A possible role for glyceraldehyde transport in the stimulation of HIT-T15 insulinoma cells

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D-Glyceraldehyde was transported into HIT-T15 cells at a linear rate for approx. 2 min and appeared to be unsaturable up to a concentration of 50 mM. Evidence was obtained for an electrogenic component of uptake of the triose. The rate of D-glyceraldehyde transport was also reduced in the absence of Na⁺, suggesting that a component of uptake was Na⁺-linked. Transport of D-glyceraldehyde could be prevented by *N*-ethylmaleimide but not significantly by *p*-chloromercuribenzenesulphonic acid, L-glyceraldehyde, nor by a number of inhibitors of known transport systems. However, D-glyceraldehyde transport was inhibited by α -cyano-4-hydroxycinnamate, an inhibitor of some anion transport systems. D-Glyceraldehyde caused a marked depolarization of HIT-T15 cells accompanied by a rise in cytosolic [Ca²⁺] and [Na⁺] and a gradual intracellular acidification. The

glyceraldehyde-induced rise in cytosolic [Na⁺] and intracellular acidification, but not the depolarization or rise in cytosolic [Ca²⁺], were reduced by dithiothreitol and 5-aminoguanidine, compounds which form chemical adducts with α -ketoaldehydes. Incubation of HIT cells with either D- or L-glyceraldehyde resulted in the formation of large amounts of D-lactate, the end product of methylglyoxal metabolism via the glyoxalase pathway. It is suggested that the depolarizing action of glyceraldehyde is the result, at least in part, of its electrogenic transport, probably via Na⁺-coupled entry into HIT cells involving an unidentified transport system. The intracellular acidification and a component of the increase in cytosolic [Na⁺] may be largely due to the presence of one or more dicarbonyl contaminants in the glyceraldehyde preparation.

INTRODUCTION

D-Glyceraldehyde has been extensively used as an *in vitro* stimulus for insulin release from the pancreatic β -cell [1,2], particularly in certain clonal insulinoma cell lines [3,4] which, due to abnormalities in glucose transport and/or phosphorylation, fail to respond to this hexose. It has been generally accepted that glyceraldehyde can be phosphorylated and metabolized in the β -cell via the glycolytic pathway, thus exerting an insulinotropic action by a mechanism analogous to that of glucose-induced insulin release [3–6]. However, the activity of triokinase, the enzyme responsible for the initial phosphorylation of glyceraldehyde, is very low in pancreatic islets [7], while we have recently shown that the rate of metabolism of glyceraldehyde either to lactate or CO₂ in HIT-T15 insulinoma cells is extremely modest compared with the rate of glucose metabolism [8]. Furthermore, complete inhibition of glyceraldehyde metabolism with iodoacetate failed to prevent activation of HIT-T15 cells by the triose [8]. We therefore proposed that an alternative mechanism to glyceraldehyde metabolism, such as transport of the triose into the β -cell, could be responsible for its stimulatory effect on β -cell function [8]. In the present study, we have studied the transport of glyceraldehyde in HIT-T15 cells and further investigated possible mechanisms whereby the triose might activate insulin-secreting cells.

MATERIALS AND METHODS

HIT-T15 cells were maintained in RPMI culture medium and used between passage numbers 70 and 90. The incubation medium used for all experiments consisted of (in mmol/l): NaCl

(135), KCl (5), MgSO₄ (1.2), CaCl₂ (1.2), NaH₂PO₄ (1) and Hepes (20) buffered to pH 7.4. In some experiments, Na⁺ was replaced completely by *N*-methyl-D-glucamine (NMDG) or, for depolarizing medium, by partial substitution with KCl.

Uptake of D-glyceraldehyde into HIT cells was measured using a rapid oil-filtration method as described previously [9]. ³H₂O was used as a marker of total volume associated with the cell pellet. In each experiment, a rapid incubation was performed on ice; the ¹⁴C counts associated with the cell pellet from this incubation were taken to represent glyceraldehyde in the extracellular compartment and were subtracted from all subsequent values.

A qualitative assessment of plasma membrane potential was obtained using stirred suspensions of cells in the presence of the fluorescent dye oxonol-V [10]. For measuring HIT-cell intracellular pH (pH_i), cells were loaded with 2',7'-bis(carboxyethyl)-5'(6')-carboxyfluorescein (BCECF; 4 μ M, 15–20 min at 37 °C) then stuck to a small glass coverslip (10 mm diam.) coated with the adhesive 'Cell-Tak' (Collaborative Biomedical Products, Bedford, MA, U.S.A.). The coverslip was in turn mounted in a metal frame and placed in a cuvette which was perfused with preheated buffer at a rate of approx. 10 ml/min in a Perkin-Elmer SP50 spectrophotometer. The excitatory wavelengths were switched between 500 and 450 nm and fluorescence measured at 530 nm. Changes in pH_i were computed from the 500/450 ratio. The signals were calibrated by perfusing the cells with buffers of different pH values containing the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, 5 μ M); [11]. Cytosolic Ca²⁺ concentration ([Ca²⁺]_i), assessed in HIT cells loaded with Fura-2 by the ratio of the fluorescence signal at 350:380 nm excitation and 510 nm emission, was measured as

Abbreviations used: AG, 5-aminoguanidine; BCECF, 2',7'-bis(carboxyethyl)-5'(6')-carboxyfluorescein; DTT, dithiothreitol; [Ca²⁺]_i, cytosolic Ca²⁺ concentration; [Na⁺]_i, cytosolic Na⁺ concentration; pH_i, intracellular pH; SBFI, Na⁺-binding benzofuran isophthalate.

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described previously [8]. Cytosolic Na^+ concentration ($[\text{Na}^+]_i$) was measured in cells loaded with the dye SBF1-AM ($7 \mu\text{M}$ with 0.02 % pluronic) for 2 h at room temperature. Excitation and emission wavelengths were as for Fura-2, an increase in the 350:380 ratio representing an increase in $[\text{Na}^+]_i$. The signals were calibrated by perfusing the cells at the end of each experiment with medium containing zero or 20 mM Na^+ in the presence of the ionophore monensin ($10 \mu\text{M}$). All experiments were performed at room temperature ($22\text{--}24^\circ\text{C}$). The formation of D-lactate from D- and L-glyceraldehyde was measured enzymically using D-lactate dehydrogenase with the fluorimetric measurement of NADH [12]. Oxonol-V and Fura-2 acetomethoxy ester were obtained from Cambridge Bioscience, Cambridge, U.K., and BCECF acetomethoxy ester from Calbiochem, Nottingham, U.K. D- and L-Glyceraldehyde, D-lactate dehydrogenase (*Staphylococcus epidermidis*) and all other biochemicals were obtained from the Sigma Chemical Co. Stock solutions of glyceraldehyde were prepared in deionized water. D-[U- ^{14}C]Glyceraldehyde (160 mCi/mmol) was purchased from American Radiolabelled Chemicals, St. Louis, MO, U.S.A.

RESULTS

Transport of 20 mM D-glyceraldehyde, a concentration which promotes maximal activation of HIT-T15 cells, is shown in Figure 1. The initial rate of uptake, which was maintained for approx. 2 min, was in the order of $3.66 \text{ nmol}/\mu\text{l}$ of intracellular space per min. Uptake of the triose continued for at least 20 min. The uptake of D-glyceraldehyde by HIT cells appeared to be an unsaturable process up to a concentration of 50 mM (Figure 2). In order to study the effects of various conditions on the rate of transport of D-glyceraldehyde, a lower concentration of 5 mM was chosen as representing the approximately half-maximally effective concentration in terms of HIT cell activation [7]. Again, uptake of 5 mM D-glyceraldehyde did not appear to reach equilibrium within 20 min (Figure 3). In fact, at this time point, the apparent intracellular concentration of the triose was approx. 7 mM, assuming a relatively low rate of metabolism of D-glyceraldehyde (less than 10 % of the rate of uptake at 10 mM D-glyceraldehyde; [7]). At least two possibilities could explain this finding. First, it is conceivable that a certain degree of intracellular binding of the triose occurs following its uptake into the cell. An alternative or additional possibility is that a component of glyceraldehyde transport occurred via a non-passive process. Such a non-passive component of transport may be relatively small, which might explain why it was less apparent at the higher concentration of the triose.

We had speculated previously that a component of glyceraldehyde transport in HIT-T15 cells might be, at least in part, Na^+ - or H^+ -coupled, thus constituting an electrogenic process which could explain the rapid depolarization elicited by the triose [8]. As shown in Figure 3 and Table 1, uptake of 5 mM D-glyceraldehyde was reduced by approx. 20–30 % (at 5–20 min) in the absence of Na^+ from the incubation medium, suggesting that D-glyceraldehyde uptake could indeed occur in part via Na^+ co-transport. In order to assess whether a component of D-glyceraldehyde transport might also be H^+ -coupled, manipulations were employed to alkalize or acidify the cell interior. Incubation of HIT cells in the presence of monensin or in citrate buffer, conditions predicted to raise pH_i , resulted, in both cases, in significant increases in D-glyceraldehyde uptake during a 5 min period of incubation. This suggests that uptake of the triose could be in part H^+ -coupled. On the other hand, the presence of amiloride, which would be expected to acidify the cell interior, did not affect uptake of the triose. In separate

Figure 1 Uptake of 20 mM D-[U- ^{14}C]glyceraldehyde in HIT-T15 cells

Each point represents the mean $\pm$ S.E.M. of three to five determinations. Incubations were at room temperature.

Figure 2 Concentration dependence of D-[U- ^{14}C]glyceraldehyde uptake in HIT-T15 cells

Each point represents the mean $\pm$ S.E.M. of four determinations. Incubations were at room temperature.

experiments, it was confirmed that these manipulations did indeed alter pH_i in the predicted manner. Thus, monensin, citrate buffer and amiloride altered pH_i by approx. +0.40, +0.45

Figure 3 Uptake of 5 mM D-[U- 14 C]glyceraldehyde in HIT-T15 cells in the presence (□) or absence (●) of Na^+

In the latter case, Na^+ was substituted by *N*-methyl-D-glucamine. Each point represents the mean $\pm$ S.E.M. of 6–11 determinations. Incubations were at room temperature.

Table 1 Uptake of D-glyceraldehyde (5 mM) in HIT-T15 cells

Cells were incubated under the above conditions for at least 5 min before addition of [14 C]glyceraldehyde. Abbreviation: DIDS; 4,4'-di-isothiocyanatostilbene-2,2'-disulphonic acid. Statistical significance: * $P < 0.01$, ** $P < 0.001$.

Conditions	Uptake (nmol/ μ l intracellular space in 5 min)
Control	3.20 ± 0.14 ($n = 55$)
<i>N</i> -Ethylmaleimide (1 mM)	0.02 ± 0.002 ($n = 4$)**
Monensin (10 μ M)	4.63 ± 0.33 ($n = 6$)*
Citrate buffer (84 mM)	5.23 ± 0.33 ($n = 4$)*
Phloretin (5 mM)	5.05 ± 0.97 ($n = 7$)
Methyl aminoisobutyrate (10 mM)	3.10 ± 0.39 ($n = 4$)
α -Cyano-4-hydroxycinnamate (5 mM)	2.25 ± 0.14 ($n = 27$)**
DIDS (0.5 mM)	3.54 ± 0.38 ($n = 8$)
L-Glyceraldehyde (10 mM)	4.28 ± 0.86 ($n = 4$)
Minus Na^+ (#NMDG)	2.51 ± 0.14 ($n = 8$)*
70 mM K^+ , 70 mM Na^+	2.31 ± 0.30 ($n = 4$)*
140 mM K^+ , 5 mM Na^+	2.15 ± 0.16 ($n = 11$)*
SCN^- (20 mM)	7.18 ± 0.54 ($n = 17$)**
Tolbutamide (1 mM)	8.31 ± 0.41 ($n = 29$)**

and -0.35 pH unit respectively. The degree of H^+ -dependence of D-glyceraldehyde uptake is therefore equivocal. The electrogenicity of D-glyceraldehyde transport is further suggested by the reduced uptake apparent in cells depolarized by incubation in high- $[\text{K}^+]$ medium and enhanced uptake in cells hyperpolarized by SCN^- (Table 1). These findings provide further support for the suggestion of a cation-linked component of D-glyceraldehyde transport. An unexpected finding was that D-glyceraldehyde

Figure 4 Effects of glyceraldehyde on $[\text{Na}^+]_i$, assessed by SBFI fluorescence, in HIT-T15 cells in the absence or presence of DTT or AG

The effect of L-alanine is shown for comparison. Each trace is representative of three or more similar experiments. All experiments were at room temperature.

uptake appeared to be markedly enhanced by the hypoglycaemic sulphonylurea tolbutamide (Table 1). While it is unlikely that this effect was a consequence of inhibition of K_{ATP} channels by tolbutamide [13], we can at present offer no explanation for this observation.

The effects of a number of potential inhibitors of transport were examined upon the uptake of 5 mM D-glyceraldehyde in HIT cells (Table 1). The thiol reagent *N*-ethylmaleimide (1 mM), an inhibitor of numerous cellular transport systems, completely blocked uptake of 5 mM D-glyceraldehyde. Transport of the triose was not significantly affected by phloretin nor by methyl-aminoisobutyrate, inhibitors of glucose and alanine transport respectively. However, uptake of D-glyceraldehyde was inhibited by 30 % by α -cyano-4-hydroxycinnamate, an inhibitor of lactic acid carriers and other anion transport systems [14] (Table 1). L-Glyceraldehyde failed to inhibit transport of the D-enantiomer, as did glycerol.

The suggestion that a component of glyceraldehyde uptake might be Na^+ -coupled was further supported by the finding that addition of either the D- or L-enantiomer caused a pronounced increase in $[\text{Na}^+]_i$, assessed by SBFI fluorescence (Figure 4). The effects of alanine, an amino acid known to depolarize β -cells by a process of Na^+ -linked uptake, is shown for comparison. For reasons at present unknown, the rise in $[\text{Na}^+]_i$ following the addition of these stimuli appeared to be largely irreversible. The effects of D-glyceraldehyde on $[\text{Na}^+]_i$ were accompanied by a

Figure 5 Effects of D-glyceraldehyde on pH_i , assessed by BCECF fluorescence, in HIT-T15 cells in the absence or presence of DTT or AG

Each trace is representative of three or more similar experiments. All experiments were at room temperature.

Figure 6 Effects of D-glyceraldehyde on plasma membrane potential, assessed by oxonol-V fluorescence, in HIT-T15 cells in the absence or presence of DTT or AG

Cells were incubated in the presence of the drugs for at least 5 min before addition of glyceraldehyde. The effect of a depolarizing concentration (25 mM) of K^+ is shown for comparison. Each trace is representative of three or more similar experiments. All experiments were at room temperature.

marked intracellular acidification (Figure 5), depolarization (Figure 6) and increase in $[\text{Ca}^{2+}]_i$ (Figure 7). The effects of D-glyceraldehyde on pH_i were inhibited by dithiothreitol (DTT; 10 mM) and aminoguanidine (AG; 5 mM), compounds which protect thiol groups and arginyl residues of cellular proteins against the actions of α -ketoaldehydes [15–17]. In addition, these compounds reduced, but did not prevent, the glyceraldehyde-induced rise in $[\text{Na}^+]_i$. Control experiments confirmed that DTT and AG did not inhibit changes in pH_i induced by 20 mM lactate, or in $[\text{Na}^+]_i$ elicited by 20 mM alanine or zero K^+ media (traces not shown). Thus the inhibitory effects of DTT and AG were probably not the result of non-specific actions on cellular pH and $[\text{Na}^+]$ homeostasis. The above observations suggest that the change in pH_i , together with a component of the rise in $[\text{Na}^+]_i$ elicited by glyceraldehyde, may be brought about by α -ketoaldehyde contaminants in the triose preparations. In contrast, the depolarization and rise in $[\text{Ca}^{2+}]_i$ in response to D-glyceraldehyde appeared to be resistant to AG and DTT (Figures 6 and 7). The effects of glyceraldehyde on membrane potential and $[\text{Ca}^{2+}]_i$ could thus correspond to the DTT- and AG-resistant component of $[\text{Na}^+]_i$ increase, and are more likely to be the result of glyceraldehyde actions per se. The DTT/AG-resistant component of the rise in $[\text{Na}^+]_i$ is comparable with that induced by alanine, which is known to be transported by a Na^+ -dependent mechanism in the β -cell [18].

Figure 7 Effects of D-glyceraldehyde on $[\text{Ca}^{2+}]_i$, assessed by the 350:380 fluorescence ratio in Fura-2-loaded HIT-T15 cells, in the absence or presence of DTT or AG

Each trace is representative of three or more similar experiments. All experiments were at room temperature.

Table 2 Formation of D-lactate in HIT-T15 cells

Conditions	D-Lactate formed (nmol/ 10^6 cells in 40 min)
Control (no addition)	0.77 ± 0.10 ($n = 4$)
D-Glyceraldehyde (10 mM)	100.0 ± 11.3 ($n = 4$)
D-Glyceraldehyde (20 mM)	284.5 ± 25.4 ($n = 4$)
L-Glyceraldehyde (10 mM)	1192.0 ± 157.0 ($n = 4$)
D-Glucose (20 mM)	0.92 ± 0.04 ($n = 4$)
Methylglyoxal (1 mM)	792.1 ± 113.2 ($n = 4$)
D-Glyceraldehyde (10 mM), no cells	19.2 ± 7.1 ($n = 4$)
L-Glyceraldehyde (10 mM), no cells	Not detectable ($n = 4$)

Table 2 shows the formation of D-lactate, the end product of methylglyoxal metabolism via the glyoxalase pathway, in HIT-T15 cells. Whereas the addition of glucose did not significantly increase the formation of D-lactate, both D- and L-glyceraldehyde resulted in the production of large amounts of this metabolite, suggesting the presence of significant quantities of methylglyoxal in the triose preparations. It was notable that L-glyceraldehyde, which had a consistently marked effect on $[\text{Na}^+]_i$, also appeared to contain particularly high levels of methylglyoxal.

DISCUSSION

Recent studies in our laboratory of the activation of HIT-T15 cells by glyceraldehyde provided evidence that this process does not depend upon glycolytic or oxidative metabolism of the triose

[8]. We speculated that the simplest mechanism which would explain our findings could be an electrogenic component of glyceraldehyde transport into the cell, resulting directly in depolarization, Ca^{2+} entry and stimulated insulin release. One purpose of the present study was to investigate the process of glyceraldehyde uptake into HIT cells in an attempt to gain further insight into its insulinotropic action.

Although glyceraldehyde has been extensively used as a β -cell secretagogue it is not a nutrient of any physiological relevance, occurring only intracellularly and in its phosphorylated form. It is therefore not surprising that little or no information exists regarding transport of the triose across mammalian cell membranes. The present findings indicate that D-glyceraldehyde is transported into HIT-T15 cells at an initial rate consistent with the kinetics of cell depolarization. While the apparent unsaturability of uptake, at least up to 50 mM D-glyceraldehyde, suggests a process of passive diffusion, the ability of *N*-ethylmaleimide to completely block uptake implies that entry of the triose into the cell is likely to be a carrier-mediated process, albeit with a very high K_m for glyceraldehyde. An attempt was made to identify the carrier system responsible for the transport of glyceraldehyde in HIT cells. While inhibitors of glucose and alanine transport were without effect on D-glyceraldehyde uptake, a significant inhibition of the latter was observed in the presence of α -cyano-4-hydroxycinnamate, an inhibitor of transporters of lactate and certain other 3-carbon substrates [14]. However, since uptake of D-glyceraldehyde was not inhibited by *p*-chloromercuribenzenesulphonic acid nor by 4,4'-di-isothiocyanato-stilbene-2,2'-disulphonic acid (DIDS), inhibitors of the lactate⁻/H⁺ co-transport and inorganic anion-exchange systems respectively, it seems unlikely that these carriers are involved in triose transport in HIT-T15 cells. Uptake of D-glyceraldehyde was also unaffected by a number of potential competitive inhibitors including L-glyceraldehyde and glycerol, possibly reflecting the apparent high K_m , or unsaturability, of D-glyceraldehyde transport. Further studies are clearly necessary to identify fully the modalities of triose uptake into mammalian cells.

Our data provide evidence that D-glyceraldehyde uptake into HIT cells involves, at least in part, an electrogenic component of transport. The impaired uptake of D-glyceraldehyde in Na⁺-free medium suggests a possible glyceraldehyde/Na⁺ co-transport process, a mechanism which would be consistent with the observed rise in $[\text{Na}^+]_i$ upon addition of the triose and also with the impaired ability of glyceraldehyde to depolarize HIT cells in the absence of the cation [8]. On the other hand, the fact that a degree of depolarization was apparent in Na⁺-free medium implies the existence of an additional process elicited by glyceraldehyde.

A serious problem which arises from the use of glyceraldehyde with biological preparations is the low degree of purity of commercially available preparations. This arises largely from the spontaneous autoxidation of hydroxyaldehydes to form α -ketoaldehydes [19], a process involving the generation of free radicals such as $\text{O}_2^{\cdot -}$. The exact nature of impurities in glyceraldehyde preparations is unknown and probably variable. However, the finding that HIT cells incubated with D- or L-

glyceraldehyde form large quantities of D-lactate, the end product of the glyoxalase pathway [15], suggests that methylglyoxal, an α -ketoaldehyde, is likely to make a significant contribution towards these impurities. The presence of α -ketoaldehyde contaminants is further supported by the ability of DTT and AG, two compounds known to protect against the actions of the former, to reduce the glyceraldehyde-induced rise in $[\text{Na}^+]_i$ and intracellular acidification. We suggest that the effects of glyceraldehyde which are resistant to these drugs, namely depolarization, a rise in $[\text{Ca}^{2+}]_i$ and a more modest rise in $[\text{Na}^+]_i$, are the effects of the triose per se. Since α -ketoaldehydes have been shown to exert profound effects on cellular processes [15], including inhibition of plasma membrane ATPases [16], it is likely that the presence of such contaminants in triose preparations could contribute significantly to changes in intracellular ion concentrations and hence cell function. In fact, recent preliminary studies in our laboratory indicate that one such α -ketoaldehyde, methylglyoxal, shares a number of actions on HIT cells with glyceraldehyde, including depolarization, increased $[\text{Na}^+]_i$ and $[\text{Ca}^{2+}]_i$ and intracellular acidification (J. Davies, S. Tomlinson, A. C. Elliott and L. Best, unpublished work). The mechanisms by which these effects of methylglyoxal on β -cell function occur, and the extent of their contribution towards the observed actions of glyceraldehyde, await further clarification.

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