Ion dependence of resting membrane potential of rat spermatids

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The membrane potential of rat spermatids was estimated as -22 ± 2 mV (mean $\pm$ SEM) using three independent methods: using oxonol as a fluorescent membrane potential sensitive probe, from the passive distribution of hydrogen ions and from whole-cell patch-clamp records. The estimated permeability ratios $P_{K^+}:P_{Cl^-}$ and $P_{Na^+}:P_{Cl^-}$ of the plasma membrane of rat spermatids were 1.0 and 0.3, respectively. These data indicate that the high luminal K^+ concentration found in seminiferous tubules could partially close voltage-sensitive calcium channels in these cells.

Introduction

In spite of the interest in spermatogenesis in animal and human reproduction, very little is known about the general physiological aspects of spermatogenic cells. Their energy metabolism has been well characterized (for example, Mita and Hall, 1982; Nakamura *et al.*, 1982; Grootegoed *et al.*, 1986; 1982; Reyes *et al.*, 1990) and some transport studies of uncharged molecules have been performed (Nakamura *et al.*, 1986). However, intracellular ion homeostasis, membrane potential, and ion transport in these cells have been only partially explored (Kierszenbaum *et al.*, 1971; Hagiwara and Kawa 1984; Reyes *et al.*, 1993).

In mammals, the differentiation stages of spermatogenesis occur in a seminiferous tubule compartment (adluminal), the composition of which is considered to be controlled by the activity of Sertoli cells and the meiotic and postmeiotic spermatogenic cells (Dym and Fawcett, 1970). There are peptides, steroids, and other factors in this adluminal compartment that could determine the permeability properties of the membranes. In this compartment, the ionic gradients responsible for the membrane potential of spermatogenic cells appear to be locally regulated and could play a role in growth and development of these cells. Potassium, an ion to which the membrane potential of many cells is very responsive, is present at a concentration of 50 mmol l^{-1} in the seminiferous tubule, that is, 10 times higher than its concentration in plasma (Setchell and Brooks, 1988).

The membrane potential of a cell is an important parameter that determines the rate of entry and the steady-state distribution of many pharmacologically active charged substances (see Scheler and Blank, 1977). It is also a trigger signal for many cellular processes controlled by voltage-sensitive sodium, potassium or calcium channels. Activation or modulation of some of these channels is a general response to the interaction of many extracellular signals with membrane receptors (Benos and Sorscher, 1992). Thus, the resting membrane potential

could determine the extent of a response by voltage sensitive channels in a cell to external stimuli.

Using zero-current whole-cell patch-clamp measurements, Hagiwara and Kawa (1984) reported that the membrane potentials of rat spermatogenic cells at different stages of development were -10 to -50 mV. However, there have been no accurate determinations of the membrane potential of mammalian spermatids. This value, and its ion dependence, is important because these cells have voltage-sensitive channels, including Ca^{2+} channels (Hagiwara and Kawa, 1984), the opening and closing of which could determine the rate of entry of Ca^{2+} , a key intracellular regulator in most cells.

We estimated the resting membrane potential of rat spermatids as -22 ± 2 mV using three independent methods: (i) using oxonol as a fluorescent membrane potential-sensitive probe; (ii) from the passive distribution of protons and (iii) from whole-cell patch-clamp recordings. The membrane potential of rat spermatids is determined predominantly by Cl^- and K^+ conductances with a minor contribution by Na^+ conductance.

Materials and Methods

Isolation of rat spermatids

Male Wistar rats (300 g) obtained either from the University of Valparaíso breeding facilities or from Charles River Laboratories (Wilmington, MA) were maintained in a 12 h light:12 h dark cycle. The rats were killed by cervical dislocation and the testicles excised, decapsulated, and treated with collagenase. The resulting seminiferous tubules were subjected to mechanical disruption. The spermatids were separated in a continuous nonlinear Percoll gradient, as described by Grootegoed *et al.* (1986) and Reyes *et al.* (1990), or by unit gravity sedimentation in a BSA gradient as described by Bellve *et al.* (1977).

Chemicals

Collagenase, DNase, Percoll, BSA, cAMP, cGMP, gramicidin, nigericin, valinomycin, carbonyl cyanide *m*-Cl-phenyl

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hydrazone (CCCP), antimycin, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) and the salts and buffers used were obtained from Sigma Chemical Co. (St Louis, MO). Bis-(1,3 diethyl) thiobarbiturate trimethine oxonol and 2',7'-bis-(2-carboxyethyl)-5-(and 6) carboxyfluorescein acetoxymethyl ester were obtained from Molecular Probes, Inc. (Eugene, OR).

Determination of the membrane potential of spermatids from fluorescence measurements

In principle, the estimation of the intra- and extracellular concentrations of any trace charged molecule that distributes at equilibrium across the membrane can be used to monitor the membrane potential of cells. The distribution of such a substance is related to the membrane potential by the Nernst diffusion potential equation:

$$V_m(i - o) = (RT/zF) \ln \{ [ion]_o / [ion]_i \} \quad (1)$$

where V_m is the membrane potential; R is the gas constant; T is the temperature in °K; z is the ion charge and F is Faraday's constant.

If this substance can be determined at very low concentrations, its contribution to the membrane potential will be minimal. This is the principle that has been used to determine the membrane potential of cells and organelles using permeable lipophilic ions, ion-selective microelectrodes, radioactively labelled ions, or fluorescent molecules (Hoffman and Laris, 1974; Rink *et al.*, 1980).

Because protons can be determined at concentrations of 100 nmol l^{-1} or less, and in a range of concentrations compatible with most cellular functions, they can also be used to estimate the value of the membrane potential. The requirement of this measurement is that the ions have to be as close as possible to equilibrium, a proton ionophore has to be provided, and contributions to transmembrane H^+ gradients from coupled acid-base transport mechanisms and internal sources of hydrogen ions should be minimal.

Membrane potential measurements using bis-(1,3 diethyl)thiobarbiturate trimethine oxonol as a negatively charged fluorescent probe. Oxonol was determined with excitation at 535 nm and emission at 560 nm using a 5 nm slitwidth in a 44 BMPF Perkin Elmer or Delta Scan PTI spectrofluorometer. The fluorescence of 1.5 nmol oxonol l^{-1} was initially measured, and 0.01 ml of a concentrated cell suspension was added to the 3 ml cuvette thermostatically regulated at 34°C (final cell concentration $0.1\text{--}0.3 \times 10^6$ cells ml^{-1}). After a steady value was achieved, gramicidin ($0.4 \mu\text{mol } l^{-1}$) was added to the cell suspension and a new steady level of fluorescence established. Measurements were performed at different K^+ , Na^+ or Cl^- concentrations, achieved by isosmotically changing the Na^+ or K^+ concentration in the external medium by replacement of Na^+ with N -methyl-D-glucamine and K^+ with 5 mmol $Na^+ l^{-1}$ and changes in Cl^- concentrations were obtained by substitution with sodium gluconate. In some cases, the measurements were performed by successive additions of aliquots of a concentrated solution of the probe in dimethylsulfoxide to obtain a fluorescence versus concentration curve. Unless stated otherwise, all the fluorescence and patch-clamp measurements

were performed at an external pH of 7.4, buffered with 20 mmol l^{-1} .

Intracellular pH measurements. The intracellular pH of the spermatids was estimated using the fluorescent probe 2',7'-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein (BCECF). The cells were loaded with the membrane permeable acetoxymethyl BCECF ($2 \mu\text{mol } l^{-1}$) for 20 min at 34°C. Fluorescence was measured by emission at 535 nm and alternate excitations at 445 and 505 nm. The probe was calibrated intracellularly in Krebs–Henseleit solution with 120 mmol $K^+ l^{-1}$ and 10 μg nigericin ml^{-1} at different pH values, and the fluorescence ratio was measured at excitations of 505:445 nm. The intracellular calibration of the probe produced, on average, only a 0.05 pH unit shift toward alkaline pH values compared with the probe in solution when the cells were permeabilized with digitonin (Owen, 1992). Values of fluorescence ratios at steady state were converted to pH, using a standard regression line for BCECF calibration with nigericin.

Extracellular pH was determined in the cell suspension before and at the end of each experiment using a combined glass electrode and a Cole–Parmer pH meter.

Whole cell patch-clamp measurements of spermatid resting membrane potentials

The zero current membrane potential was determined in current clamp mode when passing from cell-attached to the whole-cell configuration of the patch-clamp technique (Warner Instruments Corp., Model PC-50, Hamden, CT). Patch pipettes were made of borosilicate glass and filled with 140 mmol KCl l^{-1} , 5 mmol $MgCl_2 l^{-1}$, 5 mmol EGTA l^{-1} and 5 mmol Hepes l^{-1} , pH 7.4. Typical values of pipette resistance were 2.5–5 Mohms. Seal resistances ranged between 5 and 10 Gohms. The cells, attached to a polylysine-covered coverslip, were viewed under phase contrast microscopy. Cells with a diameter of 10–12 μm were chosen which showed the typical nucleus contour of rat spermatids.

Typical cell input resistances and cell capacitances were 2–5 Gohms and 3–4 pF, respectively.

Ion content of rat spermatids and solutions

The ion content of the rat spermatids was determined by preincubation of the cells in Krebs–Henseleit buffer–lactate medium at 34°C under an atmosphere of 95% O_2 :5% CO_2 for 15 min. Five microcuries $^3H_2O ml^{-1}$ and 10 μCi of [^{14}C]polyethylene glycol ml^{-1} (M_r 4000) were added to the cells, and 30 s later the cells were centrifuged through dibutylphthalate at 14 000 g for 1 min. The microfuge tube above the dibutylphthalate was washed with deionized water. The pellets were dissolved in 500 μl of 0.1% (v/v) cation-free Triton X-100 and 10 mmol LiCl l^{-1} , and aliquots were taken for liquid scintillation counting of 3H and ^{14}C , and for Na^+ , K^+ and Cl^- determination. Na^+ and K^+ were determined by atomic absorption spectrophotometry and chloride was determined using a coulombimeter. The ion concentration in the cells was

Fig. 1. Fluorescence intensity of Krebs–Henseleit solutions with different K^+ concentrations in the presence ($\blacktriangle$: 0.1, $\triangle$: 4.6, $\circ$: 20, and $\square$: 120 mmol K^+ l^{-1}) and absence ($\circ$: 0.1, $\bullet$: 20, and Δ : 120 mmol K^+ l^{-1}) of valinomycin ($0.8 \mu\text{mol } l^{-1}$) or with (∇) digitonin-treated rat spermatids ($20 \mu\text{g digitonin } ml^{-1}$, $0.4 \times 10^6 \text{ cells } ml^{-1}$) in suspension as a function of oxonol concentration.

calculated using the ion content in the pellets and the determination of intracellular water (see also Reyes *et al.*, 1993).

Statistical analyses

Unless stated otherwise, the values reported are expressed as means $\pm$ SD. The data were tested using Bartlett's test for equality of variances, and subsequently tested in a two-tailed Student's *t* test or Welch's modified *t*-like test or one-way ANOVA (Li, 1964). Nonlinear regression analysis of the membrane potential estimates versus ion concentration, using the Goldman–Hodgkin–Katz equation (Goldman, 1943; Hodgkin and Katz, 1949), were performed using the Enzfitter software package (Elsevier Biosoft, Cambridge, UK). The data were considered significant at $P < 0.05$.

Results

Measurements with oxonol

Adding cells to an oxonol solution in Krebs–Henseleit buffer increased the fluorescence, as expected from previous studies in which fluorescence was enhanced by binding of the dye to cell components (for example, Rink *et al.*, 1980).

Oxonol appears to form ion pairs in the presence of valinomycin and K^+ , as has been described for the interaction of valinomycin and 8-anilino-1-naphthalenesulfonate (Feinstein and Felsenfeld, 1971). The formation of an ion pair is accompanied by an increase in fluorescence. The fluorescence yield increased when the K^+ concentration was increased at a given concentration of valinomycin ($0.8 \mu\text{mol } l^{-1}$, Fig. 1). This interaction between K^+ –valinomycin and oxonol results in an overestimate of the amount of dye bound to the cells owing to the fluorescence yield by extracellular oxonol– K^+ –valinomycin.

When the cells were added after interaction of oxonol with K^+ –valinomycin, a slow increase in fluorescence was observed (Fig. 2a), suggesting that the dye binds to the cells with higher affinity than to K^+ –valinomycin, and that oxonol was being displaced from the ion pairs formed. Conversely, if the cells were exposed to the dye first, the increase in fluorescence occurred faster (Fig. 2b), again suggesting that dye desorption from ion pairs causes the slow increase in fluorescence observed in the previous case. When valinomycin was added after the cells, a further increase in fluorescence was observed that gradually returned toward basal values (Fig. 2b).

The cation ionophore gramicidin does not interact with oxonol (Grinstein *et al.*, 1984) and hence can be used to calibrate the dye in the cells. The calibration of oxonol requires the estimation of the membrane potential as established by adding gramicidin to the cell suspension. The gramicidin-induced potential in rat spermatids was estimated using the ion content of the cells, and permeability ratios for $P_{K^+}:P_{Na^+}$ and

Fig. 2. Time course of the fluorescence intensity of $1.5 \text{ nmol oxonol } l^{-1}$ in Krebs–Henseleit medium and its interaction with valinomycin ($0.8 \mu\text{mol } l^{-1}$) and rat spermatids. (a) When spermatids were added after valinomycin, the increase in fluorescence occurred more slowly than (b) when the cells were added before valinomycin.

$P_{\text{Cl}^-}:P_{\text{K}^+}$ of 1.6 (Lauger, 1973) and 0.6, respectively (see below). The ion content of rat spermatids (in mmol l^{-1}) were 107 ± 11 ($n = 10$) for K^+ , 45 ± 7 ($n = 11$) for Na^+ , and 60 ± 10 ($n = 10$) for Cl^- . A representative curve for the calibration of oxonol fluorescence by varying the ion composition in the external medium in the presence of $0.4 \mu\text{mol}$ gramicidin l^{-1} is given (Fig. 3a). The reported fluorescence values in Fig. 3a are those obtained 30 s after gramicidin was added to the cells in a stirred cuvette. Calibration curves were performed on each batch of cells used in the membrane potential measurements, because of the difficulties in precisely reproducing the cell density in different preparations.

The resting membrane potential of rat spermatids in Krebs–Henseleit medium was estimated by interpolation in the calibration curve of the fluorescence values after equilibration of the cells with oxonol. The membrane potential of rat spermatids in these conditions was $-25 \pm 6 \text{ mV}$ ($n = 13$). The ion dependence of the spermatid membrane potential when extracellular Na^+ , K^+ or Cl^- were varied and the rest of the ion composition was constant is shown (Fig. 3b). Fitting of the data for variable Na^+ to the Goldman–Hodgkin–Katz equation gave values for $P_{\text{Na}^+}:P_{\text{Cl}^-}$ and $P_{\text{K}^+}:P_{\text{Cl}^-}$ of 0.3 ± 0.1 and 0.7 ± 0.2 , respectively. The value of $P_{\text{Na}^+}:P_{\text{Cl}^-}$ obtained above was used to estimate $P_{\text{K}^+}:P_{\text{Cl}^-}$ for various K^+ or Cl^- concentrations (three different ion concentrations each). The values obtained were 0.9 ± 0.3 and 1.0 ± 0.1 for variable K^+ and Cl^- , respectively.

The resting membrane potential of rat spermatids was not modified either by dibutyryl cyclic AMP or by GMP (0.2 mmol l^{-1} each), or by $200 \mu\text{mol}$ DIDS l^{-1} , an inhibitor of epithelial chloride channels (not shown).

Estimation of membrane potentials using the equilibrium distribution of protons in rat spermatids

The distribution of a univalent cation at equilibrium should follow Eqn (1), which, for protons becomes:

$$V_m = 60(\text{pH}_i - \text{pH}_o) \quad \text{at } 34^\circ\text{C}.$$

Thus, from measurements of intracellular pH, using fluorescence ratio measurements with BCECF and extracellular pH measurements performed with a pH electrode, it is possible to estimate membrane potentials from distribution of protons at equilibrium.

The proton permeability of the spermatid membranes was increased by the addition of $1 \mu\text{mol}$ carbonyl cyanide *m*-Cl-phenylhydrazone l^{-1} (CCCP) to increase the rate of proton equilibration. Higher CCCP concentrations ($10 \mu\text{mol l}^{-1}$) produced a depolarization (5 mV) estimated with oxonol measurements. Furthermore, a high-affinity blocker of the $\text{Na}^+ - \text{H}^+$ exchanger, ethyl isopropyl amiloride ($16 \mu\text{mol l}^{-1}$), was used to inhibit this transport system in the spermatids. In addition, to avoid some of the important sources of metabolic acid in these cells (Reyes *et al.*, 1990), solutions without glucose and bicarbonate and including antimycin ($0.3 \mu\text{g ml}^{-1}$) were used.

Addition of CCCP, a proton ionophore, lowered the intracellular pH and a new steady-state was reached after approximately 10 min (Fig. 4a). Subsequent addition of valinomycin

Fig. 3. (a) Calibration curve for oxonol fluorescence versus gramicidin-induced membrane potential of rat spermatids at 34°C . Oxonol and gramicidin concentrations were 1.5 nmol l^{-1} and $0.4 \mu\text{mol l}^{-1}$, respectively. Cell concentration was approximately 0.2×10^6 cells ml^{-1} . Measurements were taken 30 s after addition of gramicidin. (b) Membrane potential of rat spermatids estimated from oxonol measurements as a function of the ion composition of the external solution. Concentrations of Cl^- (∇), Na^+ ($\circ$) and K^+ ($\bullet$) were varied by isosmotic replacement with gluconate or *N*-methyl-D-glucamine, maintaining the rest of the ion composition and pH constant.

Fig. 4. (a) Fluorescence intensity of intracellular 2',7'-bis-(2-carboxyethyl)-5-(and 6) carboxy fluorescein (BCECF, 0.4×10^6 cells ml^{-1}) added to rat spermatids versus time at an extracellular K^+ concentration of 4.6 mmol l^{-1} , pH 7.35 and 34°C . Carbonyl cyanide *m*-chloro-phenyl hydrazone (CCCP), a proton ionophore, valinomycin ($0.8 \mu\text{mol l}^{-1}$), and digitonin ($20 \mu\text{g ml}^{-1}$) were added sequentially. (b) Fluorescence intensity of intracellular BCECF versus time at an extracellular K^+ concentration of 140 mmol l^{-1} .

made the intracellular pH even lower, indicating that the protons distribute across the membrane according to the expected hyperpolarized potential established by valinomycin. This fact was confirmed in experiments with $140 \text{ mmol K}^+ \text{l}^{-1}$ in the external medium (Fig. 4b), where the addition of valinomycin shifted the intracellular pH to higher values.

Adding digitonin ($20 \mu\text{g ml}^{-1}$) drove the fluorescence ratio towards values of extracellular pH, as expected from the leak of the probe from the cells or equilibration of protons at zero membrane potential. Control measurements of fluorescence performed in the supernatant after centrifugation of the cells, and at all K^+ concentrations, showed that extracellular BCECF

Fig. 5. Calculated membrane potentials (V_m) of rat spermatids and the changes induced by extracellular potassium (●) alone or (○) with valinomycin added, or (▲) extracellular chloride. The continuous lines represent the fitting of the Goldman-Hodgkin-Katz equation for $P_{Na^+}:P_{Cl^-} = 0.3$ and $P_{K^+}:P_{Cl^-}$ of 1.0 (K^+), 5.5 (K^+ plus valinomycin) and 0.8 (Cl^-). The dotted line (....) represents the Nernst equation with a slope of 60 mV per decade.

fluorescence contributed less than 10% to the total fluorescence (not shown).

The membrane potential of rat spermatids calculated from proton distribution experiments was -21 ± 7 ($n = 4$) at very low extracellular Na^+ (< 0.5 mmol l^{-1} , *n*-methyl-D-glucamine replacement).

When Cl^- was replaced by an impermeant anion (gluconate), a marked effect on the membrane potential of the spermatids was observed (Fig. 5). These results suggest that the membrane potential of rat spermatids is given by a Cl^- conductance with some degree of contribution from a K^+ permeability. The continuous line was calculated for a variable Cl^- concentration using the Goldman-Hodgkin-Katz equation, the basal ion content of rat spermatids, and fitted $P_{K^+}:P_{Cl^-}$ and $P_{Na^+}:P_{Cl^-}$ of 0.8 ± 0.1 and 0.3 ± 0.1 , respectively.

The membrane potential of spermatid calculated from proton distribution, as a function of the extracellular K^+ concentration (Na^+ replacement) in the presence and absence of valinomycin, is also shown (Fig. 5). At K^+ concentrations higher than 60 mmol l^{-1} , the H^+ distribution reported V_m changes in the expected range for a K^+ equilibrium potential. At low K^+ concentrations it is likely that the Cl^- conductance (see below) became a major factor in determining the membrane potential of rat spermatids. The continuous line represents the Goldman-Hodgkin-Katz equation using the basal ion content of rat spermatids, a $P_{Na^+}:P_{Cl^-} = 0.3$ and a $P_{K^+}:P_{Cl^-}$ of 5.5. The resting membrane potential of rat spermatids in Krebs-Henseleit solution estimated from proton distribution measurements was -20 ± 4 mV ($n = 6$).

In the absence of valinomycin, the reported membrane potential was less sensitive to changes in the extracellular K^+ concentration (Fig. 5), except at 140 mmol l^{-1} K^+ , where the estimated V_m was significantly smaller ($P < 0.05$, two tailed Student's *t* test) than at any other K^+ concentration tested. The continuous line in the absence of valinomycin

was calculated for a variable K^+ concentration using the Goldman-Hodgkin-Katz equation, the basal ion content of rat spermatids, a $P_{Na^+}:P_{Cl^-} = 0.3$ and a $P_{K^+}:P_{Cl^-}$ ratio of 1.0.

Estimations from proton distribution of the membrane potential of rat spermatids in the presence of 0.4 μ mol gramicidin l^{-1} in solutions of different ion compositions, and assuming a $P_{K^+}:P_{Na^+}$ of 1.6, allows an estimation of a $P_{Cl^-}:P_{Na^+}$ ratio of 0.6 ± 0.2 ($n = 4$) under these conditions. This value was used throughout our theoretical estimations of gramicidin-induced membrane potentials (see oxonol measurements).

Estimations of resting membrane potentials of rat spermatids using whole-cell patch-clamp measurements

The membrane potential of rat spermatids was measured after passing from cell-attached to whole-cell configuration in zero current clamp mode in five cells from four different cell preparations. The resting membrane potential was -27 ± 18 mV (mean $\pm$ SEM).

Discussion

The use of lipophilic ions for membrane potential determinations presents some drawbacks, for example binding of the lipophilic ions to membranes or intracellular components, and metabolic effects of the lipophilic ions (see Johnstone *et al.*, 1982 and Smith, 1982 for discussion). Similarly, estimation of the true membrane potential using H^+ distribution is subject to uncertainties. The cells are in the presence of a proton ionophore (and antimycin) which inhibits mitochondrial ATP production and ATP production could be a necessary cell regulator for some ion conductive pathways in the cell membrane. In addition, a decrease of intracellular pH when protons become distributed according to the membrane potential could close or open pH sensitive ion pathways; and there is always some uncertainty about whether the new pH_i reached is a new steady-state or true equilibrium. Steps were taken to minimize sources of metabolic protons by removing glucose as a likely source of acid production, using antimycin to stop acid production in Krebs' cycle, by removing bicarbonate as a buffer in the cells, and blocking the Na^+-H^+ exchanger with ethyl isopropyl amiloride. Hence, the membrane potential values reported using the above mentioned methods should represent good estimates. However, the use of patch-clamp techniques permits a direct measurement of the resting membrane potential of cells. Our data using patch-clamp recordings are in good agreement with the values obtained using proton distribution measurements or oxonol distribution data. Statistical analysis of the data between groups shows that they are not significantly different from each other, thus validating the use of non-invasive dye and H^+ distribution ratios as adequate ways of estimating resting V_m in rat spermatids. However, as pointed out by Hagiwara and Kawa (1984), the determination of the membrane potential in small cells has a systematic error because of the comparable values between the cell membrane resistance and the seal resistance of the patch pipette. Thus, our measurements using zero-current patch-clamp recordings of the membrane potential would be about 5–50% less negative than

the true membrane potential of the cells. The permeability ratios $P_{K^+}:P_{Cl^-}$ and $P_{Na^+}:P_{Cl^-}$ obtained using oxonol measurements (1.0 ± 0.1 and 0.3 ± 0.1 , respectively) were not significantly different ($P < 0.05$) from the ratios obtained using intracellular pH measurements (0.8 ± 0.1 and 0.3 ± 0.1 , respectively). We also attempted to use the cationic fluorescent probe 3,3'-dipropylthio-dicarbocyanine iodide (DiSC₃) at 200 nmol l⁻¹ (Hoffman and Laris, 1974) to estimate the membrane potential of rat spermatids. However, addition of valinomycin to the cell suspension equilibrated with the dye produced an increase in the dye fluorescence at all the K⁺ concentrations used (0.1–140 mmol l⁻¹) (not shown). This effect seems to reflect mitochondria-related depolarization induced by valinomycin. DiSC₃ has been shown to enter mitochondria and inhibit mitochondrial respiration (Montecucco *et al.*, 1979). For these reasons, DiSC₃ was rejected as a suitable probe for estimations of the membrane potential in rat spermatids.

If the potential in rat spermatids is set to -25 mV by the Cl⁻ and K⁺ conductances, it can be predicted that the equilibrium intracellular Cl⁻ concentration should be 57 mmol l⁻¹, a value close to that found from cell chloride content measurements by coulombimetry. Steady-state values of intracellular K⁺ of 110 mmol l⁻¹ coupled with a measurable K⁺ electrogenic permeability, and a membrane potential of -25 mV ($V_m - V_{K^+} = +58$ mV) implies the operation of active transport systems for potassium when the cells are bathed in solutions of low (4.6 mmol l⁻¹) extracellular K⁺. However, increasing extracellular K⁺ to 40–50 mmol l⁻¹ would place this ion in conditions close to equilibrium. Whether the high K⁺ content of the seminiferous tubule (Setchell and Brooks, 1988) could be an energy-saving strategy for the spermatozoa being released to the seminiferous tubule lumen remains to be determined.

It can also be predicted that the high luminal K⁺ concentration in the seminiferous tubule (50 mmol l⁻¹) would induce a decrease of the membrane potential of rat spermatids to -17 mV. This depolarization could trigger the closing of Ca²⁺ channels in the plasma membranes of rat spermatids (Hagiwara and Kawa, 1984). The closing of Ca²⁺ channels in the plasma membrane can induce a decrease in intracellular Ca²⁺, which, in turn, could be both a signal for differentiation processes in these cells or a signal that determines a metabolically dormant state, until external regulators (for example, epididymal peptides or egg-envelope molecules) could again activate Ca²⁺ entry.

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