Na⁺-requiring mechanisms modulate capacitation and acrosomal exocytosis in mouse spermatozoa

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Mouse spermatozoa require extracellular Na⁺ for both capacitation and acrosomal exocytosis, but the minimum concentrations differ widely: $>1 \leq 25 \text{ mmol Na}^+ \text{ l}^{-1}$ will support capacitation, but $>125 \text{ mmol Na}^+ \text{ l}^{-1}$ is needed for acrosomal exocytosis in capacitated cells. Our conclusions are based on evidence obtained from sperm cells preincubated in iso-osmotic media with differing concentrations of Na⁺ and then analysed for occurrence of the acrosome reaction, capacitation-related changes in chlortetracycline (CTC) fluorescence and *in vitro* fertilization. The modified Tyrode's medium used as the control medium in these experiments contained $150 \text{ mmol Na}^+ \text{ l}^{-1}$ and supported full sperm function. At least some of the Na⁺ needs to be internalized to promote the functional changes, as evidenced by the ability of the monovalent cation ionophore monensin to accelerate capacitation and trigger acrosomal exocytosis in control medium. However, in low Na⁺ (25 mmol l^{-1}) medium, monensin could only modulate the transition to the capacitated state, assessed with CTC, indicating that higher concentrations of extracellular Na⁺ are required for initiation of acrosomal exocytosis. We suggest that changes in the composition of the female reproductive tract fluids serve to control expression of sperm functional potential. Before ovulation in the mouse, sufficient Na⁺ and Ca²⁺ are present to promote capacitation. However, the Na⁺ concentration is marginal for support of acrosomal exocytosis and the relatively high K⁺ reinforces an inhibition of exocytosis. At ovulation, the release of follicular fluid would increase the Na⁺ and decrease the K⁺ concentrations, thereby permitting full expression of fertilizing potential. Possible mechanisms that might be involved in the Na⁺-related responses, including a Na⁺–Ca²⁺ exchanger, a Na⁺–K⁺ ATPase and a Na⁺–H⁺ exchanger, were also investigated. If a Na⁺–Ca²⁺ exchanger has a role to play, it is not during capacitation *per se*. Incubation of sperm cells in high Na⁺, low Ca²⁺ ($90 \mu\text{mol CaCl}_2 \text{ l}^{-1}$) medium that supports capacitation, followed by introduction of monensin, which would have promoted an influx of Na⁺ and could have, in turn, activated a Na⁺ out, Ca²⁺ in response, did not accelerate transition to the capacitated state (B pattern of CTC fluorescence). In contrast, it is possible that a Na⁺–K⁺ ATPase may play a role during capacitation. Incubation of suspensions in control medium plus ouabain, which would inhibit the ATPase, significantly accelerated the transition from the uncapacitated to the capacitated state, although it did not trigger acrosomal exocytosis. Thus, a decline in activity of this enzyme may be an important part of capacitation. Finally, we have obtained evidence that a Na⁺–H⁺ exchange mechanism may be involved in initiation of acrosomal exocytosis. Incubation of suspensions in low Na⁺ medium for a time sufficient to promote capacitation, followed by introduction of $25 \text{ mmol NH}_4\text{Cl l}^{-1}$ for 10 min to raise intracellular pH, significantly stimulated the acrosome reaction even though the Na⁺ concentration was not increased to provide permissive Na⁺ conditions for exocytosis. We suggest that during fertilization an influx of Na⁺ into the fertilizing spermatozoon causes a rise in intracellular pH that in turn opens Ca²⁺ channels, thereby permitting the influx of Ca²⁺ needed to trigger acrosomal exocytosis.

Introduction

It has long been known that, upon release from the male reproductive tract, mammalian spermatozoa are incapable of fertilizing oocytes immediately, but acquire functional ability during

a species-dependent period of residence in the female reproductive tract (Austin, 1951; Chang, 1951). Collectively, the changes in the spermatozoa that underlie this functional 'switching on' are termed 'capacitation'; once capacitated, sperm cells can undergo acrosomal exocytosis (the acrosome reaction) in response to oocyte-associated factors, and can penetrate the

Received 11 June 1992.

zona pellucida and fuse with the oocyte plasma membrane (Yanagimachi, 1988). Although capacitation and fertilization normally occur within the female reproductive tract, the fact that permissive conditions can be provided *in vitro* has allowed analysis of specific requirements for these two distinct phases. For example, Ca^{2+} is obligatory for both capacitation and acrosomal exocytosis, but the amount required for each may differ markedly (Fraser, 1990). In the case of mouse spermatozoa, micromolar Ca^{2+} (minimum of $90 \mu\text{mol l}^{-1}$) will support full capacitation, but maximal fertilizing ability is achieved only in millimolar Ca^{2+} (1.80 mmol l^{-1}) (Fraser, 1987).

Although attention has focussed especially on Ca^{2+} , particularly because a large influx of Ca^{2+} into capacitated cells appears to be the trigger for the molecular events that culminate in acrosomal exocytosis (e.g. Roldan and Harrison, 1990), requirements for other ions have also been investigated. A major ionic constituent of both reproductive tract fluids *in vivo* and tissue culture media *in vitro* is Na^+ . Limited studies to date, primarily in guinea-pigs, have provided some evidence that Na^+ plays a role in the acquisition of sperm fertilizing ability (e.g. Hyne *et al.*, 1984; Bhattacharya *et al.*, 1986; Murphy *et al.*, 1986).

In the present study we have investigated the specific requirements for Na^+ during mouse sperm capacitation *per se* and spontaneous acrosomal exocytosis, the latter usually correlating closely with proven functional ability of sperm suspensions (e.g. Fraser, 1987). We have used a variety of approaches, including simple assessment of presence or absence of the acrosomal cap, chlortetracycline fluorescence patterns (e.g. Fraser and McDermott, 1992) and fertilization *in vitro*. In addition to determining minimum Na^+ requirements for capacitation and acrosomal exocytosis, we have also explored some of the possible mechanisms whereby Na^+ might exert influences on changes in the functional state of spermatozoa. These include action via a $\text{Na}^+ - \text{K}^+$ ATPase, a $\text{Na}^+ - \text{Ca}^{2+}$ exchanger and a $\text{Na}^+ - \text{H}^+$ exchanger. In so doing, we have obtained evidence that these mechanisms may indeed have roles to play, but only at specific stages in the events leading to successful fertilization. Given that sperm cells maintain intracellular concentrations of various ions that differ from the extracellular concentrations (e.g. high Na^+ and low K^+ outside, low Na^+ and high K^+ inside; Hyne *et al.*, 1985), ion fluxes provide numerous mechanisms for controlling cell activities and responses. A preliminary report of some of this work was published earlier (Umar and Fraser, 1991).

Materials and Methods

Media

The standard medium was a modified Tyrode's with the following composition (all in mmol l^{-1}): NaCl, 124.23; NaHCO_3 , 25.00; KCl, 2.68; NaH_2PO_4 , 0.36; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.49; CaCl_2 , 1.80; glucose, 5.56. It also contained Na penicillin at 100 units ml^{-1} , BSA (crystalline, Sigma, Poole) at 4 mg ml^{-1} and phenol red (0.5% in 154 mmol $\text{NaHCO}_3 \text{ l}^{-1}$) at 0.1 ml per 100 ml medium. The total concentration of Na^+ was approximately 150 mmol l^{-1} . For the low Na^+ medium (about 25 mmol $\text{Na}^+ \text{ l}^{-1}$), choline chloride replaced NaCl. For the very low

Na^+ medium (about 1 mmol $\text{Na}^+ \text{ l}^{-1}$), NaCl and NaHCO_3 were both replaced, the former with choline chloride and the latter with KHCO_3 ; in addition, the K^+ salt of penicillin was used rather than the Na^+ salt. Calcium-deficient medium was prepared by omitting CaCl_2 ; despite trace amounts of free Ca^{2+} ($< 20 \mu\text{mol l}^{-1}$), this medium cannot support complete capacitation (Fraser, 1987). Stocks of Tyrode's medium containing high concentrations of CaCl_2 were prepared to introduce Ca^{2+} into this medium. The initial stock contained 22.5 mmol $\text{CaCl}_2 \text{ l}^{-1}$; an aliquot of this was diluted 20-fold. When 20 μl of these stock solutions were added to 230 μl Ca^{2+} -deficient medium or sperm suspension, the final CaCl_2 concentration was 1.80 mmol l^{-1} ($+\text{Ca}^{2+}$) and 90 $\mu\text{mol l}^{-1}$ (low Ca^{2+}), respectively.

Drug stock solutions

Monensin (Calbiochem, Nottingham) at 2 mmol l^{-1} was prepared in absolute ethanol and aliquots were stored at -20°C . For use, an aliquot of this stock solution was removed and diluted in 0.9% NaCl to produce substocks of 125, 25 and 5 $\mu\text{mol l}^{-1}$. Ouabain (Sigma) was prepared as required in 0.9% NaCl at 5 mmol l^{-1} . Nigericin (Sigma) at 1 mmol l^{-1} was prepared in absolute ethanol and aliquots were stored at -20°C . For use, this stock solution was diluted fourfold in 0.9% NaCl. Amiloride (Sigma) at 100 mmol l^{-1} was prepared as required in dimethylsulfoxide; this was then diluted with 0.9% NaCl to produce substocks of 25, 7.5 and 2.5 mmol l^{-1} . All drug solutions were used at a 1/50 dilution.

Sperm suspension preparation

The contents of the epididymides from one or more mature (> 8 weeks) TO male mice (Harlan OLAC, Bicester) were released into medium at the ratio of two epididymides ml^{-1} ; this gives a sperm concentration of approximately $2-3 \times 10^7$ cells ml^{-1} . When different concentrations of Na^+ were being compared, one epididymis was released into 0.5 ml of each composition. Suspensions were incubated at 37°C in an atmosphere of 5% CO_2 -5% O_2 -90% N_2 ; the duration of incubation depended on the experimental design. Plastic culture dishes, 30 mm diameter (Sterilin, Teddington), were used and all media were overlaid with autoclaved liquid paraffin (Boots, Nottingham).

In vitro fertilization

Mature TO female mice were induced to superovulate by injecting 7.5 iu pregnant mares' serum gonadotrophin (PMSC) (Folligon, Intervet, Cambridge, UK) and, 48-54 h later, 5 iu hCG (Chorulon, Intervet), both i.p. At 14 h after hCG, cumulus clots were released from ampullae into 4 ml of appropriate medium. Suspensions were prepared as above, incubated and then diluted approximately 1/10 to a final concentration of about 2×10^6 cells ml^{-1} ; 400 μl droplets were added to paraffin-containing culture dishes. Approximately equal numbers of freshly obtained oocytes were added to all droplets. Oocytes and sperm suspensions were co-cultured for 65 min; oocytes were then transferred to fresh droplets of medium and,

at 75 min, fixed with buffered formalin (4% formaldehyde). Oocytes were stained with 0.75% aceto-orcein, mounted and assessed for fertilization. They were assumed to be fertilized if they had resumed the second meiotic division and contained a decondensing sperm head (Fraser, 1983).

Assessment of spontaneous acrosome reactions

Sperm suspensions were filtered to select motile cells and then assessed for the presence or absence of the acrosomal cap. Filtration removes the majority of non-motile (frequently dead and degenerating) cells, many of which have lost the acrosomal cap by degeneration (see Fraser, 1982). Mouse spermatozoa do not appear to become non-motile immediately upon undergoing the acrosome reaction (Fraser, 1977), so the procedure is preferentially removing non-functional cells. Short columns of Sephadex G-25 (Medium) were equilibrated and, after application of sperm suspensions, eluted with media of the appropriate composition; this procedure yields about 90% motile cells. Eluted cells were fixed in buffered formalin, drops were placed on clean slides and coverslips were added. After they were completely dry, samples were rehydrated by introducing a minimal volume of formalin under the coverslips. Two hundred cells were assessed for the presence or absence of the acrosomal cap (see Fraser, 1987) in each treatment group in each replicate experiment.

Chlortetracycline assessment

Chlortetracycline (CTC) was used to assess the capacitation state of sperm cells in some of the experiments. The method is a modification of that described by Ward and Storey (1984). The CTC solution, containing 750 $\mu\text{mol CTC l}^{-1}$ (Sigma) in a buffer of 130 mmol NaCl l^{-1} , 5 mmol cysteine l^{-1} , 20 mmol Tris-HCl l^{-1} (final pH 7.8), was prepared fresh and kept wrapped in foil to exclude light. Sperm cells were filtered through Sephadex as above, then stained and fixed in suspension to permit accurate timing. Sperm suspension (45 μl) was added to a 0.5 ml foil-wrapped microcentrifuge tube containing 45 μl CTC solution. After mixing well, 8 μl of 12.5% (w/v) paraformaldehyde in 0.5 mol Tris-HCl l^{-1} (pH 7.4) was added with mixing. Slides were prepared by placing 10 μl of this mix on a clean slide. One drop of 0.22 mol 1,4-diazabicyclo [2.2.2] octane l^{-1} (Sigma) in glycerol plus PBS (9:1) was mixed in carefully to retard fading of fluorescence. A coverslip was added, and the slide was then firmly compressed between tissues to remove excess fluid. Slides were sealed with colourless nail varnish and stored in a light-proof box in the cold. Assessment was done on the same or the following day; no fading was detected after overnight storage. Cells were assessed on an Olympus BHS microscope equipped with phase contrast and epifluorescent optics. The Hg excitation beam was passed through a 405 nm band pass filter and CTC fluorescence emission was observed through a DM 455 dichroic mirror.

One hundred cells in each sample were classified as expressing one of three CTC staining patterns: 'F', with uniform fluorescence which is characteristic of acrosome-intact uncapacitated cells; 'B', with a fluorescence-free band in the postacrosomal region, which is characteristic of acrosome-intact capacitated cells; 'AR', with dull or absent head fluorescence, which is

Fig. 1. Acrosome loss in mouse sperm suspensions incubated for 120 min in low Na⁺ (25 mmol l^{-1}) or high Na⁺ (150 mmol l^{-1}) media; an aliquot of the low Na⁺ suspension was then transferred to high Na⁺ medium (low → high Na⁺; 138 mmol Na⁺ l^{-1}) and all three suspensions were incubated for a further 10 min. Data are presented as mean ± SEM ($n = 4$). ** $P < 0.025$, *** $P < 0.01$ compared with high Na⁺ control suspensions.

characteristic of acrosome-reacted capacitated cells. The presence or absence of the acrosomal cap on each cell was verified under phase contrast illumination (Fraser, 1987).

Statistical analysis

Data were analysed using Cochran's test for combination of 2 × 2 contingency table (Snedecor and Cochran, 1980).

Experimental Details and Results

Series I: Will low Na⁺ (25 mmol l^{-1}) support capacitation and the acrosome reaction?

In these experiments, the ability of Tyrode's medium containing reduced Na⁺ of about 25 mmol l^{-1} (low Na⁺) to support capacitation and the acrosome reaction was compared with standard medium containing about 150 mmol Na⁺ l^{-1} (high Na⁺). The contents of one epididymis were released into 0.5 ml of low Na⁺ medium and the other into 0.5 ml of high Na⁺ medium. After 120 min incubation, an aliquot of the low Na⁺ suspension was diluted tenfold in high Na⁺ medium (low → high Na⁺), gassed and incubated for 10 min. The final Na⁺ concentration was about 138 mmol l^{-1} . All three treatment groups were filtered, fixed and assessed for the presence or absence of the acrosomal cap. Four replicate experiments were carried out ($n = 4$).

Spontaneous acrosome loss was very low ($4.6 \pm 1.4\%$, mean ± SEM) in the low Na⁺ suspension and significantly lower ($P < 0.01$) than that in the control high Na⁺ suspension ($22.5 \pm 5.4\%$) (Fig. 1). Within 10 min of raising the Na⁺ concentration (low → high Na⁺), the proportion of acrosome-reacted cells increased markedly to give a mean value ($30.8 \pm 8.7\%$), actually significantly higher ($P < 0.025$) than that in the high Na⁺ suspensions. However, this mainly reflects a large difference between the two treatment groups in a single replicate. From this we conclude that mouse sperm cells can

Table 1. Effect of extracellular Na^+ concentration on fertilization *in vitro* in mice

[Na^+] (mmol l^{-1})		Oocytes fertilized/ total oocytes	%	Range (%)
Preincubation ^a	Fertilization			
25	25	2/71	2.8****	0–16
25	138	84/85	98.8	97–100
150	150	69/69	100.0	—

Values are for three replicates.

^aPreincubated for 120 min, then diluted into medium containing either 25 or 150 $\text{mmol Na}^+ \text{l}^{-1}$.

**** $P < 0.001$ compared with high Na^+ suspensions.

undergo capacitation in low Na^+ medium, but cannot proceed to acrosomal exocytosis until the extracellular Na^+ is increased. Sperm cells were highly motile in both low and high Na^+ media.

The validity of this conclusion was examined by assessing similarly treated cells with CTC in two replicate experiments. After 130 min in low Na^+ medium, the mean distribution of CTC patterns was: F, 20%; B, 74%; AR, 6%. After 120 min in low Na^+ and 10 min in high Na^+ , a marked change in the proportion of B and AR patterns was observed: F, 19%; B, 41%; AR, 41%. From these data it can be seen that the large majority of cells exhibited the B pattern of fluorescence (acrosome-intact, capacitated) after incubation in low Na^+ and hence our earlier conclusion is confirmed. Likewise, progression through acrosomal exocytosis required high extracellular Na^+ . The replacement of NaCl with choline chloride had no detectable effect on capacitation: a similar proportion of cells in both low and high Na^+ media exhibited the F pattern of fluorescence.

As a final test of our conclusions, we compared the *in vitro* fertilizing ability of spermatozoa incubated in the three conditions: low Na^+ , low $\rightarrow$ high Na^+ , high Na^+ ($n = 3$). As seen in Table 1, low $\rightarrow$ high Na^+ cells were as fertile as those in continuous high Na^+ , while those in continuous low Na^+ were essentially non-fertilizing (the two fertilized oocytes probably had cracked zonae which permitted spermatozoa through). All these approaches therefore indicate that mouse spermatozoa can undergo capacitation in 25 $\text{mmol Na}^+ \text{l}^{-1}$ but require high Na^+ for the acrosome reaction.

Series II: Will very low Na^+ (1 mmol l^{-1}) support capacitation?

The experimental design was similar to that in Series I, with suspensions being incubated in very low Na^+ and Na^+ (standard) media. After incubation for 120 min, an aliquot of the very low Na^+ suspension was diluted tenfold in high Na^+ medium, giving a final Na^+ concentration of about 135 mmol l^{-1} . Ten minutes later all three samples were filtered, fixed and then assessed ($n = 5$).

Very few of the very low Na^+ -incubated cells had lost the acrosomal cap, whether or not the Na^+ was increased during the final 10 min ($1.6 \pm 0.6\%$ for continuous very low Na^+ and $2.6 \pm 1.3\%$ for very low $\rightarrow$ high Na^+) as shown in Fig. 2. These values were significantly lower ($P < 0.001$) than those obtained in the continuous high Na^+ group ($29.3 \pm 4.4\%$). These results suggest that mouse spermatozoa cannot complete capacitation in medium containing 1 $\text{mmol Na}^+ \text{l}^{-1}$, hence the

Fig. 2. Acrosome loss in mouse sperm suspensions incubated for 120 min in very low Na^+ (1 mmol l^{-1}) or high Na^+ (150 mmol l^{-1}) media; an aliquot of the very low Na^+ suspension was then transferred to high Na^+ medium (very low $\rightarrow$ high Na^+ ; 135 $\text{mmol Na}^+ \text{l}^{-1}$) and all three suspensions were incubated for a further 10 min. Data are presented as mean % $\pm$ SEM ($n = 5$). **** $P < 0.001$ compared with high Na^+ control suspensions.

failure to undergo acrosomal exocytosis when Na^+ was raised. The fact that the medium had a high K^+ concentration, due to the inclusion of KHCO_3 rather than NaHCO_3 , would not have inhibited capacitation (Fraser, 1987).

It is unlikely that the small difference in the final Na^+ concentration when reduced- Na^+ medium was added to high Na^+ medium (138 mmol l^{-1} for low $\rightarrow$ high Na^+ and 135 mmol l^{-1} for very low $\rightarrow$ high Na^+) accounted for the difference in response to high Na^+ . To be certain, however, we assessed similarly-treated cells with CTC ($n = 2$). The distribution of CTC patterns was very similar in both very low Na^+ groups. In continuous very low Na^+ , the proportions were 84% F, 16% B, 0% AR and in very low $\rightarrow$ high Na^+ they were 75% F, 25% B, 0% AR. Thus the CTC analysis, with a majority of uncapacitated F pattern cells, is consistent with our conclusion that mouse spermatozoa cannot complete capacitation in about 1 $\text{mmol Na}^+ \text{l}^{-1}$.

Series III: What is the minimum extracellular Na^+ concentration required to support both capacitation and acrosomal exocytosis?

In these experiments, we incubated mouse sperm suspensions in media with Na^+ concentrations of 25, 50, 75, 100, 125 and 150 mmol l^{-1} . Two suspensions were prepared, one in low

Fig. 3. Acrosome loss in mouse sperm suspensions incubated for 120 min in media with increasing concentrations of Na⁺ (25–150 mmol l⁻¹). Data are presented as mean % ± SEM (*n* = 3). ****P* < 0.01, *****P* < 0.001 compared with control (150 mmol Na⁺ l⁻¹) suspensions.

Fig. 4. Acrosome loss in mouse sperm suspensions incubated in control medium (150 mmol Na⁺ l⁻¹) for 30 min; monensin (0.1–2.5 μmol l⁻¹) was added and suspensions were incubated a further 10 min. Data are presented as mean % ± SEM (*n* = 3). ****P* < 0.01, *****P* < 0.001 compared with control (0 monensin) suspensions.

Na⁺ medium (25 mmol l⁻¹) and one in high Na⁺ medium (150 mmol l⁻¹), and appropriate proportions of the two were quickly mixed together to produce the desired final Na⁺ concentrations. Suspensions were then incubated for 120 min, filtered, fixed and assessed (*n* = 3).

The results (Fig. 3) indicate that none of the Na⁺ concentrations evaluated, except for 150 mmol l⁻¹, supported the acrosome reaction; fewer than 10% of cells in 25–125 mmol Na⁺ l⁻¹ had lost the acrosomal cap. Since about 10% of filtered cells are non-motile and could have begun to lose the acrosomal cap through degenerative changes, only values >10% are deemed to represent a positive response. We therefore conclude that a concentration of >125 mmol extracellular Na⁺ l⁻¹ is required to support acrosomal exocytosis; all conditions tested would have supported capacitation (see Fig. 1).

Series IV: Does the extracellular Na⁺ need to be internalized to promote capacitation and the acrosome reaction?

Here we used the monovalent cation ionophore monensin which has greater selectivity for Na⁺ than K⁺ (Pressman, 1976) to determine whether the effects observed in high extracellular Na⁺ involved internalization of Na⁺. In standard Tyrode's medium, with 150 mmol Na⁺ l⁻¹, monensin would transport Na⁺ into the cells as the intracellular Na⁺ concentration would be considerably lower (Hyne *et al.*, 1985).

In the first experiments, sperm suspensions were prepared in high Na⁺ medium and incubated for 30 min. Four aliquots were removed, three receiving monensin to give final concentrations of 2.5, 0.5 and 0.1 μmol l⁻¹ and the fourth receiving medium only (control). After gassing, samples were incubated for 10 min, then filtered, fixed and assessed (*n* = 3). The results (Fig. 4) indicate that there is a significant, concentration-dependent stimulation of the acrosome reaction by monensin (*P* < 0.01–0.001, depending on monensin concentration). Although a mean of only 6% of control cells had lost the acrosomal cap, the mean values in the monensin-treated samples were 22% in 0.1, 42% in 0.5 and 55% in 2.5 μmol l⁻¹. From these results we conclude that extracellular Na⁺ is internalized during capacitation and acrosomal exocytosis.

Fig. 5. Chlortetracycline (CTC) fluorescence patterns in mouse sperm suspensions incubated for 30 min in low Na⁺ (25 mmol l⁻¹) medium; monensin (0.5 μmol l⁻¹) was added to one aliquot and suspensions were incubated for a further 10 min. ▨ acrosome intact, uncapacitated cells, ■ acrosome intact, capacitated cells. Data are presented as mean % ± SEM (*n* = 3). ****P* < 0.01 compared with control (0 monensin) suspensions.

We next investigated the effect of monensin treatment on capacitation and exocytosis in sperm suspensions incubated in low Na⁺ medium. Suspensions were prepared in low Na⁺ and high Na⁺ media, the latter serving as a control, and incubated for 30 min. Two aliquots of each suspension were transferred into small sterile plastic tubes, one receiving monensin to give a final concentration of 0.5 μmol l⁻¹ and the other medium alone. All tubes were gassed and incubated for 10 min, then filtered, fixed and prepared for CTC assessment (*n* = 3).

Distribution of the CTC patterns after treatment with and without monensin are shown (Fig. 5). In continuous low Na⁺ after 40 min most of the cells were exhibiting the uncapacitated F pattern, with only 7.3 ± 1.5% of the cells in the capacitated, acrosome-intact B pattern and no AR cells. In contrast, the 10 min incubation in monensin promoted a significant (*P* < 0.01) shift in the distribution to 44.0 ± 5.3% of cells in

the B pattern, but still no AR cells. From these results we conclude that sufficient extracellular Na^+ is present in low Na^+ medium to be internalized by monensin and to promote capacitation, but insufficient to stimulate acrosomal exocytosis. In all experiments with monensin, treated cells exhibited reduced motility.

Series V: Does the requirement for Na^+ indicate a role for a $\text{Na}^+-\text{Ca}^{2+}$ exchanger?

Given that internalization of extracellular Na^+ appears to be required for capacitation and the acrosome reaction in mouse spermatozoa, is the Na^+ acting via a direct or an indirect mechanism? One possibility is that increasing intracellular Na^+ might activate a $\text{Na}^+-\text{Ca}^{2+}$ exchanger such that Na^+ is pumped out of the cell and Ca^{2+} in. The resulting increase in intracellular Ca^{2+} might be the direct effector of the responses observed. To investigate this possibility, we incubated sperm suspensions in media containing $150 \text{ mmol Na}^+ \text{ l}^{-1}$ plus differing amounts of CaCl_2 for 30 min, then added monensin, incubated for a further 10 min and assessed the CTC patterns. The conditions chosen were: Ca^{2+} -deficient, $90 \mu\text{mol CaCl}_2 \text{ l}^{-1}$ and $1.80 \text{ mmol CaCl}_2 \text{ l}^{-1}$. The Ca^{2+} -deficient medium itself does not support complete capacitation and the introduction of millimolar Ca^{2+} does not trigger a rapid spontaneous acrosome reaction (Fraser, 1983, 1987; Fraser and McDermott, 1992). The micromolar Ca^{2+} will support capacitation *per se* and provides sufficient extracellular Ca^{2+} for the Ca^{2+} ionophore A23187 to promote a rapid transition from the uncapacitated to the capacitated, acrosome-intact state as assessed by CTC; however, there is insufficient Ca^{2+} to promote acrosomal exocytosis (Fraser and McDermott, 1992). The millimolar Ca^{2+} supports complete capacitation and the acrosome reaction.

A sperm suspension was prepared in Ca^{2+} -deficient medium ($-\text{Ca}^{2+}$); after 5 min to allow dispersal of cells, three aliquots were transferred into sterile tubes and medium or Ca^{2+} -containing stock solutions were added to produce the three conditions assessed: Ca^{2+} -deficient ($-\text{Ca}^{2+}$); $90 \mu\text{mol Ca}^{2+} \text{ l}^{-1}$ (low Ca^{2+}); $1.80 \text{ mmol CaCl}_2 \text{ l}^{-1}$ (control Ca^{2+}). After mixing and gassing, suspensions were incubated for 30 min and then monensin stock was added to give a final concentration of $0.5 \mu\text{mol l}^{-1}$. After a further 10 min incubation, cells were prepared for CTC assessment ($n = 3$).

Results (Fig. 6) indicated that monensin had no effect on suspensions incubated in either $-\text{Ca}^{2+}$ or low Ca^{2+} media: $94.0 \pm 0.7\%$ and $93.9 \pm 0.7\%$ of cells, respectively, exhibited the F pattern after 10 min in monensin, with the remaining cells in the B pattern (no AR cells observed). In marked contrast, significant differences ($P < 0.01$ – 0.001) in the distribution of CTC patterns were observed in the control Ca^{2+} medium plus monensin: $17.0 \pm 4.6\%$ F pattern cells, $36.3 \pm 7.1\%$ B pattern cells and $46.0 \pm 2.5\%$ AR pattern cells. There was sufficient Na^+ present in all groups to support capacitation and the acrosome reaction and sufficient Ca^{2+} in the low Ca^{2+} medium to support capacitation *per se* if Ca^{2+} had been brought into the cell in exchange for extruded Na^+ . The fact that monensin could not promote capacitation in low Ca^{2+} , as evidenced by no change in the proportion of B cells compared with $-\text{Ca}^{2+}$ medium, suggests that a $\text{Na}^+-\text{Ca}^{2+}$ exchanger does not play a role during capacitation.

Fig. 6. CTC fluorescence patterns in mouse sperm suspensions incubated for 30 min in high Na^+ (150 mmol l^{-1}) medium containing no added Ca^{2+} ($-\text{Ca}^{2+}$), $90 \mu\text{mol Ca}^{2+} \text{ l}^{-1}$ (low Ca^{2+}) or $1.80 \text{ mmol Ca}^{2+} \text{ l}^{-1}$ (control Ca^{2+}); monensin ($0.5 \mu\text{mol l}^{-1}$) was added to all three suspensions and the incubation continued for a further 10 min. ▨ acrosome intact, uncapacitated cells, ■ acrosome intact, capacitated cells, ▤ acrosome reacted capacitated cells. Data are presented as mean $\% \pm \text{SEM}$ ($n = 3$). *** $P < 0.01$, **** $P < 0.001$ compared with $-\text{Ca}^{2+}$ and low Ca^{2+} suspensions, respectively.

Series VI: Does the requirement for Na^+ indicate a role for a Na^+-K^+ ATPase?

Several studies have provided evidence for a Na^+-K^+ ATPase that pumps Na^+ out and K^+ into the cell. To investigate the possibility that the requirement for Na^+ reflects an action via this pump, we incubated cells in the presence of ouabain, an inhibitor of the Na^+-K^+ ATPase (Robinson and Flashner, 1979). In the same experiments we also assessed the responses to nigericin, a monovalent cation ionophore with greater specificity for K^+ than for Na^+ . However, because the K^+ gradient is the reverse of the Na^+ gradient (with low extracellular K^+ and high intracellular K^+), the net effect of nigericin is to move K^+ out and Na^+ in, just as monensin does (e.g. Hyne, 1984).

A sperm suspension was prepared in standard Tyrode's medium; after 5 min for dispersal, an aliquot was transferred to a second paraffin-containing culture dish and ouabain stock solution was added to give a final concentration of $100 \mu\text{mol l}^{-1}$. Both suspensions ($\pm$ ouabain) were incubated for 30 min, and an aliquot was then removed from each and transferred to a small sterile plastic tube; nigericin stock was added to give a final concentration of $5 \mu\text{mol l}^{-1}$. After gassing, all four treatment groups were incubated for a further 10 min, then filtered, fixed and assessed ($n = 3$).

Ouabain had no detectable effect on the acrosome reaction: $+\text{Na}^+$ control, $4.2 \pm 1.0\%$ reacted cells and $+\text{Na}^+ + \text{ouabain}$, $1.7 \pm 1.1\%$ (Fig. 7). In contrast, nigericin significantly ($P < 0.001$) increased the proportion of acrosome-reacted cells in both groups: $+\text{Na}^+ \rightarrow \text{nigericin}$, $71.8 \pm 7.8\%$ and $+\text{Na}^+ + \text{ouabain} \rightarrow \text{nigericin}$, $65.2 \pm 20.6\%$. Nigericin treatment also markedly reduced forward progression in the treated spermatozoa, leaving the majority just twitching or moving feebly.

Fig. 7. Acrosome loss in mouse sperm suspensions incubated in control (150 mmol Na⁺ l⁻¹) medium with and without ouabain (100 µmol l⁻¹) for 30 min; aliquots from each suspension received 5 µmol nigericin l⁻¹ and all four suspensions were incubated for a further 10 min. Medium composition: 1, +Na⁺; 2, +Na⁺ + ouabain; 3, +Na⁺ + nigericin; 4, +Na⁺ + ouabain + nigericin. Data are presented as mean % ± SEM (*n* = 3). *****P* < 0.001 compared with control (+Na⁺) suspensions.

The fact that nigericin stimulated a marked response was not surprising, given that it has the same net effect on the cell as monensin, which also stimulated the acrosome reaction. As ouabain failed to elicit a response, the results suggested that a Na⁺-K⁺ ATPase does not play a role in triggering the acrosome reaction. However, this method of assessment does not indicate whether there was an effect on capacitation *per se*. We therefore incubated sperm suspensions in the presence of 100 µmol ouabain l⁻¹ for 40 min, as above; the cells were then filtered and assessed with CTC (*n* = 3).

Results (Fig. 8) indicated that ouabain promoted a significant (*P* < 0.01) shift in the distribution of CTC patterns towards the B (capacitated, acrosome-intact) pattern: control suspensions with 81.7 ± 4.5% Fs and 16.3 ± 3.6% Bs compared with ouabain-treated suspensions having 63.7 ± 5.4% Fs and 35.3 ± 4.8% Bs. As observed in previous experiments (Fig. 7), very few acrosome-reacted cells were found. Thus, these CTC data indicate that capacitation was accelerated in the presence of ouabain, suggesting that a Na⁺-K⁺ ATPase may be involved in capacitation but possibly not in acrosomal exocytosis. Since the net effect of the inhibitor would be to reduce the Na⁺ efflux, it is possible that during normal capacitation enzyme activity declines as capacitation progresses, thereby allowing the intracellular Na⁺ concentration to rise.

Series VII: Does the requirement for Na⁺ indicate an effect mediated by a rise in intracellular pH?

It is possible that the Na⁺ requirement for support of capacitation and the acrosome reaction reflects a role for a Na⁺-H⁺ exchange mechanism, leading to Na⁺ in, H⁺ out and thus a rise in intracellular pH (pH_i). To investigate this possibility, we incubated spermatozoa in complete medium for various periods, and then introduced NH₄Cl. The latter has been used to increase intracellular pH in various cell types (Winkler and Grainger, 1978).

Fig. 8. CTC fluorescence patterns in mouse sperm suspensions incubated for 40 min in control (+Na⁺) medium in the absence or presence of 100 µmol ouabain l⁻¹. ▨ acrosome intact, uncapacitated cells, ■ acrosome intact, capacitated cells, ▩ acrosome reacted, capacitated cells. Data are presented as mean % ± SEM (*n* = 3). ****P* < 0.01 compared with control (0 ouabain) suspensions.

Initially, a sperm suspension was prepared in standard Tyrode's medium and incubated for 30 min; NH₄Cl was added (both 5 and 10 mmol l⁻¹ were investigated) and the incubation continued for 20 min. As this approach had no detectable effect on the acrosome reaction, suspensions were incubated for 120 min and then given 10 mmol NH₄Cl or NaCl l⁻¹; a medium-only control was also included. After 20 min further incubation, all three suspensions were filtered, fixed and assessed (*n* = 4). Results indicated a significantly (*P* < 0.025) higher proportion of acrosome-reacted cells in the NH₄Cl-treated group (45.4 ± 14.3%) than in the NaCl- (31.9 ± 11.3%) and control medium-treated (30.1 ± 10.3%) groups.

The fact that a response to NH₄Cl was elicited only in capacitated suspensions suggests that a mechanism involving an increase in pH_i is likely to play an important role only in events associated with acrosomal exocytosis and not during capacitation *per se*. To test this further, we prepared suspensions in low Na⁺ and high Na⁺ media. After incubation for 120 min, an aliquot of low Na⁺ suspension was removed and received NH₄Cl to give a final concentration of 25 mmol l⁻¹. Two other aliquots were removed from the same suspension, one receiving choline chloride at a final concentration of 25 mmol l⁻¹ (to serve as a control for the increase in osmolality) and the other, low Na⁺ medium. An aliquot of the control suspension received a small quantity of standard medium; these last two served as controls for the dilution effected in the experimental groups. After 10 min, cells were filtered and prepared for CTC assessment (*n* = 3).

The introduction of NH₄Cl to the low Na⁺ suspensions caused a significant (*P* < 0.01) increase in the proportion of AR cells and a concomitant decrease (*P* < 0.05) in the proportion of B cells (Fig. 9). The values obtained in this group were very similar to those in the standard Na⁺ control group, with about 45% B and about 24% AR cells. The response to NH₄Cl was not due simply to a change in osmolality since the choline chloride control did not differ significantly from the low Na⁺ control, exhibiting < 10% AR cells. Given that the NH₄Cl effect was observed in the presence of low Na⁺, these results provide further that the Na⁺ requirement for acrosomal exocytosis may involve

Fig. 9. CTC fluorescence patterns in mouse sperm suspensions incubated for 120 min in low Na⁺ (25 mmol l⁻¹) or high Na⁺ (150 mmol l⁻¹) media. Aliquots of the low Na⁺ suspensions received 25 mmol NH₄Cl or choline Cl l⁻¹ and all suspensions were incubated for a further 10 min. □ acrosome intact, uncapsulated cells, ▨ acrosome intact, capacitated cells, ■ acrosome reacted, capacitated cells. Data are presented as mean % ± SEM (*n* = 3). **P* < 0.05, ****P* < 0.01 compared with low Na⁺ suspensions.

a Na⁺-H⁺ exchange and a consequent increase of intracellular pH_i.

A sperm suspension prepared in low Na⁺ medium was incubated for 30 min, filtered and NH₄Cl (25 mmol l⁻¹) was added to one aliquot to make certain that NH₄Cl treatment only affected capacitated cells. After 10 min, both suspensions were prepared for CTC assessment (*n* = 3). Results indicated that NH₄Cl treatment at this early time had no effect on either the capacitation state or acrosomal exocytosis. The values for control and NH₄Cl-treated suspensions, respectively, were as follows: F, 68 and 67%; B, 29 and 26%; AR, 3 and 7%.

Series VIII: What is the effect of amiloride on acrosomal exocytosis in capacitated cells?

Since amiloride has been shown to inhibit Na⁺ fluxes in various somatic cells (e.g. Smith *et al.*, 1982; Krieger and Kim, 1988), we investigated the effect of amiloride on acrosomal exocytosis in mouse spermatozoa. A sperm suspension was prepared in standard Tyrode's medium. After 5 min for dispersal, three aliquots were transferred to culture dishes containing paraffin oil and amiloride stock solutions were added to produce final concentrations of 50, 150 and 500 μmol l⁻¹. All four suspensions were incubated for 120 min, then filtered, fixed and assessed (*n* = 3). Results indicated a concentration-dependent stimulation of acrosomal exocytosis (Fig. 10), with both 150 and 500 μmol amiloride l⁻¹ having a significant effect (*P* < 0.05 and *P* < 0.001, respectively). The highest concentration of amiloride also substantially reduced sperm motility.

Discussion

In this study we investigated the requirements for extracellular Na⁺ during capacitation and the acrosome reaction in mouse spermatozoa incubated *in vitro*. Using a variety of analytical

Fig. 10. Acrosome loss in mouse sperm suspensions incubated for 120 min in control (150 mmol Na⁺ l⁻¹) medium and increasing concentrations of amiloride. Data are presented as mean % ± SEM (*n* = 3). **P* < 0.05, *****P* < 0.001 compared with control (0 amiloride) suspensions.

approaches, we demonstrated that a relatively low concentration of Na⁺, > 1 ≤ 25 mmol l⁻¹, will support capacitation. Sperm cells incubated for 120 min in medium containing 25 mmol Na⁺ l⁻¹ would not undergo spontaneous acrosomal exocytosis, but within 10 min of increasing the Na⁺ to about 138 mmol l⁻¹ a significant increase in the proportion of acrosome-reacted cells was observed. As only capacitated cells can undergo such rapid exocytosis, these results indicate that incubation in low Na⁺ does support capacitation, a conclusion confirmed by CTC evaluation. Most cells incubated in low Na⁺ exhibited the capacitated, acrosome-intact B pattern; the introduction of high Na⁺ caused a decrease in B pattern cells and an increase in AR pattern cells. Finally, sperm suspensions preincubated in low Na⁺ medium were non-fertilizing when tested *in vitro*, but were highly fertile as soon as the Na⁺ concentration was increased (Table 1). In contrast, incubation of suspensions for 120 min in medium containing 1 mmol Na⁺ l⁻¹ did not support capacitation. These cells could undergo acrosomal exocytosis in response to increased Na⁺ and CTC analysis revealed that most exhibited the uncapsulated, acrosome-intact F pattern.

Whereas extracellular Na⁺ requirements for capacitation are quite low, those for occurrence of the acrosome reaction are much higher. By incubating suspensions in media with increasing amounts of Na⁺, ranging from 25 to 150 mmol l⁻¹, we found that capacitation plus acrosomal exocytosis required > 125 mmol Na⁺ l⁻¹. From the first experimental series, 138 mmol l⁻¹ proved to be sufficient, suggesting that the minimum is > 125 ≤ 138. Extracellular Na⁺ is also required to support the acrosome reaction in other spermatozoa, including those of invertebrates (e.g. sea urchin, Schackmann *et al.*, 1978; Schackmann and Shapiro, 1981) and guinea-pigs (Hyne *et al.*, 1984; Murphy *et al.*, 1986). Hyne *et al.* (1984) reported that at least 125 mmol Na⁺ l⁻¹ were needed for the acrosome reaction to occur, whereas Murphy *et al.* (1986) obtained responses at lower concentrations, with 60 mmol Na⁺ l⁻¹ supporting exocytosis in most cells. Requirements for capacitation were not addressed directly.

Elemental analyses of female murine reproductive tract fluids (Borland *et al.*, 1977) indicated fluctuation in the mean concentrations of sodium, with the lowest value (114 ± 6 mmol l⁻¹)

for uterine fluid just after mating. From the present study, such conditions would promote only capacitation. Values for ampullary fluid were slightly higher, at $121 \pm 7 \text{ mmol l}^{-1}$, and are near the threshold where acrosomal exocytosis and hence fertilization would also be supported. Values for bursal sac fluid were considerably higher, at $156 \pm 3 \text{ mmol l}^{-1}$, and would probably contribute to the immediate environment of recently ovulated oocytes. Thus there should be sufficient Na⁺ present in the female tract to promote sperm function including fertilization. The low values for uterine fluid probably reflect the introduction of seminal plasma which had $88 \text{ mmol sodium l}^{-1}$ (Borland *et al.*, 1977).

These female tract fluids also have concentrations of potassium that differ from that found in serum. In the mouse, the value for ampullary fluid shortly after mating was $28.6 \pm 2.2 \text{ mmol l}^{-1}$, whereas in serum it was 5.26 ± 0.15 and in bursal sac fluid it was $6.93 \pm 0.66 \text{ mmol l}^{-1}$ (Borland *et al.*, 1977). Experimental investigation of iso-osmotic medium with high K⁺ (27.7 mmol l^{-1}) and reduced Na⁺ (125 mmol l^{-1}), similar to the composition experienced by the spermatozoa *in vivo*, indicated that mouse sperm capacitation could proceed with kinetics indistinguishable from those in control medium (2.7 and 150 mmol l^{-1} for K⁺ and Na⁺, respectively). However, the cells could neither undergo spontaneous acrosomal exocytosis nor fertilize oocytes *in vitro* until the K⁺ was reduced to $<10 \text{ mmol l}^{-1}$; this last adjustment would also result in an increase in Na⁺ (Fraser, 1983). Looking again at these results in the light of the present study, it can be seen that the iso-osmotic, high K⁺ medium had insufficient Na⁺ to support acrosomal exocytosis and this, then, at least partially explains the inhibitory effects of that medium. Sperm motility was quite sluggish in the presence of high K⁺ (Fraser, 1983), an effect not attributable to reduced Na⁺, as in the present study cells were vigorously motile in both low and high Na⁺ media.

We have previously proposed that endogenous changes in the composition of female tract fluids, with particular reference to K⁺, may serve to modulate and control expression of sperm functional potential (Fraser, 1983). This has been further strengthened by our observations regarding Na⁺. The composition of the fluid in which the ejaculated spermatozoa find themselves is one that will support capacitation: there are adequate concentrations of Na⁺, K⁺ and Ca²⁺. The Na⁺ is probably just below the necessary threshold and the K⁺ is too high to promote the acrosome reaction and fertilization. At ovulation, however, follicular and bursal sac fluids, with a Na⁺ and K⁺ composition similar to serum, would be released and provide a permissive environment for the full expression of sperm fertilizing potential.

Having established that there is a requirement for extracellular Na⁺ to support the acquisition and expression of fertilizing potential, we next explored the possibility that the ion needed to be internalized to exert its effects. Addition of the monovalent cation ionophore monensin to sperm suspensions preincubated for only 30 min in complete medium containing $150 \text{ mmol Na}^+ \text{ l}^{-1}$ promoted a rapid, concentration-dependent increase in the proportion of acrosome-reacted cells (Fig. 4). Because the majority of acrosome-intact cells at this time would still be uncapacitated, as determined by CTC fluorescence (e.g. Fig. 6), this response indicates an acceleration of capacitation preceding the exocytotic event. In contrast, when suspensions

were preincubated in low Na⁺ medium, monensin did not trigger acrosomal exocytosis. It did, however, accelerate capacitation as evidenced by a significant increase in the proportion of cells exhibiting the B pattern of CTC fluorescence. Although we have not measured intracellular concentrations of Na⁺ in mouse spermatozoa, published values for guinea-pig spermatozoa are less than 10 mmol l^{-1} (Hyne *et al.*, 1985); this is sufficiently low to ensure an inward concentration gradient with 25 mmol l^{-1} in the extracellular compartment, a necessary requirement for monensin to effectively promote internalization of Na⁺. Similarly, Hyne (1984) observed that a high Na⁺ concentration was required for monensin to stimulate acrosome reactions in guinea-pig spermatozoa. From these results we conclude that extracellular Na⁺ must be internalized to support both capacitation and acrosomal exocytosis. The concentrations required, even with an ionophore to facilitate the internalization, differ for the two stages: relatively low concentrations suffice for capacitation but only much higher ones for exocytosis. This then suggests that different mechanisms may control intracellular Na⁺ concentrations needed for these separate events.

There is evidence for the presence of a Na⁺-Ca²⁺ exchanger in spermatozoa of several mammalian species: rams (Bradley and Forrester, 1980), bulls (Rufo *et al.*, 1984), boars (Ashraf *et al.*, 1982). Although in somatic cells this system usually functions to pump Ca²⁺ out and hence to maintain low intracellular Ca²⁺, it has been proposed that in sperm cells it may operate in the opposite direction (e.g. Rufo *et al.*, 1984). If intracellular Na⁺ increases, activation of the exchanger could cause Na⁺ to be extruded and Ca²⁺ to enter. We investigated the possibility that such a mechanism might operate during capacitation by incubating suspensions in media with differing concentrations of Ca²⁺, but all with high Na⁺, and then introducing monensin for 10 min. The Ca²⁺-deficient medium used will not support capacitation, whereas the low Ca²⁺ ($90 \mu\text{mol l}^{-1}$) medium will (Fraser, 1987). In the latter, the ionophore A23187 can promote a rapid change from the uncapacitated to the capacitated acrosome-intact state, but there is insufficient Ca²⁺ to trigger acrosomal exocytosis (Fraser and McDermott, 1992). If the Na⁺-Ca²⁺ exchanger does function during capacitation, the rise in intracellular Na⁺ caused by monensin should trigger an influx of Ca²⁺, there being sufficient Ca²⁺ present to support capacitation. Our data, however, clearly indicate that under these conditions monensin did not promote a rapid transition to the capacitated, acrosome-intact state: in both Ca²⁺-deficient and low Ca²⁺ suspensions most (>90%) cells still exhibited the uncapacitated F pattern of CTC fluorescence (Fig. 6). We conclude, therefore, that a Na⁺-Ca²⁺ exchanger does not operate to increase intracellular Ca²⁺ concentrations during capacitation *per se*. However, our results do not exclude the possibility that such an exchange mechanism plays a role during acrosomal exocytosis.

Another mechanism whereby Na⁺ concentrations might alter sperm function is a Na⁺-K⁺ ATPase that acts to pump Na⁺ out and K⁺ in. When suspensions were incubated in the presence of ouabain, a Na⁺-K⁺ ATPase inhibitor (Robinson and Flashner, 1979), there was no effect on acrosomal exocytosis but CTC analysis revealed a significant increase in the proportion of B pattern cells (Fig. 8). Thus inhibiting the ATPase, which would normally function to maintain a low intracellular

Na^+ concentration, accelerated capacitation. We therefore propose that, if this ATPase does play a role during mouse sperm capacitation, its endogenous activity must either be low, with a minimal effect on the intracellular Na^+ concentration, or decrease as capacitation proceeds. This would allow the slight increase in intracellular Na^+ required for capacitation. In contrast, Mrsny *et al.* (1984) have reported that ATPase activity that can be inhibited by ouabain, assumed to represent the Na^+-K^+ ATPase, increased during hamster sperm capacitation *in vitro*. They therefore proposed a positive role for the enzyme during capacitation.

Low or declining Na^+-K^+ ATPase activity would also affect the intracellular K^+ concentration. However, it has been demonstrated that extracellular K^+ is not required for mouse sperm capacitation *in vitro* although it is required for the acrosome reaction and fertilization (Fraser, 1983). Our proposal that this ATPase would be relatively inactive is therefore consistent with the known ionic requirements for mouse sperm capacitation. We have not addressed the possibility that the ATPase may play a role in initiating the acrosome reaction, given our focus on Na^+ and the demonstrable requirement for a large Na^+ influx to achieve acrosomal exocytosis. In other species, this ATPase has been investigated, particularly in conjunction with K^+ requirements. Mrsny and Meizel (1981) reported that ouabain could inhibit the acrosome reaction in capacitated hamster sperm suspensions and proposed that the ATPase played an important role in modulating a K^+ influx needed for exocytosis. In contrast, Hyne *et al.* (1984) demonstrated that the presence of ouabain did not inhibit guinea-pig acrosomal exocytosis which would argue against the enzyme playing an obligatory role in the initiation of the acrosome reaction in that species.

Lastly, we investigated the possibility that Na^+ might act via a Na^+-H^+ exchange mechanism, causing a rise in pH_i and consequently altering sperm functional state. In these experiments we introduced NH_4Cl , a compound that has been used successfully to increase pH_i in somatic cells (Winkler and Grainger, 1978). Our results indicated that while NH_4Cl did not obviously alter the kinetics of capacitation *per se*, it could trigger acrosomal exocytosis in capacitated suspensions. This was most convincingly demonstrated when the addition of 25 mmol NH_4Cl l^{-1} to suspensions capacitated in low Na^+ medium promoted acrosomal exocytosis to the same extent as observed in control (high Na^+) suspensions (Fig. 9); low Na^+ does not usually permit the cells to progress from the capacitated, acrosome-intact state to the acrosome-reacted state (e.g. Fig. 1). Thus, it is not high Na^+ *per se* that is obligatory for acrosomal exocytosis, but rather a rise in pH_i . We therefore suggest that when the intracellular Na^+ concentration reaches a threshold in capacitated cells, it plays a role in acrosomal exocytosis by causing an efflux of H^+ and a consequent rise in pH_i .

There is clear evidence that an increase in pH_i is associated with the initial stages of acrosomal exocytosis (e.g. Meizel and Deamer, 1978; Garcia-Soto *et al.*, 1985, 1987; Florman *et al.*, 1989; Lee and Storey, 1989). It is also known that monensin causes an initial Na^+ in and H^+ out exchange followed by H^+ in and K^+ out, resulting in an overall electroneutral Na^+ in and K^+ out exchange (Hyne, 1984). Thus, the temporary rise in pH_i might play a role in monensin-induced responses. On the other hand, nigericin which has the same final effect as monensin with

Na^+ in and K^+ out, causes an initial H^+ in and K^+ out followed by Na^+ in and H^+ out exchange (Hyne, 1984). It is therefore less likely that these transient changes in pH_i are important, but the ensuing increased intracellular Na^+ might promote a more sustained efflux of H^+ . Indeed, Garcia-Soto *et al.* (1987) observed that nigericin treatment of sea urchin spermatozoa causes a rise in pH_i of 0.51 pH units. When the pH_i reaches a threshold, a Ca^{2+} influx is triggered and acrosomal exocytosis ensues.

There is evidence that such a rise in pH_i initiates Ca^{2+} influx by activating Ca^{2+} channels in sea urchin spermatozoa (Garcia-Soto *et al.*, 1985; Guerrero and Darszon, 1989). Our data suggest that a similar series of events may occur in mouse spermatozoa. In an earlier study we showed that dihydropyridine-sensitive Ca^{2+} channels play a role in modulating the Ca^{2+} influx associated with mouse sperm acrosomal exocytosis (Fraser and McIntyre, 1989). The fact that NH_4Cl treatment was effective only in capacitated cells is consistent with our observation that Ca^{2+} channels do not appear to play a role during capacitation *per se* (Fraser and McIntyre, 1989). Furthermore, the response of capacitated cells to amiloride may reflect the action on Ca^{2+} channels rather than on Na^+ transport. Breitbart *et al.* (1990) reported that amiloride analogues stimulate Ca^{2+} uptake in epididymal bull spermatozoa by activating Ca^{2+} channels. This interpretation of our data is consistent with the recent report by Florman *et al.* (1992) that membrane depolarization of mouse, ram and bull spermatozoa in alkaline K^+ -based media, conditions that would promote an increase in pH_i , promoted both an increase in intracellular Ca^{2+} concentrations and subsequent acrosomal exocytosis. Exocytosis could be inhibited by preincubation with dihydropyridines, strongly suggesting that activation of voltage-dependent Ca^{2+} channels played a role in events leading to exocytosis.

In conclusion, the data presented in the present study indicate that Na^+ plays an important role during both capacitation and acrosomal exocytosis. The intracellular Na^+ concentration appears to rise moderately during capacitation, perhaps aided by a reduction in the activity of the Na^+-K^+ ATPase and hence a decline in the rate at which Na^+ is pumped out. In capacitated cells, we suggest that a larger rise in intracellular Na^+ activates Na^+-H^+ exchange, leading to an increase in pH_i and consequent opening of Ca^{2+} channels. The resulting influx of Ca^{2+} then triggers exocytosis. Current evidence indicates that initiation of acrosomal exocytosis in the fertilizing mouse spermatozoon is triggered by interaction with ZP3, one of the glycoproteins making up the zona pellucida (Wassarman, 1990). Assessment of individual capacitated bull sperm cells exposed to solubilized zona material revealed rises in pH_i and intracellular Ca^{2+} (Florman *et al.*, 1989). Furthermore, Florman *et al.* (1992) demonstrated that zona-induced exocytosis in mammalian spermatozoa could be inhibited by dihydropyridines, again implicating Ca^{2+} channels in the signal transduction pathway. It may be that one of the first responses to ZP3 is a rise in intracellular Na^+ which would lead to increased pH_i and subsequent activation of Ca^{2+} channels.

We thank Susan Adeoya-Osiguwa for stimulating discussions. This study was supported in part by a grant from the Medical Research Council.

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