Involvement of hormone processing in insulin-activated glucose transport by isolated cardiac myocytes

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Isolated muscle cells from adult rat heart were used to study the relationship between myocardial insulin processing and insulin action on 3-*O*-methylglucose transport at 37 °C. Internalization of the hormone as measured by determination of the non-dissociable fraction of cell-bound insulin increased linearly up to 10 min, reaching a plateau by 30–60 min at 3 nM-insulin. At this hormone concentration the onset of insulin action was found to be biphasic, with a rapid phase up to 8 min, followed by a much slower phase, reaching maximal insulin action by 30–60 min. Insulin internalization was totally blocked by phenylarsine oxide, whereas dansylcadaverine had no effect on this process. Initial insulin action (5 min) on glucose transport was not affected by chloroquine and dansylcadaverine, but was completely abolished by treatment of cardiocytes with phenylarsine oxide. This drug effect was partly prevented by the presence of 2,3-dimercaptopropanol. Under steady-state conditions (60 min), the stimulatory action of insulin was decreased by about 60 % by both chloroquine and dansylcadaverine. This study demonstrates that insulin action on cardiac glucose transport is mediated by processing of the hormone. The data suggest dual pathways of insulin action involving initial processing of hormone–receptor complexes and lysosomal degradation.

INTRODUCTION

Receptor-mediated endocytosis and lysosomal association of insulin has now been recognized to follow the initial binding event in a variety of target cells (Carpentier *et al.*, 1979; Marshall & Olefsky, 1979). The physiological significance of this process remains obscure. Although its main function appears to be related to degradative processing of the hormone (Marshall, 1985b), internalized insulin has been suggested to serve as its own intracellular messenger (Goldfine, 1977). Subsequent findings from different laboratories lend further support to this hypothesis. Thus, inhibition of protein degradation (Draznin & Trowbridge, 1982; Trowbridge & Draznin, 1986) and stimulation of alanine transport (Peavy *et al.*, 1984) in isolated hepatocytes have been found to be functionally related to insulin internalization. Moreover, internalized insulin–receptor complexes exhibit significant physiological activities, as reported by Ueda *et al.* (1985).

In contrast with the studies cited above, very rapid actions of insulin, such as stimulation of glucose transport, have been assumed to be exclusively mediated by the initial insulin–receptor interaction at the cell surface (Marshall & Olefsky, 1980; Häring *et al.*, 1981) and to be independent of the lysosomal pathway. More recent reports in the literature, however, cast doubt on this assumption, suggesting a possible relationship between insulin processing and stimulation of glucose transport in isolated adipocytes (Frost & Lane, 1985; Jochen & Berhanu, 1987; Hashimoto *et al.*, 1987).

In the light of these controversial findings, attempts have been made to rule out a possible relationship between insulin internalization and activation of glucose transport. Freshly isolated cardiocytes from adult rat, which we have shown to possess specific insulin receptors

(Eckel & Reinauer, 1980, 1984) mediating insulin internalization and lysosomal processing (Eckel & Reinauer, 1982) and a very rapid activation of glucose transport (Eckel *et al.*, 1983), have been used to approach these problems. The data suggest a functional relationship between insulin processing and stimulation of cardiac glucose transport.

MATERIALS AND METHODS

Chemicals

Pig [A14-¹²⁵I]iodotyrosine[insulin (sp. radioactivity 2200 Ci/mmol) was from New England Nuclear, Dreieich, Germany. 3-*O*-[¹⁴C]Methyl-D-glucose (sp. radioactivity 57.2 Ci/mol), L-[1-¹⁴C]glucose (sp. radioactivity 58 Ci/mol) and [³H]triphenylmethylphosphonium ([³H]TPMP) iodide (sp. radioactivity 31 Ci/mmol) were purchased from Amersham, Braunschweig, Germany. Pig monocomponent insulin was from Novo, Bagsvaerd, Denmark. Collagenase (EC 3.4.24.3) was obtained from Boehringer, Mannheim, Germany. Bovine serum albumin (fraction V, reagent grade) was purchased from Miles, Frankfurt, Germany. Hyaluronidase (EC 3.2.1.35), EDTA, chloroquine, dansylcadaverine, phenylarsine oxide (PhAsO), 2,3-dimercaptopropanol and isoprenaline (isoproterenol) were from Sigma, München, Germany. Hepes was obtained from Serva, Heidelberg, Germany. All other chemicals were analytical grade and supplied by 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. Ca²⁺-tolerant myocytes were isolated by

Abbreviations used: PhAsO, phenylarsine oxide; TPMP⁺, triphenylmethylphosphonium cation.

perfusion of the heart with collagenase as detailed previously (Eckel *et al.*, 1983). The final cell suspension was washed three times 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) and stored in silicone-treated 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 was checked by determination of the percentage of rod-shaped cells. Cellular ATP content was monitored in trichloroacetic acid extracts by using a Luminometer 1250 (LKB) and the firefly luciferase reaction, as previously described (Eckel *et al.*, 1983).

Insulin-binding assay

Isolated cardiac myocytes (2×10^5 cells/ml) were incubated with ^{125}I -insulin and unlabelled insulin (final total concn. 3 nM) in Hepes buffer, pH 7.4, at 37 °C in a final volume of 0.5 ml. Incubations were terminated by the oil-centrifugation technique as detailed by Eckel & Reinauer (1980). All binding studies were carried out in triplicate; duplicate tubes containing an excess of unlabelled insulin (1 μM) were incubated in parallel to determine non-specific binding. Specific binding was calculated by subtracting the amount of ^{125}I -insulin bound in the presence of unlabelled insulin from total binding. All binding data reported in this paper have been corrected for non-specific binding, which never exceeded 20–30 % of total binding.

The extent of insulin internalization was estimated by measuring the non-dissociable fraction of cell-bound insulin after various times of incubation. Specifically, at the end of the association period, excess unlabelled insulin (1 μM) plus EDTA (5 mM) was added, and incubation was continued for 5 min at 37 °C (final volume 0.55 ml). EDTA was included since it significantly decreases receptor affinity (Eckel & Reinauer, 1984). This method removes essentially all cell-surface-located ligand, as verified by control experiments with cells treated with PhAsO, a drug which is known to block insulin internalization (Knutson *et al.*, 1983) and endocytosis in mammalian cells (Low *et al.*, 1981).

3-O-Methylglucose-transport assay

Transport experiments were carried out at 37 °C in Hepes buffer, pH 7.4, containing D-glucose (5 mM). The reaction was started by addition of 50 μl of the cell suspension (2×10^4 cells) to 50 μl of Hepes buffer containing 3-O-[^{14}C]methyl-D-glucose (final concn. 100 μM). Incubations were terminated by using a stopping solution and the oil-centrifugation technique, as outlined by Eckel & Reinauer (1984). All data for sugar 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 experiments were carried out in triplicate; duplicate tubes containing L-[^{14}C]glucose were run in parallel to determine non-specific uptake.

The above technique was also used for monitoring uptake of [^3H]TPMP⁺, a lipophilic cation which may be used for determination of membrane potentials in isolated cells (Cheng *et al.*, 1980). All data for [^3H]TPMP⁺ uptake were corrected for extracellular trapping of radioactivity by performing a 'zero-time'

Fig. 1. Binding of ^{125}I -insulin to isolated heart cells as a function of time, and determination of the non-dissociable fraction

Cardiac myocytes (2×10^5 cells/ml) were incubated with ^{125}I -labelled and unlabelled insulin (final concn. 3 nM) in Hepes buffer. At the indicated times one set of tubes (■) was used for the determination of specific insulin binding, whereas a second set of tubes (□) was incubated for a further 5 min in the presence of EDTA and excess insulin (1 μM) as detailed in the Materials and methods section. All data reported have been corrected for non-specific binding, and represent mean values $\pm$ S.E.M. for four separate experiments.

assay. This consisted of direct addition of cells to cold stopping solution containing labelled TPMP⁺, and immediately centrifuging portions of the resulting suspension as described above. Calculation of membrane-potential changes was based on equilibrium uptake values (60 min at 37 °C) and the Nernst relation, as described in detail by Cheng *et al.* (1980).

All data analysis was run on an IBM Personal Computer by using Statgraphics (STSC, Rockville, MD, U.S.A.) statistical software. Significance of reported differences was evaluated by using the null hypothesis and *t* statistics for paired data. Corresponding significance levels are indicated in the legends to Figures.

RESULTS

Kinetics of insulin internalization

Acid or proteolytic dissociation steps are commonly used for determination of insulin internalization in isolated cells (Kahn & Baird, 1978). Both methods proved to be inappropriate with cardiac myocytes, either because of a large increase in non-specific binding or because of incomplete dissociation of bound ligand. The method used in the present investigation (excess insulin plus EDTA) removes all cell-surface-located insulin within 5 min of incubation, with an unaltered non-specific binding. As shown in Fig. 1, the non-dissociable fraction of cell-associated insulin increases linearly up to 10 min, reaching a plateau by 30–60 min at an insulin concentration of 3 nM. This concentration was used since it results in maximal stimulation of glucose transport (Eckel *et al.*, 1983), and thus a direct correlation between insulin internalization and insulin action could be achieved. In contrast with insulin internalization, insulin binding to the myocytes was very rapid under these

Fig. 2. Time course of the onset of insulin action on 3-*O*-methylglucose transport

Cardiocytes (2×10^5 /ml) were incubated in Hepes buffer at 37 °C. At zero time insulin (3 nM) was added, and incubation was continued for time periods of 30 s up to 60 min. At the indicated times 3-*O*-methylglucose transport (■) was determined over a 10 s assay period. Insulin internalization (●) was calculated from data in Fig. 1. Data represent means $\pm$ S.E.M. for three different experiments: *significantly different from transport stimulation at 8 min ($P < 0.018$).

conditions, with a plateau reached by at least 5 min (Fig. 1). Under steady-state conditions about 50% of cell-bound insulin was found to be non-dissociable, in agreement with previous reports on isolated adipocytes (Kahn & Baird, 1978) and with more recent autoradiographic observations in cardiac myocytes (Junger *et al.*, 1986).

As presented in Fig. 2, the kinetics of insulin internalization were comparable with the time course of the onset of insulin action on 3-*O*-methylglucose transport when incubated under identical conditions. Thus two phases can be clearly distinguished for both curves. A rapid phase up to 8 min resulted in stimulation of transport by about 80% and a 30% non-dissociable fraction of cell-bound insulin. After that time point a much slower phase was observed, but the stimulatory action of insulin still significantly increased by 57%, reaching a maximal transport stimulation of about 130% after 30–60 min of incubation (Fig. 2, upper curve). A proportionate (56%) increase in insulin internalization between 10 and 60 min paralleled the slower phase of glucose-transport stimulation. This striking kinetic similarity points to a possible functional relationship between the two reactions.

Inhibition of insulin processing

Insulin processing by isolated cells can be selectively studied by using a variety of specific inhibitors (Marshall & Olefsky, 1980; Peavy *et al.*, 1984; Ueda *et al.*, 1985). Effects of chloroquine, a lysosomotropic agent, on isolated cardiac myocytes have been reported by us (Eckel & Reinauer, 1982). The data were consistent with receptor-mediated internalization and lysosomal degradation of insulin in these cells. In order to block the initial post-binding events, two other inhibitors, PhAsO and dansylcadaverine, have now been examined. The former, a trivalent arsenical, irreversibly blocks endocytosis (Low *et al.*, 1981), whereas the latter is a known inhibitor of transglutaminase, an enzyme which has been

Fig. 3. Effect of PhAsO and dansylcadaverine on the non-dissociable fraction of cell-associated insulin

Myocytes (2×10^5 cells/ml) were preincubated for 30 min with PhAsO (1 μ M) or dansylcadaverine (0.1 mM). 125 I-insulin (3 nM) was then allowed to associate for 5 min, followed by determination of specific insulin binding (■) and the non-dissociable fraction (▨), as outlined in the legend to Fig. 1. Data presented are mean values $\pm$ S.E.M. for three different experiments; *total binding not significantly different from non-specific binding ($P > 0.05$).

thought to be involved in the process of polypeptide-hormone internalization (Davies *et al.*, 1980). Treatment of cardiac myocytes with PhAsO (1 μ M) totally abolished the non-dissociable fraction of insulin bound to the cells after 5 min of incubation, with no significant effects on the amount of specific cell-associated insulin (Fig. 3). In contrast, dansylcadaverine was without effect on both fractions. This drug, however, prevented the increase in cell-associated radioactivity produced by chloroquine (results not shown), suggesting that dansylcadaverine inhibits insulin processing by blocking the transfer of internalized insulin to the lysosomal compartment.

The use of inhibitors may be limited by changes in cell viability, a decrease in cellular energy or general non-specific perturbations of cellular function. As shown by the ATP profiles presented in Fig. 4, the maximal decrease in content of this nucleotide never exceeded 45%, corresponding to a value of 82 nmol of ATP/ 10^6 cells. As reported previously (Eckel *et al.*, 1983), an amount of at least 60 nmol of ATP/ 10^6 cells has been found necessary for insulin to activate fully the cardiac glucose-transport system. It should be noted that the viability of cardiac myocytes remained unaltered at all incubation conditions indicated in Fig. 4. The selectivity of PhAsO was ruled out by additional control experiments described in Table 1. The data show that isoprenaline-induced depolarization of cardiac myocytes is not affected by PhAsO treatment. Retention of this β -receptor-specific effect (J. Eckel & H. Reinauer, unpublished work) strongly suggests that the effects of this drug are not due to gross structural perturbations of the cell.

Insulin processing and insulin action

Insulin action on glucose transport was studied after incubation for 5 and 60 min after modification of hormone processing at different levels by using selective inhibitors as described above. Treatment of cardiocytes

Fig. 4. Effect of inhibitors on ATP content of isolated cardiac myocytes

Myocytes (10^5 cells/ml) were incubated in the absence (●) or the presence of PhAsO ($1 \mu\text{M}$) (■), dansylcadaverine (0.1 mM) (▼) or chloroquine (0.1 mM) (▲). Cellular ATP was determined in portions of the incubation mixture as outlined in the Materials and methods section. Data are means of four separate experiments.

Table 1. Effect of PhAsO on isoprenaline-induced depolarization of cardiac myocytes

Myocytes (4×10^5 cells/ml) were preincubated for 15 min with PhAsO. TPMP⁺ uptake was then determined after incubating 2×10^4 cells with [³H]TPMP⁺ (final concn. 69 nM) for 60 min in the absence or the presence of isoprenaline. Depolarization was calculated as outlined in the Materials and methods section. Data are means $\pm$ S.E.M. for three separate experiments.

Condition	TPMP ⁺ uptake (c.p.m./60 min per 1.2×10^4 cells)	Depolarization (mV)
Control	68538 ± 2173	
Isoprenaline ($10 \mu\text{M}$)	59417 ± 2214	-3.72 ± 0.82
PhAsO ($1 \mu\text{M}$)	77956 ± 5267	
PhAsO + isoprenaline	64893 ± 4329	-4.70 ± 1.66

with PhAsO resulted in a total loss of initial (5 min) insulin action on the glucose-transport system, whereas chloroquine and dansylcadaverine were without effect under these conditions (Fig. 5). A possible site of PhAsO action may be related to vicinal thiol groups (Webb, 1966), which may form stable ring complexes with this compound. This was tested by treating the myocytes with PhAsO in the presence of 2,3-dimercaptopropanol ($160 \mu\text{M}$), a dithiol compound which was shown to reverse completely the PhAsO effects on insulin-stimulated glucose transport in isolated adipocytes (Frost & Lane, 1985). In our hands about 20–30 % of the initial insulin action was maintained by addition of 2,3-dimercaptopropanol (results not shown).

Under steady-state conditions (60 min) the stimulatory action of insulin was significantly decreased from 63 to only 24 % after treatment of cells with chloroquine (Fig. 6). A qualitatively similar inhibition was observed in the

Fig. 5. Effect of PhAsO, chloroquine and dansylcadaverine on initial insulin action on 3-O-methylglucose transport

Heart cells (2×10^5 cells/ml) were incubated in the absence or the presence of PhAsO ($1 \mu\text{M}$), chloroquine (0.1 mM) or dansylcadaverine (0.1 mM) for 30 min at 37°C . Incubation was then continued for a further 5 min in the absence (□, basal) or presence (▨) of insulin (3 nM), followed by determination of 3-O-methylglucose transport over a 10 s assay period. The data shown are mean values $\pm$ S.E.M. obtained from five or six separate experiments: *significantly different from basal transport rates ($P < 0.05$).

Fig. 6. Effect of chloroquine and dansylcadaverine on steady-state insulin action on 3-O-methylglucose transport

Myocytes were incubated for 30 min in the absence or presence of chloroquine (0.1 mM) or dansylcadaverine (0.1 mM). Incubation was continued for 60 min in the absence (□, basal) or presence (▨) of insulin (3 nM). At the end of the incubation period, glucose transport activity was determined as outlined in the legend to Fig. 5. Data reported are means $\pm$ S.E.M. for six separate experiments.

presence of dansylcadaverine, though basal transport rates were decreased under these conditions (Fig. 6). These data suggest involvement of both insulin internalization and lysosomal processing in insulin action on cardiac glucose transport.

DISCUSSION

Initial insulin binding to cell-surface receptors is followed by a complex chain of events which has been

collectively termed 'processing of hormone-receptor complexes' (Baldwin *et al.*, 1980). Subsequent evidence indicates involvement of these processes in some, but not all, physiological actions of insulin (Trowbridge & Draznin, 1986). Specifically, very rapid actions of the hormone on membrane-located transport systems have been exclusively ascribed to initial insulin-receptor interactions (LeCam *et al.*, 1979; Marshall & Olefsky, 1980; Häring *et al.*, 1981), since insulin internalization was found to proceed much slower than the onset of insulin action and was even absent at lower temperatures. However, these authors provided only a rough estimate of overall insulin internalization by either using microscopic techniques (LeCam *et al.*, 1979) or measuring insulin accumulation in chloroquine treated cells (Marshall & Olefsky, 1980; Häring *et al.*, 1981).

Improved techniques (Wiley & Cunningham, 1982; Marshall, 1985a) have now established that surface-derived insulin can be identified within cells as early as 30 s. In the present investigation, insulin internalization was determined by measuring the non-dissociable fraction of cell-bound insulin. By using this method, the half-time of insulin internalization in cardiac myocytes was found to be 7 min, in agreement with a value of 5.3 min reported for isolated hepatocytes (Draznin *et al.*, 1984). Clearly, the present method involves no indication of the site of internalized ligand. It should be noted, however, that even after 60 min nearly all cell-associated insulin was in an intact form (Eckel & Reinauer, 1982). Thus the non-dissociable fraction may represent insulin covalently bound to the receptor (Clark & Harrison, 1982), insulin compartmentalized within the sarcolemma, or insulin residing inside the cell. On the other hand, covalent binding was found to represent a rather small fraction of specifically bound insulin (4–6% after 10 min at 37 °C) (Clark & Harrison, 1982). It may therefore be concluded that determinations of the non-dissociable fraction provide a good estimate of insulin internalization when performed under carefully controlled conditions. Consistently, the extent of steady-state internalization in cardiac myocytes (50%) agrees favourably with autoradiographic findings in this cell system (Junger *et al.*, 1986).

In the present investigation, a biphasic onset of insulin action on glucose transport was observed, with maximal insulin action reached by 30–60 min. This contrasts with our previous report (Eckel *et al.*, 1983), where transport stimulation was found to be very rapid, reaching its maximal value after 60 s, and with similar observations in isolated adipocytes (Whitesell & Gliemann, 1979). Those studies, however, were performed with very high concentrations of insulin (up to 1 μ M), which may have masked the second, slower, phase of the onset of insulin action. It should be noted that insulin binding was not rate-limiting under the conditions used in the present investigation. Since insulin internalization exhibited a time course very similar to the onset of insulin action, we speculated on a possible functional relationship between these two processes. This relationship was substantiated by using selective inhibitors of hormone processing.

Initial (5 min) post-binding events could be specifically blocked by PhAsO, whereas dansylcadaverine did not affect this process, in agreement with findings in isolated hepatocytes (Peavy *et al.*, 1984). The site of PhAsO action is at present unclear. Frost & Lane (1985) reported on a completely reversible reaction with vicinal thiol

groups, whereas Wiley & Cunningham (1982) observed irreversible inhibition of endocytosis by this drug. As shown in the present study, insulin action on glucose transport in cardiac myocytes was totally blocked by PhAsO, but only a minor part of this effect could be attributed to the interaction with vicinal thiol groups. It is noteworthy that PhAsO has been shown to be without effect on insulin-activated autophosphorylation of the insulin receptor (Frost & Lane, 1985), indicating that this putative signalling pathway remains intact. Thus, despite suggesting a precise molecular mechanism, our data show that the initial part of insulin action on cardiac glucose transport is essentially related to a very early processing of the hormone, involving formation of covalent hormone-receptor complexes, or subsequent steps of insulin internalization which are still to be identified. Lysosomal processing, however, appears not to be involved in the rapid onset of insulin action, since chloroquine did not block initial transport stimulation by insulin.

On the other hand, steady-state insulin action (60 min) was decreased by lysosomal inhibitors to an extent comparable with the initial transport stimulation. This finding completely disagrees with previous observations by Marshall & Olefsky (1980) and Häring *et al.* (1981) in isolated adipocytes. One possible explanation stems from the fact that in the work of Marshall & Olefsky (1980) insulin degradation was only partially (about 50%) inhibited by chloroquine treatment. It cannot be excluded, however, that insulin action is mediated by different pathways in different cell types. Consistently, Plas & Desbuquois (1982) reported a decrease in insulin-stimulated incorporation of [14 C]glucose into glycogen by chloroquine treatment of cultured fetal hepatocytes, whereas this same response was found to be independent of insulin internalization in freshly isolated hepatocytes (Trowbridge & Draznin, 1986). It may be speculated that insulin fragments generated by lysosomal degradation of the hormone mediate (at least partially) insulin action, as originally proposed by Steiner (1977). This hypothesis, however, was doubted, since anti-(insulin receptor) antibodies were found to mimic insulin action (Kahn *et al.*, 1977). Thus not insulin degradation but a degradative step in general may be involved in mediating steady-state insulin action. Specifically, since the action of chloroquine is not limited to the lysosome, but may also affect endosomes (Posner *et al.*, 1982), our data would also be consistent with the involvement of a membrane-associated degradative, most probably proteolytic, step in the transmission of the insulin signal (Seals & Czech, 1980; Cherqui *et al.*, 1985).

Taken together, two pathways of insulin action on cardiac glucose transport have been clearly distinguished in the present investigation, demonstrating that this hormonal action is not simply due to the initial binding event. Instead, very early processing of insulin-receptor complexes and subsequent degradative steps both appear to be involved in mediating the stimulatory action of insulin on the glucose-transport system of the isolated cardiac cell.

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