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Measurement of Intracellular Calcium Concentration and Plasma Membrane Potential in Human Spermatozoa Using Flow Cytometry

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ABSTRACT: We report 2 novel approaches using flow cytometry to measure intracellular calcium concentration and plasma membrane potential in human spermatozoa. Both approaches have the potential to measure different responses in subpopulations of cells, which is particularly useful when studying heterogeneous populations such as human spermatozoa. Intracellular calcium concentration ($[Ca^{2+}]_i$) was measured using the probe indo-1/AM. This allowed measurements to be made that were independent of variation in cell size and dye loading. It also enabled dead cells to be directly identified and excluded from the analyses without the need for counterstaining. Mean basal $[Ca^{2+}]_i$ was determined as 50 nM (25–75 nM range) and, in response to the agonist progesterone (20 μM), this increased transiently to 195 nM (125–285 nM range) before declining to approximately half the maximal level within 2 minutes (values in parentheses correspond to the range of values typically found within a sperm population from 1 sample). These results are comparable with previously published data on whole sperm populations. Sperm membrane potential (V_M) was assayed using the probe $DiOC_6(3)$. In carefully controlled experiments, a marked depolarization of the plasma membrane potential of capacitated spermatozoa was observed in response to progesterone (20 μM). Following in vitro capacitation, the sperm plasma membrane potential became hyperpolarized compared with the noncapacitated state. Therefore, this technique may be used to assay for sperm capacitation in vitro.

Key words: Sperm, FACS, indo-1, cyanine, capacitation, progesterone.

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Calcium fluxes are central to several key events in mammalian spermatozoa essential for fertilization. In particular the development of hyperactivated motility during sperm capacitation involves modest increases in intracellular calcium concentration ($[Ca^{2+}]_i$) and both zona pellucida- and progesterone-induced acrosomal exocytosis have been shown to be triggered by a marked increase in $[Ca^{2+}]_i$ in the sperm head (Blackmore et al, 1990; Yanagimachi, 1994; Brucker and Lipford, 1995; Suarez and Dai, 1995; Aitken, 1997; Brewis and Moore, 1997; Foresta and Rosatta, 1997).

The significance of membrane potential (V_M) to sperm function has also been recognized (Florman et al, 1998) even though it has been studied in much less detail than calcium. Spermatozoa maintain a negative resting plasma membrane potential and in vitro capacitation causes the plasma membrane to become hyperpolarized (Chou et al, 1989; Florman et al, 1995; Zeng et al, 1995). In contrast, progesterone and zona pellucida proteins have been shown to depolarize the plasma membrane (Calzada et al, 1988; Calzada et al, 1991; Foresta et al, 1993; Florman et al, 1995; Zeng et al, 1995; Arnoult et al, 1996).

Intracellular calcium concentration and membrane potential are closely linked phenomena in mammalian spermatozoa. For example, ZP3 is believed to initiate several different intracellular signals during gamete adhesion (Brewis and Moore, 1997). One pathway is believed to involve the activation of a cation channel (voltage-insensitive) channel and conductance through this channel produces a depolarizing current. This membrane depolarization is believed to cause activation of a voltage-sensitive, T-type calcium channel and there is good evidence that it is the resulting increase in $[Ca^{2+}]_i$ that triggers the acrosome reaction (Florman et al, 1998).

Although the importance of calcium to sperm function has been appreciated for many years (Yanagimachi and Usui, 1974), it is only in the last decade that researchers have been able to directly assay $[Ca^{2+}]_i$ in mammalian spermatozoa. This is the result of the development and use of a variety of membrane permeable ion-selective fluorescent calcium probes that measure the intracellular concentration of free (ie, unbound) calcium (Grynkiewicz

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et al, 1985; Minta et al, 1989; Thomas and Delaville, 1991).

The mean $[Ca^{2+}]_i$ of the whole sperm population has been most commonly measured using fluorescence spectrometry, but these assays cannot detect variation in $[Ca^{2+}]_i$ in different sperm subpopulations (see for example, Thomas and Meizel, 1988; Blackmore et al, 1990; Tesarik et al, 1993; Brewis et al, 1996). Assays based on the measurement of individual cells using fluorescence microscopy have recently been reported (Florman, 1994; Plant et al, 1995; Suarez and Dai, 1995; Tesarik et al, 1996). Although they are technically difficult and time-consuming, advances in low-level light detector systems and image processing have resulted in this approach becoming more widely employed (Florman et al, 1995). However, a major concern is that cells need to be immobilized, which may interfere with the normal functioning of the spermatozoon. Two studies have used flow cytometry with the probe fluo-3/AM to study calcium in boar and human spermatozoa (Harrison et al, 1993; Giojolas, 1998).

Voltage-sensitive lipophilic fluorescent cyanine dyes are routinely used to study cell membrane potential and these techniques have also been used to study spermatozoa (Shapiro, 1990; Florman et al, 1998). Measurements have generally been achieved on whole sperm populations using fluorescence spectrometry (see for example, Chou et al, 1989; Florman et al, 1992; Espinosa and Darszon, 1995; Silvestroni et al, 1997). However, there has been one report of using cyanine dyes with flow cytometry, in which the assay was performed to identify live versus dead spermatozoa rather than measure changes in membrane potential in a live sperm population (Szollosi et al, 1986). Unlike calcium studies, the membrane potential of single sperm cells has never been reported (Florman et al, 1998).

In this paper we describe flow cytometric methodologies using indo-1/AM to measure $[Ca^{2+}]_i$ and changes in plasma membrane potential in human spermatozoa. The benefit of these methods is that they allow differential responses of sperm subpopulations to be distinguished.

Materials and Methods

Chemicals

Minimal essential medium (MEM) and RPMI 1640 (with L-glutamine) were used from Gibco Life Technologies, Paisley, Scotland; Lymphosep™ Lymphocyte Separation Medium was supplied by ICN Biochemicals Ltd (Thame, Oxon, United Kingdom); fetal calf serum was supplied by Advanced Protein Products (Brierley Hill, West Mids, United Kingdom); carbonyl cyanide chlorophenylhydrazone (CCCP), dimethyl sulfoxide (DMSO), ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA), gramicidin D, 1-2[2-amino-5-(6-carboxyindol-2-yl)-phenoxy]-2-(2′-amino-5′-methylphenoxy)-ethane-N,N,N′,N′-tetraacetic acid/acetoxymethyl ester (indo-1/AM), ionomycin, human serum albumin, progesterone, and valinomycin were supplied by the Sigma Chemical Company (Poole, Dorset, United Kingdom); and 3-3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) and propidium iodide (PI) were supplied by Cambridge Bioscience (Newmarket, Cambs, United Kingdom, distributors for Molecular Probes Inc, Eugene, Ore). Unless otherwise stated, all chemicals and reagents were from Sigma Chemical Company.

Preparation of Cells

Lymphocytes were prepared from peripheral human blood using Lymphosep™ Lymphocyte Separation Medium and centrifugation as described by Ray (1989). The cell pellet was then resuspended into 1 mL of RPMI 1640 (with L-glutamine) containing 1% (vol/vol) fetal calf serum (FCS; medium designated as RPMI + FCS). The cell concentration was determined using a Neubauer haemocytometer (2 aliquots of 10 μL) and the sample was adjusted to approximately 5×10^6/mL using RPMI + FCS.

Freshly ejaculated semen was obtained from proven fertile donors by masturbation after 48 to 72 hours of sexual abstinence, and was allowed to liquefy for at least 30 minutes at 37°C. All donors were appropriately screened and shown to be free from sexually transmitted infections, including human immunodeficiency virus (Barratt et al, 1990). Following liquefaction the sperm motion characteristics were assessed as described previously (Barratt et al, 1993; Zhu et al, 1994a,b) using a Hamilton Thorn Research Motility Analyser (HTM Model 2030; Hamilton Thorn Research, Danvers, Mass). Semen used was selected from donors by using the following characteristics: sperm concentration ≥60 × 10/mL, morphologically ideal forms ≥30%, sperm forward progressive motility ≥50%, and no evidence of antisperm antibodies.

Spermatozoa were prepared using a direct swim-up technique. Aliquots (0.5 mL) of liquefied semen were gently overlaid with minimal essential medium (MEM; with Earle's salts without L-glutamine, 1.8 mM Ca^{2+}; 0.5 mL) containing 0.03% (wt/vol) human serum albumin (HSA) and left for 1 hour at 37°C in 5% CO_2 in air. The upper fraction (0.35 mL) of the MEM overlay from each aliquot was carefully removed by aspiration and those from the same donor were pooled. The sperm concentration was determined using a Neubauer haemocytometer on 2 aliquots (10 μL) from each pooled sample. Noncapacitated cells were adjusted to a final sperm concentration of approximately 5×10^6/mL and used immediately. Capacitated spermatozoa were adjusted to 3.5% (wt/vol) HSA and incubated for 3 hours at 37°C in 5% CO_2 in air. A 10-μL aliquot of the latter sample was observed under a microscope to confirm that the spermatozoa displayed hyperactivated motility and were therefore capacitated.

Measurement of Intracellular Calcium Concentration

Following in vitro capacitation, 1-mL aliquots of spermatozoa were centrifuged at 500 × *g* for 10 minutes at room temperature and the pellet was resuspended into RPMI (0.4 mM Ca^{2+}) + FCS (1 mL). A 1-mM stock of indo-1/AM was prepared in dimethyl sulfoxide (DMSO) and, for each incubation, 7 μL of indo-1/AM stock solution was mixed with 173 μL of RPMI +

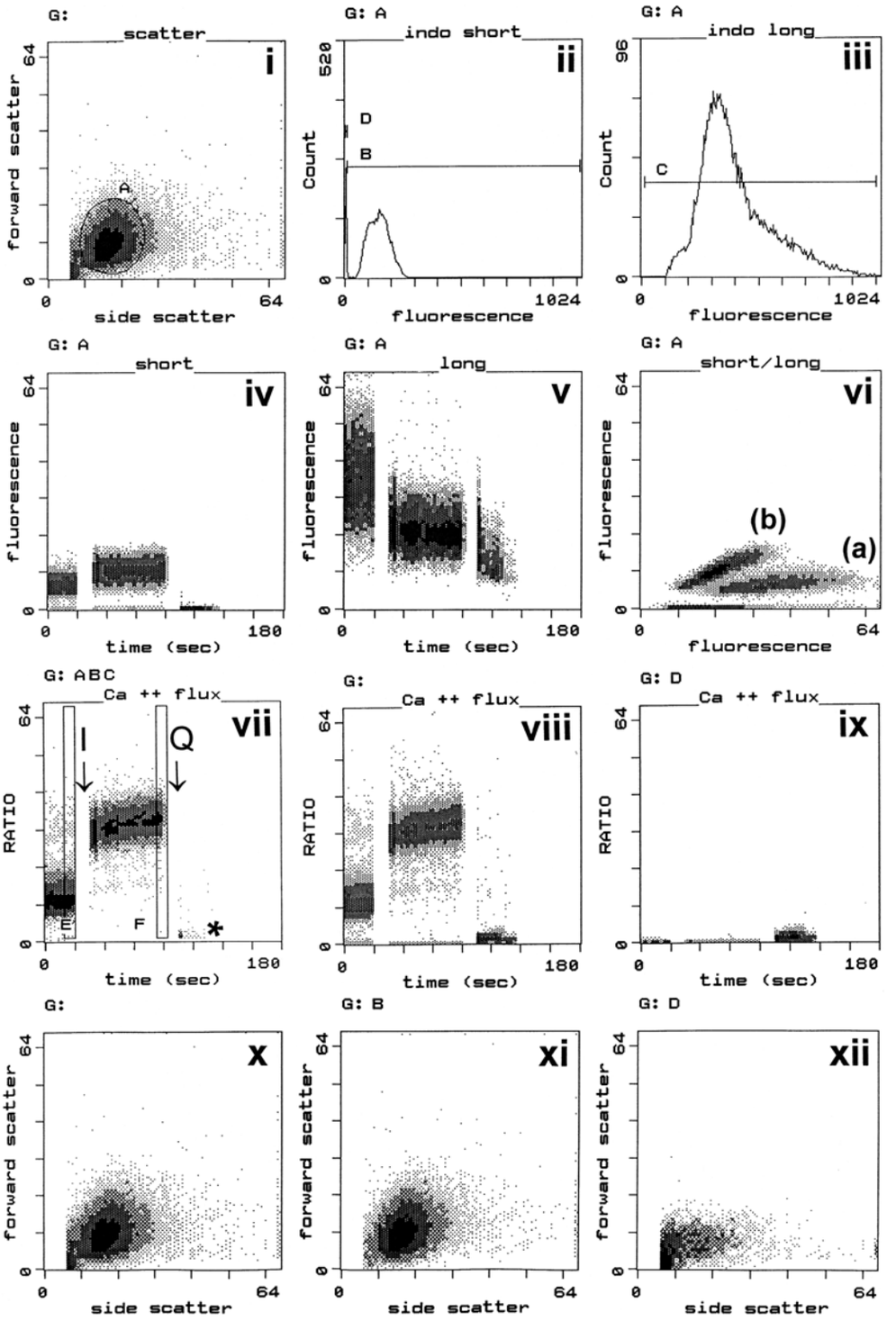

Figure 1. Calibration of the experimental system to determine intracellular calcium concentration in human spermatozoa. The results presented are from a typical experiment with indo-1–loaded capacitated spermatozoa. **(Panel i)** represents the forward and side scatter of the whole cell population. A gate was set to exclude debris and cell aggregates (all except Region A). **(Panel ii)** displays short wavelength fluorescence (calcium bound dye) of the Region A cell population. A gate was set to include stained and, therefore, live cells (Region B) and the remaining unstained cells were considered to be dead (Region D; approximately 5% of the total population). **(Panel iii)** represents long-wavelength fluorescence (calcium-free dye) of the Region A cell population. Any unusually weak or strong residual fluorescence was excluded (all except Region C). **(Panel iv)** and **(Panel v)** show the change over time of the short- and long-wavelength fluorescence, respectively of the Region A population. **(Panel vi)** shows the short- and

FCS (prewarmed to 37°C) in a microcentrifuge tube. This was then added to a 1-mL aliquot of cells (either spermatozoa or lymphocytes; 6 μM of final indo-1/AM concentration). The suspension was then gently mixed and incubated for 30 minutes at 37°C in 5% CO_2 in air. Following incubation the sample was stored in the dark at room temperature for 15 minutes. Before flow cytometric analysis, an aliquot (100 μL, 5×10^5 cells) of the indo-1 loaded cells was diluted with 400 μL of RPMI + FCS containing 1.8 mM $CaCl_2$ (overall, 1.84 mM Ca^{2+}) and incubated for 2 minutes at 37°C in a water bath. A 10-μL aliquot of sperm samples was assessed under an epifluorescence microscope to confirm loading of the dye (visualized as fluorescence in the head) and continued motility of the sample was confirmed.

Flow cytometric analysis was performed using an EPICS Elite instrument (Beckman Coulter Inc, Fullerton, Calif) fitted with a 10-mW HECD laser with an excitation wavelength of 325 nm (Coulter Elite software, Version 4.02). Emitted fluorescence wavelengths were measured with the following band pass filters: 381 ± 10 nm (*indo short*) for calcium-bound dye, and 525 ± 15 nm (*indo long*) for calcium-free dye. Cells were maintained at 37°C during analysis by the use of an attached water jacket. Debris and cell aggregates were initially excluded from analysis by a gate set on the forward versus side scatter cytogram. Dead cells do not metabolize indo-1/AM into indo-1, hence, the dye does not become caged (Thomas and Delaville, 1991). Consequently, dead cells that remain unstained can be removed from the analysis by gating out nonfluorescent cells or particles (Figure 1). Instrument settings were adjusted to establish a ratio baseline response for unstimulated cells (Figure 1). Agonist and controls were introduced after approximately 30 s by quick removal and reattachment of the sample tube. Response was then recorded for up to 4 minutes.

Unless otherwise stated, the following additions were routinely used. Loaded lymphocytes were analyzed first in order to validate the system. A 1.5-μL aliquot of an ionomycin stock solution (10 mM in DMSO) was added to the sample to give a final concentration of 30 μM. Shortly after the response maxima, 26 μL of a freshly made EGTA stock solution (200 mM in 1M NaOH) was added (approximately 10 mM final concentration) in order to quench the response. Loaded spermatozoa were similarly tested after validation; however, responses to both ionomycin and progesterone were recorded. Progesterone was made up fresh as a 1 mM stock in DMSO and 10 μL was added to achieve a final concentration of 20 μM. In addition, we also tested both the RPMI + FCS medium and DMSO as negative controls to confirm that they did not affect the experimental system.

The ratio of the fluorescence intensities at the 2 wavelengths (indo short [violet]: indo long [blue]) was used to calculate absolute values for $[Ca^{2+}]_i$ (Grynkiewicz et al, 1985; June and Rabinovitch, 1990; Rabinovitch and June, 1990). The following equation was employed:

$$[Ca^{2+}]_i = Kd(R - R_{min})\ S_{f2} / (R_{max} - R)\ S_{b2}$$

where Kd is the effective dissociation constant (250 nM at 37°C); R, R_{min}, and R_{max} are the fluorescence intensity ratios at the point of analysis, zero (EGTA quenched) and saturating (with ionomycin), respectively. S_{f2}/S_{b2} is the ratio of blue fluorescence intensity of the calcium-free to calcium-bound dye. June and Rabinovitch (1990) and Rabinovitch and June (1990) have reported that S_{f2}/S_{b2} and R_{min} cannot be reliably calculated using flow cytometry; consequently, these values were obtained from similar experiments using a spectrofluorimeter (Perkin-Elmer LS5, Perkin-Elmer Analytical Instruments, Seer Green, Bucks, United Kingdom) incorporating the same excitation wavelengths and band-pass filters as those used in flow cytometry. Values for S_{f2}/S_{b2} and R_{min} were found to be constant in different experiments and were 1.1 ± 0.04 and 0.375 ± 0.015, respectively (mean ± standard error of the mean, n = 6).

Measurement of Membrane Potential

The following stock solutions were prepared in DMSO unless otherwise stated: 10 μM 3-3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$), 1mM valinomycin, 0.1% (wt/vol) gramicidin D, 1 mM carbonyl cyanide chlorophenylhydrazone (CCCP), and 0.635 mM propidium iodide (PI) in PBS. Spermatozoa were prepared under capacitating or noncapacitating conditions as described earlier. The concentration of HSA of the capacitated samples was then adjusted to 0.03% (wt/vol) to ensure that it was the same as that of the noncapacitated sample. This was achieved by centrifugation at 500 × *g* for 10 minutes and resuspending the pellet in MEM + 0.03% (wt/vol) HSA. The sperm concentration was then adjusted to 1×10^6/mL.

From each of these samples, 8 aliquots (200 μL each) with 2 μL of PI added to each (14 μM final concentration) were prepared in sealed microcentrifuge tubes and maintained at 37°C in a water bath. All but one of the aliquots were simultaneously loaded with 1 μL of $DiOC_6(3)$ (50 nM final concentration) and 1 μL of CCCP (5 μM final concentration) for 30 minutes at 37°C. The use of the uncoupler CCCP caused mitochondrial depolarization; hence, only plasma membrane potential was measured. The unloaded sample was used as a negative control following dye loading to confirm loading in the other samples. One of the loaded aliquots was also viewed under a light microscope to confirm continued motility of the spermatozoa.

A series of incubations to validate that the experimental system was functioning appropriately were performed. The following treatments were carried out, in order, with one of the aliquots used each time: control (1 μL DMSO), valinomycin (1 μL; causes hyperpolarization), gramicidin D (1 μL; causes depolariza-

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long-wavelength fluorescence of the Region A population plotted against each other. The population of cells before and after addition of ionomycin are labeled (a) and (b), respectively. **(Panel vii)** indicates the ratio of indo short:indo long plotted against time (s) of the selected live cell population (cells not in Regions A, B, and C excluded). The addition of 30 μM ionomycin (labeled I) and 10 mM EGTA (labeled Q) and the quenching as a result of EGTA addition (*) is indicated. The mean ratio of the sperm population before and after the addition of ionomycin (regions E and F, respectively) was determined using flow cytometry software. **(Panel viii)** is the same analysis as Panel vii except that it is of the entire population of cells without any gating. **(Panel ix)** indicates the ratio of indo short:indo long plotted against time (s) of Region D. **(Panels x, xi,** and **xii)** represent the forward and side scatter of whole cell populations, Region B (stained and, therefore, live cells) and Region D (unstained and, therefore, dead cells), respectively.

tion), control (1 μL DMSO), control (1 μL DMSO), valinomycin (1 μL) followed by gramicidin D (1 μL) on the same aliquot (causes hyperpolarization then depolarization to ensure that any changes in membrane potential are dynamic). Each treatment was incubated for 10 minutes at 37°C, except for the last treatment, which was incubated 10 minutes for valinomycin and a further 10 minutes following addition of gramicidin D. An additional pair of aliquots of the relevant sperm sample were prepared to determine the effect of a potential agonist (1 for a control and 1 for the agonist), loaded with $DiOC_6(3)$ and CCCP and analyzed after incubation. Progesterone was made as a stock as described earlier, and 4 μL was added (final concentration 20 μM) and incubated for 2 minutes and 10 minutes (the control was therefore 4 μL of DMSO).

Flow cytometric analysis was performed with an excitation wavelength of 488 nm and an emission wavelength of 525 ± 15 nm (Shapiro, 1990). Cell debris and clumps were excluded from the analysis as previously described. Counterstaining with PI was employed to gate dead cells. We were precise with the timing of loading and incubation of the samples so that these were the same for each of the aliquots at the point of flow cytometric analysis. Hence, it was necessary to stagger the additions (both for loading and incubation) for each of the aliquots. Cells were maintained at 37°C during analysis by the use of an attached water jacket.

Results

Measurement of Sperm Intracellular Calcium Concentration by Flow Cytometry

Whenever we investigated $[Ca^{2+}]_i$ in human spermatozoa we initially performed a control experiment using freshly isolated human lymphocytes. Typically, a large (8- to 10-fold) increase in the fluorescence ratio (equivalent to calcium flux) was observed following the addition of ionomycin (data not shown). This was fully quenched with the addition of EGTA. This experiment was performed as a positive control to confirm that the experimental system was functioning optimally. The results presented in Figures 1 and 2 are from a typical experiment measuring $[Ca^{2+}]_i$ of indo-1 loaded in vitro capacitated spermatozoa. The results presented in Figures 1 and 2 are typical results from one experiment, although we performed the experiment 4 times with similar results to confirm consistency.

Figure 1 shows the calibration of the experimental system to enable absolute values of calcium to be determined. Panel i represents the forward and side scatter of cells, panel ii displays short wavelength fluorescence (calcium-bound dye), and panel iii represents long wavelength fluorescence (calcium-free dye). Gates were set to exclude debris and cell aggregates (all except Region A), any unstained and therefore dead cells (all except Region B), and any unusually weak or strong residual fluorescence (all except Region C). Approximately 5% of the total population were dead cells and were excluded from the analysis (Region D).

Panel vii plots the ratio of indo short: indo long (corresponds to intracellular calcium levels) against time (180 s). There was an increase in this ratio following the addition of ionomycin (Region F, y-axis; mean = 32.3) compared with basal levels (region E, y-axis; mean = 12.1) and the addition of EGTA caused the free calcium to be quenched. Panels iv and v show the change over time of the short and long wavelength fluorescence, respectively. Following the addition of ionomycin it was important to observe an increase in the former and a decrease in the latter to ensure that the experimental system was functioning appropriately. Panel vi shows the short and long wavelength fluorescence plotted against each other and 2 distinct populations, representing cells before (labeled a) and after (labeled b) incubation with ionomycin.

Panel viii displays the same analysis as panel vii except that it is of the entire population of cells without gating. A population of cells that did not respond was also observed. In addition, an analysis was also performed on the population of unstained cells (Region D; panel ix), and this population was shown not to respond to ionomycin, confirming that these were indeed dead, nonresponding cells. Panel x corresponds to the forward and side scatter of the whole population (the same as panel i). Panels xi and xii are the forward and side scatter of the Region B and Region D populations, respectively. It is important to note that there is overlap in the scatter characteristics of the nonstained (dead) cells (Region D) and the stained (live) cells.

The result presented in Figure 2 shows the effect of the addition of progesterone (20 μM) on $[Ca^{2+}]_i$ over time (data equivalent to Figure 1a, panels i–vi not shown). There was a rapid increase in calcium flux following the addition of progesterone (region F, y-axis; mean = 17.3) compared with basal levels (region E, y-axis; mean = 8.8). Although not shown, the appropriate negative controls (RPMI + FCS; DMSO alone) were also run to confirm that any effect was solely caused by progesterone. From the values obtained in panel vii of Figure 1 and from spectrofluorometric data, it was possible to quantify the progesterone-mediated change in $[Ca^{2+}]_i$ in Figure 2a. Following equilibration (after approximately 40 s) the mean basal $[Ca^{2+}]_i$ was 50 nM (25–75 nM range) and, in response to progesterone (20 μM), this increased transiently to 195 nM (125–285 nM range) before declining to approximately half the maximal level within 2 minutes (Region G). The values in parentheses correspond to the range of values found within this single sperm population (range in y-axis for regions E and F). The distribution of indo short: indo long in the selected sperm population at each of the times (Regions E, F, and G) is illustrated in

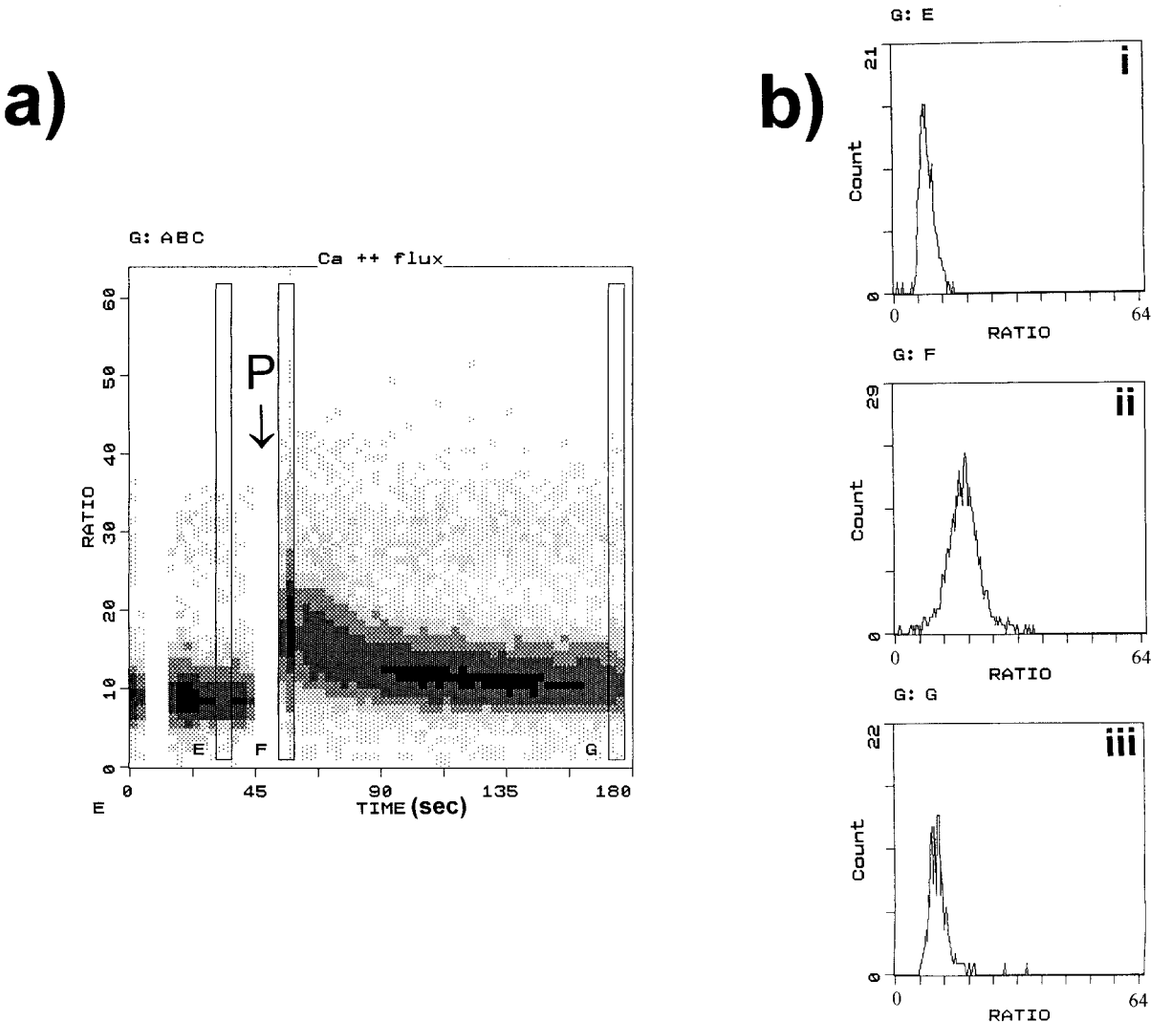


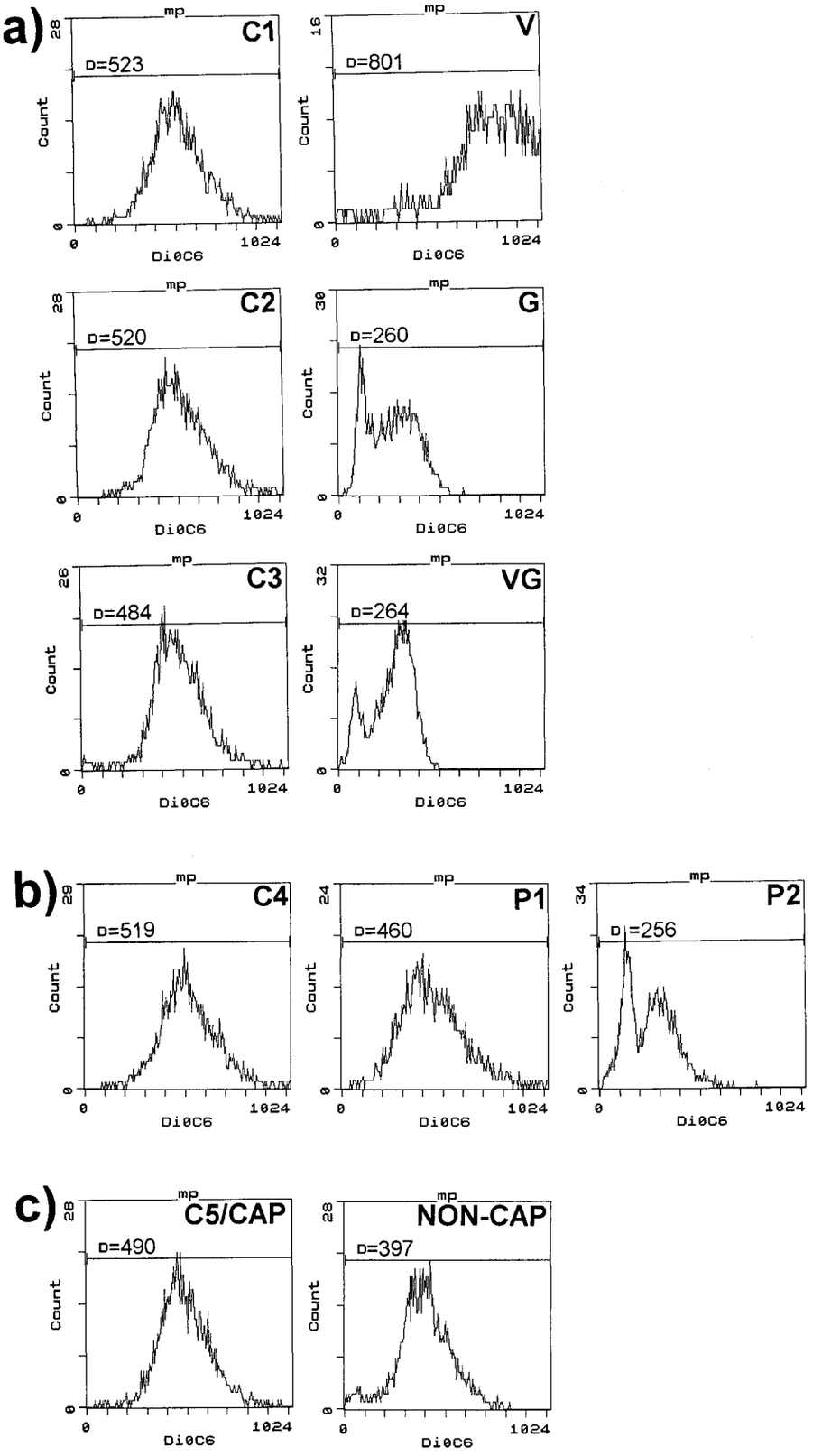
Figure 2. The effect of progesterone on intracellular calcium levels in human spermatozoa. The results presented are from a typical experiment with indo-1–loaded capacitated spermatozoa. **(a)** Is the ratio of indo short:indo long plotted against time (in seconds). The mean ratio of the sperm population before, immediately after the addition of progesterone (20 μM; labeled P), and after 2 minutes' incubation with progesterone was calculated from the cells in regions E, F, and G, respectively. **(b)** Histograms show the distribution of the indo short:indo long ratio in the sperm population (Figure 2) before and after incubation with progesterone. **(Panels i, ii,** and **iii)** correspond to Regions E (before), F (immediately after the addition of progesterone), and G (after incubation with progesterone for 2 minutes), respectively. The indo short:indo long ratio (x-axis) of the cells is plotted against the relative number of cells displaying that ratio (y-axis).

Figure 2b and clearly shows the increase in ratio following the addition of progesterone.

Measurement of Sperm Plasma Membrane Potential by Flow Cytometry

We employed a rapid flow cytometric assay to measure plasma membrane potential in human spermatozoa and an example of the results typically obtained is presented in Figure 3. In each of the results shown, a small proportion (less than 5% in each case) of dead or deteriorated cells were excluded by counterstaining with propidium iodide. Figure 3a shows the results that were necessary to ensure that the system was responding appropriately and in a dynamic fashion. It was necessary to perform this series at the start of each experiment to validate the experimental system. Plots of the relative membrane potential of capacitated human spermatozoa are shown under control (DMSO; panels C1, C2, and C3), hyperpolarizing (valinomycin, panel V), depolarizing (gramicidin D, panel G), and hyperpolarizing then depolarizing (valinomycin/gramicidin D, panel VG) conditions. The samples were analyzed in the order presented in Figure 3 (from left to right, then top to bottom) to ensure that the experiment was properly controlled, especially for cell viability and responsiveness.

We investigated the effect of the addition of an appro-


a)
C1
D=523
V
D=801
C2
D=520
G
D=260
C3
D=484
VG
D=264
b)
C4
D=519
P1
D=460
P2
D=256
c)
C5/CAP
D=490
NON-CAP
D=397
Count
Di0C6
mp
0
1024

priate physiological agonist (progesterone) on the membrane potential of in vitro capacitated spermatozoa (Figure 3b). Addition of progesterone (20 μM) was found to cause depolarization of the sperm plasma membrane potential, which was small after 2 minutes (panel P1). However, the same sample was assayed again after 15 minutes (panel P2) and the depolarization was more marked at this time compared with the control sample (panel C4). After 15 minutes of incubation with progesterone (panel P2), a subpopulation was much more depolarized than the majority of the cells.

Figure 3c shows the relative membrane potential of noncapacitated spermatozoa (panel NON-CAP) compared with in vitro capacitated spermatozoa (panel C5/CAP). Following in vitro capacitation, the sperm membrane potential became hyperpolarized compared with the membrane potential in noncapacitated spermatozoa. The results presented in Figures 3a–c are typical results from one experiment, although this experiment was repeated 3 times with similar results. Each of the control samples (C1–C5) displayed similar mean fluorescence values, which confirms that the experiment was properly controlled, and therefore validates the data from Figures 3b and 3c.

Discussion

Following the first successful measurement of $[Ca^{2+}]_i$ in human spermatozoa (Irvine and Aitken; 1986), Thomas and Meizel (1988) were the first to demonstrate that a potentially physiological stimulus (human follicular fluid) could cause an increase in $[Ca^{2+}]_i$ and that this increase was responsible for initiating the acrosome reaction. Progesterone was demonstrated to be the active component in follicular fluid and many studies have since observed that progesterone causes increased $[Ca^{2+}]_i$ in human spermatozoa (Osman et al, 1989; Blackmore et al, 1990). In addition, Plant et al (1995) demonstrated that the majority of individual spermatozoa from fertile donors are able to respond to this agonist. We therefore decided to use progesterone in this study to enable the data obtained to be compared with these other reports that used different techniques.

Our attention was drawn to the possibility of using flow cytometry to measure changes in $[Ca^{2+}]_i$ in spermatozoa by the work of Harrison et al (1993), who used boar spermatozoa and the probe fluo-3/AM. The advantage of this technique is that it allows the response of the whole population to be monitored but, in addition, allows differential responses of subpopulations to also be detected. This is particularly relevant for human spermatozoa because they are a relatively heterogeneous population (Giojolas, 1998). However, the use of dual wavelength dyes, such as fura-2/AM and indo-1/AM, has now largely superseded the use of fluo-3/AM as the fluorescent probe of choice to assay for calcium (Thomas and Delaville, 1991; Thomas and Meizel, 1988; Tesarik et al, 1993; Florman, 1994; Suarez and Dai, 1995). Dual wavelength probes allow a ratiometric approach to be used to calculate $[Ca^{2+}]_i$ and, importantly, this means that the amount of dye loaded into the cells does not need to be known or constant. Hence, any variability caused by different levels of dye loading because of cellular size or other factors will not affect the results (Grynkiewicz et al, 1985; June and Rabinovitch, 1990; Rabinovitch and June, 1990; Thomas and Delaville, 1991). Furthermore, because dead cells do not retain the hydrophilic impermeant dye, they can be directly identified and excluded from the analyses without the need for additional live or dead staining (eg, with propidium iodide; June and Rabinovitch, 1990). The data shown in panels vii, viii, and ix of Figure 1 clearly show that there is a nonresponding population and that only nonresponding cells are found in Region D. In addition, the forward and side scatter patterns for the whole population, Regions B and D (Figure 1, panels x, xi, and xii) exhibit a degree of overlap in their scatter characteristics. This demonstrates that, in this instance, live and dead cells could not be easily separated on the basis of scatter alone.

Considering that motile spermatozoa were obtained following direct swim-up, that debris and cell aggregates were gated out, and that dead (unstained) cells were also gated out, it is clear that a certain amount of preselection of the original sperm population took place. Such prese-

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Figure 3. The effects of in vitro capacitation and progesterone on the membrane potential of human spermatozoa. Presented are the results from a typical experiment with $DiOC_6$-loaded spermatozoa and each of the histograms shows the distribution of the relative membrane potential in the sperm population following the relevant treatment. The membrane potential (in arbitrary fluorescence units; x-axis) of the cells is plotted against the relative number of cells displaying that fluorescence (y-axis). The D value on each panel refers to the mean fluorescence (relative membrane potential) of the whole sperm sample. **(a)** Validation of the experimental system using capacitated spermatozoa. The panels correspond to the relative membrane potential of spermatozoa incubated under control (DMSO; panels **C1**, **C2**, and **C3**), hyperpolarizing (valinomycin; **panel V**), depolarizing (gramicidin D; **panel G**) and depolarizing following hyperpolarizing (valinomycin/gramicidin D; **panel VG**) conditions. **(b)** The effect of the addition of progesterone on capacitated spermatozoa. Panel **C4** corresponds to the relative membrane potential of spermatozoa incubated under control (DMSO) conditions. Following the addition of progesterone (20 μM), the membrane potential was measured at 2 minutes (**panel P1**) and 15 minutes (**panel P2**). **(c)** The effect of in vitro capacitation. **(Panel C5/CAP)** corresponds to the relative membrane potential of capacitated spermatozoa and panel **(NON-CAP)** corresponds to noncapacitated spermatozoa.

lection is normal when experimenting on mammalian spermatozoa but, nonetheless, should still be considered when interpreting results.

A good deal of preliminary work was performed in the development of the assay and we found that the conditions detailed in the Materials and Methods section were optimal. In particular, the temperature and the timing of the incubations was critical and should be the same for each of the samples. Spermatozoa were capacitated in the presence of 1.8 mM of calcium and were then indo-1 loaded in 0.4 mM of calcium because it is usual to perform this loading in the presence of lower calcium levels. The cells were then re-equilibrated to 1.84 mM of calcium for sufficient time (2 minutes in an incubator). This was confirmed in preliminary experiments in which the calcium level of samples remained the same at the start of flow cytometry. In addition, when they were left longer, the cells still had a similar starting calcium ratio. Hence, it may be concluded that the cells were equilibrated to the calcium concentration of the media directly before flow cytometry.

In the preliminary experiments, we had difficulty in achieving consistent data for similar experiments performed on different days and therefore decided to assess the experimental system using isolated human lymphocytes to ensure that the system was functioning optimally (data not shown). Consequently, before each series of experiments on spermatozoa we also performed an experiment with lymphocytes. This acted as a point of reference for the sperm data that were found to be more variable in the preliminary experiments. A technical problem that we encountered was variability in baseline because of possible contamination, especially following the addition of ionomycin. Hence, we found it necessary to clean the flow cytometer sample line carefully between experimental runs. In the experiments presented there was a small time period (about 5 s) where data were not recorded when the agonist was added to the sample. This was not believed to be crucial, although it is possible to continually record data by using an apparatus that allows the addition of the agonist to take place by direct injection into the sample stream. It is also possible to calculate and display the mean ratio values during an experiment using Multitime software (Phoenix Flow Systems), although we did not use the software in these experiments.

An important consideration with cells loaded with fluorescent probes is compartmentalization of the dye into intracellular organelles. This problem may be minimized by loading for as short a time as possible and even at lower temperatures than the usual 37°C (June and Rabinovitch, 1990; Rabinovitch and June, 1990; Thomas and Delaville, 1991). In these experiments the cells were loaded for 30 minutes at 37°C and were then left at room temperature in the dark for 15 minutes. This was optimal in allowing the dye to load without significant compartmentalization compared with a number of other loading protocols examined. Furthermore, for the duration of the experiments, the indo-1 exerted no obvious toxic effects because the spermatozoa remained motile. In addition, we also experimented with the concentration of indo-1/AM and found that 6 μM was optimal (1–10 μM indo-1/AM assessed).

The methods for determining absolute values for calcium using flow cytometry and indo-1/AM are well established (June and Rabinovitch, 1990; Rabinovitch and June, 1990). Spectrofluorimetric measurements for R_{min} and S_{f2}/S_{b2} were found to vary little and we now routinely use the mean values obtained in preliminary experiments. A key criterion in determining that an increase in $[Ca^{2+}]_i$ was real and not the result of dye leakage was to observe that both an increase in short wavelength fluorescence and a decrease in long wavelength fluorescence (Figure 1, panels iv and v, respectively) contributed to the ratiometric increase (Figure 1, panel vii and Figure 2a). The results obtained from a typical experiment to assess the effect of progesterone on $[Ca^{2+}]_i$ are presented in Figure 2. In this experiment we used a high concentration of progesterone (20 μM) to illustrate that the increase in $[Ca^{2+}]_i$ was maximal. However, we also observed similar maximal increases in $[Ca^{2+}]_i$ using 10 μM of progesterone as well as smaller increases with 5 μM of progesterone (data not shown). The absolute calcium values and the pattern of change in calcium levels in the presented experiment are consistent with previously published data on whole sperm populations. For example, Blackmore et al (1990) measured basal levels of free cytosolic calcium to be about 80 nM and showed that progesterone caused a transient rise in $[Ca^{2+}]_i$ to approximately 200 nM, which was not sustained. From this we conclude that this method may be used to measure absolute values of intracellular calcium in spermatozoa over time (intracellular calcium activity). As for all methods to assay calcium in mammalian spermatozoa, this approach is technically difficult and requires perseverance to achieve consistent results. Having performed a good deal of preliminary work to optimize the methodology we are now able to perform the assay and achieve consistency in different experiments using the same sperm sample and same treatment regime.

Sperm membrane potential has been studied in much less detail than calcium levels have, even though the significance to sperm function is well recognized (Florman et al, 1998). It was first measured in human spermatozoa using the radiolabeled lipophilic cation triphenylmethylphosphonium ($TPMP^+$) and some workers continue to use this approach (Glander and Rehorek, 1987; Calzada et al, 1988; Calzada et al, 1991; Calzada et al, 1992; Calzada and Tellez, 1997). More recently, the use of voltage-sen-

sitive lipophilic fluorescent cyanine dyes has become the preferred method (Chou et al, 1989; Florman et al, 1992; Foresta et al, 1993; Espinosa and Darszon, 1995; Silvestroni et al, 1997). These dyes work on the principle that, following cell equilibration with the cationic dye, electrical depolarization (a decrease in membrane potential) will cause the dye to be released from the cell, whereas hyperpolarization will cause cells to take up more dye (Shapiro, 1990).

Membrane potential in somatic cells is routinely measured by flow cytometry and we were therefore interested in applying this technique to human spermatozoa (Shapiro, 1991). Changes in membrane potential measured by flow cytometry have never been reported for live spermatozoa, although one study used cyanine dyes with flow cytometry to identify live versus dead spermatozoa (Szollosi et al, 1986). The technique was relatively straightforward to develop but much attention was paid to ensure that the experiments were carefully controlled and that each of the sample treatments was similar other than the specific treatment itself. The concentration of albumin in the capacitated samples was adjusted to be the same as that of the noncapacitated samples. This is because cyanine dyes bind to protein in solution and cause less availability of the dye, which can result in artifactual apparent depolarization. In this case, the membrane potential of the capacitated spermatozoa would otherwise appear to be more depolarized than it actually was (Shapiro, 1991).

We were precise with the timing of loading and incubation of the samples so that these were the same for each of the aliquots at the point of flow cytometric analysis. Hence, it was necessary to stagger the additions (both for loading and incubation) for each of the aliquots. Before each experiment, a series of incubations confirmed that the experimental system was functioning appropriately (Figure 3a). Treatments were performed to ensure that depolarization and hyperpolarization of the spermatozoa occurred and, importantly, that the changes were dynamic. A control was included between each of these test incubations and also between the experimental incubations. This was done to confirm that the sample was at a similar resting membrane potential before each treatment. In this technique the results obtained are for single cells only because cell debris and clumps were excluded from the analysis by gating on forward versus side scatter. In addition, counterstaining with propidium iodide was employed to gate dead cells from the analyses. In our experiments we used the uncoupler CCCP, which causes mitochondrial depolarization. Hence, only changes in plasma membrane potential, rather than mitochondrial membrane potential, were measured. However, we have achieved broadly similar data for total (plasma and mitochondrial) membrane potential changes in spermatozoa that were loaded with $DiOC_6(3)$ only and not CCCP (data not presented).

Sperm plasma membrane potential became hyperpolarized following in vitro capacitation compared with the noncapacitated state (Figure 3c). To the best of our knowledge, this is the first time this has been reported in human spermatozoa, although it has been well documented in other model systems (Zeng et al, 1995, in the bull and mouse; Chou et al, 1989, in the mouse). Although Calzada et al (1988) stated that they had measured membrane potential in capacitated human spermatozoa, these spermatozoa were in fact acrosome-reacted rather than capacitated because of the addition of the ionophore A23187 and the researchers observed membrane depolarization rather than hyperpolarization. A key application of this approach is that it may, therefore, be used as an assay to monitor capacitation of human sperm samples in vitro, either on its own or in concert with other currently used methods, such as the assessment of hyperactivated motility or the use of the chlortetracycline fluorescence assay (Fraser, 1993).

We decided to use progesterone as a potential agonist for similar reasons as those outlined for the calcium work. Progesterone caused a marked depolarization of the plasma membrane potential of capacitated spermatozoa after 15 minutes, which was consistent with previously described data (Figure 3b; Calzada et al, 1988; Calzada et al, 1991; Foresta et al, 1993). It is interesting that after incubating for 15 minutes with progesterone, a subpopulation of live cells was much more depolarized than the majority of the cells. Incubation with 20 μM of progesterone for 15 minutes will cause a proportion of cells to become acrosome-reacted (Osman et al, 1989; Brucker and Lipford, 1995). It is therefore likely that the subpopulation represents those spermatozoa that are acrosome-reacted, and hence, further depolarized.

As with flow cytometric measurement of $[Ca^{2+}]_i$ the key advantage of this technique is that it allows the response of the whole population to be monitored but, in addition, allows differential responses of subpopulations also to be detected. The main disadvantage of this technique is that the accuracy is more limited than the calcium assay because of a greater variation in fluorescence within each sample. Hence, small differences in membrane potential may not be detected. It should also be noted that this assay measures relative changes rather than absolute values of membrane potential (Shapiro, 1991). We have performed these experiments 3 times on different occasions and are confident that we have achieved technical consistency. However, we have not yet examined variations between ejaculates from different sperm donors in any detail.

In summary, we have presented 2 novel techniques using flow cytometry to measure intracellular calcium levels

and plasma membrane potential in human spermatozoa. The many advantages conferred over previous approaches should ensure that these techniques become more widely adopted where flow cytometry facilities are available.

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