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THE MEMBRANE POTENTIAL CHANGES POLARITY DURING CAPACITATION OF MURINE EPIDIDYMAL SPERM

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The membrane potential in murine epididymal sperm was determined with a voltage-sensitive, fluorescent probe. In freshly collected sperm, the potential was inside-negative, viz., -13 mV, and was associated with an intracellular K^+ concentration of about 122 mM. Following incubation of sperm in a medium capable of sustaining capacitation and fertilization efficacy, the potential became gradually positive. An inside-positive potential, +24 mV, was obtained after 40 min of incubation, concomitant with an intracellular K^+ concentration of approximately 30 mM. At this time, about 70 percent of sperm had capacitated. An inside-positive membrane potential may play a role in facilitating the acrosome reaction.

Mammalian spermatozoa first released from testes are not fertile unless the cells undergo maturation in the epididymides (1,2) and, after ejaculation, capacitation in the female reproductive tract. Epididymal maturation and the process of capacitation involve a sequence of structural and functional changes in sperm, including modifications in thiol content (3), and low molecular weight polypeptides (4) in epididymidal maturation, and depletion of sterols and enrichment in phospholipids during capacitation (5). These changes probably play a role in stabilizing the sperm plasma membrane in the epididymides and prepare sperm for the acrosome reaction after ejaculation. Given the limited biosynthetic capabilities of sperm and the critically-timed processes of sperm-egg interaction, initiation and temporal arrangements of sperm structures mostly depend on signals received from the extracellular environment, e.g., variations in ionic concentrations in the epididymal fluid (6), and hormonally regulated factors in the female repro-

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ductive tract (7). Evidently, information is needed to understand temporal processes to get a grasp on control mechanisms of fertilization.

In the present communication, we are therefore describing temporal membrane potential changes taking place in murine epididymal sperm incubated in a chemically defined medium (modified Brinster's medium). As monitored by a potentiometric fluorescence probe, the transmembrane potential gradually changes its polarity when sperm are subjected to capacitating conditions.

MATERIAL AND METHODS

Laboratory animals and reagents: Throughout the experiments 12 week old mice of the B_6D_2-F_1 strain were employed. The voltage-sensitive dye, 3,3'-dipropyl-thiacarbocyanine iodide, diC_3-(5), was purchased from Molecular Probes (Eugene, OR). Valinomycin was purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals were of the highest grade available.

Sperm collection: Murine epididymal sperm were collected in Brinster's medium (8) modified to contain 2 percent bovine serum albumin (9). Each pair of epididymides was punctured with a 25G precision glide needle and the sperm cells were squeezed with a forceps into 0.5 ml medium, saturated with carbon dioxide, at 37°C, pH 6.4. The sperm suspension was centrifuged, and the supernatant was discarded. The precipitate was resuspended, either in modified Brinster's medium for a fixed time period (15, 25, 40 min), or in the case of "zero incubation time" directly into MOPS buffer containing 30 mM KCl and 120 mM NaCl. Then cells were centrifuged and resuspended into MOPS buffer after discarding the supernatant. Modified Brinster's medium reportedly permits in vitro capacitation and fertilization to occur in murine gametes (2). The sperm concentration was adjusted to 3×10^7 cells/ml.

Buffers: While maintaining the osmolarity at 260 mos, a series of assay buffers was employed solely containing K^+ and Na^+ in MOPS buffer, pH 7.0. [K^+], and accordingly [Na^+], were prepared to zone in on the respective "null point". Doubly glass-distilled water was used to prepare the buffer systems.

Spectral analysis: 0.5 ml of the resuspended sperm cells and 2 ml of one of the assay buffer solutions were transferred to a quartz cuvette. Fluorescence spectra of the voltage-sensitive dye, di$S$-C_3-(5), were recorded on a Perkin-Elmer luminescence spectrometer, model LS-5B, at 37°C. The final dye concentration was 2×10^{-7} M. The validity of applying potentiometric dyes to measure membrane potentials has been substantiated by numerous studies on various cell types (8). The excitation wavelength was set at 622 nm, the emission was recorded at 670 nm, with both slit widths set at 10 nm. Ten seconds after initiating the time course measurements, the fluorescence probe, diC_3-(5), was added to the stirred cellular suspension in the cuvette. When the fluorescence intensity reached an equilibrium, valinomycin (final concentration 2 uM) was added. Spectral intensities were sampled every 10 seconds for a total of 5 min.

RESULTS

Determination of "null point": In response to the addition of the potentiometric dye to the cellular suspension, the fluorescence intensity immediately increased, followed by a decrease. This behavior indicated that the

cells have a membrane potential which was responsible for shifting the dye's equilibrium distribution towards the cytoplasmic side or membrane-bound state as evidenced by the decrease in fluorescence intensity (9,10). The changes in fluorescence intensity reached an equilibrium within 2 min after applying the dye to the cell suspension. Following administration of valinomycin, the fluorescence intensity changed, dependent on the external K^+ concentration, $[K^+]_o$, of the assay buffer (Fig. 1) and the intracellular K^+ concentration, $[K^+]_i$. By varying $[K^+]_o$, the "null point" can be determined, i.e., that concentration at which no fluorescence change can be detected after application of valinomycin. The ratio $[K^+]_i/[K^+]_o$ is close to unity at the "nullpoint". Fluorescence intensities lower than those representative of the "null point" reflect membrane hyperpolarization generated by $[K^+]_o$ lower than that present at the "null point". Similarly, higher fluorescence intensities are indicative of depolarization resulting from a higher $[K^+]_o$.

Linearity of response: Following valinomycin application, the fluorescence intensity was linearly related to log $[K^+]_o$ in the range from 0 to 130 mM (Fig. 2). These data therefore suggested a potential-dependent partition

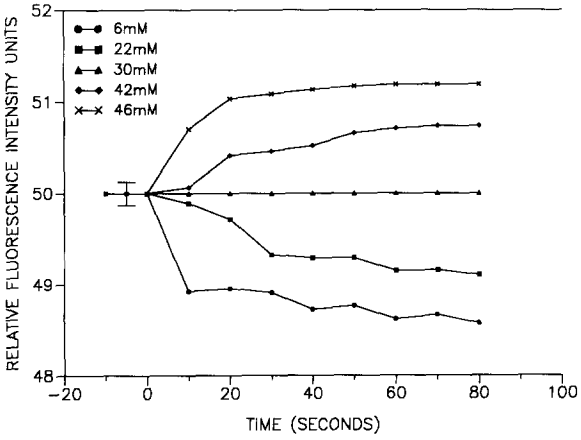

Fig. 1. Typical time course of fluorescence intensity of the voltage-sensitive probe diS-C_3-(5) in murine epididymal sperm (3 x 10^7 cells/ml), preincubated for 40 min in a medium capable of sustaining capacitation and fertilization, at 37°C, and pH 7.0. After establishing an equilibrium level, valinomycin is applied (final concentration 2 uM), at 0 seconds. The fluorescence intensity of the dye (final concentration 0.2 uM) attains new steady state levels which are dependent on the K^+ concentration (in mM) of the sperm's suspension medium.

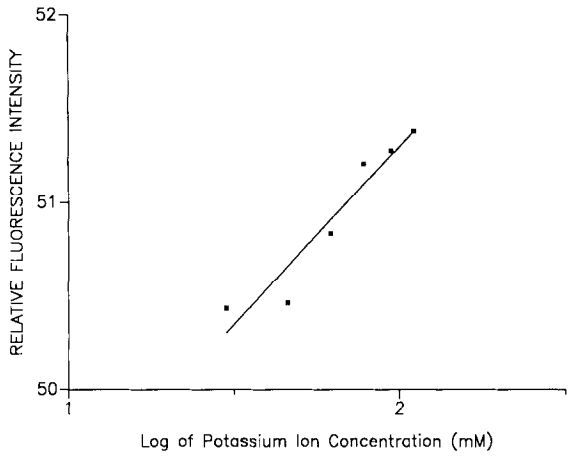

Fig. 2. Fluorescence intensity of the voltage-sensitive dye (final concentration 0.2 uM) in murine epididymal sperm suspension (3 x 10^7 cells/ml), in the presence of valinomycin (final concentration 2 uM), as a function of log[K^+]. The cells had been preincubated for 15 min in a medium capable of sustaining capacitation and fertilization.

of the dye between sperm and the medium; fluorescence intensity changes can thus be used to monitor transmembrane potentials. Given these circumstances, the Nernst equation was applicable to calculate the equilibrium potential (Table 1).

This potential showed an approximately linear dependence on the duration of incubation in modified Brinster's medium (Fig. 3). As the sperm incubation time in the modified Brinster's medium was lengthened, the "null point" decreased from about 122 mM of [K^+] for spermatozoa, at "zero incubation

Table 1. Effect of incubation time on the intracellular K^+ concentration

Incubation time (min)	$[K^+]$ (mM)	E (mV)
0	122 ± 4	-12 ± 1
15	81 ± 4	-2 ± 1
25	42 ± 1	15 ± 1
40	30 ± 1	24 ± 1

Murine epididymal sperm were incubated in modified Brinster's medium in the presence of a voltage-sensitive fluorescent probe. The intracellular concentration was found from examining the "null point". The membrane potential, E, was calculated from the Nernst relation.

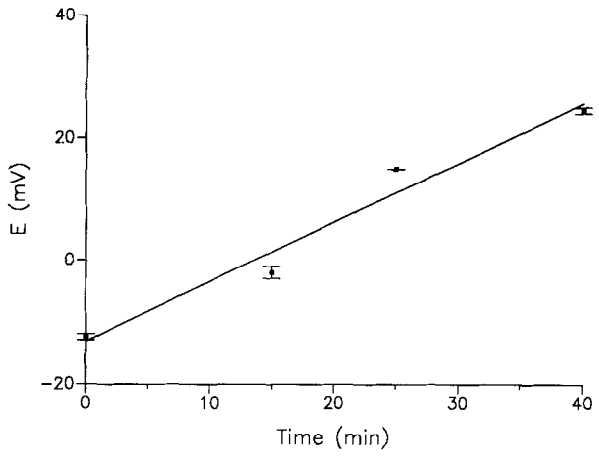

Fig. 3. Temporal changes of the membrane potential, E, of murine epididymal spermatozoa. Following incubation (0 to 40 min) of sperm in a medium capable of sustaining capacitation and fertilization, the membrane potential was derived from the fluorescence intensity of a voltage-sensitive probe. To illustrate the quasilinear dependence, a linear correlation line has been drawn.

time", to about 30 mM [K^+] for cells incubated for 40 min in modified Brinster's medium (Table 1).

Dye compartmentation: Since a spermatozoon represents a multicompartment system (1), the question arises as to the distribution of the membrane potential dye. We therefore examined the distribution of the voltage-sensitive dye in murine sperm under the microscope. The sperm head was stained by the dye. Whether dye accumulated along the sperm tail could not be detected. Following administration of the dye to the sperm sample, the dye accumulated in less than 1 min consistent with the rate of appearance of a fluorescence signal in the fluorimeter. These findings on the dye distribution are supported by experiments on murine epididymal spermatozoa where a chemically related carbocyanine dye was shown to be homogeneously associated (not patchy) with the plasma membrane. Most intensely stained was the anterior region of the sperm head (13).

DISCUSSION

From the results described in these experiments we conclude : (a) Murine epididymal sperm have a transmembrane potential which is dominated by the distribution of K^+ ions across the membrane. (b) The intracellular K^+

concentration drastically declines as epididymal sperm are capacitating in vitro. (c) In freshly isolated epididymal sperm, the transmembrane potential is inside negative, viz., -12 mV, and becomes internally positive, viz., +24 mV, in sperm exposed to capacitating conditions.

Our results demonstrate that the membrane potential varied by about 35 mV comparing the potential at "zero incubation time" to that 40 min later. Furthermore, employing the chlorotetracycline fluorescence assay on murine epididymal sperm (9) under identical conditions to those in the present experiments, about 70 percent of sperm were found to be capacitated after 40 min (14). Our data are also in accordance with previous investigations on the membrane potential although temporal changes of epididymal sperm and the progress of capacitation had not been taken into account. Using the same voltage-sensitive probe, a potential of +13 mV was found in guinea pig epididymal spermatozoa (15). This value is in accord with that observed in our studies at about 25 min (Table 1). Moreover, in bovine epididymal sperm, $[K^+]_i$ reportedly was 120 mM (6), a value in agreement with that found in our studies at "zero incubation time" (Table 1).

At physiological conditions, sperm capacitation is a slow process compared with the more rapid acrosome reaction (16). The in vitro acrosome reaction, however, can be triggered by applying the ionophore, A23187, or digitonin, to enhance Ca^{2+} uptake (17, 18). An increase in cytosolic Ca^{2+} reportedly precedes the acrosome reaction (19). Using calcium channel antagonists like verapamil, recent studies demonstrated the presence of membrane potential-sensitive channels in murine spermatozoa (20). These channels become apparently operational towards the end of capacitation. In light of our findings we therefore suggest that during the capacitation phase a positive potential is established which, at the onset of the acrosome reaction, participates in the activation of voltage-gated channels, thus facilitating a rapid Ca^{2+} influx.

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