receptors to the glucose-transport system has been studied by using a 5 s transport assay and high concentrations of insulin (270 nM). Under these conditions insulin–receptor association is assumed to be not rate-limiting (Ciaraldi & Olefsky, 1979), and the observed delay in the transport activation must be due to the coupling process. As shown in Fig. 5, after the addition of insulin there is a time lag of at least 20 s before the transport activity increases above basal, whereas 60 s are required to obtain the maximal response. These data demonstrate that stimulation of cardiac glucose transport, such as that in adipocytes (Whitesell & Gliemann, 1979), represents a very rapid process and one of the early effects of insulin.

Effect of insulin on the transport parameters

The concentration-dependence of the initial velocity of methylglucose transport in basal and insulin-stimulated cells was measured under zero-trans conditions and is presented in Fig. 6 (Hanes plot of the data). It appears that insulin increases the

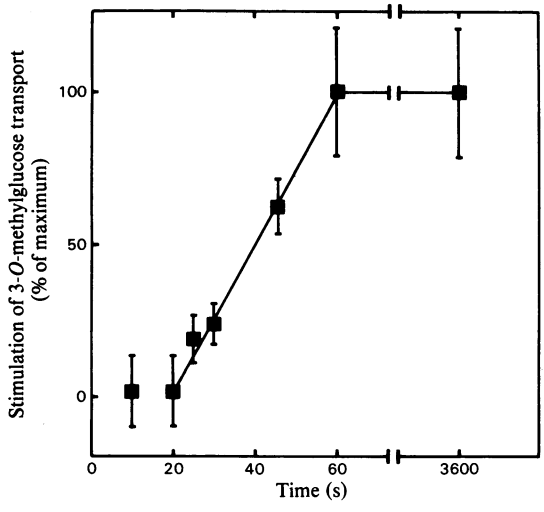

Fig. 5. *Time course of the onset of insulin action on 3-O-methylglucose transport*

2×10^5 cells/ml were incubated in Hepes buffer. At time zero, insulin (0.27 μM) was added and subsequently 3-*O*-methylglucose transport was determined over a 5 s assay period at the indicated time intervals. Each point is the mean ± S.E.M. of six separate experiments.

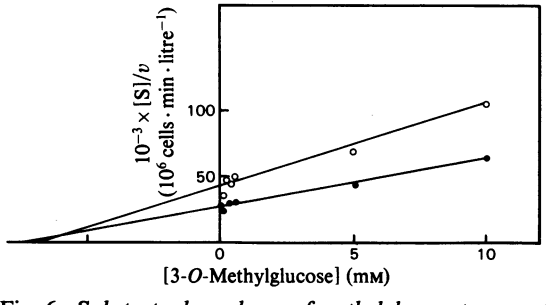

Fig. 6. *Substrate dependence of methylglucose transport under zero-trans conditions*

Cardiocytes were incubated for 30 min in Hepes buffer (without D-glucose) in the absence (○) or in the presence (●) of insulin (0.27 μM). Sugar transport was then monitored by incubating 2×10^5 cells/ml for 20 s with 3-*O*-[^{14}C]methylglucose (100 μM) and increasing concentrations of unlabelled methylglucose. Data are presented according to the Hanes form of the flux equation and represent mean values of four experiments. The lines shown have been obtained by linear regression analysis of the data points (coefficient of correlation 0.989 and 0.999 for basal and insulin-treated cells respectively).

$V_{max.}$ without significantly affecting the K_m of the carrier system. The K_m values for basal and insulin-treated cells were found to be 6.8 and 7.7 mM respectively. The corresponding $V_{max.}$ values were 160 and 287 nmol/min per 10^6 cells.

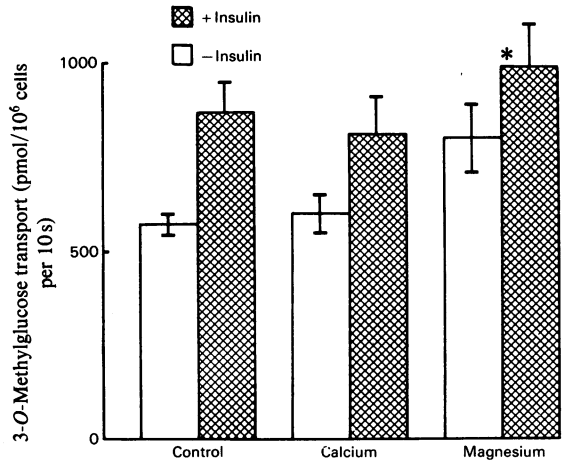

Fig. 7. *Effect of extracellular calcium and magnesium on basal and insulin-stimulated transport of 3-O-methylglucose*

Heart cells were incubated for 15 min in the absence or presence of calcium (2.5 mM) or magnesium (1.2 mM). Incubation was then continued for an additional 15 min period in the absence or presence of insulin (0.35 μM). Transport of 3-*O*-methylglucose was then determined by using a 10 s assay period. Data represent mean ± S.E.M. of six to nine different experiments. * Indicates a significant difference from the basal value at $P < 0.05$.

Effect of calcium and magnesium on the glucose transport stimulation by insulin

Both calcium and magnesium have been suggested to be involved in the glucose transport stimulation by insulin in heart and skeletal muscle (Gould & Chaudry, 1970; Bihler, 1980; Clausen, 1980). In the case of calcium, either extracellular and intracellular calcium have been implicated in insulin's stimulatory action (Schudt *et al.*, 1976). However, the role of calcium in this process is not unequivocal. As recently shown by Haworth *et al.* (1982), insulin stimulates cardiac deoxyglucose transport in the absence of calcium. We have therefore investigated the effect of extra- and intra-cellular cations on basal and insulin-stimulated sugar influx in cardiac myocytes.

As shown in Fig. 7, extracellular calcium does not significantly affect basal and insulin-stimulated transport. However, addition of magnesium induces an increase in the basal transport rate of 3-*O*-methylglucose by about 40%, resulting in a lower, but still significant, response to insulin. Removal of extracellular and presumably membrane-bound calcium and magnesium by treatment of cells with the

Table 1. *Effect of EDTA, EGTA and ionophore A23187 on basal and insulin-stimulated transport of 3-O-methylglucose in isolated cardiac myocytes*

Myocytes (4×10^5 cells/ml) were pre-incubated for 30 min with ionophore A23187 (20 μM), EDTA (1 mM), EGTA (1 mM) or a combination of these drugs as indicated. Incubation was then continued for 15 min in the absence and in the presence of insulin (0.27 μM). Subsequently, portions of the cell suspension were incubated with 3-*O*-methylglucose (final concentration 50 μM; 2×10^5 cells/ml) for 10 s for the determination of initial transport rates as outlined in the Materials and methods section. Data are means ± S.E.M. of three separate experiments. All calculated stimulations are significant with $P < 0.05$ unless otherwise indicated.

Treatment	Insulin	Transport rate (pmol/10^6 cells per 10 s)	Stimulation (%)
Control	–	331 ± 60	
	+	515 ± 54	56
EDTA	–	365 ± 15	
	+	596 ± 13	63
Ionophore A23187	–	229 ± 33*	
	+	409 ± 69	79
Ionophore A23187 + EDTA	–	302 ± 56	
	+	323 ± 58†	7
Ionophore A23187 + EGTA	–	264 ± 49	
	+	371 ± 79	41

* Not significantly different from untreated basal transport rate at $P > 0.05$.

† Not significantly different from basal value at $P > 0.05$.

chelator EDTA had no effect on basal 3-*O*-methylglucose transport and the stimulatory action of insulin (Table 1). It is noteworthy that the ATP content of cardiocytes is not affected by treatment with EDTA; insulin binding, however, has been found to be significantly reduced under these conditions (results not shown).

It has been suggested that the regulation of glucose transport by insulin involves the participation of a specific intracellular calcium pool rather than extracellular calcium (Bihler *et al.*, 1980). One possible approach to modify cytoplasmic calcium concentrations is represented by using the ionophore A23187 (Schudt *et al.*, 1976; Bihler *et al.*, 1980). Treatment of cardiocytes with this drug resulted in an insignificant depression of basal hexose transport with an unaltered stimulation by insulin (Table 1). To deplete the cells of intracellular calcium and magnesium we have used a combined treatment with the ionophore and the chelator EDTA. As shown in Table 1, the stimulatory action of insulin was totally abolished by this treatment with an unaltered basal rate of sugar transport. The use of EGTA, which has a much lower affinity for magnesium than for calcium (Yu *et al.*, 1980), instead of EDTA, revealed an apparent specificity of this effect for magnesium (Table 1). The magnesium content of cardiac cells, as determined by atomic-absorption spectrophotometry, was found to be decreased by 50% when treated with ionophore plus EDTA, but only by 15–20% when treated with ionophore plus EGTA (results not shown). It is noteworthy that the viability and the ATP content of treated and untreated cells was not significantly altered under all conditions. In contrast with previous work on the myocardium our data strongly suggest that instead of calcium intracellular magnesium may be involved in the stimulatory action of insulin on cardiac glucose transport.

Discussion

Several laboratories have recently reported on the preparation of calcium-tolerant myocytes from adult rat hearts. Procedures involved either the use of complex isolation media (Kao *et al.*, 1980; Montini *et al.*, 1981) or tryptic digestion along with variations of the calcium concentration during the enzymic treatment (Haworth *et al.*, 1980). We have modified our previously published procedure by using low concentrations of calcium (less than 10 μM) and an additional trypsin treatment after the collagenase digestion. This preparation exhibits an excellent tolerance to physiological concentrations of calcium. Moreover, sufficient amounts of ATP can be retained even after longer incubation periods in the absence of exogenous substrates (see Fig. 2). In the light of viability, calcium tolerance and retention of specific insulin receptors our preparation of cardiac myocytes appears to represent an adequate model for the study of insulin action.

The sensitivity of cardiac myocytes towards glucose transport stimulation (half-maximal stimulation at an insulin concentration of 0.1 nM) and the energy requirements of this process compare well with the recent reports on isolated adipocytes (Whitesell & Gliemann, 1979; Siegel & Olefsky, 1980). Maximal transport stimulation in the cardiac cell, however, is much lower (average 50–80%) when compared with adipocytes, in agreement with previous work on the perfused heart (Morgan *et al.*, 1961; Fischer, 1971) and with the data of Haworth *et al.* (1982) on the uptake of deoxyglucose by isolated adult rat heart cells.

The mechanisms involved in translating the signal of insulin receptor occupancy into the biological response of glucose transport stimulation are not adequately understood. Two types of coupling have been suggested. First, the receptor is directly coupled to the effector system analogous to an allosteric enzyme (Cuatrecasas *et al.*, 1975). Secondly, a

physical or chemical signal is generated upon receptor occupancy by the hormone (Ciaraldi & Olefsky, 1979). The latter type would involve a delay between binding and onset of the biological response. As shown in the present paper, insulin action on cardiac glucose transport is preceded by a short time lag of about 20s, after which insulin action gradually increases, reaching maximal stimulation by 60s. This observation is in excellent agreement with the results of Whitesell & Gliemann (1979) on the transport of 3-*O*-methylglucose in isolated adipocytes. Thus it appears that in cardiac muscle, like in adipose tissue, the stimulation of glucose transport by insulin involves a physical or chemical signal, which may be generated upon binding of the hormone to its receptor.

The effect of insulin on the kinetic constants of cardiac glucose transport appears to be controversial. Working on the perfused rat heart, Morgan *et al.* (1961) reported an increase in both K_m and $V_{max.}$; more detailed kinetic experiments by Cheung *et al.* (1978) under both zero-trans and equilibrium exchange conditions demonstrated that insulin increases the maximal rate of sugar transport with no effect on the K_m. In agreement with the latter studies we have found that in isolated cardiac myocytes insulin action on the glucose transport system is exclusively due to an increase in $V_{max.}$ with an unaltered affinity of the carrier. These observations are consistent with the suggestion that insulin action is due to a translocation of glucose transport systems from a specific intracellular pool to the plasma membrane (Karnieli *et al.*, 1981). In contrast with our findings Gerards *et al.* (1982), in their studies on isolated cardiocytes, reported an increase in the K_m value by a factor of 3 and an increase in the $V_{max.}$ value by a factor of 9, resulting in a stimulation of 3-*O*-methylglucose transport by 500–800% at a temperature of 23°C. This large effect of insulin, however, is open to question, since at this temperature only an insignificant stimulation (about 15%) of glucose uptake by insulin has been observed in the perfused rat heart preparation (Fischer, 1971).

A variety of recent studies have focused on the role of calcium in the insulin-induced stimulation of glucose transport (Clausen, 1980). As suggested by Bihler (1980), sugar transport may be regulated by calcium binding to a specific regulatory site. The findings reported in the present paper give no support for these assumptions. Two lines of evidence argue against the involvement of calcium in the activation of the glucose transporter in the cardiac cell. First, variations in the extracellular calcium concentration as well as depletion of intracellular calcium by use of ionophore A23187 in combination with EGTA (Yu & Gould, 1981) did not interfere with the stimulatory action of insulin. Secondly, ionophore A23187, which has been shown to increase cytoplasmic calcium concentrations (Hainaut & Desmedt, 1974; Murray *et al.*, 1975), did not affect basal and insulin-stimulated hexose influx. Our findings agree with a very recent report by Haworth *et al.* (1982) on isolated heart cells and the work of Reeves (1977) on rat thymocytes. In contrast, Bihler (1980) in guinea-pig atria and Schudt *et al.* (1976) in developing muscle cells demonstrated a direct involvement of calcium in the stimulatory action of insulin. The reasons for these discrepancies are presently unclear. Preliminary observations in our laboratory suggest that the sensitivity of cardiac cells is strictly dependent on the presence of extracellular calcium with an unaltered maximal response at very high concentrations of insulin. This shift in sensitivity may result in an apparent loss of insulin action at the insulin concentrations used in the perfusion experiments described by Bihler (1980).

Recently, Yu & Gould (1981) proposed that muscle sugar transport is regulated by an intracellular magnesium pump and stimulated through an increase in cytoplasmic magnesium concentration. Our observations on cardiac myocytes lend further support to this hypothesis. Removal of intracellular bivalent cations by treatment of cells with ionophore A23187 and EDTA resulted in a loss of insulin action. On replacement of EDTA by EGTA the effect of insulin became evident again. In the light of the same affinity of both chelators for calcium but a 2×10^4 lower affinity of EGTA for magnesium (Yu *et al.*, 1980), the involvement of intracellular magnesium in the regulation of cardiac glucose transport by insulin is strongly suggested. The site of magnesium action is presently unclear, one possibility being the involvement in transphosphorylation reactions (Bowen-Pope & Rubin, 1977). Addition of extracellular magnesium resulted in an insulin-like stimulation of cardiac glucose transport, which might be related to an increase in cytoplasmic magnesium concentrations. However, in the light of the slow exchange of magnesium across the sarcolemmal membrane (Shine, 1979), other mechanisms, such as a direct effect of magnesium on potassium channels (Shine & Douglas, 1975) or on the $(Na^+ + K^+)$-stimulated ATPase activity (Seller, 1971), have to be considered.

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