An investigation of glucose uptake in relation to steroidogenesis in rat testis and tumour Leydig cells

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The mechanisms of the requirement of glucose for steroidogenesis were investigated by monitoring the uptake of the glucose analogue 2-deoxy-D-glucose by rat testis and tumour Leydig cells. The characteristics of glucose transport in both of these cell types were found to resemble those of the facilitated-diffusion systems for glucose found in most other mammalian cells. The Leydig cells took up 2-deoxy-D-glucose but not L-glucose, and the uptake was inhibited by both cytochalasin B and forskolin. In the presence of luteinizing hormone, the rate of 2-deoxy-D-glucose uptake by both cell types was increased by approx. 50%. In addition to D-glucose, it was shown that the Leydig cells could also utilize 3-hydroxybutyrate or glutamine to maintain steroidogenesis.

INTRODUCTION

Little is known about the rates of utilization of different metabolic fuels in steroidogenic cells. In an early study, Rommerts *et al.* (1973) reported that glucose is required for testosterone production by rat testis Leydig cells. However, it has not previously been demonstrated whether or not other fuels are able to support steroidogenesis. Nor has the mechanism and regulation of glucose transport into Leydig cells been investigated. Most mammalian cells take up glucose by the process of facilitated diffusion, catalysed by glucose-transport proteins in the plasma membrane (Elbrink & Bihler, 1975). Transport is stereospecific for D-glucose and is potentially inhibited by the mould metabolite cytochalasin B (Baldwin & Lienhard, 1981) and by the diterpene forskolin (Kashigawa *et al.*, 1983; Sergeant & Kim, 1985; Shanahan *et al.*, 1986). In many cell types, the rate of glucose uptake is regulated by hormones such as insulin and catecholamines (Simpson & Cushman, 1986). Follicle-stimulating hormone (FSH) enhanced hexose accumulation by cultured Sertoli cells (Hall & Mita, 1984), and thyroid-stimulating hormone (TSH) has been shown to increase glucose transport by rat thyroid cells by a cyclic AMP-dependent mechanism (Filetti *et al.*, 1987).

The aim of the present study was to investigate the mechanism of glucose transport in rat testis and tumour Leydig cells in relation to the requirement for steroidogenesis. The effect of luteinizing hormone (LH), which controls steroidogenesis in these cells, on the rate of glucose uptake was also investigated. In addition, the ability of substrates other than glucose to support steroidogenesis was examined.

MATERIALS AND METHODS

Materials

[1,2,6,7-³H]Testosterone (92 Ci/mmol) was obtained from Amersham International (Amersham, Bucks, U.K.). 2-Deoxy-D-[1,2-³H]glucose (30.2 Ci/mmol) and L-[1-³H]glucose (10.6 Ci/mmol) were obtained from New England Nuclear (Southampton, U.K.). Forskolin was from Calbiochem (La Jolla, CA, U.S.A.) and cytochalasin B from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). Ovine LH (1.9 i.u. of NIH-SI/mg) was a gift from the Endocrinology Study Section, National Institutes of Health, Bethesda, MD, U.S.A. Percoll was from Pharmacia. Chemicals for the production of Dulbecco's modified Eagle's minimum essential medium (DMEM) were purchased from Gibco. Other chemicals were from Sigma and were the highest grade available.

Cell preparation and treatment

Rat testis Leydig cells and tumour Leydig cells were isolated and purified on linear 0–90% (w/v) Percoll gradients as previously described (Aldred & Cooke, 1982; Dix & Cooke, 1981). Staining of the cells for 3β-hydroxysteroid dehydrogenase by the method of Levy *et al.* (1959) as modified by Aldred & Cooke (1983) indicated that at least 70% of the cells in the preparations were steroidogenic. The cells were then incubated at 34 °C in DMEM containing 1 mg of bovine serum albumin (BSA)/ml and 1 μg of Kanamycin/ml for the times indicated in the text in the presence or absence of LH, before assay of 2-deoxyglucose uptake. Cell viability during the preincubation and during the deoxyglucose uptake assay was measured by histochemical staining for

Abbreviations used: LH, luteinizing hormone (lutropin); FSH, follicle-stimulating hormone (follitropin); TSH, thyroid-stimulating hormone (thyrotropin); DMEM, Dulbecco's modified Eagle's minimum essential medium; BSA, bovine serum albumin.

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Table 1. Effect of cytochalasin B on 2-deoxyglucose uptake into Leydig cells

Leydig cells were prepared and isolated as described in the Materials and methods section. The cells were preincubated for 3 h with or without 100 ng of LH/ml (testis Leydig cells) or 1 μ g of LH/ml (tumour Leydig cells) as shown. They were then assayed for 2-deoxyglucose uptake in the presence or absence of 10 μ M-cytochalasin B. Results are expressed as means $\pm$ S.E.M. ($n = 3$, testis Leydig cells) or means $\pm$ ranges ($n = 2$, tumour Leydig cells).

Leydig cells	Preincubation conditions	2-Deoxy-D-glucose uptake (pmol/h per μ g of cell protein)		
		Control	+Cytochalasin B	Inhibition (%)
Testis	-LH	2.9 $\pm$ 0.1	0.5 $\pm$ 0.1	83 $\pm$ 3
	+LH	3.7 $\pm$ 0.2	0.7 $\pm$ 0.3	81 $\pm$ 5
Tumour	-LH	3.7 $\pm$ 1.7	0.3 $\pm$ 0.1	92 $\pm$ 3
	+LH	5.9 $\pm$ 0.7	0.1 $\pm$ 0.1	98 $\pm$ 2

NADH dehydrogenase (diaphorase) as described by Aldred & Cooke (1983).

Assay for hexose uptake

Before assay, cells were washed three times by centrifugation for 10 min at 110 *g* in glucose-free DMEM containing 1 mg of BSA/ml. Uptake was initiated by resuspending the cells (5×10^5 testis Leydig cells or 10^6 tumour Leydig cells) in 0.75 ml of glucose-free DMEM containing 1 mg of BSA/ml and 0.1 mM-2-deoxy-D-[1,2- 3 H]glucose or 0.1 mM-L-[1- 3 H]glucose (10 Ci/mol for each sugar) and the test compounds at the appropriate concentrations. After various times of incubation at 37 °C, uptake was terminated by the addition of 2 ml of ice-cold glucose-free DMEM lacking BSA, followed by centrifugation at 100 *g* for 5 min. This washing procedure was repeated twice with 1 ml of ice-cold medium, and then the cell pellets were solubilized in 400 μ l of 0.1 M-NaOH. A sample (250 μ l) was taken for determination of radioactivity by liquid-scintillation counting, and duplicate samples (70 μ l) were assayed for protein by the method of Lowry *et al.* (1951) as modified by Markwell *et al.* (1978). Values for cell-associated radioactivity at zero time were measured by adding the ice-cold washing medium to the cells before the addition of radiolabelled sugar. All uptake values have been corrected for this zero-time uptake. Results are expressed as pmol of sugar taken up per μ g of cell protein, and are given as means $\pm$ S.D.

Assay for testosterone production

Rat testis Leydig cells were plated out in Costar culture wells at a density of 5×10^4 cells/well in 0.5 ml of DMEM containing 1 mg of BSA/ml and 0.1 μ g of Kanamycin/ml. After incubation for 1 h at 34 °C, the cells were washed three times in glucose- and glutamine-free DMEM to remove extracellular glucose, and then incubated for 2 h at 34 °C in 0.5 ml of DMEM containing various metabolic fuels with and without 10 ng of LH/ml. After incubation, testosterone was extracted from the cells and medium by addition of HClO₄ (final concn. 0.5 M). The supernatants were neutralized by the addition of K₃PO₄ (final concn. 0.27 M), and testosterone was determined by radioimmunoassay by the method of Verjans *et al.* (1973).

RESULTS AND DISCUSSION

Preliminary experiments showed that the uptake of 2-deoxyglucose by both testis and tumour Leydig cells was linear for at least 1 h, in the presence or absence of LH. An uptake period of 1 h was therefore used for all subsequent experiments, so that sufficient radioactive hexose could be taken up into the cells to enable its accurate quantification. Diaphorase histochemistry (Aldred & Cooke, 1983) indicated that there was little or no loss of cell viability during the assay (results not shown). In the absence of LH, the rate of 2-deoxyglucose uptake varied in different preparations from 3.7 to 20.4 pmol/h per μ g of cell protein for tumour Leydig cells and from 2.7 to 11.2 pmol/h per μ g of cell protein for testis Leydig cells. A similar variability in the maximal rate of steroidogenesis has been reported for different preparations of rat testis Leydig cells (Hunter *et al.*, 1982; Sullivan & Cooke, 1984). Therefore, in each experiment where the effect of LH or other substances on transport was examined, a single preparation of cells was used.

The uptake of 2-deoxy-D-glucose by both testis and tumour Leydig cells was stereospecific: the uptake of L-glucose did not exceed 2% of that seen for 2-deoxyglucose during a 1 h incubation. Because this non-facilitated hexose-uptake rate was so low, the 2-deoxyglucose-uptake data were not corrected for this non-specific component of sugar entry. The uptake of 2-deoxyglucose was potently inhibited in both cell types by cytochalasin B (Table 1). This compound is an inhibitor of the facilitated diffusion of glucose in most mammalian cells (Baldwin & Lienhard, 1981). Similarly, the diterpene forskolin, which inhibits glucose transport by binding to the transport protein in other cell types (Kashigawa *et al.*, 1983; Sergeant & Kim, 1985; Shanahan *et al.*, 1986, 1987), inhibited 2-deoxyglucose uptake by tumour Leydig cells at all concentrations tested (Fig. 1). The concentration of forskolin required to inhibit uptake by 50% was about 10 μ M, a value similar to that reported for inhibition of transport in platelets (Kim *et al.*, 1986) and in erythrocytes (Sergeant & Kim, 1985). It is therefore likely that the glucose-transport system in Leydig cells resembles that found in most other mammalian cells.

Forskolin is known to be an activator of adenylate cyclase (Seamon & Daley, 1981) and potentiates the

Fig. 1. Effects of forskolin on the uptake of 2-deoxy-D-glucose by rat tumour Leydig cells

Rat tumour Leydig cells were prepared as described in the Materials and methods section and then incubated for 3 h in the absence of LH. 2-Deoxy-D-glucose uptake was then assayed in the presence of various concentrations of forskolin. Results are shown as means $\pm$ S.D.

action of LH on Leydig-cell cyclic AMP production and steroidogenesis (Sullivan & Cooke, 1984). However, it has previously been reported that, at high concentrations of forskolin ranging from 12.5 to 100 μ M, testosterone production by Leydig cells is less than that observed with 6.25 μ M-forskolin, despite the concentration of cyclic AMP in the cells being greater at the higher forskolin concentrations (Sullivan & Cooke, 1984). Our findings that forskolin inhibits glucose transport in Leydig cells, and that glucose is required for steroidogenesis (see below), provide an explanation for the biphasic effects of the diterpene on testosterone production.

The results shown in Table 1 indicate that pre-incubation of both testis and tumour Leydig cells for 3 h with LH before measurement of 2-deoxyglucose

Table 3. Abilities of metabolic fuels to support testosterone production by testis Leydig cells

Cells were prepared and then incubated for 2 h in the presence of 10 ng of LH/ml and the metabolic fuels shown, as described in the Materials and methods section. Results are means $\pm$ S.D. ($n = 6$). In the absence of LH and in the presence of 25 mM-D-glucose, basal testosterone production was 1.3 ± 1.7 ng/2 h per 10^6 cells.

Metabolic fuel	Testosterone production (ng/2 h per 10^6 cells)
None	0
D-Glucose (25 mM)	87.7 ± 2.4
Glutamine (4 mM)	65.9 ± 37.2
3-Hydroxybutyrate (0.2 mM)	92.3 ± 2.0
3-Hydroxybutyrate (5 mM)	88.8 ± 3.4

uptake leads to an increase in the rate of hexose entry. However, the extent of the increase was found to be rather variable from experiment to experiment (results not shown). A more consistent increase in the rate of hexose uptake was obtained if LH was present during both the preincubation period (2 h) and during the 1 h period of the uptake assay itself (Table 2). Indeed, a similar increase in uptake rate was seen even if LH was present only during the uptake period (Table 2), indicating that the stimulatory effect of the hormone is of rapid onset. In these cases LH produced an increase in the rate of uptake of about 50% in both tumour and testis Leydig cells. Because LH is known to stimulate adenylate cyclase activity, it is possible that the increase in hexose uptake caused by incubation with LH is mediated by cyclic AMP. Therefore the effect of dibutyryl cyclic AMP on hexose transport was examined. When this compound was present at a concentration of 2 mM during the uptake assay, the rate of 2-deoxyglucose uptake into tumour Leydig cells was increased by about 40% (Table 2).

In the absence of D-glucose it was found that no steroidogenesis took place (Table 3). However, there was

Table 2. Effects of LH and dibutyryl cyclic AMP on 2-deoxyglucose uptake by Leydig cells

Cells were preincubated for 3 h in the absence of LH before assay of 2-deoxyglucose uptake. In one case, tumour Leydig cells were incubated for 1 h in the absence of LH and then for 2 h in the presence of 1 μ g of LH/ml before assay. Values are means $\pm$ S.D., for the numbers of replicates shown in parentheses; * $P < 0.01$, ** $P < 0.001$, *** $P < 0.05$ (significance values by two-sided t test).

Leydig cells	Length of preincubation with LH (h)	Substance present during uptake assay	2-Deoxy-D-glucose uptake (pmol/h per μ g of cell protein)	
			Basal	+ LH/dibutyryl cyclic AMP
Tumour	2	LH (1 μ g/ml)	20.7 ± 0.8 (3)	29.7 ± 2.0 (3)*
	0	LH (1 μ g/ml)	18.2 ± 2.0 (6)	27.5 ± 4.3 (6)**
	0	Dibutyryl cyclic AMP (2 mM)	18.2 ± 2.0 (6)	25.3 ± 1.4 (6)**
Testis	0	LH (100 ng/ml)	2.7 ± 0.5 (3)	3.8 ± 0.1 (3)***
	0	LH (100 ng/ml)	11.2 ± 0.4 (7)	18.1 ± 1.4 (7)**

not a specific requirement for D-glucose, because both glutamine and 3-hydroxybutyrate were also found to be equally effective in maintaining steroidogenesis. It is probable that all three substances are acting as energy sources for steroidogenesis, although it cannot be excluded that they are also providing a source of carbon units for the biosynthesis of cholesterol which is required for testosterone production.

In summary, the present study has demonstrated that Leydig cells can take up glucose by a transport system that appears to be similar to the facilitated-diffusion systems for glucose uptake in other mammalian cells. Hexose uptake is significantly stimulated by LH, a phenomenon that may be related to an increased requirement for glucose to support steroidogenesis. The increase in transport can be mimicked by dibutyryl cyclic AMP. In the absence of glucose, we have confirmed the original finding (Rommerts *et al.*, 1973) that Leydig cells cannot produce testosterone. However, in addition to glucose, glutamine and 3-hydroxybutyrate are also capable of supporting steroidogenesis.

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