Dynamics of intracellular calcium induced by lactate and glucose in rat pachytene spermatocytes and round spermatids

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Glycolytic metabolism in meiotic and post-meiotic spermatogenic cells shows differentiation-related changes. The developmental and physiological significance of these metabolic changes is not known. The aim of the present study was to test the hypothesis that glucose and lactate metabolism can modulate intracellular calcium $[Ca^{2+}]_i$ in spermatogenic cells in an opposing and dynamic manner. Fluorescent probes were used to measure $[Ca^{2+}]_i$ and pH_i, and HPLC was used to measure intracellular adenine nucleotides and mitochondrial sensing of ATP turnover. $[Ca^{2+}]_i$ in pachytene spermatocytes and round spermatids was modulated by changes in lactate and glucose concentrations in the media. The kinetics and magnitude of the $[Ca^{2+}]_i$ changes induced by lactate and glucose were different in meiotic and post-meiotic spermatogenic cells.

The presence of glucose in the medium induced a decrease in pH_i in spermatogenic cells. This glucose-induced pH_i decrease occurred later than the changes in $[Ca^{2+}]_i$, which were also observed when the pH_i decrease was inhibited, indicating that the glucose-induced $[Ca^{2+}]_i$ increase was not a consequence of pH_i changes. Hexose phosphorylation in glycolysis was part of the mechanism by which glucose metabolism induced a $[Ca^{2+}]_i$ increase in spermatogenic cells. The sensitivity of $[Ca^{2+}]_i$ to carbohydrate metabolism was higher in round spermatids than in pachytene spermatocytes. Thus, differentiation-related changes in carbohydrate metabolism in spermatogenic cells determine a dynamic and differential modulation of their $[Ca^{2+}]_i$ by glucose and lactate, two substrates secreted by the Sertoli cells.

Introduction

The functional relationships between germ and Sertoli cells in the seminiferous tubules may occur either through physical contact (adhesion molecules) or through molecules secreted into the extracellular space in the adluminal compartment (Jegou, 1993). The precise composition of the extracellular environment of germinal cells in the seminiferous tubules has not been determined. However, it is expected that lactate is secreted into this compartment by Sertoli cells as a product of glucose metabolism (Robinson and Fritz, 1981; Mita *et al.*, 1982; Le Gac *et al.*, 1983). The glycolytic activity of Sertoli cells is stimulated by FSH, β -adrenergic agonists, IL1- β and tumour necrosis factor α (TNF- α ; Mita *et al.*, 1982; Le Gac *et al.*, 1983; Hall and Mita, 1984; Riera *et al.*, 2001) and a similar regulation is expected of the lactate secretion rate toward the adluminal compartment.

Hexose transporters are present inside the seminiferous tubules (Angulo *et al.*, 1998) and Sertoli cells can take up glucose from the external medium (Hall and Mita, 1984). Furthermore, a non-metabolizable analogue of glucose can

cross the blood–testis barrier (Turner *et al.*, 1983), indicating that glucose can enter the adluminal compartment through Sertoli cells. The rate of glucose secretion by Sertoli cells toward the adluminal compartment is expected to vary inversely with the rate of metabolic glucose consumption by these cells.

Pachytene spermatocytes and round spermatids can take up glucose from their external medium (Nakamura *et al.*, 1986) and metabolize this sugar through glycolytic and oxidative metabolism (for example, see Grootegeod *et al.*, 1984). Carbohydrate metabolism of spermatogenic cells undergoes differentiation-related changes (Bajpai *et al.*, 1998). This metabolic change determines that meiotic spermatogenic cells have a high glycolytic flux (Bajpai *et al.*, 1998) and that round spermatids respond to glucose metabolism with a decrease in ATP content (Nakamura *et al.*, 1982; Grootegeod *et al.*, 1986). These differentiation-related changes in glycolytic metabolism of spermatogenic cell have been perplexing in terms of their physiological implications. Herrera *et al.* (2000) reported that long-term incubation with glucose induced a differential increase in $[Ca^{2+}]_i$ in round spermatids of rats. As glucose and lactate fluxes toward the adluminal compartment are expected to

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vary according to the FSH-, catecholamine- or cytokine-modulated glycolytic activity of Sertoli cells, in the present study the hypothesis that spermatogenic cells can rapidly translate extracellular lactate and glucose changes to a dynamic behaviour of $[Ca^{2+}]_i$, linking external substrate supply by Sertoli cells to intracellular signalling in spermatogenic cells was tested. In addition, some of the mechanisms by which glucose can induce changes in $[Ca^{2+}]_i$ in round spermatids and pachytene spermatocytes were investigated.

Materials and Methods

Materials

Fluo-3 and fura-2 acetoxymethyl esters were obtained from Molecular Probes (Eugene, OR). Glucose, sodium L-lactate, 3-O-methyl-D-glucopyranoside (OMG), 2-deoxy glucose (DOG), iodoacetate, ionomycin, the enzymes, salts and buffers were obtained from Sigma Chemicals Co. (St Louis, MO). Thapsigargin was obtained from Calbiochem (La Jolla, CA).

Preparation of rat spermatogenic cells

Rat spermatogenic cells were prepared from the testes of adult (50–70 days of age) Wistar rats as described by Romrell *et al.* (1976). Rats were housed with free access to food and water under a 12 h light:12 h dark photoperiod. The animals were exposed to CO₂ for 45 s and then killed by cervical dislocation. A 95% O₂ and 5% CO₂ atmosphere was maintained throughout the tissue enzymatic digestion procedure. The pachytene spermatocyte (85 ± 5% purity) and round spermatid fractions (92 ± 4% purity) were identified both by their size and by the typical aspect of their nucleus stained with H₃₃₃₄₂ (Molecular Probes; Reyes *et al.*, 1997). The round spermatid fraction contained cells between stages one and seven. Our method of vital cell identification did not allow a sub-classification of rat spermatids at these stages of development. The isolated cells were used within 4 h after the purification procedures.

Intracellular Ca²⁺ measurements of spermatogenic cells in suspension

Measurements of $[Ca^{2+}]_i$ in pachytene spermatocytes and round spermatids in suspension were performed in cells loaded with fura-2. Cells were loaded with the dye by incubation of approximately 5×10^6 cells ml⁻¹ with 5 µmol acetoxymethyl fura-2 I⁻¹ in a medium containing 140.0 mmol Na⁺ I⁻¹, 4.6 mmol K⁺ I⁻¹, 1.6 mmol Mg²⁺ I⁻¹, 1.6 mmol phosphate I⁻¹, 0.5 mmol Ca²⁺ I⁻¹, 10 mmol Hepes I⁻¹, pH 7.4 (KH), 24 mmol bicarbonate (KHB) I⁻¹ and 5 mmol L-lactate (KHB-lactate) I⁻¹ for 1 h at room temperature and in an atmosphere of 95% O₂ and 5% CO₂. The cells were then washed three times in KH medium and maintained at 4°C until used. The measurements were performed in a Fluoromax-2 (Jobin Ivon-Spex, Edison, NJ) fluorometer using a ratiometric method as described by Grynkiewicz *et al.* (1985). Calibration of fura-2 was performed by lysis of

the cells with digitonin (20 µg ml⁻¹) in medium that contained 0.5 mmol Ca²⁺ I⁻¹ (F_{max}) and subsequent addition of a final concentration of 5 mmol EGTA I⁻¹ (pH 7.4) (F_{min}). The K_d value for fura-2/Ca²⁺ was 250 nmol I⁻¹ and was interpolated from the data in Larsson *et al.* (1999) at 33°C. The addition of glucose and lactate to cells in different metabolic conditions was performed to examine the effects of these substrates on $[Ca^{2+}]_i$ dynamics. The first condition was depletion of external substrates. This condition was reached by incubation of the cell suspension for 20 min at 33°C in the absence of external substrates and with abundant O₂. This condition also allowed characterization of the ability of lactate to decrease $[Ca^{2+}]_i$ and to maintain a low steady-state of $[Ca^{2+}]_i$ in the cells. The second condition was minimal lactate supply (0.5 mmol L-lactate I⁻¹). This condition allowed maintenance of low concentrations of $[Ca^{2+}]_i$ and revealed the ability of glucose to increase $[Ca^{2+}]_i$. All experiments performed in the nominal absence of external Ca²⁺ were made with KH medium without Ca²⁺, and with the addition of 0.5 mmol EGTA I⁻¹. This condition gives an estimated free Ca²⁺ concentration of approximately 3 nmol I⁻¹. Control measurements for possible quenching or enhancement of fura-2/Ca²⁺ fluorescence by glucose, L-lactate, DOG, OMG (5 mmol I⁻¹ each) or 1 mmol iodoacetate I⁻¹ were performed with 0.5 µmol fura-2 I⁻¹ and 20 or 100 nmol free Ca²⁺ I⁻¹. In each case, the different substances induced changes in fluorescence equivalent to < 3 nmol free Ca²⁺ I⁻¹ changes in the cells.

Measurements of $[Ca^{2+}]_i$ in single cells

The variations of $[Ca^{2+}]_i$ in single cells were estimated using the fluorescent probe fluo-3 (excitation wavelengths 450–490 nm, emission wavelengths > 510 nm). Acetoxymethyl fluo-3 (fluo-3 AM; 5 µmol I⁻¹) was incubated with the cells for 30 min at room temperature (18 ± 2°C). The cells were allowed to settle on the glass coverslip bottom of a microscope chamber (0.7 ml) on a temperature-regulated microscope stage at 33°C for 10 min and were then washed with KH-lactate buffer. The medium surrounding the cells in the microscope chamber was changed by perfusing 10 ml of the corresponding solution using a peristaltic pump at a rate of approximately 3 ml min⁻¹. The cell fluorescence was quantified using videomicroscopy in an inverted Nikon Diaphot microscope with epifluorescence and a × 40 fluo objective (NA 0.85). A cooled CCD, 12-bit videocamera (Spectrasource, Los Angeles, CA) was attached to the videoport of the microscope. The image analysis was performed with appropriate software using a personal computer. Background fluorescence, standardized for the appropriate window size, was taken from a zone in the image without cells and subtracted from the fluorescence in each cell.

Oxygen consumption measurements

Oxygen consumption (QO₂) measurements were performed using a Clark type O₂ sensing electrode (YSI, Yellow

Fig. 1. (a) Intracellular $[Ca^{2+}]_i$ of round spermatids loaded with fura-2 in rats. The measurements were made in a spectrofluorometer at 33°C in 140.0 mmol Na⁺ l⁻¹, 4.6 mmol K⁺ l⁻¹, 1.6 mmol Mg²⁺ l⁻¹, 1.6 mmol phosphate l⁻¹, 0.5 mmol Ca²⁺ l⁻¹, 10 mmol Hepes l⁻¹, pH 7.4 (KH) buffer. The cells were incubated previously for 20 min without substrates (●) or with 0.5 mmol L-lactate l⁻¹ (○) in KH and 24 mmol bicarbonate l⁻¹ (KHB) in a 95% O₂ and 5%

Springs, OH) in a sealed glass chamber (0.9 ml) surrounded by a water jacket kept at 33°C. Isolated spermatogenic cells were pre-incubated for 1 min in KH buffer at 33°C before being transferred to the QO₂ chamber. QO₂ values were obtained from the slope of the O₂ tension versus time curve between 0.5 and 2.0 min after each experimental addition of substrate.

Determination of the intracellular adenine nucleotide content of rat round spermatids

Rat round spermatids in suspension were incubated at a concentration of approximately 6 mg cell protein ml⁻¹ in KHB-lactate at 33°C and under an atmosphere of 95% O₂ and 5% CO₂ for 10 min. Subsequently, the cells were washed in KH buffer and subjected to different conditions for several time periods. After the reported incubation times, 400 µl of the cell sample was added to an ice-cold micro-centrifuge tube and pelleted by centrifugation at 16 000 *g* for 30 s. The supernatant was then removed and the pellet was processed as described by Herrera *et al.* (2000). A procedure and storage control was made by taking duplicate 200 µl samples of 2 mmol ATP and ADP solutions l⁻¹ and subjecting them to the previously mentioned sample processing and storage conditions. The samples were maintained at -20°C until they were thawed and analysed by an HPLC equipped with high-pressure pumps coupled to an automated gradient controller, UCK injector and UV detector (model 501; Millipore, Waters). Nucleotide separation was obtained with an anion exchange column (MA7Q, 50.0 × 7.8 mm; BioRad) at a flow of 1.5 ml min⁻¹ and at room temperature. The mobile phases were 0.01 mol MOPS l⁻¹ + 0.01 mol KCl l⁻¹, pH 7.0 and 0.1 mol MOPS l⁻¹ + 0.5 mol KCl l⁻¹, pH 7.0. Calibration was performed with diluted standards of ATP, ADP and AMP (2 mmol l⁻¹).

CO₂ atmosphere. At the indicated times (arrows), different concentrations of glucose or L-lactate were added. The dotted or continuous trace corresponds to controls without or with 0.5 mmol L-lactate l⁻¹, respectively. The effect of the addition of glucose and lactate on intracellular $[Ca^{2+}]_i$ was similar in at least 15 different cell preparations. (b) Steady-state intracellular $[Ca^{2+}]_i$ of rat round spermatids loaded with fura-2. The measurements were made in a spectrofluorometer at 33°C in KH. The cells were incubated previously for 20 min without substrates in KHB buffer in a 95% O₂ and 5% CO₂ atmosphere. L-lactate (○) or glucose (●) was added at the indicated concentrations and $[Ca^{2+}]_i$ was monitored for 15 min until a steady-state value was obtained. The measurements shown were obtained from five or more cell preparations. (c) Intracellular $[Ca^{2+}]_i$ of round spermatids in rats loaded with fura-2. The measurements were made in a spectrofluorometer at 33°C in KH buffer supplemented with 0.5 mmol EGTA l⁻¹. The cells were incubated previously for 10 min with 0.5 mmol L-lactate l⁻¹ and the measurements were performed in the absence (○) and presence (●) of 0.5 mmol external free Ca²⁺ l⁻¹. At the indicated times (arrows), 5 mmol glucose l⁻¹ was added. This behaviour of intracellular $[Ca^{2+}]_i$ in response to the addition of glucose was similar in at least three different cell preparations.

Intracellular pH measurements

Intracellular pH of round spermatids was determined using the fluorescent probe 2',7'-bis-(2-carboxyethyl-5-(and -6)-carboxyfluorescein (BCECF). The cells in suspension were incubated in KHB-lactate in an atmosphere of 95% O₂ and 5% CO₂ with 0.5 µmol BCECF-AM l⁻¹ for 30 min at room temperature (20 ± 2°C) and subsequently washed in KH medium three times. Intracellular pH measurements were performed at a cell density of approximately 2 × 10⁶ cells ml⁻¹ in the appropriate solution. Calibration of intracellular BCECF was performed as described by Rink *et al.* (1982).

Statistical analysis

The data were analysed by *t* tests. Where indicated, a paired *t* test was performed to document relative differences in a variable. Non-linear regression was performed with the ORIGIN™ software package. The values reported are mean ± SD unless stated otherwise.

Results

Kinetics and steady-state effects of glucose and lactate on [Ca²⁺]_i in rat round spermatids and pachytene spermatocytes of rats

Round spermatids. The absence of exogenous substrates in round spermatids produced a steady increase in [Ca²⁺]_i in the absence and presence of external Ca²⁺. The addition of L-lactate prevented this increase in [Ca²⁺]_i in round spermatids. Thus, round spermatids incubated with 0.5 mmol L-lactate l⁻¹ in KH buffer had relatively low [Ca²⁺]_i (53 ± 11 nmol l⁻¹, *n* = 30) and gained [Ca²⁺]_i at a rate of 3.5 ± 2.5 nmol min⁻¹ (*n* = 30). The addition of glucose to these cells produced an increase in [Ca²⁺]_i (Fig. 1a) until a higher steady-state value was attained. Addition of L-lactate (1 mmol l⁻¹) counteracted the effect of glucose (Fig. 1a). In cells incubated previously in the absence of external substrates, the addition of lactate lowered [Ca²⁺]_i and subsequent addition of glucose produced an increase in [Ca²⁺]_i to a new steady-state value (Fig. 1a). The glucose-induced increase in [Ca²⁺]_i was dependent on the glucose concentration in the media with a *K*_{0.5} value of 0.7 mmol l⁻¹ (Fig. 1b). In the absence of other metabolic substrates, the presence of lactate produced a decrease in [Ca²⁺]_i with a *K*_{0.5} value of 0.89 ± 0.25 mmol l⁻¹ (Fig. 1b). The effect of the addition of glucose (at 0.5 mmol l⁻¹ external L-lactate) on [Ca²⁺]_i was similar in the presence (0.5 mmol l⁻¹) and absence (3 nmol l⁻¹) of external Ca²⁺ (55 ± 13 and 46 ± 15 nmol l⁻¹, respectively, *n* = 6; Fig. 1c). In the absence of external Ca²⁺, the interpretation of the data was more directly linked to glucose-induced changes in intracellular Ca²⁺ homeostatic mechanisms. Thus, unless stated otherwise, the experiments described below were performed in the absence of external Ca²⁺. The effects of glucose on [Ca²⁺]_i were less marked at higher lactate concentra-

Fig. 2. Steady-state intracellular [Ca²⁺]_i of round spermatids in rats as a function of the lactate:glucose ratio in the incubation medium. The cells were incubated previously in 140.0 mmol Na⁺ l⁻¹, 4.6 mmol K⁺ l⁻¹, 1.6 mmol Mg²⁺ l⁻¹, 1.6 mmol phosphate l⁻¹, 0.5 mmol Ca²⁺ l⁻¹, 10 mmol Hepes l⁻¹, pH 7.4 (KH), 24 mmol bicarbonate (KHB) l⁻¹ and 5 mmol L-lactate (KHB-lactate) at 33°C in a 95% O₂ and 5% CO₂ atmosphere, washed three times in KH medium with 0.5, 2.0 or 5.0 mmol glucose l⁻¹ (Δ, ■ or ●, respectively) and added to the fluorometer cuvette. After a steady state of intracellular [Ca²⁺]_i was obtained, lactate (0.2–10.0 mmol l⁻¹) was added to reach the different ratios studied, and a new steady-state of intracellular [Ca²⁺]_i was reached. The dotted line represents a sigmoidal curve and was drawn for reference purposes. The measurements shown were obtained from at least 12 different cell preparations.

tions. Thus, glucose (5 mmol l⁻¹) induced changes of 46 ± 15 nmol l⁻¹ (*n* = 6) in [Ca²⁺]_i at 0.5 mmol L-lactate l⁻¹ but induced changes in [Ca²⁺]_i of 21 ± 10 nmol l⁻¹ (*n* = 6) at 1 mmol L-lactate l⁻¹. Thus, [Ca²⁺]_i appears to respond to the ratios of glycolytic and oxidative metabolism in post-meiotic spermatogenic cells. The steady-state of [Ca²⁺]_i correlated in a non-linear manner with the external lactate:glucose ratio, and values of [Ca²⁺]_i ranged between approximately 120 and 40 nmol l⁻¹ (Fig. 2).

Pachytene spermatocytes. When pachytene spermatocytes were incubated in 0.5 mmol L-lactate l⁻¹, they had relatively low [Ca²⁺]_i (41 ± 8 nmol l⁻¹, *n* = 15) and gained [Ca²⁺]_i at a rate of approximately 1.8 ± 1.5 nmol min⁻¹ (*n* = 15). The addition of glucose to these cells produced an increase in [Ca²⁺]_i (Fig. 3) that was smaller than that in round spermatids. The addition of 1 mmol L-lactate l⁻¹ after the addition of glucose induced a rapid decrease in [Ca²⁺]_i similar to that observed before the addition of glucose. The addition of glucose in the presence of 1 mmol L-lactate l⁻¹ produced only a small increase in [Ca²⁺]_i (2.5 ± 1.2 nmol l⁻¹, *n* = 6). In the absence of metabolic substrates, L-lactate produced a decrease in [Ca²⁺]_i in pachytene spermatocytes with a *K*_{0.5} value of 1.25 ± 0.42 mmol l⁻¹. At 0.5 mmol L-lactate l⁻¹, addition of 5 mmol glucose l⁻¹ produced a

Fig. 3. Intracellular $[Ca^{2+}]_i$ of pachytene spermatocytes loaded with fura-2 in rats. The measurements were made in a spectrofluorometer at 33°C in 140.0 mmol Na^+ l^{-1} , 4.6 mmol K^+ l^{-1} , 1.6 mmol Mg^{2+} l^{-1} , 1.6 mmol phosphate l^{-1} , 0.5 mmol Ca^{2+} l^{-1} , 10 mmol Hepes l^{-1} , pH 7.4 (KH) buffer. The cells were incubated previously for 20 min in KH and 24 mmol bicarbonate l^{-1} (KHB) either without substrates (●) or with 0.5 mmol lactate l^{-1} (○) in a 95% O_2 and 5% CO_2 atmosphere. At the indicated times (arrows), different concentrations of glucose or L-lactate were added. The effect of the addition of glucose and lactate on intracellular $[Ca^{2+}]_i$ was similar in at least 15 different cell preparations.

significantly smaller change in $[Ca^{2+}]_i$ (19 ± 8 , $n = 7$) in pachytene spermatocytes than in round spermatids ($P < 0.05$ in a paired t test). Furthermore, pachytene spermatocytes showed a transient response to glucose in contrast to the high steady-state values of $[Ca^{2+}]_i$ reached in round spermatids (Fig. 4a). Thus, a differential response of $[Ca^{2+}]_i$ to glucose was observed in round spermatids compared with pachytene spermatocytes.

Effects of glucose on intracellular Ca^{2+} homeostasis in round spermatids

Similar to other mammalian cells, $[Ca^{2+}]_i$ in round spermatid and pachytene spermatocytes of rats was actively regulated by sarco-endoplasmic reticulum and plasma membrane Ca^{2+} -ATPases. In these cells, intracellular Ca^{2+} stores seemed to be either permanently leaking Ca^{2+} or the Ca^{2+} exit pathways in intracellular Ca^{2+} stores were rapidly activated when sarco-endoplasmic reticulum ATPase activity was decreased (Berrios *et al.*, 1998; Treviño *et al.*, 1998; Herrera *et al.*, 2000). The lack of a significant difference between the glucose-induced increase in $[Ca^{2+}]_i$ in the presence and absence of external Ca^{2+} strongly indicates that most of the cytosolic $[Ca^{2+}]_i$ increase induced by glucose came from intracellular Ca^{2+} stores. The following experiments were designed to determine the source of intracellular Ca^{2+} responsible for the glucose-induced changes in $[Ca^{2+}]_i$ in round spermatids. In the absence or

Fig. 4. Intracellular $[Ca^{2+}]_i$ of rat (a) round spermatids and (b) pachytene spermatocytes loaded with fura-2. The measurements were made in a spectrofluorometer at 33°C in 140.0 mmol Na^+ l^{-1} , 4.6 mmol K^+ l^{-1} , 1.6 mmol Mg^{2+} l^{-1} , 1.6 mmol phosphate l^{-1} , 0.5 mmol Ca^{2+} l^{-1} , 10 mmol Hepes l^{-1} , pH 7.4 (KH) buffer. The cells were incubated previously for 20 min in KH, 24 mmol bicarbonate l^{-1} (KHB) and 5 mmol L-lactate (KHB-lactate) in a 95% O_2 and 5% CO_2 atmosphere, washed in KH medium and added to the fluorometer cuvette. At the indicated times (arrows), 5 mmol 2-deoxyglucose (DOG, ○) l^{-1} or 5 mmol 3-O-methyl D-glucopyranoside (OMG, ●) l^{-1} was added. Thapsigargin (tsg, 500 nmol l^{-1}) was added to release intracellular Ca^{2+} stores. These measurements were representative of experiments performed in three different cell preparations.

presence of external Ca^{2+} , intracellular Ca^{2+} stores were released with thapsigargin and the transient $[Ca^{2+}]_i$ increase was allowed to subside towards a new steady-state. At this point (complete release of thapsigargin-sensitive intracellular Ca^{2+} stores), glucose was added (Fig. 5). The addition of glucose did not produce a further increase in $[Ca^{2+}]_i$ ($n = 4$) in the presence or absence of external Ca^{2+} . Furthermore, the subsequent addition of ionomycin induced the

Fig. 5. Intracellular $[Ca^{2+}]_i$ of round spermatids of rats loaded with fura-2 in rats. The measurements were made in a spectrofluorometer at $33^\circ C$ in $140.0 \text{ mmol Na}^+ \text{ l}^{-1}$, $4.6 \text{ mmol K}^+ \text{ l}^{-1}$, $1.6 \text{ mmol Mg}^{2+} \text{ l}^{-1}$, $1.6 \text{ mmol phosphate l}^{-1}$, $0.5 \text{ mmol Ca}^{2+} \text{ l}^{-1}$, $10 \text{ mmol Hepes l}^{-1}$, pH 7.4 (KH) buffer. The cells were washed and maintained in KH with $0.5 \text{ mmol EGTA l}^{-1}$. The cells were either without (●) or with (○) $1 \text{ mmol external Ca}^{2+} \text{ l}^{-1}$, giving approximate extracellular free Ca^{2+} concentrations of 3 nmol l^{-1} and 0.5 mmol l^{-1} , respectively. L-lactate (0.5 mmol l^{-1}) was added at zero time. Thapsigargin (tsg, 500 nmol l^{-1}) was added to release intracellular Ca^{2+} stores. Glucose (5 mmol l^{-1}) was added at the indicated times (arrows). Ionomycin (Ionom; $1 \mu\text{g ml}^{-1}$) was added to release intracellular Ca^{2+} stores not released by tsg. Digitonin ($15 \mu\text{g ml}^{-1}$) was added to release fura-2 from the cytoplasm of the cells. These measurements were representative of experiments performed in three different cell preparations.

release of thapsigargin-insensitive Ca^{2+} stores present in these cells (for an example, see Pizzo *et al.*, 1997; Berrios *et al.*, 1998), indicating that thapsigargin-insensitive intracellular Ca^{2+} stores had a supply of Ca^{2+} susceptible to release if their Ca^{2+} permeability was increased, as shown with ionomycin. These measurements were terminated with digitonin, the addition of which permeabilized the cell plasma membrane and allowed confirmation that the $[Ca^{2+}]_i$ increase induced by thapsigargin or ionomycin was not a consequence of Ca^{2+} entry. These results indicate that glucose induced the release of Ca^{2+} exclusively from thapsigargin-sensitive intracellular Ca^{2+} stores in rat round spermatids, rather than through activation of Ca^{2+} entry from the extracellular space or release of Ca^{2+} from thapsigargin-insensitive intracellular Ca^{2+} stores.

The glucose-induced increase in $[Ca^{2+}]_i$ may arise either from an increased rate of Ca^{2+} release from thapsigargin-sensitive intracellular Ca^{2+} stores or from a decreased activity of sarco-endoplasmic reticulum-mediated Ca^{2+} uptake. The first possibility was tested by the addition of glucose to round spermatids in the absence of external Ca^{2+} . After $[Ca^{2+}]_i$ began to increase, thapsigargin was added. Activation by glucose metabolism of Ca^{2+} release pathways from intracellular Ca^{2+} stores (for an example, see Takasawa

et al., 1993) was expected to produce a higher rate of increase in thapsigargin-induced $[Ca^{2+}]_i$ in the presence rather than in the absence of glucose. No significant differences in the rates of thapsigargin-induced $[Ca^{2+}]_i$ release in the absence and presence of glucose were found (paired experiments, $n = 3$, not shown). Hence, the results of the present study are consistent with the contention that glucose-induced changes in $[Ca^{2+}]_i$ can be attributed to glucose-induced changes in sarco-endoplasmic reticulum-ATPase transport activity in round spermatids. On average, glucose was able to induce the release of approximately 25% of thapsigargin-sensitive intracellular Ca^{2+} stores.

Glycolytic metabolism and glucose-induced changes in $[Ca^{2+}]_i$ in rat pachytene spermatocytes and round spermatids

A transported but non-metabolizable analogue of glucose (3-O-methyl-D-glucopyranoside: OMG) and a transported and hexokinase-metabolizable analogue of glucose (2-deoxy glucose: DOG) were used to test whether glucose metabolism was needed for the glucose-induced increase in $[Ca^{2+}]_i$ in spermatogenic cells. Addition of OMG (5 mmol l^{-1}) did not produce a significant change in $[Ca^{2+}]_i$ in pachytene spermatocytes ($0.8 \pm 1.1 \text{ nmol l}^{-1}$, $n = 4$) or round spermatids ($0.9 \pm 1.2 \text{ nmol l}^{-1}$, $n = 4$) (Fig. 4a,b). In contrast, addition of DOG (5 mmol l^{-1}) induced a clear increase in $[Ca^{2+}]_i$ in both pachytene spermatocytes ($16 \pm 3 \text{ nmol l}^{-1}$, $n = 6$) and round spermatids ($33 \pm 11 \text{ nmol l}^{-1}$, $n = 5$; $P < 0.01$). After the addition of DOG or OMG, thapsigargin (inhibitor of the intracellular Ca^{2+} - Mg^{2+} -ATPase) produced a large increase in $[Ca^{2+}]_i$ ($112 \pm 25 \text{ nmol l}^{-1}$, $n = 6$) above the concentrations of $[Ca^{2+}]_i$ induced by the glucose analogues. This result shows that the Ca^{2+} content of intracellular Ca^{2+} stores was not limiting the response to OMG or DOG.

In round spermatids that were incubated with 5 mmol l^{-1} L-lactate l^{-1} , washed and treated with $1 \text{ mmol iodoacetate}$ (inhibitor of glyceraldehyde 3-P dehydrogenase, GPDH) l^{-1} , the increase in $[Ca^{2+}]_i$ after the addition of glucose was significantly augmented (Fig. 6a; $61 \pm 8 \text{ nmol l}^{-1}$ versus $22 \pm 4 \text{ nmol l}^{-1}$, $n = 4$, $P < 0.01$, paired test). In pachytene spermatocytes, and in the absence of iodoacetate, $[Ca^{2+}]_i$ reached a maximum after the addition of glucose and then slowly decreased toward basal amounts. This phase of decreasing $[Ca^{2+}]_i$ seemed to be related to the metabolism of glucose beyond GPDH in spermatocytes, as it was abolished by treatment with iodoacetate (Fig. 6b).

Effects of glucose on adenine nucleotide pools in round spermatids

As shown above, glycolytic metabolism and specifically sugar phosphorylation appear to be essential for the glucose induction of $[Ca^{2+}]_i$ changes in pachytene spermatocytes and round spermatids. Hence, either intermediaries of glucose metabolism or changes in adenine nucleotide pools

Fig. 6. Intracellular $[Ca^{2+}]_i$ of (a) round spermatids and (b) pachytene spermatocytes of rats loaded with fura-2. The measurements were made in a spectrofluorometer at 33°C in 140.0 mmol Na⁺ l⁻¹, 4.6 mmol K⁺ l⁻¹, 1.6 mmol Mg²⁺ l⁻¹, 1.6 mmol phosphate l⁻¹, 0.5 mmol Ca²⁺ l⁻¹, 10 mmol Hepes l⁻¹, pH 7.4 (KH) buffer with 0.5 mmol EGTA l⁻¹. The cells were incubated previously for 20 min in KH, 24 mmol bicarbonate l⁻¹ (KHB) and 5 mmol L-lactate (KHB-lactate) in a 95% O₂ and 5% CO₂ atmosphere, washed in KH-EGTA medium and added to the fluorometer cuvette. At the indicated times (arrows), glucose alone (●) or iodoacetate (IA) followed by glucose (○) were added to the cuvette. These measurements were representative of experiments performed in three different cell preparations.

(Nakamura *et al.*, 1982; Grotegoed *et al.*, 1986; Reyes *et al.*, 1990) could be involved in the glucose-induced modulation of Ca²⁺ uptake by intracellular Ca²⁺ stores. Two approaches were used to test the possibility that glucose metabolism could trigger rapid changes in adenine nucleotide pools. Firstly, in conditions of abundant O₂, inorganic phosphate and reducing equivalent supply to the mitochondria, these organelles should respond with changes in O₂ consumption to changes in the concentrations of cytosolic ADP. In agreement with the predicted changes in adenine nucleotides induced by activation of glycolysis, addition of glucose in the presence of 1 mmol L-lactate l⁻¹ induced a transient increase in QO₂ (21%

Table 1. Relative adenine nucleotide content in rat round spermatids in the presence of glucose

Time (min)	[ATP]	[ADP]	[AMP]
0	1.00	1.00	1.00
0.5	0.90 ± 0.09	1.02 ± 0.16	0.86 ± 0.24
2.0	1.25 ± 0.48	1.00 ± 0.19	0.83 ± 0.20
4.0	0.82 ± 0.35	1.31 ± 0.08*	1.89 ± 0.10*

Round spermatids were incubated previously in a shaker waterbath in 140.0 mmol Na⁺ l⁻¹, 4.6 mmol K⁺ l⁻¹, 1.6 mmol Mg²⁺ l⁻¹, 1.6 mmol phosphate l⁻¹, 0.5 mmol Ca²⁺ l⁻¹, 10 mmol Hepes l⁻¹, pH 7.4 (KH), 24 mmol bicarbonate (KHB) l⁻¹ and 5 mmol L-lactate l⁻¹ (KHB-lactate) under an atmosphere of 95% O₂ and 5% CO₂ at 33°C. The spermatids were subsequently washed in a medium devoid of substrates. Samples were taken at time 0 and glucose (5 mmol l⁻¹) was added at 0.5, 2.0 and 4.0 min. ATP, ADP and AMP are expressed as the ratio between the concentrations per mg of cell protein at the indicated times and the concentrations at time 0. The measurements were performed in three cell preparations. Results are expressed as the mean ± SD. *Significantly different (paired test, *P* < 0.01) from the values at time 0.

average, range 15–35%, *n* = 4) in round spermatids. This glucose-induced increase in QO₂ indicates that glucose metabolism induced a transient burst of ADP followed by a new lower steady-state level of this nucleotide. Secondly, HPLC determinations were used to estimate the amounts of ATP, ADP and AMP at short intervals after the addition of glucose to a suspension of round spermatid cells. Intracellular ATP concentrations did not change significantly at 4 min after glucose was added to round spermatids (Table 1). As a comparison, the addition of antimycin (inhibitor of the mitochondrial electron chain) induced a decrease to 10% of the original concentrations of ATP in round spermatids in a similar time (Herrera *et al.*, 2000). However, intracellular ADP and AMP concentrations did show a significant increase at 4 min after addition of glucose to the cells, indicating an increased turnover of ATP in round spermatids in the presence of glucose. However, it should be noted that as a result of the relatively large variance and the averaging character of nucleotide measurements by HPLC, this method would fail to detect small or localized changes in nucleotide pools in the cells.

Intracellular Ca²⁺ content of round spermatids and changes in the external lactate and glucose concentrations

It is well known that Sertoli cells respond to FSH, catecholamines and cytokines with an increase in their glycolytic flux and lactate output (Mita *et al.*, 1982; Le Gac *et al.*, 1983; Hall and Mita, 1984; Riera *et al.*, 2001). FSH pulses in male mammals have a periodicity of approximately 2–3 h (Pincus *et al.*, 1997). Hence, it is expected that the glycolytic activity of Sertoli cells and their release of lactate and glucose to the seminiferous tubule adluminal micro-environment would vary according to the endocrine and paracrine status of these cells. The putative dynamics of

Fig. 7. Relative fluorescence of fluo-3-loaded single cells obtained from fluorometric imaging at 33°C. F_0 is the fluorescence of each cell at time zero. The cells were perfused with 140.0 mmol Na^+ l^{-1} , 4.6 mmol K^+ l^{-1} , 1.6 mmol Mg^{2+} l^{-1} , 1.6 mmol phosphate l^{-1} , 0.5 mmol Ca^{2+} l^{-1} , 10 mmol Hepes l^{-1} , pH 7.4 (KH)-EGTA and 5 mmol L-lactate l^{-1} or 5 mmol glucose solution l^{-1} as indicated on the bottom lines. Heavy lines indicate the time taken for complete changes in the solutions bathing the cells. The data shown are from experiments performed in three different cell preparations. Inserts show measurements performed in similar conditions but with two cycles of glucose and L-lactate solution changes (insert, a) or with changes in glucose and L-lactate solutions followed by glucose solution maintained until the end of the experiment (insert, b). Lact: lactose; glu: glucose.

glucose and lactate concentrations in the spermatogenic cell extracellular environment in the seminiferous tubule were mimicked by varying the external lactate and glucose concentrations by passing solutions containing lactate or glucose on single round spermatids. Round spermatid $[\text{Ca}^{2+}]_i$ followed the pulses in lactate and glucose with changes in $[\text{Ca}^{2+}]_i$, setting a pattern of pulsed changes in $[\text{Ca}^{2+}]_i$ (Fig. 7). Approximately 80% of the cells responded to glucose with changes in fluo-3 fluorescence of $> 20\%$. However, the magnitude of the response was clearly heterogeneous. As our method of vital cell identification does not allow sub-classification of rat spermatids at these stages of development, it was not possible to ascribe this diversity of glucose sensitivities in round spermatids $[\text{Ca}^{2+}]_i$ unequivocally to a differentiation-related phenomenon.

Glucose-induced changes in pH_i and their relation to $[\text{Ca}^{2+}]_i$ changes

The addition of glucose to round spermatids and pachytene spermatocytes induced a decrease in pH_i of approximately 0.5 pH units (Fig. 8). There was a delay in these changes in pH_i (measured from the time of glucose addition to the extrapolated initial velocity of pH_i change)

Fig. 8. Intracellular pH of pachytene spermatocytes (●) and round spermatids (○, Δ) of rats. The cells were incubated previously in 140.0 mmol Na^+ l^{-1} , 4.6 mmol K^+ l^{-1} , 1.6 mmol Mg^{2+} l^{-1} , 1.6 mmol phosphate l^{-1} , 0.5 mmol Ca^{2+} l^{-1} , 10 mmol Hepes l^{-1} , pH 7.4 (KH), 24 mmol bicarbonate l^{-1} (KHB) and 5 mmol L-lactate (KHB-lactate) under a 95% O_2 and 5% CO_2 atmosphere, and subsequently washed three times in KH medium without substrates. The measurements were performed at 33°C with constant stirring. At the indicated times (arrows), 2 mmol glucose l^{-1} was added to the cell suspension. NH_4Cl (15 mmol l^{-1} , Δ) was added to show that the glucose-induced decrease in pH below 7.3 was prevented by NH_4Cl . These measurements are representative of experiments performed in three different cell preparations. Intracellular $[\text{Ca}^{2+}]_i$ of rat round spermatids loaded with fura-2 (insert). The measurements were made in a spectrofluorometer at 33°C in KH buffer with 0.5 mmol EGTA l^{-1} . NH_4Cl (15 mmol l^{-1} , ●) was added before addition of glucose to the cells.

of 56 ± 28 s ($n = 7$) in round spermatids and 42 ± 14 s ($n = 7$) in pachytene spermatocytes. In contrast, the changes in $[\text{Ca}^{2+}]_i$ in these cells were initiated approximately 10 s after glucose addition. These results provide a kinetic argument to state that $[\text{Ca}^{2+}]_i$ changes induced by glucose did not derive from changes in pH_i in round spermatids or pachytene spermatocytes. Furthermore, the addition of NH_4Cl , which increased pH_i in these cells and prevented the decrease in pH induced by glucose (Fig. 8), did not modify the kinetics or the magnitude of the changes in $[\text{Ca}^{2+}]_i$ induced by glucose (Fig. 8, insert).

Discussion

There is extensive experimental evidence supporting the idea that glucose and lactate concentrations are regulated by the glycolytic activity of Sertoli cells in the micro-environment surrounding meiotic and post-meiotic spermatogenic cells (Robinson and Fritz, 1981; Mita *et al.*, 1982; Le Gac *et al.*, 1983; Hall and Mita, 1984). FSH, catecholamines and cytokines increase the glycolytic activity of

Sertoli cells (Mita *et al.*, 1982; Le Gac *et al.*, 1983; Hall and Mita, 1984; Riera *et al.*, 2001) indicating an endo–paracrine modulation of glucose and lactate release and changes of the concentrations of these substrates in the adluminal compartment of the seminiferous tubules. The results of the present study demonstrating that changes in extracellular glucose and lactate concentrations can induce a dynamic behaviour of $[Ca^{2+}]_i$ in meiotic and post-meiotic spermatogenic cells provide a hypothetical link between endo–paracrine status in the seminiferous tubules, the functional state of Sertoli cells and intracellular signalling in spermatogenic cells.

The lack of a significant difference in the glucose-induced increase in $[Ca^{2+}]_i$ in the presence and absence of external Ca^{2+} does not support an important role for Ca^{2+} entry in the glucose-induced $[Ca^{2+}]_i$ changes in meiotic and post-meiotic spermatogenic cells. The results of the present study indicate that most of the cytosolic $[Ca^{2+}]_i$ increase induced by glucose came from thapsigargin-sensitive intracellular Ca^{2+} stores. Furthermore, glucose was able to inhibit sarco-endoplasmic reticulum ATPase Ca^{2+} uptake by intracellular Ca^{2+} stores in round spermatids, and this effect was not mediated by changes in intracellular pH. Nucleotide turnover could be involved in this phenomenon.

Both kinetically and in magnitude, the $[Ca^{2+}]_i$ of pachytene spermatocytes and round spermatids responded in different ways to the addition of glucose. In the presence of lactate, $[Ca^{2+}]_i$ in round spermatids correlates with the concentration ratios of lactate and glucose. Thus, a differential and developmentally expressed intracellular Ca^{2+} sensitivity to glucose and lactate was demonstrated in post-meiotic spermatogenic cells. The metabolism of glucose was necessary to observe the effects of glucose on $[Ca^{2+}]_i$ in both pachytene spermatocytes and round spermatids, as no effect of a non-metabolizable glucose analogue (OMG) was observed. The effects of 2-deoxy glucose and the enhancement of the increase in $[Ca^{2+}]_i$ induced by glucose after inhibition of GPDH with iodoacetate indicate that glucose phosphorylation is an essential mechanism involved in the glucose-dependent changes in $[Ca^{2+}]_i$. Glucose metabolism can induce a net ATP hydrolysis in spermatids after several minutes of exposure of the cells to this sugar, apparently by sugar phosphorylation and activation of a substrate cycle catalysed by phosphofructokinase and fructose 1,6 bisphosphatase (Nakamura *et al.*, 1982; Grotegoed *et al.*, 1986; Reyes *et al.*, 1990). A decrease in intracellular [ATP] was not observed 4 min after exposure of round spermatids to glucose. However, intracellular [ADP] and [AMP] were significantly increased at this time. These changes in intracellular adenine nucleotides and the rapid changes in QO_2 induced by glucose demonstrate a glucose-stimulated rapid increase in ATP turnover in round spermatids. These results strongly indicate that the ATP consuming steps in glycolysis and a likely limiting flux in GPDH in round spermatids (for an example, see Nakamura *et al.*, 1982) can be responsible for the differential response to glucose in

round spermatids. The idea that addition of glucose could increase $[Ca^{2+}]_i$ in round spermatids by increasing the turnover of ATP is in agreement with previous findings that inhibition of mitochondrial metabolism, which induced rapid changes in intracellular adenine nucleotides, also rapidly induced an increase in $[Ca^{2+}]_i$ in these cells (Herrera *et al.*, 2000). The transient increase in $[Ca^{2+}]_i$ induced by glucose in pachytene spermatocytes could be thought to arise from the fact that these cells have a larger glycolytic flux (as compared with round spermatids) leading to pyruvate and lactate production that, in turn, would be able to enter oxidative metabolism (Bajpai *et al.*, 1998). Thus, these cells would present a transient glucose phosphorylation (increased ATP turnover) followed by a relatively larger (as compared with round spermatids) flux through ATP generating steps in glycolysis and oxidative phosphorylation.

In light of the results of the present study, the metabolic properties of post-meiotic cells emerge as a differentiation-related process that causes $[Ca^{2+}]_i$ of post-meiotic cells to be more responsive to the external supply of oxidative and glycolytic substrates. Local pulses of cytokines or catecholamines, or cyclic FSH (for example, Pincus *et al.*, 1997) could translate to changes in glycolytic fluxes in Sertoli cells and a dynamic (but inverse) supply of glucose and lactate to meiotic and post-meiotic spermatogenic cells. In the present study it was shown that steady-state or cyclic changes in lactate and glucose can produce a dynamic of $[Ca^{2+}]_i$ composed of sustained or oscillatory changes. These dynamics of intracellular Ca^{2+} are presently considered to form the basis for intracellular Ca^{2+} signalling in most cells (Berridge *et al.*, 2000). Thus, the present study provides a physiological interpretation for the differentiation-related changes in carbohydrate metabolism in spermatogenic cells, linking Sertoli cell metabolism, their endocrine and paracrine control, and intracellular signalling in spermatogenic cells. Along these lines, major aspects of this working hypothesis to be determined would be whether there is a dynamic secretion of glucose and lactate in the seminiferous tubules, whether these changes can induce $[Ca^{2+}]_i$ changes in spermatogenic cells *in situ* and, finally, how these $[Ca^{2+}]_i$ changes could affect growth and differentiation of spermatogenic cells.

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