Measurement of anionic sites of rat epididymal spermatozoa using tritiated polycationized ferritin

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Summary. Anionic sites of rat epididymal spermatozoa were measured at pH 7.4 using tritiated polycationized ferritin. The spermatozoa from the caput region had $1.25 \pm 0.06 \times 10^6$ anionic sites per cell and a binding constant of $1.26 \pm 0.01 \times 10^6 \text{ M}^{-1}$. Spermatozoa from the cauda region had $1.50 \pm 0.09 \times 10^6$ anionic sites per cell and a binding constant of $4.84 \pm 0.82 \times 10^6 \text{ M}^{-1}$. The values were mean $\pm$ s.d. The anionic sites were partly sensitive to treatments with neuraminidase, trypsin and Triton X-100.

Introduction

Increase in the net negative charges on the sperm surface during epididymal transit has been demonstrated by electrophoresis (for review, see Bedford & Cooper, 1978) and by an isoelectrofocussing technique (Moore, 1979; Hammerstedt, Keith, Hay, Deluca & Amann, 1979). Since the increase could be due to an increase in the negative charge residues or to a decrease in the positive charge residues on the sperm surface, it is necessary to quantitate each type of charged residue separately.

The binding of colloidal iron hydroxide has been used to demonstrate the increase in the negative charges on maturing spermatozoa (Bedford & Cooper, 1978; Holt, 1980). However, the acidic pH required for binding colloidal iron hydroxide is non-physiological and likely to alter the negatively charged residues to various degrees, depending on their pK_a values (Hughes, 1976). It is therefore unsatisfactory to relate such binding data to the negative charges on the sperm surface at physiological pH. The negative charges or anionic sites on cell surfaces at neutral pH can now be measured by techniques involving cationized ferritin (Lin-Liu & Bondareff, 1981) and tritiated polycationized ferritin (Grinnell & Hays, 1979). We have used the latter method to re-examine the increase in the sperm surface negative charges during epididymal transit.

Materials and Methods

Spermatozoa were extruded from adult albino rats into phosphate-buffered saline (PBS) containing 0.15 M-NaCl in 5 mM-phosphate, pH 7.4 at 4°C as previously described (Chulavatnatol, Hasibuan, Yindepit & Eksittikul, 1977). The cells were washed 5 times by centrifugation at 600 *g* for 15 min at 4°C and finally resuspended in ice-cold PBS. The cell suspension was then inspected under a phase-contrast microscope and found to be free of contaminating debris. The spermatozoa were counted in a haemocytometer after washing.

Tritiated polycationized ferritin ($[^3\text{H}]\text{PCF}$) was prepared according to the method of Grinnell

& Hays (1979). A mixture (1 ml) of 30 mg ferritin, 30 μ Ci 2,3-[N- 3 H]putrescine-HCl (sp. act. 25.4 Ci/mmol; New England Nuclear, Boston, Massachusetts, U.S.A.) and 80 mg 1-ethyl-3-(3-dimethyl)-carbodiimide-HCl (EDC; Pierce Chemical, Rockford, Illinois, U.S.A.) was adjusted to pH 6.5 with HCl and incubated at room temperature (27°C) with gentle constant shaking. After 30 min, a solution (4 ml) containing 2 M-putrescine-HCl (Sigma, St Louis, Missouri, U.S.A.) and 320 mg EDC was added. The incubation was allowed to continue for another 2 h, during which the pH of the solution was maintained at 6.5 by periodic additions of HCl. Then the solution was left at room temperature overnight. Excess putrescine was removed by exhaustive dialysis at 4°C for 24 h against 3 changes of 4 litres 0.15 M-NaCl. The [3 H]PCF was stored at 4°C until use. The protein concentration in the [3 H]PCF preparation was determined by its absorbance at 279 nm and calculated using an extinction coefficient value of a 1% solution at 279 nm of 79.9. From three preparations, the average specific radioactivity was 194 c.p.m./ μ g protein or 9.1×10^{13} c.p.m./mol [3 H]PCF, based on the M_r of PCF being 467 000.

Binding of [3 H]PCF (100 μ g) with washed spermatozoa (1.2×10^7) in PBS was performed in a total volume of 250 μ l at room temperature for 10 min. At the end of the incubation, the spermatozoa were centrifuged through 1 ml of 10% Ficoll-PBS in a Pasteur pipette with sealed tip at 600 g for 10 min at room temperature. The packed spermatozoa were collected by breaking the tip of the pipette into 10 ml of 25% Triton X-114/xylene scintillation fluid and the bound radioactivity was determined using a Packard liquid scintillation counter.

Treatments of the spermatozoa by protease-free neuraminidase (*Vibrio cholera*, 0.1 unit/ml), by trypsin (0.3 mg/ml) or by Triton X-100 (1%, v/v) containing 2 mM-dithiothreitol before the binding with [3 H]PCF were performed as described previously (Toowicharanont & Chulavatnatol, 1983).

Results

Binding conditions

To measure the anionic sites of the spermatozoa correctly, the incubation time, sperm concentration, amount of [3 H]PCF added and intactness of the spermatozoa were considered. Using 100 μ g [3 H]PCF and the PBS-washed spermatozoa from the cauda epididymidis (1.2×10^7 cells), binding was rapid, reaching half equilibrium in 0.5 min and full equilibrium within 10 min (data not shown). Using 100 μ g [3 H]PCF and an incubation time of 10 min, the binding increased linearly with increasing amount of spermatozoa used up to 1.5×10^7 cells (Text-fig. 1). The amount

Text-fig. 1. Binding of [3 H]PCF to various amounts of rat spermatozoa from the caput (O) and cauda (●) epididymidis. The spermatozoa were washed in PBS and incubated at room temperature with 100 μ g [3 H]PCF for 10 min in PBS. Each point was an average of 2 determinations.

of added [^3H]PCF required to saturate the binding sites of 1.2×10^7 spermatozoa in 10 min was found to be between 80 and 100 μg (Text-fig. 2). The intactness of the spermatozoa was suggested by the observation that the binding curves of the PBS-washed spermatozoa (Text-fig. 2) were identical to those of cells washed by centrifugation through Ficoll (Harrison, 1976) or sucrose (Jones, Pholpramool, Setchell & Brown, 1981) (data not shown). These results led to the binding technique for the PBS-washed spermatozoa as described in 'Materials and Methods'.

Text-fig. 2. Binding of [^3H]PCF to rat spermatozoa from the caput and cauda epididymidis. Before binding, PBS-washed spermatozoa ($\circ$, $\bullet$) were treated with neuraminidase ($\triangle$, $\blacktriangle$), trypsin (∇ , $\blacktriangledown$) or Triton X-100 ($\square$, $\blacksquare$). After treatment, the spermatozoa were washed twice in PBS and then 1.2×10^7 spermatozoa were incubated with various amounts of [^3H]PCF in PBS at room temperature for 10 min. Each point was an average of 2 determinations.

Anionic sites

The amount of [^3H]PCF bound to spermatozoa from the caput epididymidis was significantly less than that bound to the spermatozoa from the cauda (Table 1). During sperm maturation, the calculated number of anionic sites per cell increased by 20% but there was a decrease in the binding constant estimated according to the method of Grinnell & Hays (1979).

Table 1. Binding of tritiated polycationized ferritin ([^3H]PCF) to rat epididymal spermatozoa

Epididymal region	[^3H]PCF bound (pmol/ 10^6 cells)	Anionic sites per cell ($\times 10^{-6}$)	Binding constant (M^{-1}) ($\times 10^{-6}$)
Caput	2.08 ± 0.10 (6)	1.25 ± 0.06 (6)	1.26 ± 0.01 (3)
Cauda	$*2.49 \pm 0.16$ (6)	$*1.50 \pm 0.09$ (6)	$**4.84 \pm 0.82$ (3)

Values are mean $\pm$ s.d. for the no. of observations in parentheses.

Values significantly different from caput values: * $P < 0.05$, ** $P < 0.025$.

Effects of enzymes and detergent

Since treatment with neuraminidase, trypsin or Triton X-100 decreases sperm-bound sialic acids (Toowicharanont & Chulavatnatol, 1983), the sensitivity of the anionic sites on the sperm surface to these treatments was examined (Text-fig. 2). The anionic sites were partly sensitive to pre-treatment by these agents with the following order of sensitivity: Triton X-100 > trypsin > neuraminidase. Furthermore, the anionic sites of spermatozoa from the cauda were less sensitive to each treatment than those of spermatozoa from the caput epididymidis.

Discussion

Polycationized ferritin has long been used to study the ultrastructural topography of cell surface anionic sites (Danon, Goldstein, Marikovsky & Skutelsky, 1972). Since a [^3H]PCF molecule is large and contains numerous positively charged amino groups from putrescine, an anionic site on the sperm surface that is capable of binding it should be a domain of the surface which consists predominantly of negatively charged residues. Therefore, an increase in anionic sites during sperm maturation (Table 1) is interpreted as an increase in negative charge residues, confirming findings with other techniques (Bedford & Cooper, 1978; Hammerstedt *et al.*, 1979; Moore, 1979; Holt, 1980).

For the rat epididymal spermatozoa, the binding was not proportional to the sperm number when a large number ($> 2 \times 10^7$) of spermatozoa was used, suggesting a possible crowding effect. Too much [^3H]PCF is also undesirable since free ferritin, which may be a contaminant of the [^3H]PCF preparation, can bind at a level of ≥ 1 mg/ml to rabbit spermatozoa (Schrader, Eng & Metz, 1981), and can probably compete for the anionic sites. Such interference is not expected in this study because the total [^3H]PCF used was 0.4 mg/ml and contaminating free ferritin, if present, would be much less.

The fact that sperm anionic sites increase (Table 1) while the sperm-bound sialic acids decrease (Toowicharanont & Chulavatnatol, 1983) during sperm maturation suggests that the decrease in sperm sialic acids must be accompanied by a greater increase of other negatively charged groups as spermatozoa mature. Such a change may modify the binding strength of the anionic sites and is reflected by a change in the binding constant (Table 1).

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