Tracer-flux analysis of sodium and potassium permeability in differentiating mouse spermatozoa

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Summary. Heterogeneous populations of developing mouse spermatozoa were isolated from the seminiferous tubules and suspended in media of varied ionic composition. Tracer-flux analysis showed that P_{Na}/P_K in developing spermatozoa is 0.7 which is relatively high and that permeability of the cells to potassium and sodium is dependent on the ionic concentration of the medium.

Introduction

One of the current major questions in reproductive biology concerns the cellular events that lead to differentiation of the spermatozoon to the mature motile condition. During differentiation, striking variations occur not only in cell shape but also in the ionic composition of the developing spermatozoa and their surrounding media, specifically in the ratio of sodium to potassium (Cragle, Salisbury & Muntz, 1958).

The membrane potential (E_m) of cells isolated from mouse testis is dependent on both sodium and potassium concentration gradients (McGrady, 1979). Cuthbert & Wong (1975) postulated a relatively high permeability ratio of sodium to potassium (P_{Na}/P_K) based on their study of membrane potential in rat seminiferous tubules. Wong (1976) studied uptake of ^{22}Na into rat seminiferous tubules and found that entry of sodium occurs rapidly and by simple diffusion. Chueng, Hwang & Wong (1976, 1978) showed a significant contribution of sodium as well as potassium to membrane potential of rat epididymal epithelial cells. Zimmerman *et al.* (1979) studied the uptake of Na^+ and K^+ in boar epididymal spermatozoa and suggested that caput cells are more permeable to sodium than are those from the cauda or in the ejaculate. In ejaculated bull spermatozoa the E_m was dependent on the intracellular–extracellular potassium ratio (McGrady & Nelson, 1972, 1973). In ejaculated boar spermatozoa the uptake of ^{42}K was relatively rapid, whereas movement of ^{22}Na was slow (Crabo, Zimmerman, Moore & Thornborough, 1976). Permeability changes concurrent with development may exist in spermatozoa; specifically, the ratio P_{Na}/P_K appears to decrease as cells progress from the testis to the epididymis and finally to the ejaculate.

The present paper explores fluxes of potassium (^{42}K) and sodium (^{22}Na) in a heterogeneous population of cells isolated from mouse testes.

Materials and Methods

Preparation of cell samples

Male mice weighing approximately 30 g were killed by intraperitoneal injection of pentobarbitone sodium. The testes were excised and their capsules removed. The tubules were

teased into a control medium with the following composition (mmol/l): fructose, 40; KCl, 50; NaHCO_3 , 35; $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$, 1.8; MgSO_4 , 1; NaCl, 69.2. This is a synthetic medium similar in composition to free flow medium (defined as that obtained by micropuncture of seminiferous tubules (Tuck, Setchell, Waites & Young, 1970)). Free flow medium was chosen as the control solution because it is considered to be nearly the natural environment of the spermatogenic cells. In the ion substitution experiments, sodium or Tris (Tris-(hydroxymethyl)-aminomethane) was substituted for potassium (sodium and chloride held constant) and Tris was substituted for sodium (potassium and chloride held constant). No effect of Tris on E_m or cell viability was apparent. In another series of experiments, ouabain ($1 \mu\text{M}$ – 1 mM) was added to the control medium. Ouabain is an inhibitor of Na^+ – K^+ -activated Mg^{2+} -dependent ATPase, an enzyme which is active in cation transport in mature and developing spermatozoa (Voglmayr, White & Quinn, 1969; O'Donnell & Ellory, 1970). Osmolarity was maintained at approximately 320 mosmol/l, and pH 7.3 in all the solutions. All experiments were carried out at room temperature (24°C) as were previous measurements of E_m and of intracellular sodium and potassium.

The teased seminiferous tubules were minced and agitated for 20 min in control medium, with 0.3% trypsin (ICN Pharmaceuticals, Inc. Life Science Division, Cleveland, Ohio, U.S.A.) and 0.002% DNase (Worthington Biochemicals, Freehold, New Jersey, U.S.A.) according to Romrell, Bellvé & Fawcett (1976). The preparation was filtered through Nitex Cloth, 102 μm mesh (Tetko, Inc., Elmsford, New York, U.S.A.) into 12 ml conical centrifuge tubes. This procedure retains cell aggregates and pieces of seminiferous tubules while allowing isolated germinal cells to pass through (Steinberger, 1975). The cells were washed twice in free flow medium to remove trypsin and centrifuged at 1200 g for 10 min in a Sorval Type NSE centrifuge. Cells treated with trypsin did not differ in membrane potential from cells not subjected to trypsin. Dye exclusion (eosin–nigrosin) was used to assess viability of the cells as previously described (McGrady & Nelson, 1972). Viability of the cells was 70–80%.

Tracer-flux measurements

The supernatant was decanted and the pellet resuspended in 3 ml of control or experimental medium. For efflux experiments, cells were preloaded for 1 h in labelled control medium containing 10 μCi ^{42}K /ml or 5 μCi ^{22}Na /ml (New England Nuclear, Boston, Massachusetts, U.S.A.). Specific activity was maintained constant at changing K^+ and Na^+ levels. The cells were centrifuged at 1200 g for 10 min and washed once in non-radioactive medium (containing 109 mM- Na^+ and 50 mM- K^+). The cells were resuspended in the control medium or in one of the experimental media, and 25 or 50 μl aliquots of cells were taken with calibrated micropipettes at predetermined time intervals. Protein was precipitated by the addition of 0.5 ml 20% trichloroacetic acid. The samples were homogenized and centrifuged for 20 min at 2000 g , and the supernatant was decanted and added to a scintillation cocktail. The amounts of ^{42}K and ^{22}Na were measured with a Beckman Biogamma Counter, and background counts were subtracted. Corrections were made for 29% extracellular space as determined previously using $[^3\text{H}]$ inulin (McGrady, 1979). This value is close to that (21%) used by Wong (1976).

For the uptake experiments, the cells were incubated for various times in labelled control or experimental media containing 10 μCi ^{42}K /ml or 5 μCi ^{22}Na /ml. Aliquots of cells were removed, washed with non-radioactive control medium and treated as described above for efflux experiments.

Influx of potassium was also studied by cellular uptake of ^{42}K in the presence of various concentrations of ouabain. Influx of sodium was determined by uptake of ^{22}Na . Although the flux measurements were made on a heterogeneous population of spermatogenic cells, previous electrophysiological analysis showed no difference in E_m (which in turn depends on the concentration of potassium and sodium) between the spermatogenic cells.

Calculations

The assumptions underlying these calculations derive from Hodgkin & Katz (1949), Solomon (1960), Tupper (1972) and Tupper & Powers (1973).

The data from ^{42}K efflux was plotted as:

$$-\ln \frac{R_i(t)}{R_i(t_0)} \quad (1)$$

where $R_i(t)$ is the c.p.m. inside the cells at various times and $R_i(t_0)$ is c.p.m. inside the cell at time zero. From the slope of this line the rate constant, k_0 , can be found. The unidirectional efflux $J_0(\text{K})$ in $\text{mol}/\text{cm}^2/\text{sec}$ was determined from:

$$J_0 = k_0 [C_i V_i] \quad (2)$$

where k_0 is the rate constant, V_i is the cell volume and C_i is the total intracellular potassium concentration. Therefore $[C_i V_i]$ is the total K^+ content. Intracellular and extracellular Na^+ and K^+ concentrations were previously determined by flame photometry as described by McGrady (1979). Although the specific activity of the external medium was normally kept low, it is well known that when these cells are suspended in low potassium medium, they lose intracellular potassium. Therefore, there is some error inherent in this calculation since it assumes no backflux. To determine the cell volume per sample, cell dimensions were measured with an ocular micrometer. Also, the developmental stage of each cell was determined on the basis of cell size and shape, density of nucleus and presence and length of the flagellum (Leblond & Clermont, 1952; Romrell *et al.*, 1976). The approximate percentage of cell types was as follows: spermatogonia, 6%; spermatocytes, 22%; spermatids, 45%; testicular spermatozoa, 17%; Sertoli and Leydig cells, <4%; other cells, 6%. These values are comparable to those described by Meistrich, Bruce & Clermont (1973) for trypsin-prepared suspensions from mouse testes. To find the total volume, the volume per cell type was multiplied by the number of that cell type per sample. The total cell volume of a 50 μl sample was $2.73 \times 10^{-2} \text{ cm}^3$.

Results for ^{22}Na uptake can be plotted as:

$$-\ln \left[1 - \frac{R_i(t)}{R_i(\infty)} \right] \text{ versus time} \quad (3)$$

where $R_i(t)$ is the intracellular c.p.m. at various times and $R_i(\infty)$ is the intracellular c.p.m. at apparent isotope equilibrium. From the slope of this line the rate constant k_i is found. The influx ($\text{mol}/\text{cm}^2/\text{sec}$) is determined by:

$$J_i = k_i [C_i V_i] \quad (4)$$

The problem of backflux also arises here, in that, as intracellular ^{22}Na increases with time, some ^{22}Na will leak or will be pumped back out of the cells. This simple interpretation of the data is justified in order to give a first approximation of the permeability constants in these cells. Furthermore, the permeability coefficients P_K and P_{Na} (cm/sec) can be calculated from the formulae of Hodgkin & Katz (1949):

$$P_K = \frac{J_0(\text{K})}{(K)_i} \cdot \frac{1 - e^{-VF/RT}}{VF/RT} \quad (5)$$

$$P_{\text{Na}} = \frac{J_i(\text{Na})}{(\text{Na})_0} \cdot \frac{1 - e^{-VF/RT}}{VF/RT} \quad (6)$$

where F is the Faraday, 96500 C/mol; R is the gas constant, 8.3 J/degree mol; and T is the absolute temperature in degrees Kelvin, i.e. 297 K. Values for V , K_i , Na_i , K_0 and Na_0 were

determined by electrophysiological and flame photometric techniques as reported previously (McGrady, 1979). Since passive diffusion is driven by the electrochemical gradient and both the chemical and electrical components will vary with varying K_0 and Na_0 , the values previously determined were used in all the calculations as appropriate.

Results

Flux of ^{42}K

The uptake of ^{42}K in control and ouabain-modified media is shown in Text-fig. 1. Uptake of ^{42}K in cells suspended in free flow medium (which contains 109 mM- Na^+ and 50 mM-K) was biphasic, increasing rapidly during the first 15 min, then continuing to increase slowly until equilibrium was reached at 2 h. When ouabain was added to the control medium, K_i decreased due to an inhibition of active transport. At apparent equilibrium there was 33% less isotope in cells treated with 10^{-6} M-ouabain and 54% less at 10^{-3} M.

Text-fig. 1. Uptake of ^{42}K by mouse testicular spermatogenic cells in control and ouabain-modified media. Values are mean $\pm$ s.e.m. for the no. of replicates indicated in parentheses.

Movement of potassium was analysed by monitoring efflux of ^{42}K from preloaded cells. The cells were re-suspended in non-radioactive media with varied potassium concentrations. Efflux was accelerated by suspension of cells in low potassium medium (1 mM- K^+), while an increase in

Table 1. Calculation of the permeability constants for mouse testicular cells suspended in control medium containing 50 mM- K^+ , 109 mM- Na^+ and free of Ca^{2+}

Previous values (McGrady, 1979)		
$E_m = -4.6 \pm 0.3$ mV		
Intracellular sodium:	74 mM	Extracellular sodium: 109 mM
Intracellular potassium:	70 mM	Extracellular potassium: 50 mM
Present study		
$J_o(K)$	3.75×10^{-7} mol/cm ² /sec	
$J_i(Na)$	4.18×10^{-7} mol/cm ² /sec	
P_K	5.45×10^{-6} cm/sec	
P_{Na}	4.2×10^{-6} cm/sec	
P_{Na}/P_K	0.72	

extracellular potassium over that in control medium (50 mM-K⁺) correspondingly decreased ⁴²K efflux (Text-fig. 2). The rate constant of the efflux (k_0) was derived from the slope of Equation 1 versus time (Text-fig. 2, insert). P_K was calculated from Equations 2 and 5 (Table 1). P_K was inversely related to the concentration of potassium in the medium (Table 2).

Text-fig. 2. (a) Efflux of ⁴²K from preloaded mouse testicular spermatogenic cells into media of different ionic composition. Cells were preloaded for 1 h in ⁴²K control medium, washed once in non-radioactive medium and re-suspended in an experimental medium. In 1 mM-K⁺ medium, Tris was substituted for potassium; in 60 mM-K⁺ medium, potassium was substituted for sodium. Values are means $\pm$ s.e.m. for the no. of replicates in parentheses. (b) Plot of

$$-\ln \frac{R_i(t)}{R_i(t_0)}$$

versus time from which the rate constant is determined.

Table 2. The effect of ion concentration on permeability of cells isolated from mouse testes

Medium	P_{Na} (cm/sec)	P_K (cm/sec)
Control (Ca ²⁺ -free), 50 mM-K ⁺ , 109 mM-Na ⁺	4.2×10^{-6}	5.45×10^{-6}
1 mM-K ⁺ (Tris replaced), 109 mM-Na ⁺	—	3.61×10^{-5}
60 mM-K ⁺ , 99 mM-Na ⁺	—	5.89×10^{-6}
50 mM-K ⁺ , 50 mM-Na ⁺ (Tris replaced)	3.3×10^{-6}	—
Control + 5 mM-Ca ²⁺	2.2×10^{-6}	—

Flux of ²²Na

The presence of 5 mM-Ca²⁺ decreased uptake of ²²Na by approximately 30%. Decreasing extracellular sodium (50 mM-Na) produced a decline in ²²Na uptake of 20% at equilibrium. The rate constant (k_i) of the influx of sodium was determined from the slope of the plot of Equation 3 (Text-fig. 3, insert). P_{Na} was calculated from Equations 4 and 6, and was 4.2×10^{-6} cm/sec in control medium (Table 1). Decreased sodium and addition of calcium lowered ²²Na influx (Table 2). Efflux of ²²Na from preloaded cells was measured in control medium and in sodium-free medium (Text-fig. 4). Considering time zero as 100%, 10% of the intracellular labelled sodium

Text-fig. 3. (a) Uptake of ^{22}Na in media of different ionic composition. Values are means $\pm$ s.e.m. for the no. of replicates indicated in parentheses. (b) Plot of

$$-\ln \left[\frac{1 - R_i(t)}{R_i(\infty)} \right]$$

versus time from which the rate constant is determined.

Text-fig. 4. Efflux of ^{22}Na from preloaded cells into control (109 mM- Na^+) and sodium-free medium (Tris replaced sodium; potassium and chloride remained constant). Cells were preloaded for 1 h in ^{22}Na control medium, washed once in non-radioactive medium, and resuspended in the experimental medium. Values are mean $\pm$ s.e.m. for the no. of replicates indicated in parentheses.

had run out in 1 h from cells suspended in control medium and 55% had run out from cells suspended in sodium-free medium. Approximately 40% of the labelled sodium remained in the cells after 4 h of incubation in both suspending media.

Discussion

It is generally believed that transmembrane potentials in non-excitable cells are similar to those in nerve and muscle, i.e. diffusion potentials are maintained by active transport (Peterson, 1976). In non-excitable cells, however, the E_m is usually less negative, cell membrane resistance is less and relative permeability to sodium is higher than in excitable cells (Williams, 1970). Accordingly, in mature (bull), epididymal (rat) and developing (mouse) spermatozoa, membrane potential and cell membrane resistance were relatively low compared to values for excitable cells (McGrady & Nelson, 1972, 1973; Cuthbert & Wong, 1975; Cheung *et al.*, 1976, 1978; McGrady, 1979). In mature spermatozoa, potassium was the major contributor to E_m , with secondary influences due to sodium and chloride. The cell membrane was permeable to all three ions whose intracellular concentration could be manipulated by changing the external concentration (McGrady & Nelson, 1972, 1973).

In mouse spermatocytes and spermatids, E_m was dependent on both potassium and sodium with E_m linearly related to the extracellular concentration of both ions. Since chloride did not affect E_m , the Goldman equation in the form

$$E_m = \frac{RT}{F} \ln \left(\frac{(K)_o + P_{Na}/P_K (Na)_o}{(K)_i + P_{Na}/P_K (Na)_i} \right)$$

was used to determine the relative contribution of sodium and potassium to E_m . The best fit of E_m versus K_o was obtained with a P_{Na}/P_K value between 0.5 and 0.6. In addition, P_{Na}/P_K varied with extracellular ion concentration (McGrady, 1979).

The present paper investigated the permeability of the membrane of isolated cells from the mouse testis by direct calculation of P_{Na}/P_K via tracer flux analysis. P_{Na}/P_K in control medium (109 mM- Na^+ , 50 mM- K^+) was 0.7, suggesting a relatively high sodium permeability (Table 1). This value was close to the predicted P_{Na}/P_K of 0.5–0.6 from electrophysiological measurements alone and calculated with the Goldman equation. In low potassium medium (1 mM- K^+ , sodium constant), P_K increased to 3.61×10^{-5} cm/sec (Table 2). Efflux of potassium under these conditions occurred very rapidly, confirming the previous findings of hyperpolarization and a decline in intracellular potassium (McGrady, 1979). With increased K_o , P_K declined. Previous electrophysiological analysis of the effects of ouabain (Cuthbert & Wong, 1975; McGrady, 1979) showed a decrease of 20–35% in membrane potential which occurred within 15 min. The present results tend to substantiate the earlier analysis in that a ouabain-induced decrease in K_i concentration occurred at 10^{-6} and 10^{-3} M-ouabain.

Wong (1976) and Reisin & Cerejido (1969) studied ^{22}Na uptake by rat seminiferous tubules but did not calculate the permeability constant. The present work corroborates their findings that decreasing external sodium decreased ^{22}Na uptake; the uptake curve over the first 20 min is similar. The presence of calcium as well as the extracellular sodium concentration affected P_{Na} (Table 2).

Data from other cell systems indicate a relationship between membrane permeability and development. In echinoderm embryos (Tupper & Powers, 1973) and in embryonic heart cells (DeHaan, 1970), the permeability to sodium declines with maturation. Developing sperm cells, whether spermatocytes or spermatids, appear to be responsive electrophysiologically to both sodium and potassium, and membrane permeability is dependent on the ion concentration of the medium. Nevertheless, differentiating spermatozoa maintain homeostasis while challenged with the variations in the Na:K ratio that occur in the reproductive tract (Tuck *et al.*, 1970; Wyker & Howards, 1977). In mature ejaculated spermatozoa (McGrady & Nelson, 1972, 1973), sensitivity to sodium was less apparent, with potassium clearly being the dominant ion. Zimmerman *et al.* (1979) found that caput spermatozoa responded more strongly to sodium than did cauda or ejaculated boar spermatozoa.

Although permeability constants for epididymal or ejaculated spermatozoa have not been calculated for mouse spermatozoa, the hypothesis that membrane permeability (P_{Na}/P_K) declines with development in spermatogenic cells must be given further consideration.

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