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Sperm Membrane Potential: Hyperpolarization during Capacitation Regulates Zona Pellucida-Dependent Acrosomal Secretion

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Membrane potential (V_M) was investigated in mouse and bovine sperm populations. V_M was determined from the fluorescence emission of the lipophilic anion, bis(1,3-diethylthiobarbituric acid)trimethine oxonol ($DiSBAC_2(3)$), and from the lipophilic cation, 3,3′-dipropylthiodicarbocyanine iodide ($DiSC_3(5)$). Fluorescent signals were corrected for contributions of mitochondrial potentials and apparent V_M values were obtained by calibrations in sperm selectively permeabilized with valinomycin or with gramicidin D. The calculated V_M values of uncapacitated mouse and bovine sperm were approximately −35 and −30 mV, respectively. In contrast, capacitated populations of mouse and bovine sperm have V_M values of −50 to −60 mV. Membrane hyperpolarization is due in part to an enhanced K^+ permeability. The development of zona pellucida-activated signal transducing mechanisms during capacitation is dependent upon hyperpolarization. It is suggested that V_M alterations regulate the activation state of sperm, thereby suppressing premature acrosome reactions in uncapacitated sperm and permitting capacitated sperm to respond to zona pellucida stimuli.

INTRODUCTION

Membrane potential (V_M)[2] controls a wide range of homeostatic events and functions as one of the primary means of coupling external signals to cellular responses (reviewed by Hille, 1992; Hall, 1992). This mechanism of regulation may be particularly important in the transcriptionally inactive sperm, in which many physiological processes are controlled by ion fluxes (reviewed by Garbers and Kopf, 1980; Trimmer and Vacquier, 1986; Shapiro, 1987; Ward and Kopf, 1993). In the sea urchin sperm, V_M-sensitive pathways mediate the activation of flagellar motility (Schackmann *et al.*, 1984), the chemotactic and metabolic responses to egg peptides (Lee and Garbers, 1986; Lee, 1988; Babcock *et al.*, 1992; Cook and Babcock, 1993; Cook *et al.*, 1994), and acrosomal secretion (the acrosome reaction; AR) following egg jelly stimulation (Schackmann *et al.*, 1981, 1984; Gonzalez-Martinez and Darszon, 1987; Gonzalez-Martinez *et al.*, 1992).

Mammalian sperm function may also be controlled by V_M. Elevations of external $[K^+]$ ($[K^+]_o$) modulate sperm motility (Babcock *et al.*, 1983), the development of fertilizing ability during capacitation *in vitro* (Mrsny and Meizel, 1981; Fraser, 1983), and the mechanisms that control intracellular $[Ca^{2+}]$ and pH ($[Ca^{2+}]_i$ and pH_i, respectively; Babcock and Pfeiffer, 1987; Florman *et al.*, 1992). The proposed mechanism by which $[K^+]_o$ regulates $[Ca^{2+}]_i$ is by membrane depolarization and activation of voltage-sensitive Ca^{2+} channels. The same class of Ca^{2+} channels are activated by adhesive contact between sperm and the egg's zona pellucida (ZP) during the early stages of fertilization, producing the Ca^{2+} influx during the AR (Florman *et al.*, 1992; Florman, 1994).

Direct analysis of V_M has proven difficult in mammalian sperm, in which the small size and cytosolic volumes complicate electrophysiological and fluorescent probe approaches. In addition, the ionic determinants of V_M have not been established. Here, we have reexamined this issue using fluorescent carbocyanine and oxonol redistribution-type probes of V_M. Mouse and bovine sperm maintain a polarized V_M (inside negative) that is determined in part by K^+ permeability. Capacitation *in vitro* hyperpolarizes V_M through a mechanism that includes a further increase in K^+

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[2] Abbreviations used: AR, acrosome reaction; BSA, bovine serum albumin; $DiSBAC_2(3)$, bis(1,3-diethylthiobarbituric acid)trimethine oxonol; $DiSC_3(5)$, 3,3′-dipropylthiodicarbocyanine iodide; $[K^+]_o$ and $[K^+]_i$, external and internal $[K^+]$, respectively; V_M, membrane potential; ZP, zona pellucida.

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permeability. ZP-dependent signal transducing pathways leading to acrosomal secretion remain inactive if membrane hyperpolarization is prevented. A model is suggested in which the activation state of sperm Ca^{2+} channels is controlled by V_M and in which capacitation-associated hyperpolarization provides a unique means for regulating acrosomal exocytosis.

EXPERIMENTAL PROCEDURES

Solutions and biological preparations. Bovine sperm were incubated in a dTALP medium containing (m*M*): NaCl (100), KCl (3), $CaCl_2$ (2.1), $MgCl_2$ (1.5), NaH_2PO_4 (0.3), $NaHCO_3$ (10), Na-Hepes (25), EGTA (0.1), Na-lactate (22.5), and Na-pyruvate (1), pH 7.4. This medium was supplemented with Na-heparin (10 μg/ml) and bovine serum albumin (0.6% w/v) for sperm capacitation (Parrish *et al.*, 1988). Mouse sperm were incubated in a Hepes-buffered CM medium containing (m*M*): NaCl (109.4), KCl (4.78), $CaCl_2$ (1.71), $MgCl_2$ (1.19), KH_2PO_4 (1.19), $NaHCO_3$ (10), Na-Hepes (25), Na-lactate (25), Na-pyruvate (1), and glucose (5.56), pH 7.4. This medium was supplemented with bovine serum albumin (2% w/v) for sperm capacitation. Polyvinylpyrrolidone (M_r 40,000) was substituted for albumin in protein-free media. Other medium alterations are described under Results. Free concentrations of divalent metal ions were calculated using the MaxC 6.5 program (Dr. Chris Patton, Hopkins Marine Station, Pacific Grove, CA).

Sperm were isolated from bovine seminal secretions and from mouse caudae epididymides, capacitated *in vitro*, and assayed for the ability to undergo zona pellucida-initiated AR and to fertilize eggs *in vitro* according to methods that have been described previously (Florman and Storey, 1982; Florman *et al.*, 1989, 1992). Fertilization *in vitro* was carried out as described previously, using mouse eggs recovered following ovulation from excised oviducts (Florman and Storey, 1982) and fully grown follicular oocytes recovered from bovine ovaries that had completed meiotic maturation *in vitro* (Florman and First, 1988). Mouse and bovine ZP were obtained from ovarian homogenates and soluble extracts were obtained as described previously (Florman *et al.*, 1992). AR were assayed using the Coomassie blue binding assay of Moller *et al.* (1990), as modified by Miller *et al.* (1993).

Membrane potential determinations. V_M was monitored with an AlphaScan II spectrofluorometer operated in the ratiometric mode and equipped with a themostated, stirred sample chamber (Photon Technology Int., South Brunswick, NJ). The deleterious effects of stirring were assessed in preliminary experiments by: (1) release of esterolytic activity, which probably represents acrosin, with the fluorogenic substrate benzoyl-L-arginine-4-methylcoumaryl-7-amide HCl following sedimentation of sperm (Cardullo and Florman, 1993); and (2) propidium iodide staining of sperm following membrane damage. Stirring at any speed damages sperm, with mouse sperm being relatively more fragile than bovine sperm. Such damage was minimized in two ways. First, forward light scattering by suspensions of hypotonically lysed sperm that had been washed by sedimentation (to remove membrane vesicles) was used to determine the minimum stirring rates required to suspend immotile sperm of each species. Second, gentle stirring was insured by covering the stir bar with a minimal layer of mineral oil and then overlaying media containing sperm samples. Stir bar rotation produces waves in the mineral oil layer that suspend sperm while minimizing exposure of cells to the more violent shear forces produced by the typical placement of stir bars in aqueous solutions. Stirring of living cells under these conditions reduced the estimated rates of sperm death (relative to unstirred populations) to <10%/hr and 10–20%/hr for bovine and mouse sperm, respectively.

A number of fluorescent indicator dyes were evaluated for their use as probes of sperm V_M. Several dyes were rejected for further use either because they did not provide adequate signals; because they accumulated extensively in intracellular compartments; or because of their toxic effects, as assessed by determinations of motility, fertilization rates *in vitro*, and ZP-initiated AR. These probes included FM 1-43, JC-1, merocyanine 540, oxonal VI, the tetramethyl and tetraethyl esters of tetramethylrhodamine, and WW781. $DiSBAC_2(3)$ and $DiSC_3(5)$ were selected for use with mammalian sperm (see Results).

$DiSBAC_2(3)$ and $DiSC_3(5)$ are lipophilic probes that distribute across cellular membranes in response to electrochemical gradients and, as a result of binding to intracellular proteins, exhibit enhanced fluorescence emission (Rink *et al.*, 1980). Similar binding and spectral responses occur when these dyes interact with the extracellular albumin that is required for sperm capacitation *in vitro* and is a component of many mammalian gamete culture media. Binding to albumin can obscure that component of fluorescent response derived from intracellular dye. V_M determinations must be carried out in the absence of added albumin.

V_M was determined as follows: capacitated sperm ($1–5 \times 10^7$/ml) were recovered by sedimentation through discontinuous Percoll gradients, as described by Tanphaichitr *et al.* (1990), and resuspended (2.5×10^6 sperm/ml protein-free medium) in 2-ml volumes. Sperm subjected to appropriate preincubation under capacitating conditions *in vitro* and subsequently transferred to protein-free media still undergo ZP-induced AR. $DiSBAC_2(3)$ (0.5 μM) or $DiSC_3(5)$ (0.25 μM) was added to sperm suspensions and fluorescence emission was monitored using wavelength pairs of 530/575 and 620/670 nm, respectively (5-nm bandpass). Fluorescence values were converted to apparent V_M by calibration of $DiSC_3(5)$ emission using valinomycin, the K^+ transporting ionophore, as described previously (Florman *et al.*, 1992) and as discussed under Results. $DiSBAC_2(3)$ calibrations are also described under Results. These values should be considered

"apparent V_M" until confirmed by direct electrophysiological recording.

Chemicals. Reagents were obtained from the following sources: $DiSBAC_2(3)$, $DiSC_3(5)$, other membrane potential-sensitive fluorescent probes (see below), rhodamine 123, nigericin, and valinomycin from Molecular Probes; gramacidin D, FCCP (carbonyl cyanide *p*-(trifluoromethyoxy)phenylhydrazone), antimycin A, polyvinylpyrrolidone (M_r 40,000), and all other chemicals from Sigma.

Statistical methods. Statistical comparison between treatment groups was performed using Student's *t* test.

RESULTS

Selection of fluorescent probes. The ideal probe of plasma membrane potential provides an uncontaminated signal and has no obvious toxic effects. $DiSBAC_2(3)$, a anionic bisoxonal, had many of the desired characteristics of a probe of plasmalemmal V_M. This dye had no apparent untoward effects on the function of sperm of either species when present at $\leq 25\ \mu M$ and it provided a signal-to-noise of $\geq 3{:}1$ at probe and sperm concentrations that were employed (see below). $DiSC_3(5)$, a cationic carbocyanine dye, was also selected for use because it will redistribute across membranes during changes in polarization in the opposite direction of the $DiSBAC_2(3)$ anion. Certain systematic artifacts can be avoided by parallel application of dyes with distinct ionic characteristics. In addition, $DiSC_3(5)$ provides a large signal and it has previously been used to determine V_M in mammalian sperm (Rink, 1977; Babcock and Pfeiffer, 1987; Chou *et al.*, 1989; Florman *et al.*, 1992).

Nevertheless, caution must be exercised in interpretation of $DiSC_3(5)$ results. This dye is more toxic than the bisoxonol probe, as evidenced from decreases in sperm motility and fertilization rates *in vitro* in the presence of $\geq 5\ \mu M$ $DiSC_3(5)$. Furthermore, signals are corrupted by mitochondrial potentials (Florman *et al.*, 1992; see below) and the probe produces complex and concentration-dependent alterations in the calculated V_M of sperm (H. M. Florman, unpublished results). Similar complications are reported occasionally in somatic cells (see Smith, 1982). Yet, when constant concentrations of sperm and probe are used, and when $DiSC_3(5)$ concentrations are $<1\ \mu M$, this dye provides reproducible estimates of V_M. Thus, we determined mouse and bovine sperm V_M with $DiSBAC_2(3)$ and confirmed these results with $DiSC_3(5)$.

Probe calibration with ionophore-treated sperm. Addition of mouse and bovine sperm to dye suspensions produced an increased $DiSBAC_2(3)$ and $DiSC_3(5)$ fluorescent emission that stabilized within 10 min. The augmented fluorescence is dependent on the presence of a polarized V_M and it is diminished in large part (range = 78–95%, $n = 3$) by subsequent addition of 5 μM gramicidin D, a pore-forming K^+/Na^+ ionophore that dissipates V_M (Wallace, 1990).

In order to apply potentiometric probes during experimental manipulations, it is essential first to establish the relationship between V_M and dye emission. This requires knowledge of $[K^+]_i$. PBFI, the K^+-selective fluorescent probe, was not well suited for use with sperm because it distributes nonuniformly within sperm. Other features of the PBFI signal suggest that cytosolic $[K^+]$ contributes only a minor component of dye emission from sperm (Oberdorf, Zeng, and Florman, unpublished).

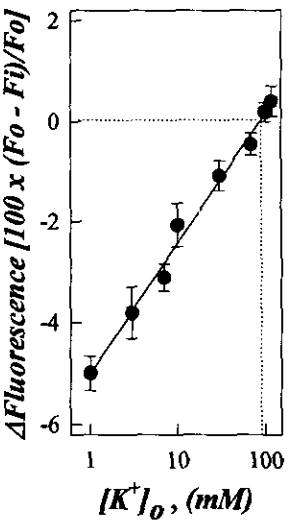

FIG. 1. Determination of $[K^+]_i$ of uncapacitated bovine sperm populations by null-point methods. Bovine sperm samples were diluted to 2.5×10^6/ml in media containing the indicated $[K^+]$ composition and incubated with $DiSC_3(5)$ and with 2 μM valinomycin, as described under Experimental Procedures and Results. Fractional change in fluorescence emission is determined from steady-state levels prior to and following valinomycin addition. Data are the mean (± standard deviation) of four separate experiments and are fit by the following equation: $\Delta F = (2.58 \cdot \log[K^+]_0) - 5.01$; solid line. Dashed lines indicate $[K^+]_0$ at the null point. Results are tabulated in Table 1 and compared to similar values from capacitated bovine sperm and from capacitated and uncapacitated mouse sperm.

Alternatively, $[K^+]_i$ was determined by interpolation from a series of null-point experiments. Sperm were suspended in media of differing $[K^+]$ and sequentially treated with $DiSC_3(5)$ and with valinomycin (2 μM), the K^+ transporting ionophore. The null point is that $[K^+]_0$ that produces no altered probe fluorescence upon addition of valinomycin and reflects the situation when $[K^+]_0 = [K^+]_i$. Figure 1 and Table 1 show that $[K^+]_i$ values for uncapacitated mouse and bovine sperm are in the range of 90–100 mM. Calculated $[K^+]_i$ decreases to 80–90 mM during incubation of sperm populations under capacitating conditions, although these values do not differ significantly from those derived from uncapacitated sperm.

A standard curve was constructed relating $DiSC_3(5)$ fluorescence to plasma membrane potential in the presence of

TABLE 1
Calculated Internal $[K^+]$ of Mouse and Bovine Sperm

Species	Calculated $[K^+]_i$	
	Uncapacitated	Capacitated
Mouse	89 ± 10 (3)	81 ± 8 (3)
Bovine	95 ± 9 (4)	87 ± 5 (4)

Note. Sperm populations were incubated under capacitating or noncapacitating conditions, diluted to a final concentration of 2.5 × 10^6 sperm/ml, and $[K^+]_i$ was determined by null-point methods, as described under Results. Mean $[K^+]_i$ values are shown (±SD; experimental replicates indicated in parenthesis). $[K^+]_i$ differences within a species during capacitation and between species are not significant ($P > 0.1$).

valinomycin (Fig. 2A). In these experiments, the contribution of mitochondrial membranes to dye-reported potentials was assessed by treating sperm with antimycin A (1 μg/10^8 sperm) or FCCP (1 μM) to deenergize mitochondria. Substrate-level phosphorylation was sustained with glucose (5.56 m*M*; Schoff and Lardy, 1987) and rhodamine 123 fluorescence was used to assess mitochondrial potentials (Chen, 1989; Graham *et al.*, 1990). Mitochondria potentials remained largely intact (25–40% reduction) when sperm were treated with ionophore in the absence of uncoupling agents. Subsequent addition of uncoupling agents reduced mitochondrial membrane potentials by >80% and also reduced $DiSC_3(5)$ fluorescence by 20–40%. Higher concentrations of valinomycin (>10 μM) had similar effects alone (not shown). Figure 2A shows that the fluorescence derived from bovine sperm treated with valinomycin and with mitochondrial uncoupling agents is a Nernstian function of $[K^+]_0$ and demonstrates that $DiSC_3(5)$ emission can be used to determine V_M. Similar results were obtained with mouse sperm (not shown).

$DiSBAC_2(3)$ fluorescence is also determined by sperm V_M. When mitochondria were deenergized exactly as described above the fluorescent signal from $DiSBAC_2(3)$ was reduced by ≤5%. Thus, $DiSBAC_2(3)$ provides a signal that is smaller than that of $DiSC_3(5)$ but is relatively free of contamination with mitochondrial potentials. This result is anticipated since cationic probes such as $DiSC_3(5)$ redistribute to mitochondria more readily than do anionic molecules such as $DiSBAC_2(3)$.

Calibration of the $DiSBAC_2(3)$ signal is complicated as this dye forms insoluble complexes with valinomycin. First, calibrations were carried out using gramicidin D in a medium in which Na^+ is replaced with gramacidin D-impermeant cations (choline$^+$ and with *N*-methyl-D-glucamine$^+$). Under these circumstances, K^+ is the major external ion capable of permeating the gramicidin D pore and V_M is determined by the K^+ equilibrium potential (Rink *et al.*, 1980). Figure 2B shows an example of this type of calibration with bovine sperm. As an alternative approach, we have found that $DiSBAC_2(3)$ emission can be calibrated by comparison to the corrected standard curve for $DiSC_3(5)$. Both calibration methods demonstrate that $DiSBAC_2(3)$ fluorescence responds to sperm V_M.

Most of the experiments reported in this study were carried out with $DiSBAC_2(3)$, since this anionic probe provides signals that were relatively free of mitochondrial contamination and since this probe had lower apparent toxicity. In all cases, parallel experiments were carried out with the cationic $DiSC_3(5)$.

Membrane potential of uncapacitated sperm. Table 2 shows the calculated V_M of uncapacitated sperm suspended in standard culture media. Both species maintain polarized V_M (inside negative). The uncorrected potentials obtained with $DiSC_3(5)$ were significantly more negative than those derived with $DiSBAC_2(3)$. Yet, when signals were corrected for mitochondrial contributions, there was reasonable correspondence between the potentials reported by these probes.

The apparent V_M reported by $DiSBAC_2(3)$ varied with $[K^+]_0$ over the range of concentrations tested (Fig. 3). The slopes were 18.0 and 23.1 mV per 10-fold change in $[K^+]_0$ for bovine and mouse sperm, respectively. These values are significantly less than that anticipated for a V_M determined exclusively by the K^+ gradient (62 mV per 10-fold change in $[K^+]_0$;

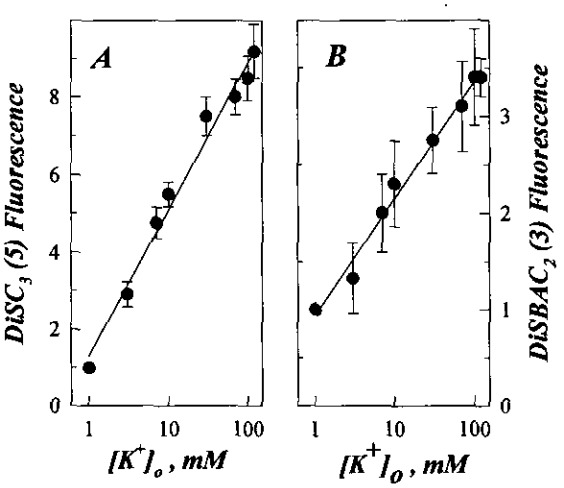

FIG. 2. Fluorescence signals from uncapacitated, ionophore-treated bovine sperm labeled with $DiSC_3(5)$ and $DiSBAC_2(3)$ are dependent upon $[K^+]_0$. Sperm were diluted to 5 × 10^6 sperm/ml in 1 m*M* $[K^+]$ medium, treated with 2 μM ionophore (A, valincomycin; B, gramacidin D) and further diluted to 2.5 × 10^6 sperm/ml in media containing the indicated final $[K^+]_0$ prior to addition of dyes: (A) 0.25 μM $DiSC_3(5)$; (B) 0.5 μM $DiSBAC_2(3)$. Fluorescence emission values are normalized to emission in 1 m*M* $[K^+]_0$. Data (± standard deviations) are the results of three to five separate experiments and are fit by the following equation: $\Delta F = (m^*\log[K^+]_0) + b$. (A) $DiSC_3(5)$: $m = 3.78$, $b = 1.3$; (B) $DiSBAC_2(3)$: $m = 1.2$, $b = 0.95$.

TABLE 2
Calculated Membrane Potentials of Mouse and Bovine Sperm

Sperm	Calculated V_M, mV			
	$DiSBAC_2(3)$		$DiSC_3(5)$	
	Uncorrected	Corrected	Uncorrected	Corrected
Mouse				
Uncapacitated	−41 ± 3 (3)	−33 ± 4 (3)[1]	−56 ± 6 (3)	−38 ± 4 (3)[1]
Capacitated	−59 ± 4 (3)	−49 ± 3 (3)[1]	−68 ± 5 (4)	−55 ± 2 (4)[1,2]
Bovine				
Uncapacitated	−34 ± 7 (3)	−28 ± 3 (3)	−49 ± 5 (3)	−33 ± 3 (3)[1]
Capacitated	−66 ± 5 (3)	−52 ± 4 (3)[1]	−79 ± 6 (3)	−61 ± 4 (3)[1,2]

Note. Capacitated mouse and bovine sperm were obtained following 1- and 5-hr incubations in media that support capacitation of these respective species, as described under Experimental Procedures. Uncapacitated sperm were incubated for 1 hr in media lacking specific capacitating agents (albumin, mouse and bovine; Na-heparin, bovine). Samples were diluted to 2.5×10^6 sperm/ml, incubated with fluorescent probes, and emission was collected as described. Corrected values are obtained by discharging mitochondrial potentials, as described under Results. Data represent means and standard deviations (replicates indicated in parenthesis) and statistical significance is indicated by superscripts:[1] corrected and uncorrected values for a single dye differ ($P < 0.01$);[2] corrected values differ between the two dyes ($P < 0.01$).

Hille, 1992). Thus, V_M of uncapacitated sperm of these species is determined in part, but not exclusively, by the K^+ permeability. In contrast, the V_M reported by both dyes was not altered significantly by addition of 5 m*M* EDTA, which reduces divalent metal ion concentrations to <1 μM (not shown). Mg^{2+} and Ca^{2+} permeabilities therefore do not contribute a major portion of the calculated V_M of uncapacitated sperm.

Membrane potential of capacitated sperm. Mammalian sperm acquire the ability to fertilize eggs during the period of capacitation (Florman and Babcock, 1990). $[K^+]_0$ alterations are associated with capacitation *in vitro* and, potentially, *in vivo* (Meizel, 1985; Fraser and Ahuja, 1988). One plausible mechanism for K^+ action is to depolarize sperm membranes. This possibility was examined directly by determining the membrane potential of capacitated bovine and mouse sperm populations.

Sperm were incubated *in vitro* under conditions shown previously to promote capacitation (Florman *et al.*, 1984; Clark *et al.*, 1993). The development of a capacitated population was confirmed using both ZP-induced AR (Ward and Storey, 1984; Florman and First, 1988) and fertilization *in vitro* (Florman and Babcock, 1990) as assays. The results of AR assays indicate that a minimum of 25–40% of the sperm of each species capacitate during incubation *in vitro* (not shown). These results are similar to those that we reported previously (Florman and Storey, 1982; Florman *et al.*, 1984, 1992; Florman and First, 1988, 1992; Clark *et al.*, 1993; Florman, 1994).

V_M determinations were carried out on capacitated sperm populations after transfer to protein-free media. Table 2 and Fig. 3 show that capacitation hyperpolarized mouse and bovine sperm V_M reported by both $DiSBAC_2(3)$ or $DiSC_3(5)$. In contrast, hyperpolarization was not observed in sperm incubated for similar time periods under conditions that do not support capacitation. Furthermore, the dependence of apparent V_M on $[K^+]_0$ increased as part of the process of capacitation, as shown from comparisons of traces in Fig. 3.

Effects of membrane potential on sperm capacitation. These findings suggest that membrane hyperpolarization may be an essential element of the capacitation process. This was tested by maintaining depolarized V_M during the preliminary incubation period. Bull and mouse sperm populations were subjected to a preliminary incubation in 9 and 12 m*M* $[K^+]_0$, respectively. The calculated V_M at the beginning and end of incubation was −20 ± 3 mV and −28 ± 9 mV, respectively, for bull sperm and was −22 ± 4 and −32 ± 5 mV, respectively for mouse sperm ($n = 3$). As shown in Fig. 3, V_M values after incubation in elevated $[K^+]_0$ were similar to those obtained from uncapacitated populations. The capacitation state was determined subsequently by assessing the ability of sperm to undergo ZP-induced acrosomal release following return to standard $[K^+]_0$ solutions (Ward and Storey, 1984; Florman and First, 1988).

As shown in Fig. 4, sperm manifest the ability to undergo ZP-initiated acrosomal release following preliminary incubation under capacitating conditions (Standard K^+; closed symbols). The fraction of sperm completing the AR increased approximately 250% during a subsequent 20-min incubation with homologous ZP (226 and 260% for mouse and bovine, respectively), while similar incubations with a control glycoprotein produced 10–15% increases. In contrast, when sperm were subjected to preliminary incubation under conditions of depolarized V_M (High K^+; open symbols) there was a significant inhibition of ZP-dependent acroso-

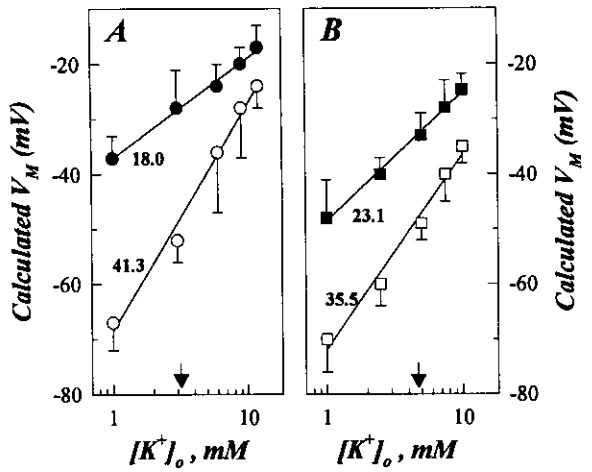

FIG. 3. The $[K^+]_0$ dependence of calculated V_M of mammalian sperm is altered by capacitation. Sperm were diluted to 10^7 sperm/ml and incubated under noncapacitating (●, ■) and under capacitating conditions (○, □), as described under Experimental Procedures and Results. Sperm samples were then diluted to 2.5×10^6 sperm/ml in media containing the indicated $[K^+]_0$ and further incubated with 0.5 μM $DiSBAC_2(3)$. Steady-state fluorescence emission values were converted to "calculated V_M" as described under Results. Data are means (± standard deviation) of three to five separate experiments and are fit by the following equation: $V_M = (m^*\log[K^+]_0) + b$. The slope of the regression line (b), which represents the $[K^+]_0$ dependency of V_M (ΔmV/10-fold change in $[K^+]_0$), is indicated in boldface beside each trace. (A) Bovine sperm, with the following fitting parameters: uncapacitated sperm (●), $m = 18$, $b = -37.1$ mV; capacitated sperm (○), $m = 41.3$, $b = -68.5$. (B) Mouse sperm, with the following fitting parameters: uncapacitated sperm (■), $m = 23.1$, $b = -48.6$; capacitated sperm (□), $m = 35.5$, $b = -71.8$. Arrow: $[K^+]_0$ during the initial incubation phase and the $[K^+]_0$ previously shown to support sperm capacitation and fertilization *in vitro* in sperm of these species.

mal secretion. The responses of mouse and bull sperm to ZP, when corrected for altered rates of spontaneous AR, are inhibited by 76 and 80%, respectively, as a result of preincubation with elevated $[K^+]_0$. These results are consistant with the hypothesis that hyperpolarization of sperm V_M is an important aspect of the capacitation process.

DISCUSSION

The central role of V_M in the control of sperm function prompted us to reexamine the ionic determinants of this potential. Mouse and bovine sperm maintain a polarized V_M (−30 to −40 mV) that is determined in part by K^+ permeability. Capacitation produces an enhanced K^+ permeability, resulting in hyperpolarization of V_M. ZP-dependent signal transduction pathways are not expressed during sperm capacitation when membrane hyperpolarization is prevented, suggesting that V_M alterations may be important control elements in the acquisition of fertility.

Fluorescent probes of sperm membrane potential. The relatively small cytosolic volumes in sperm have complicated efforts to determine V_M. For example, the total cell water in bovine sperm is <20 fl (Hammerstedt *et al.*, 1978) and minimizes the utility of intracellular electrodes (Lindemann and Rikmenspoel, 1971). The strategy of the present study was to utilize redistribution-type fluorescence reporters of V_M. Probes of this class are lipophilic and partially charged structures that redistribute across biological membranes according to their electrical and chemical gradients. The spectral characteristics of probe molecules are altered following binding to intracellular proteins, providing a fluorescent signal of V_M (Rink *et al.*, 1980; Freedman and Laris, 1988; Loew, 1988).

Redistribution-type probes have been applied to the determination of mammalian sperm V_M previously. Results varied from highly polarized (>50 mV), inwardly negative V_M values (Florman *et al.*, 1992) to moderately polarized values (<30 mV) that were in some cases inwardly negative (Chou

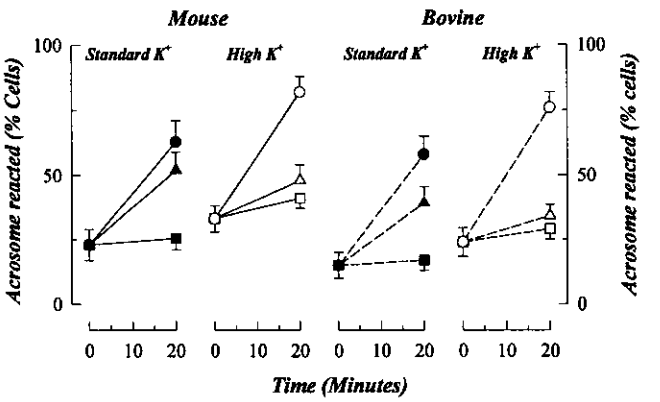

FIG. 4. Sustained depolarization prevents development of zona pellucida-dependent acrosomal secretion during sperm capacitation. Mouse and bovine sperm were subjected to preliminary incubation under capacitating conditions (Normal K^+, 4.78 and 3 mM $[K^+]$, respectively; closed symbols) or in the presence of elevated $[K^+]$ (High K^+, 12 and 9 mM, respectively; open symbols). $[K^+]$ values for High K^+ conditions are those that produce in capacitated populations the depolarized V_M characteristic of uncapacitated sperm (obtained from Fig. 3). Incubation conditions are otherwise those required to capacitate sperm of these species. Following preliminary incubation, the acrosomal status of mouse (—) and bovine sperm (---) was determined immediately after return to standard media (0 min) or following 20 min additional incubation in the presence of control glycoprotein (fetuin; ■, □), solubilized zonae pellucidae (▲, △), or 5 μM ionomycin (●, ○). Concentrations of control and zona pellucida glycoproteins were 40 μg/ml for mouse sperm and 100 μg/ml for bovine sperm. Data represent the mean (± standard deviation) of three to four separate experiments, with each experiment consisting of triplicate samples and with 100–200 sperm assayed/sample.

et al., 1989) and in other cases of reversed polarity (Rink, 1977). Several features of the redistribution-type probes and of their specific application to sperm may account for these differences. First, spectral characteristics of probe fluorescence are altered by binding to extracellular protein: these responses cannot readily be distinguished from those occurring with intracellular protein and complicate V_M determinations. This is a particular problem with sperm, where BSA is required for capacitation (Yanagimachi, 1994) and where this protein may adsorb to the cell surface (Blank *et al.*, 1976). In the present study we have attempted to remove residual protein by density gradient sedimentation.

Second, application of redistribution probes to sperm is also complicated by the relative fragility of these cells. Spectral shifts occur upon probe efflux from cells, irrespective of whether probe loss is due to V_M alterations or to cell death. We attempted to control for these effects by developing a minimally disruptive method for stirring sperm and by consistantly using two probes with distinct ionic characteristics. Thus, the cationic $DiSC_3(5)$ and the anionic $DiSBAC_2(3)$ redistribute in opposite directions during shifts in V_M, whereas cell death produces release of both dyes. Other systematic dye effects, such as the mitochondrial interactions and toxicity of $DiSC_3(5)$, are obviated by parallel application of these two probes.

Third, previous studies of sperm V_M have relied extensively on $DiSC_3(5)$ responses (Rink, 1977; Chou *et al.*, 1989; Florman *et al.*, 1992) and are complicated by the well-known contribution of mitochondrial potentials to probe signals (Rottenberg, 1979). Mitochondrial potentials were accounted for both by selectively discharging this potential and by using an anionic probe that does not interact with mitochondria extensively.

We conclude that fluorescent probes can be used to determine sperm V_M, provided that extensive efforts are made to control for the diverse spurious factors that contaminate these signals. Using these probes we derived inwardly negative values for mammalian sperm. Caution is advised though in comparing these calculated values to V_M derived by other methods. Several factors contribute to V_M: bulk-phase asymmetric distribution of ions, surface charge, and dipole moment in the membrane (Hille, 1992). It is unlikely that the surface charges on mammalian sperm membranes are detected by the redistribution probes used here since removal of divalent metal cations, which screen such charges, had no effect on the dye signal. The response of redistribution probes is dominated by the bulk-phase ion asymmetries. Other approaches to determining V_M, such as the use of electrochromic indicator dyes and electrophysiological techniques, may respond to different components of V_M.

V_M in uncapacitated and capacitated sperm. Mammalian sperm are maintained in a functionally quiescent state during storage in caudae epididymides and require activation, or capacitation, either *in vivo* or *in vitro* in order to express fertility (reviewed by Florman and Babcock, 1990; Yanagimachi, 1994). We observed that sperm maintain a moderately polarized V_M (−25 to −35 mV) during incubation under conditions that do not support capacitation. K^+ permeability is a component of the reported V_M, as indicated by the effects of altered $[K^+]_0$. In this regard, K^+ channels are present in mammalian spermatogenic cells (Hagiwara and Kawa, 1984) and in sea urchin sperm (Lievano *et al.*, 1985; Babcock *et al.*, 1992). Yet, the K^+ equilibrium potential calculated from determined $[K^+]_i$ values in these cells is in the range of −75 to −90 mV (Hille, 1992). The permeability of ions with more positive equilibrium potentials (such as Na^+) must also contribute to the observed V_M.

V_M of sperm populations becomes hyperpolarized during capacitation *in vitro*. An enhanced K^+ permeability is at least one component of this change, as indicated by the increased dependence of V_M on $[K^+]_0$ (Fig. 3). These results represent spatially averaged values for sperm populations. Only 25–40% of the sperm population complete the capacitation process, as assessed by the secretory response to ZP (Table 3, Fig. 4). Thus, the observed membrane alterations may reflect a moderate hyperpolarization (to −50 mV) in all cells or a larger hyperpolarization (to $\geq$ −80 mV) that is restricted to the subpopulation that completes capacitation. The relative contribution of K^+ and other ion permeabilities to capacitation-associated V_M alterations differs between these models. In order to discriminate between these possible models, it is necessary to determine the respective time courses of capacitation and of hyperpolarization in single cells. Such studies are in progress.

Significance of V_M alterations during capacitation. One feature of regulated secretory systems is the suppression of constitutive exocytosis, with extrinsic cues initiating secretion by removing inhibitory modulation (Popov and Poo, 1993). The case for inhibitory modulation of spontaneous secretion can also be made for mammalian sperm, in which the acrosome is the solitary secretory vesicle. Completion of the AR at a distance from eggs results in a decreased ability to penetrate the cumulus oophorus matix or to adhere to zonae pellucidae (reviewed in Florman and Babcock, 1990; Yanagimachi, 1994). Capacitation produces an enhanced rate of the spontaneous AR and also places the secretory pathway under regulation by ZP3 (Ward and Storey, 1984; Florman and First, 1988; Florman and Babcock, 1990; Florman, 1994).

In sperm, ZP-dependent secretion is not observed when hyperpolarization during a "capacitating" preincubation is prevented (Fig. 4). Membrane hyperpolarization may provide one means for the release of inhibitory modulation during capacitation. Sperm have voltage-sensitive Ca^{2+} channels with some of the characteristics of somatic, L-type channels (Babcock and Pfeiffer, 1987; Florman *et al.*, 1992). L-like channels are activated by stimulatory glycoproteins in the ZP, or by experimental manipulations, and produce a Ca^{2+} influx that initiates the AR (Florman *et al.*, 1989, 1992; Storey *et al.*, 1992; Clark *et al.*, 1993; Bailey and Storey, 1994; Florman, 1994). One characteristic of the

TABLE 3
Indicators of Sperm Capacitation

	Fertilization	Acrosome reacted, %	
	% (Total eggs)	Control (*n*)	+Zona pellucida (*n*)
Mouse			
Uncapacitated	12 ± 4 (74)	19 ± 6 (2)	24 ± 9 (2)
Capacitated	62 ± 8 (61)[1]	27 ± 8 (3)	56 ± 12 (3)[1,2]
Bovine			
Uncapacitated	5 ± 5 (57)	11 ± 5 (2)	16 ± 6 (2)
Capacitated	72 ± 8 (69)[1]	19 ± 7 (2)	43 ± 9 (2)[1,2]

Note. Mouse and bovine sperm were incubated in media that support capacitation and fertilization *in vitro* for 1 and 5 hr, respectively (see Experimental Procedures). Sperm were diluted to 10^6 sperm/ml and cocultured with 10–20 eggs in 200-μl volumes under mineral oil. Fertilization was assayed after 4 h by the presence of sperm head and tail within the ooplasm. AR were determined in after 20-min incubations in 50-μl volumes containing 10^5 sperm/ml and solubilized extracts of mouse or bovine ZP (40 and 100 μg protein/ml, respectively). Control samples for AR assays were incubated with equivalent additions of BSA. Data represent means (± standard deviation) of three to four fertilization experiments and two to three AR determinations, with each AR assay consisting of triplicate samples and >200 sperm examined/sample. *Superscripts indicate statistical significance:*[1] *different from uncapacitated sample of the same species* ($P < 0.01$);[2] different from capacitated sperm samples incubated with control proteins ($P < 0.01$).

somatic L-type channel, as well as other classes of Ca^{2+} channels, is a voltage-dependent inactivation. The characterized classes of voltage-sensitive Ca^{2+} channels can be activated to varying extents from holding potentials equivalent to the V_M of capacitated sperm (−50 to −60 mV) but cannot be activated when the holding potential is shifted to values typical of the V_M of uncapacitated sperm (Hille, 1992; Zhang *et al.*, 1993; McDonald *et al.*, 1994).

A similar mechanism may account for some aspects of inhibitory modulation of acrosomal secretion. The relatively depolarized V_M of uncapacitated sperm is expected to maintain channels in an inactivated state, from which positive shifts in V_M are unlikely to evoke channel opening or produce Ca^{2+} conductance. Hyperpolarization may prime sperm Ca^{2+} channels by recruiting them out of an inactivated state, from which they cannot be opened efficiently, to a closed state from which they are readily opened by subsequent depolarization. Thus, shifts in V_M during capacitation are well suited to act as a regulatory switch and may relieve inhibitory modulation of the AR.

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