Temperature dependence of intracellular Ca^{2+} homeostasis in rat meiotic and postmeiotic spermatogenic cells

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The hypothesis that intracellular $[\text{Ca}^{2+}]$ is a cell parameter responsive to extreme temperatures in rat meiotic and postmeiotic spermatogenic cells was tested using intracellular fluorescent probes for Ca^{2+} and pH. In agreement with this hypothesis, extreme temperatures induced a rapid increase of cytosolic $[\text{Ca}^{2+}]$ in rat pachytene spermatocytes and round spermatids. Oscillatory changes in temperature can induce oscillations in cytosolic $[\text{Ca}^{2+}]$ in these cells. Intracellular $[\text{Ca}^{2+}]$ homeostasis in round spermatids was more sensitive to high temperatures compared with pachytene spermatocytes. The calculated activation energies for SERCA ATPase-mediated fluxes in pachytene spermatocytes and

round spermatids were 62 and 75 kJ mol⁻¹, respectively. The activation energies for leak fluxes from intracellular Ca^{2+} stores were 55 and 68 kJ mol⁻¹ for pachytene spermatocytes and round spermatids, respectively. Together with changes in cytosolic $[\text{Ca}^{2+}]$, round spermatids undergo a decrease in pH_i at high temperatures. This temperature-induced decrease in pH_i appears to be partially responsible for the increase in cytosolic $[\text{Ca}^{2+}]$ of round spermatids induced by high temperatures. This characteristic of rat meiotic and postmeiotic spermatogenic cells to undergo an increment in cytosolic Ca^{2+} at temperatures > 33°C can be related to the induction of programmed cell death by high temperatures in these cells.

Introduction

Scrotal temperature in mammals is 2–10°C lower than the abdominal temperature. In rats, the abdominal–scrotal temperature difference is approximately 8°C and exposure of the testis to temperatures $\geq 37^\circ\text{C}$ results in increased death of germ cells (Kandeel and Swerdloff, 1988). Histological studies in rats have shown that heating of the testis or surgically induced cryptorchidism (abdominal testis) resulted in increased death of spermatogenic cells. Pachytene spermatocytes and early spermatids were the first spermatogenic cells to degenerate after a single short-term (15 min) exposure to 43°C (Chowdhury and Steinberger, 1964; Collins and Lacy, 1969; Jones *et al.*, 1977). Induction of cell death by cryptorchidism or exposure of the testis to high temperature appears to occur via an apoptotic pathway (Yin *et al.*, 1997; Lue *et al.*, 1999). Yamamoto *et al.* (2000) suggested that Bax and bcl-2 proteins might be involved in this process. The early cellular and molecular events involved in the activation of germ cell death have not been elucidated.

In rat meiotic and postmeiotic spermatogenic cells a fine balance between Ca^{2+} uptake and release by intracellular stores maintains intracellular $[\text{Ca}^{2+}]$ homeostasis (Herrera *et al.*, 2000). As derangement of intracellular $[\text{Ca}^{2+}]$

homeostasis is the initiating event leading to programmed cell death in many cells, the hypothesis that impairment of intracellular Ca^{2+} homeostasis is an early event induced by extreme temperatures in spermatogenic cells was investigated.

Materials and Methods

Materials

Fura-2 acetoxymethyl ester was obtained from Molecular Probes (Eugene, OR). Thapsigargin (tsg) was obtained from Calbiochem (La Jolla, CA). Other chemicals were obtained from Sigma Chemical Co. (St Louis, MO).

Preparation of rat spermatogenic cells

Rat spermatogenic cell populations were prepared from the testicles of adult (aged 60 days) Sprague–Dawley rats as described by Romrell *et al.* (1976). The rats were housed with free access to food and water with a 12 h light:12 h dark cycle. The animals were narcotized lightly by exposure to CO_2 for 45 s and then killed by cervical dislocation. The pachytene spermatocyte ($85 \pm 5\%$ purity, $17 \pm 2 \mu\text{m}$, range 15–20 μm) and round spermatid ($92 \pm 4\%$ purity, $11 \pm 2 \mu\text{m}$, range 8–13 μm) fractions were identified by size and by the typical aspect of their nucleus stained with H_{33342} (Reyes *et al.*, 1997). The contaminating cells in the pachytene spermatocyte fraction were small cells (spermatids: 50%;

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residual bodies: 20%; and leptotene and zygotene spermatocytes: 30% of the contaminating cells). Owing to the large size of pachytene spermatocytes, their volume was calculated to be approximately 96% of the total cell volume in this fraction. The contaminating cells in the round spermatid fraction were spermatocytes and residual bodies (20 and 80% of the contaminating cells, respectively). The calculated volume of round spermatids corresponded to approximately 94% of the total cell volume in this fraction. The integrity of the cell membrane was estimated by incubating the cell suspension with 4 μmol ethidium bromide l^{-1} and examining the cells under a fluorescence microscope. The cell membrane integrity was $> 90\%$ under all the conditions tested in this study.

Intracellular Ca^{2+} measurements of spermatogenic cells in suspension

Measurements of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) 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 1 mg cell protein ml^{-1} (5×10^6 cells ml^{-1}) with 5 μmol acetoxymethyl fura-2 l^{-1} for 1 h at room temperature in a 95% O_2 and 5% CO_2 atmosphere. The cells were washed three times in Krebs–Henseleit (KH) medium supplemented with 10 mmol Hepes l^{-1} and 5 mmol l -lactate l^{-1} . The measurements were performed in a Fluoromax 2 fluorimeter using a ratiometric method as described by Grynkiewicz *et al.* (1985). Calibration of fura-2 was performed at 4, 15, 30 and 40°C by lysis of the cells with digitonin (20 μg ml^{-1}) in the medium that contained 0.5 mmol Ca^{2+} l^{-1} (F_{max}) and subsequent addition of a final concentration of 10 mmol EGTA l^{-1} (pH 7.4) (F_{min}). As described by Larsson *et al.* (1999), no significant differences in F_{max} or F_{min} were found at the temperatures tested. The apparent K_d value for the Ca^{2+} –fura-2 complex (224 nmol at 37°C ; Grynkiewicz *et al.*, 1985) was corrected at each temperature according to Larsson *et al.* (1999).

The rates of Ca^{2+} leakage and SERCA-mediated uptake from intracellular Ca^{2+} stores were estimated from the initial rate of $[\text{Ca}^{2+}]_i$ increase after addition of thapsigargin (a specific inhibitor of SERCA ATPases) to the cells in suspension (J_{leak}), from the basal rate of $[\text{Ca}^{2+}]_i$ increase (J_{net}) and from the relationship $J_{\text{net}} = J_{\text{leak}} - J_{\text{pump}}$. These measurements were performed in the nominal absence of external Ca^{2+} (3 nmol free Ca^{2+} l^{-1}) in a fluorometer cuvette with temperature control. Temperature was monitored by a thermistor probe attached to the side of the cuvette and kept insulated from the ambient temperature. The temperature reading agreed with the temperatures estimated using a temperature microprobe inside the cuvette.

Determination of the concentrations of intracellular adenine nucleotides in rat round spermatids

Rat round spermatids in suspension were incubated at a concentration of approximately 6 mg cell protein ml^{-1} in

KH calcium lactate at 33°C in a 95% O_2 and 5% CO_2 atmosphere for 10 min. Subsequently, the cells were subjected to different temperatures for several time periods. After the reported incubation times, 400 μl cells was added to an ice-cold microcentrifuge tube and pelleted by centrifugation for 30 s at 16 000 *g*. The supernatant was 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 l^{-1} and 2 mmol ADP l^{-1} solutions and subjecting them to the sample processing and storage conditions. The samples were maintained frozen at -20°C until defrosted 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 mm $\times$ 7.8 mm; BioRad, Hercules, CA) at a flow rate 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 2 mmol l^{-1} standards of ATP, ADP and AMP (Herrera *et al.*, 2000).

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 with 0.5 μmol BCECF-AM l^{-1} for 30 min at room temperature ($20 \pm 2^\circ\text{C}$) and washed subsequently in KH medium three times. Intracellular pH measurements were performed at a cell density of approximately 2×10^6 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. Linear or non-linear regression was performed with the ORIGINTM software package. The values reported are mean $\pm$ SD unless stated otherwise.

Results

Temperature-dependent changes in $[\text{Ca}^{2+}]_i$ in pachytene spermatocytes and round spermatids

Changes in temperature can induce changes in $[\text{Ca}^{2+}]_i$ in both pachytene spermatocytes and round spermatids (Fig. 1a,b). These temperature-dependent changes in $[\text{Ca}^{2+}]_i$ were reversible within the time frame shown (Fig. 1). Cycling in temperature can induce oscillations in $[\text{Ca}^{2+}]_i$ in these cells. The sensitivity of $[\text{Ca}^{2+}]_i$ to high temperatures, especially the kinetics of changes in $[\text{Ca}^{2+}]_i$, was higher in round spermatids compared with pachytene spermatocytes (Fig. 1a,b). For example, at approximately 38°C , the $[\text{Ca}^{2+}]_i$ of pachytene spermatocytes was about 30–40 nmol l^{-1} ,

whereas at the same temperature, $[Ca^{2+}]_i$ in round spermatids was $> 80\text{--}100\text{ nmol l}^{-1}$.

Temperature dependence of intracellular Ca^{2+} fluxes in pachytene spermatocytes and round spermatids

In the absence of external Ca^{2+} , addition of $50\text{ }\mu\text{mol l}^{-1}$ vanadate I^{-1} to the cells did not significantly affect the initial rate of basal cytosolic $[Ca^{2+}]$ changes at the different temperatures (data not shown; for example see Berrios *et al.*, 1998). This result indicates that under these conditions, the plasma membrane Ca^{2+} -ATPase was contributing only marginally to intracellular Ca^{2+} homeostasis in these cells. Under low cytosolic $[Ca^{2+}]$, mitochondrial uptake does not contribute to removal of cytosolic Ca^{2+} (Herrera *et al.*, 2000). Hence, at each temperature and in the absence of external Ca^{2+} , the initial rate of change in basal $[Ca^{2+}]_i$ concentration (J_{net}) represents the difference between the leakage fluxes (J_{leak}) and the uptake fluxes by intracellular Ca^{2+} stores (J_{up}). Thus, the initial rate of Ca^{2+} release from intracellular stores after addition of thapsigargin represents the leakage flux from intracellular Ca^{2+} stores (J_{leak}) and, therefore, J_{up} can be equated to the Ca^{2+} uptake by SERCA ATPases (J_{pump}). From the relationship $J_{net} = J_{leak} - J_{pump}$, and assuming flux independence, the pump flux could be calculated at each temperature for pachytene spermatocytes and round spermatids (Figs 2a and 3a, respectively). The data for the logarithm of J_{leak} and J_{pump} were plotted against T^{-1} to estimate the apparent activation energy (E'_a) for these processes. These data are shown for pachytene spermatocytes (Fig. 2b) and round spermatids (Fig. 3b). E'_a of SERCA ATPase-mediated fluxes had calculated values of 62 ± 8 and $74 \pm 5\text{ kJ mol}^{-1}$ in pachytene spermatocytes and round spermatids, respectively. E'_a of intracellular Ca^{2+} stores leakage fluxes had calculated values of 55 ± 8 and $68 \pm 7\text{ kJ mol}^{-1}$ in pachytene spermatocytes and round spermatids, respectively.

Intracellular adenine nucleotide concentrations in spermatogenic cells at different temperatures

Intracellular Ca^{2+} homeostasis is very sensitive to the energy metabolic state of pachytene spermatocytes and round spermatids (Herrera *et al.*, 2000). Thus, to determine whether changes in the energy state of spermatogenic cells could account for the observed changes in $[Ca^{2+}]_i$ homeostasis at different temperatures, intracellular adenine nucleotide (ATP, ADP and AMP) concentrations were measured in these cells at 4, 33 and 40°C in KH medium supplemented with 10 mmol l^{-1} Hepes I^{-1} and 5 mmol l^{-1} L-lactate I^{-1} in a $95\%\text{ O}_2$ and $5\%\text{ CO}_2$ atmosphere. None of ATP, ADP or AMP concentrations in round spermatids changed significantly after 0–10 min incubation at 4, 33 or 40°C (Table 1).

Temperature-induced changes in intracellular pH in round spermatids

SERCA ATPase-mediated Ca^{2+} transport is affected by pH in different tissues, indicating that the limitation of SERCA

Fig. 1. Changes of cytosolic $[Ca^{2+}]_i$ (●) induced by changes in temperature (○) in (a) rat pachytene spermatocytes in suspension and (b) rat round spermatids in suspension. The cells were loaded with fura-2 and suspended in Krebs–Henseleit buffer supplemented with 10 mmol l^{-1} Hepes I^{-1} and 5 mmol l^{-1} L-lactate I^{-1} and stirred constantly. The temperature was monitored on the wall of the fluorometer cuvette.

ATPase-mediated uptake into intracellular Ca^{2+} stores in spermatogenic cells at high temperatures might be mediated by changes in pH_i in these cells. As greater changes in cytosolic $[Ca^{2+}]$ with high temperatures were observed in round spermatids in the present study, we decided to focus on these cells to explore the involvement of pH_i changes in cytosolic $[Ca^{2+}]$ in these conditions. An increase in temperature from 33 to 40°C also induced a decrease in pH_i in round spermatids (Fig. 4). On average, this decrease in pH_i was 0.04 ± 0.01 pH units from 33 to 40°C . pH_i was titrated with NH_4Cl after application of the temperature increase to determine whether the changes in cytosolic $[Ca^{2+}]$ induced by high temperatures were related to the changes in pH_i . Addition of 5 mmol l^{-1} NH_4Cl I^{-1} increased pH_i above basal values after induction of the temperature increase (Fig. 5a). Cytosolic $[Ca^{2+}]$ was

Fig. 2. (a) Initial rate of basal cytosolic $[Ca^{2+}]_i$ changes (● and inset) and intracellular Ca^{2+} store uptake (Δ) and leakage (○) in rat pachytene spermatocytes. Intracellular Ca^{2+} store leakage fluxes were estimated from the rate of intracellular Ca^{2+} store release of Ca^{2+} after addition of a saturating concentration of thapsigargin (500 nmol l^{-1}). Uptake fluxes were calculated as the difference between the leak fluxes and the basal changes in $[Ca^{2+}]_i$. The lines for leakage and uptake fluxes represent the exponential non-linear fit of the data. Y axis in inset has same units as in main figure. (b) Arrhenius plot of the natural logarithm of leakage (○) and pump (Δ) fluxes for pachytene spermatocytes (from Fig. 2a) against the inverse of the temperature. The lines represent the linear fit of the data.

monitored after addition of $5 \text{ mmol NH}_4\text{Cl l}^{-1}$ 1 min after the temperature increase was applied (Fig. 5b). Under these conditions, cytosolic $[Ca^{2+}]_i$ still increased to values of $120 \pm 30 \text{ nmol l}^{-1}$ ($n = 3$). However, the rate of cytosolic $[Ca^{2+}]_i$ increase was decreased by an average 68% (range 40–85%, $n = 4$) in the presence of NH_4Cl , indicating that the intracellular pH decrease induced by temperature was affecting the rate of SERCA ATPase-mediated Ca^{2+} removal from the cytoplasm. Isobutyrate (a non-metabolizable

Fig. 3. (a) Initial rate of basal cytosolic $[Ca^{2+}]_i$ changes (● and inset) and intracellular Ca^{2+} stores uptake (Δ) and leakage (○) in round spermatids. Leakage fluxes were estimated from the rate of intracellular Ca^{2+} store release of Ca^{2+} after addition of a saturating concentration of thapsigargin (500 nmol l^{-1}). Uptake fluxes were calculated as the difference between the leak fluxes and the basal changes in $[Ca^{2+}]_i$. The lines for leakage and uptake fluxes represent the exponential non-linear fit of the data. Y axis in inset has same units as in main figure. (b) Arrhenius plot of the natural logarithm of leakage (○) and pump (Δ) fluxes for round spermatids (from Fig. 3a) against the inverse of the temperature. The lines represent the linear fit of the data.

permeant weak acid) was added at 10 mmol l^{-1} to cells in KH media with $10 \text{ mmol L-lactate l}^{-1}$ at 33°C to test further the hypothesis that a temperature-induced decrease in pH_i could induce the release of Ca^{2+} from intracellular Ca^{2+} stores. Isobutyrate induced a decrease of pH_i of approximately 0.08 pH units (Fig. 5c). In agreement with the notion that a decrease in pH_i could determine release of Ca^{2+} from intracellular Ca^{2+} stores, cytosolic $[Ca^{2+}]_i$ increased steadily after addition of isobutyrate.

Table 1. Adenine nucleotide concentrations in rat round spermatids at three different temperatures

Temperature (°C)	Adenine nucleotide	Time (min)		
		0	5	10
4	ATP	*	122 ± 9	113 ± 4
	ADP	*	35 ± 6	29 ± 2
	AMP	*	13 ± 1	10 ± 1
33	ATP	134 ± 26	—	155 ± 21
	ADP	31 ± 1	—	43 ± 17
	AMP	12 ± 1	—	13 ± 2
40	ATP	*	141 ± 10	148 ± 30
	ADP	*	32 ± 3	16 ± 9
	AMP	*	16 ± 3	15 ± 2

Round spermatids were incubated in a shaker waterbath in Krebs–Henseleit buffer supplemented with 10 mmol Hepes I^{-1} , 5 mmol L-lactate I^{-1} and in 95% O_2 and 5% CO_2 at 33°C. At time 0 the round spermatids were transferred in the same media and similar conditions to the temperatures shown. ATP, ADP and AMP concentrations are expressed in nmol (mg cell protein) $^{-1}$. Results are mean ± SD ($n = 3$).

*See data at 33°C.

Discussion

The results of the present study indicate that extreme temperatures impair the ability of pachytene spermatocytes and round spermatids to maintain $[Ca^{2+}]_i$ homeostasis. These cells began to gain cytoplasmic Ca^{2+} at temperatures < 15°C or > 30°C. This behaviour of cytoplasmic Ca^{2+} was reversible in the time frame shown in this study. The kinetics of pachytene spermatocyte $[Ca^{2+}]_i$ changes induced by high temperatures was slower than that of round spermatids (see below). Thus, at a temperature of about 38°C, cytoplasmic Ca^{2+} in pachytene spermatocytes was kept at 30–40 nmol I^{-1} , whereas cytoplasmic Ca^{2+} of round spermatids increased to > 80 nmol I^{-1} in similar conditions.

These data also show that $[Ca^{2+}]_i$ homeostasis was well maintained between 15°C and 30°C by a balance between Ca^{2+} leakage and uptake by intracellular Ca^{2+} stores in both pachytene spermatocytes and round spermatids. At temperatures lower or higher than this range, leakage from intracellular stores surpassed uptake, leading to a net gain in cytosolic Ca^{2+} in these cells. The use of vanadate at 50 μ mol I^{-1} and in the presence of external Ca^{2+} revealed the participation of a plasma membrane Ca^{2+} -ATPase (Berrios *et al.*, 1998). Under the conditions presented in this study (3 nmol external Ca^{2+} I^{-1}), 50 μ mol vanadate I^{-1} only marginally (not significantly) affected the basal or thapsigargin-induced rate of cytosolic $[Ca^{2+}]$ changes at the temperatures tested. This result limits the control of cytosolic $[Ca^{2+}]$ to Ca^{2+} movements in intracellular Ca^{2+} stores. Herrera *et al.* (2000) showed that under basal conditions (< 50 nmol $[Ca^{2+}]_i$ I^{-1}) it is unlikely that mitochondrial Ca^{2+} uptake or release contributed significantly to $[Ca^{2+}]_i$ homeostasis in these cells, allowing the interpretation of the initial velocities of cytosolic Ca^{2+} changes as leakage and uptake from non-mitochondrial intracellular Ca^{2+} stores. The calculated apparent activation energies (E'_a) for SERCA ATPase-mediated and intracellular Ca^{2+} stores leakage fluxes in pachytene spermatocytes and

Fig. 4. Changes in intracellular pH (●) of rat round spermatids induced by a gradual change in temperature (○) from 33 to 40°C. Cells were loaded with 2',7'-bis-(2-carboxyethyl-5-(and -6)-carboxyfluorescein (BCECF) and suspended in Krebs–Henseleit buffer supplemented with 10 mmol Hepes I^{-1} and 5 mmol L-lactate I^{-1} and stirred constantly. The temperature was monitored on the wall of the fluorometer cuvette.

round spermatids were not different from each other. These E'_a values are in the range of E'_a values reported for SERCA ATPase-mediated Ca^{2+} transport (96 kJ mol $^{-1}$; Black *et al.*, 1980) and other transport ATPases, such as Na $^{+}$ -K $^{+}$ -ATPase (80 kJ mol $^{-1}$; Friedrich and Nagel, 1997) or Fo-ATPase (55 kJ mol $^{-1}$; Richard and Graber, 1992). E'_a for Ca^{2+} leakage fluxes is relatively high, but in the range of E'_a for some ion channels, such as Ca^{2+} -activated Cl $^{-}$ channel (66 kJ mol $^{-1}$; Helliwell and Large, 1995) or a voltage-dependent Ca^{2+} channel (74 kJ mol $^{-1}$; Cota *et al.*, 1983).

At 5°C, a small imbalance between leakage fluxes and uptake could explain the basal rate in cytosolic Ca^{2+} increase of 2.6 ± 0.4 and 4.0 ± 1.3 nmol per l per min that

Fig. 5. (a) Intracellular pH_i (●) of rat round spermatids at 33°C in Krebs–Henseleit buffer with 5 mmol L-lactate l^{-1} . 5 mmol NH_4Cl l^{-1} , pH 7.4, was added to the cuvette at the time indicated. (b) Fura-2 fluorescence ratio from fura-2-loaded rat round spermatids in Krebs–Henseleit buffer with 5 mmol L-lactate l^{-1} in the absence (●) and presence (○) of 5 mmol NH_4Cl l^{-1} , pH 7.4. As the fura-2- Ca^{2+} dissociation constant is temperature-dependent, the data are expressed as the fluorescence ratio divided by the dissociation

was observed in both pachytene spermatocytes and round spermatids, respectively (not significantly different). However, at 40°C, pachytene spermatocytes gained cytosolic Ca^{2+} at a rate (1.3 ± 0.7 nmol per l per min) that was significantly lower than that of round spermatids (6.3 ± 1.0 nmol per l per min) ($P < 0.002$). Both the leakage fluxes and pump fluxes at 40°C were larger in round spermatids compared with pachytene spermatocytes ($P < 0.05$). It is unclear why SERCA ATPase is unable to cope with the increased leakage from intracellular Ca^{2+} stores at high temperatures, especially in round spermatids. Herrera *et al.* (2000) showed that SERCA ATPases in round spermatids behaved as though they were regulated by intracellular adenine nucleotide concentration. However, the data for ATP, ADP and AMP concentrations in round spermatids at 40°C obtained in the present study do not indicate that a decrease in intracellular ATP or an increase in ADP or AMP concentrations is limiting the rate of SERCA-mediated uptake in intracellular Ca^{2+} stores at 40°C. High temperatures also induced a decrease in pH_i in round spermatids. This finding indicates that pH-related changes in Ca^{2+} homeostasis may be taking place at high temperatures. A decrease in pH_i could produce a decrease in affinity constants of intracellular Ca^{2+} buffers. These changes in affinity constants are expected to occur rapidly. However, a rapid decrease in pH_i induced by isobutyrate did not produce a stepped increase in cytosolic $[Ca^{2+}]_i$, thus indicating that it is unlikely that changes in intracellular Ca^{2+} -buffering capacity are a major mechanism involved in the steady increase in cytosolic $[Ca^{2+}]_i$ induced by high temperatures. Low pH lowers SERCA ATPase-mediated transport in different tissues, and SERCA3 ATPase is especially sensitive to a decrease in pH in the range of physiological pH (Lytton *et al.*, 1992; Wolosker *et al.*, 1997). Consistent with this notion, restoration of the basal pH at high temperatures in round spermatids affected the kinetics of cytosolic $[Ca^{2+}]_i$ increase, but did not significantly prevent the extent of the increase in intracellular $[Ca^{2+}]_i$. Furthermore, and in agreement with the notion of a pH-dependent effect of temperature on intracellular Ca^{2+} store homeostasis, decreasing cytosolic pH by addition of a non-metabolizable weak acid induced a release of intracellular Ca^{2+} from intracellular stores. Whatever the mechanism involved, round spermatids appear to express a high temperature limitation of intracellular Ca^{2+} store uptake to a higher degree than do pachytene spermatocytes, indicating developmental changes in intracellular Ca^{2+} homeostasis regulation during rat spermatogenesis. The results of Herrera *et al.* (2000) also predict that rat spermatogenic cell mitochondria take up Ca^{2+} in relation to the cytosolic $[Ca^{2+}]_i$ increase induced by high temperature.

constant to avoid biasing the estimate of the $[Ca^{2+}]_i$ in the transition temperatures. (c) Intracellular pH_i (○) and $[Ca^{2+}]_i$ (●) of rat round spermatids at 33°C in Krebs–Henseleit buffer with 5 mmol L-lactate l^{-1} . 10 mmol isobutyrate l^{-1} , pH 7.4, was added to the cuvette at the times indicated. The pH_i and $[Ca^{2+}]_i$ measurements were obtained in separate determinations.

Several lines of evidence indicate that spermatogenic cell death induced by testicular exposure to high temperature or experimental cryptorchidism is a complex event that depends strongly on paracrine interactions in the testicles (Kerr and Sharpe, 1989; Lue *et al.*, 1999). Our findings indicate that changes in intracellular Ca^{2+} homeostasis can be the initiating event in high temperature induction of programmed cell death in meiotic and postmeiotic spermatogenic cells. In fact, an increase in cytosolic $[Ca^{2+}]$ has been implicated in chemically induced apoptosis by activation of Ca^{2+} entry (Li *et al.*, 1997) or by inhibition of SERCA ATPase (Hughes *et al.*, 2000) in spermatogenic cells. Two likely targets for Ca^{2+} action can be envisaged: firstly, a Ca^{2+} -dependent endonuclease, which appears to be involved in apoptotic events in germ cells (Wine *et al.*, 1997); and secondly, spermatogenic cell mitochondria, where intramitochondrial Ca^{2+} could trigger the aperture of a permeability transition pore leading to activation of a caspase cascade (for a review see Crompton, 1999). As a working hypothesis, we would like to propose that these mechanisms could be activated by the temperature-induced cytosolic $[Ca^{2+}]$ changes shown in the present study. The differentiation-related change in intracellular Ca^{2+} homeostasis and its associated differential sensitivity to high temperature also provide a mechanism by which different spermatogenic cells could have differential sensitivities to high temperature-induced cytotoxicity.

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