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Voltage-dependent calcium influx in human sperm assessed by simultaneous optical detection of intracellular calcium and membrane potential

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Abstract

There are several physiological and pharmacological evidences indicating that opening of voltage dependent calcium channels play a crucial role in the induction of the acrosome reaction in mammalian sperm. In mature sperm, physiological inductors of the acrosome reaction such as ZP3, a zona pellucida protein, and the steroid hormone progesterone, induce depolarization and calcium influx, which are required for the acrosome reaction. In this paper, we describe a voltage-dependent calcium influx present in human sperm. We report an experimental procedure that allows measurement of intracellular calcium and membrane potential simultaneously using the fluorescent dyes $DiSC_3(5)$ and Fura-2. We found that in human uncapacitated sperm, depolarization induces a nifedipine-insensitive calcium influx that, in most cases, was transient. Calcium influx was observed in the range of -60 to -15 mV (the range tested). At resting membrane potential (around -40 mV), potassium addition depolarized and induced calcium influx, but when the depolarization was preceded by a hyperpolarization (induced with valinomycin), calcium influx was remarkably enhanced, suggesting that at -40 mV, channels are in a putative inactivated state. When sperm was incubated in medium without calcium, calcium restoration caused calcium influx that depended on voltage, and decayed between 1 and 2 min after depolarization. Unlike ram, mouse or bovine sperm, in which an alkalinization is required to induce calcium influx with potassium, the voltage-dependent calcium influx observed in human sperm did not require an increase in internal or external pH. However, we observed that ammonium, which increases intracellular pH, enhanced the voltage-dependent calcium influx about 90%. Furthermore,

Abbreviations: DiSC3(5), Diisopropylthiodicarbocyanine iodide; BCECF-AM, Biscarboxyethylcarboxyfluoresceine acetoxy methyl ester; HEPES, (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]); PIPES, (Piperazine-*N*,*N*′-bis[2-ethanesulfonic acid; 1,4-piperazinediethanesulfonic acid); CCCP, Carbonyl cyanide *m*-chlorophenyl hydrazone; HSM, Human sperm medium; HSMm, HSM without lactate and pyruvate

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depolarization by itself caused a small increase in intracellular pH suggesting that pH can be regulated by membrane potential in human sperm.

Keywords: Sperm; Acrosome reaction; Intracellular calcium; Calcium channel blocker; Nifedipine; Nickel; Intracellular calcium; Membrane potential; Membrane potential sensitive dye; Fura-2; DiSC3(5); (Human)

1. Introduction

In most species, the acrosome reaction (AR) in sperm is a necessary step for egg fertilization. It consists of the exocytosis of the acrosome that leads to the release of lytic enzymes and to the exposure of its inside membrane. This reaction is a necessary step for the further fusion of the sperm equatorial segment with the egg plasma membrane [1]. In human sperm, the AR is induced by a zona pellucida protein, which, in mouse, is called ZP3 [2,3], and by progesterone, a steroid hormone that is present in the follicular fluid [4].

In mammalian sperm, the acrosome reaction is mediated by an intracellular calcium increase caused by calcium influx from the medium. The ion transport systems that cause intracellular calcium increment, as well as its regulation, are not well established yet in mature sperm. A major inconvenience to explore ion currents in these cells is probably the size and shape of the sperm head that make it difficult to use electrophysiological methods.

Alternatively, undifferentiated spermatogenic cells have been useful to investigate calcium channels with the whole cell patch-clamp method. There are several reports that have demonstrated the presence of a T-type calcium channel in mouse spermatocytes [5–8]. This channel is blocked by the calcium channel blockers amiloride, nifedipine and nickel, and is stimulated by tyrosine dephosphorylation [9]. Since mature sperm lack competence for protein synthesis, it has been proposed that this channel should be present in the mature cell. Furthermore, the fact that the acrosome reaction is inhibited by the same channel blockers has lead to the hypothesis that this channel serves as the calcium permeability pathway, which should open during the ZP3-induced acrosome reaction [6,7,10].

In mature sperm, the use of membrane potential-sensitive dyes and calcium fluorescence detectors have allowed to estimate, indirectly though, the presence of voltage-dependent calcium channels. Indeed, studies made in *Litechinus pictus* sea urchin sperm [11] have demonstrated that these cells have voltage-dependent calcium channels whose activation induces the acrosome reaction. As for mammalians, it has been suggested that bovine and mouse sperm have voltage-dependent calcium channels since potassium, added at alkaline pH or at normal pH with NH_4Cl (which increase intracellular pH), induces an increase in intracellular calcium and the acrosome reaction [12–14]. Also, it has been shown that ZP3 induces a depolarization [10], and an increase in internal pH and calcium that are inhibited by pertussis toxin [13]. In this regard, it has been proposed that in mouse and bovine sperm, an alkalinization and a depolarization are required for voltage-dependent calcium influx and for the acrosome reaction induced by the physiological inductor ZP3 [9,10,14]. In human sperm, Brandelli et al. [15] have suggested that voltage-dependent calcium channels should open during the acrosome reaction induced by potassium at alkaline pH, since this reaction is blocked by calcium channel blockers.

In this report, we present data supporting the notion that human sperm are endowed with voltage-dependent calcium channels. In non-capacitated human sperm, we observed a voltage-dependent calcium influx that occurs at normal pH, and is insensitive to nifedipine but blocked by nickel. Apparently, at resting membrane potential, these channels are partially in an inactivated state. We support this hypothesis based on studies made with the cyanine $DiSC_3(5)$, a positively charged, membrane potential sensitive dye, in combination with the calcium channel indicator Fura-2. We used an experimental approach that allows to measure both parameters simultaneously in the same sperm sample.

2. Experimental procedures

2.1. Materials

Fura-2-AM, BCECF-AM and $DiSC_3(5)$ were obtained from Molecular Probes; nifedipine, verapamil, CCCP, ionomycin, nigericine and valinomycin from Sigma. The other reagents were also obtained from Sigma.

2.1.1. Sperm collection and purification

Human semen was obtained by masturbation from a group of 16 healthy donors (between 18- and 25-years-old). Normospermic men were selected according to WHO's protocol (men whose semen had: >50 million sperm cells/ml semen, $>50\%$ motility, $>50\%$ viability) (World Health Organization, WHO Laboratory Manual for the examination of human semen and semen–cervical mucus interaction, 1987. ed 2. Ed. Panamericana. pp 9–77). Semen was left 30 min at 37°C to allow liquefaction and sperm was separated by Percoll gradients according to Suarez et al. [16]. Two milliliters of semen were layered on a 50% and 75% discontinuous Percoll gradient (buffered at pH 7.4 with HEPES 10 mM, NaCl 150 mM) and centrifuged at $300 \times g$, 20 min. The sperm pellet was separated and washed twice by centrifugation at 300 g, 10 min, in HSM medium (NaCl 117.5 mM, KCl 8.6 mM, $CaCl_2$ 2.5 mM, $MgCl_2$ 0.49 mM, NaH_2PO_4 0.3 mM, glucose 2 mM, Na-pyruvate 0.25 mM, Na-lactate 19 mM, HEPES 25 mM, pH 7.5–7.6). After the last centrifugation, sperm was resuspended in 1 ml HSM and kept at 37°C and 5% CO_2–95% air. Immediately, cells were loaded with Fura-2 (see below).

2.1.2. Simultaneous detection of $[Ca^{2+}]_i$ and membrane potential

To detect membrane potential and intracellular calcium simultaneously, we used the membrane potential-sensitive dye $DiSC_3(5)$ and the calcium detector Fura-2 in the same sample. Since this study was done with non-capacitated sperm, the fluorescence recordings were performed in HSM without lactate and pyruvate (HSMm). Fura-2 loaded sperm (see below) were added to a fluorescence cuvette containing HSMm medium $+0.5$ μM $DiSC_3(5)$. The cuvette was kept at 37°C and under constant magnetic stirring. The fluorescence was measured with a PTI spectrofluorometer (Photon Technology International) which has two 810 and 814 PMTs (photomultipliers) placed in front each other at 90°with respect to the excitation Xenon source. To collect both signals simultaneously, we used one PMT with an interference filter of 488 nm to detect Fura-2 fluorescence, and the other PMT with a 670-nm interference filter to detect $DiSC_3(5)$ fluorescence. Fura-2 was excited at 340 and 380 nm with the excitation monochromator of the PTI system, as described below. $DiSC_3(5)$ was excited at 600 nm with an additional source of light (Tri-lite, WPI) with a filterholder to hold a 600-nm filter (Hansatech Instruments). This additional source of light was placed just in front of the Xenon light source of the PTI system. Therefore, both fluorescence recordings, that is, the ratiometric readings of Fura-2 and the single reading of $DiSC_3(5)$, were detected simultaneously at 90° with respect of their light sources. Simultaneous recordings were obtained at 0.85 Hz, and data was collected and analyzed with the PTI computer interface. All measurements were performed within 2 h after sperm Fura-2 loading.

2.1.3. Measurement and calibration of $[Ca^{2+}]_i$

Cells ($1–2 \times 10^8$) were loaded in 1 ml HSM with 3 μM Fura 2-AM and incubated 40 min at 37°C under 5% CO_2–95% air. Cells were washed by centrifugation ($300 \times g$, 10 min) and used immediately for calcium determination. Ratios of 340/380 nm excitation (emitted at 488 nm) were obtained with automatic monochromator adjustments at 0.85 Hz. Usually, we obtained a basal ratio among 1.4 and 2.3 that stabilized within 1 min. The ratios were calibrated according to Ref. [17] using the following equation $[Ca^{2+}] = K_d\ (R - R_{min})/(R_{max} - R)$ (Ff380/Fb380). The ratiometric 340/380 value R_{max} and Fb380 (fluorescence at 380 excitation) when calcium saturated, were obtained upon 1 μM ionomycin addition, then, R_{min} and Ff380, which measure fluorescence without calcium, were obtained by adding EGTA 15 mM (stock solution 1 M at pH 9; this pH was adjusted to avoid acidification). The K_d, obtained from Grynkiewicz et al. [17], was 224 nM.

2.1.4. Measurements and calibration of the membrane potential

$DiSC_3(5)$ signal was calibrated according to García-Soto et al. [18] with some modifications. This is a cationic permeant dye that senses membrane potential changes in seconds. Negative membrane potential drives positively charged $DiSC_3(5)$ molecules into the cell. In the cytoplasm, the dye concentrates to a higher value than in the external medium, and tends to form non-fluorescent aggregates [19,20]. So, when cells hyperpolarize, there is an electrophoretical influx of the positive dye, a further formation of non-fluorescent aggregates, and therefore fluorescence quenching. On the contrary, when cell depolarizes, the less negative (or more positive) membrane potential drives positively charged molecules out of the cell (an electrophoretical efflux of the dye), the non-fluorescent aggregates fall; consequently, there is an increase in fluorescence.

An electrochemical potassium potential was induced to Fura-2 loaded sperm with 1 μM valinomycin. In normal conditions, and most cases, the potassium ionophore caused a decrease in fluorescence consistent with a hyperpolarization. Subsequent addition of potassium increased fluorescence to stable values indicating depolarization. Since $DiSC_3(5)$ is positive and permeant, it can be electrophoretically uptaken into the mitochondrial by the negative mitochondrial potential causing a further quenching of fluorescence [18–20]. However, in most cases, the contribution of the incapacitated sperm mitochondrial potential to the fluorescence signal was undetectable, since 5 mM NaCN or 1 μM CCCP (a proton ionophore) that collapse mitochondrial potential did not affect $DiSC_3(5)$ fluorescence (not shown). The lack of effect of cyanide and CCCP indicated that incapacitated sperm had mitochondrial potential close to zero and agreed with the fact that in human sperm, the main metabolic process that supplies energy is glycolysis with low mitochondrial contribution [21,22]. Therefore, all experiments presented here were done without cyanide or mitochondrial uncouplers.

To calibrate the membrane potential signal we plotted $F_x - F_{8.6}/F_{val} \times 100$ vs. E_k, where $F_{8.6}$ is the fluorescence value with valinomycin in HSMm (that is, at $[K]_{ext} = 8.6$ mM), and F_x, is the fluorescence obtained upon x amount of external K (in the presence of valinomycin). The E_k, the Nernst electrochemical potential for K distribution, at 37°C, is, $E_k = -61.54$ mV log $[K]_i/[K]_{ext}$. The extracellular potassium concentration, $[K]_{ext}$, ranged from 2 to 68.6 and the internal potassium concentration, $[K]_i$, determined as described below, was 120 mM. We obtained a linear relationship in the range of -70 to -14 mV ($r = 0.997$), however, the calibration points at -91 mV and -109 mV did not fall into the linear relationship. The slope indicated that there was 1.51 units of percentage fluorescence change per mV (see inset of Fig. 2B) which agreed with calibration values

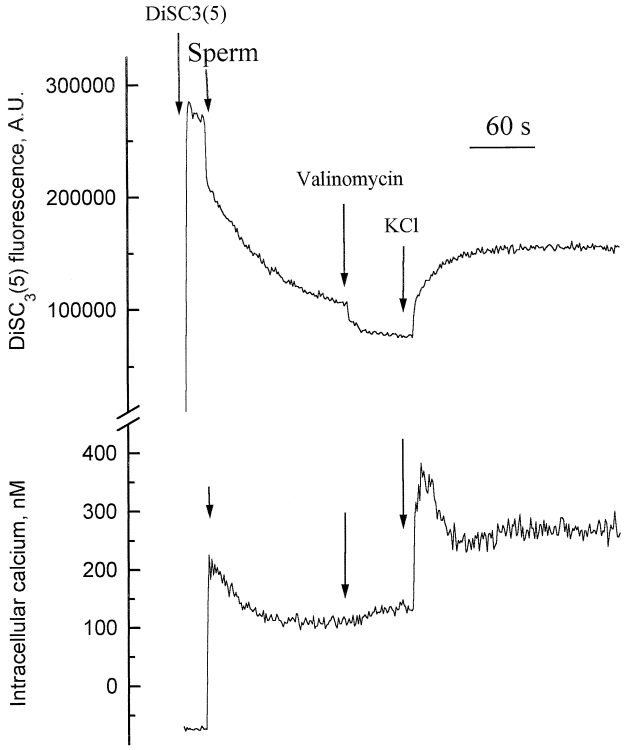

Fig. 1. Simultaneous detection of membrane potential and intracellular calcium in incapacitated human sperm. Fura-2 fluorescence (340/380–488 nm) (lower part) and $DiSC_3(5)$ fluorescence (600–670 nm) (upper part) were detected simultaneously as described in Section 2. Five hundred nanomolar $DiSC_3(5)$ was added to the fluorescence cuvette containing 2 ml HSMm, at 37°C and under constant magnetic stirring, then Fura-2 loaded sperm (1×10^{-7}) was added. Once both signals equilibrated (DiSC3(5) fluorescence took 3–4 min), 1 μM Valinomycin and 60 mM KCl were added as indicated. $DiSC_3(5)$ fluorescence is reported in arbitrary units (A.U.) and intracellular calcium as nanomolar (nM).

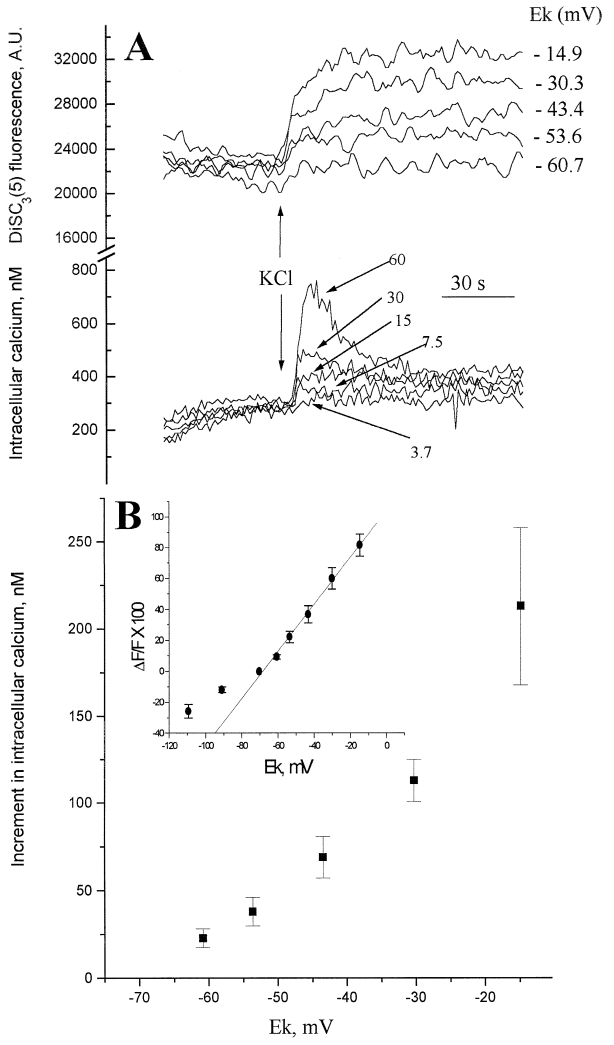

Fig. 2. Effect of KCl addition on the membrane potential and intracellular calcium detected simultaneously in incapacitated human sperm. (A) Fura-2 loaded sperm were added to 2 ml HSMm containing 500 nM $DiSC_3(5)$. At 4 min, 1 μM valinomycin was added (not shown). One minute later, KCl was added at the indicated amounts. The upper part of the curve shows the membrane potential recordings and the E_k value reached at the corresponding $[K]_{ext}$. The lower part shows the simultaneous intracellular calcium recordings. The numbers are the amount of potassium addition in mM. In (B), the increase at the peak in $[Ca^{2+}]_i$ (in nanomolar) triggered by depolarization is plotted against the theoretical E_k (bars are S.E.M., with $n=7$). The inset shows the calibration curve, that is, the percentage of change in fluorescence (referred to the basal fluorescence in HSMm, that is, in 8.6 mM potassium) as a function of the theoretical E_k. The slope of the curve was 1.51 and correlation coefficient was 0.997. Bars are S.E.M. with $n=6$. $DiSC_3(5)$ fluorescence is reported in arbitrary units (A.U.) and Other conditions are described in Section 2 and Fig. 1.

previously reported [18]. This indicated that the fluorometrical detection can be used as a semiquantitative estimation of membrane potential in human sperm at the indicated voltage range.

2.1.5. Simultaneous detection of intracellular pH and membrane potential

We used the procedure described by Reynaud et al. [23] with some modifications. Purified sperm ($1–2 \times 10^8$) were loaded in 1 ml HSM with 3 μM BCECF AM and incubated 40 min at 37°C under 5% CO_2–95% air. Cells were washed by centrifugation ($300 \times g$, 10 min) and used immediately for pHi and membrane potential determination. About 1×10^7 BCECF loaded cells were added to a thermostatized (37°C) fluorescence cuvette containing 2 ml HSMm medium plus 0.5 μM $DiSC_3(5)$. pHi and membrane potential were recorded simultaneously using the same setup and procedures described above for intracellular calcium and membrane potential simultaneous measurements, except that we substituted settings for Fura-2 detection for those for BCECF. BCECF was excited at the ratiometric 500/439 nm, and the

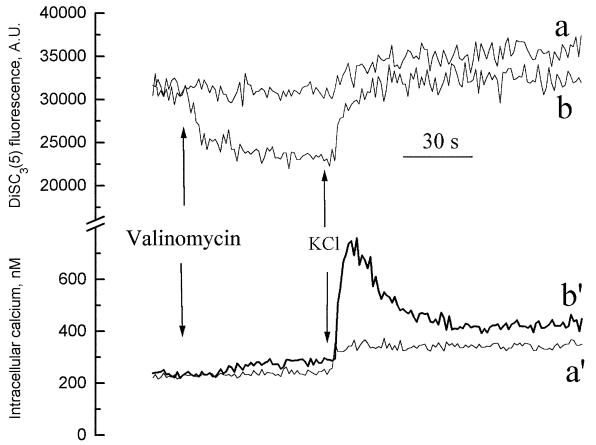

Fig. 3. Voltage-dependent calcium influx is enhanced when depolarization is preceded by valinomycin addition. Traces a and a′ are the simultaneous recordings of membrane potential (upper trace) and intracellular calcium (lower trace) of incapacitated human sperm under resting conditions, that is, no treated with valinomycin. Traces b and b′ show the effect of valinomycin (added as indicated; the intracellular calcium trace is drawn bold). In both conditions, membrane was depolarized with 60 mM KCl as indicated. $DiSC_3(5)$ fluorescence is reported in arbitrary units (A.U.) and intracellular calcium as nanomolar (nM). The other conditions are described in Section 2 and Fig. 1.

emission collected at 550 nm, at a frequency 0.6 Hz. The intracellular pH was calibrated in situ as described in Ref. [24].

2.1.6. Determination of intracellular [K$^+$]

To evaluate the E_k, $[K^+]_i$ was determined by the null point method according to Babcock [25]. This method takes into consideration that $[K^+]_i$ equals $[K^+]_{ext}$ when nigericine, a K^+/H^+ exchanger, does not produce any change in internal pH. Briefly, BCECF loaded cells ($1–5 \times 10^6$, see above) were added to a fluorescence cuvette containing 2 ml medium composed with 2.5 mM $CaCl_2$, 0.49 mM $MgCl_2$, 2 mM glucose and different KCl (from 100 to 130 mM) and LiCl concentrations. To maintain the ionic strength constant the sum of LiCl + KCl was kept 130 mM. The pH was buffered with 10 mM HEPES-Li at 6.8–6.9 (which was the human sperm internal pH obtained in HSMm). pHi was determined as described above. K^+ and H^+ exchange was induced with 2 μM nigericine at different potassium concentrations. The $[K^+]_i$, obtained at the external $[K]_{ext}$ concentration, at which there was no pHi change upon nigericine addition, was 120 mM. This value was the same to that obtained by Babcock [25] in bovine sperm.

3. Results

Fig. 1 shows a simultaneous recording of membrane potential and intracellular calcium in incapacitated human sperm (see Section 2). As expected, in HSMm without cells, the fluorescence observed upon addition of $DiSC_3(5)$ did not produce any signal at the ratiometric 488 nm recording. In the absence of $DiSC_3(5)$, addition of Fura-2 loaded sperm did not affect the reading at 670 nm either (not shown). Upon Fura-2 loaded sperm addition to the cuvette containing HSMm + $DiSC_3(5)$, an expected slow decrease in $DiSC_3(5)$ fluorescence was observed. This decrease in fluorescence was due to the electrophoretical dye uptake, which equilibrated in around 3 min. The Fura-2 ratiometric recording reached a value, in this experiment, corresponding to $\sim$120 nM $[Ca^{2+}]_i$. Valinomycin addition caused a clear decrease in $DiSC_3(5)$ fluorescence, indicating hyperpolarization and a slight increase in $[Ca^{2+}]_i$ (see also Fig. 3). A

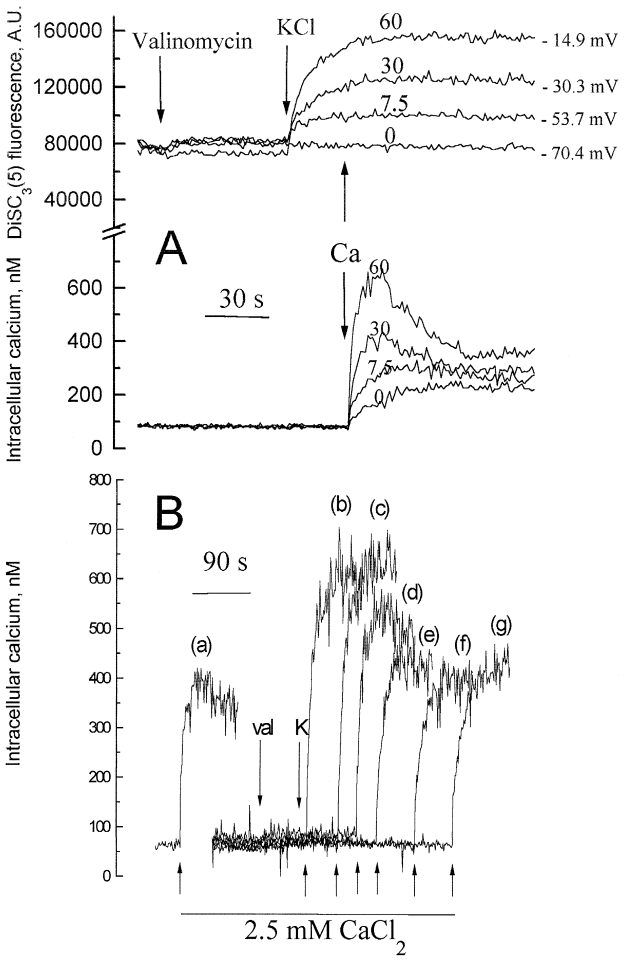

Fig. 4. Effect of membrane depolarization on intracellular calcium of human incapacitated sperm incubated in HSM without calcium. Simultaneous recordings were performed in HSMm with no calcium + EGTA 0.5 mM (0CaHSMm). In (A), traces show the effect of adding calcium to sperm incubated in 0CaHSMm at different membrane potentials (upper part) on intracellular calcium (lower part). One micromolar valinomycin was added to sperm in 0CaHSM; 1 min later, KCl was added at the indicated amounts to depolarize at the indicated values (right side of the membrane potential traces). After 30 s, 2.5 mM $CaCl_2$ was added to restore 0CaHSMm to its normal calcium concentration. The number on the traces are the amounts of K added previously as indicated. These traces are representative of 5 experiments. (B) shows the effect of 1 μM valinomycin and the subsequent addition of 60 mM KCl on intracellular calcium in sperm incubated in 0CaHSMm. $CaCl_2$ (2.5 mM) was added at (b) 10, (c) 60, (d) 90, (e) 120, (f) 180 and (g) 240 s after KCl addition, as indicated. In trace (a) sperm was no depolarized with KCl and calcium was added 1 min after valinomycin. Other conditions are described in Section 2 and Fig. 1. These traces are representative of 5 experiments.

further addition of 60 mM KCl caused an increase in $DiSC_3(5)$ fluorescence consistent with a depolarization and a high, transient increase in intracellular calcium with a peak of ~380 nM. After the peak, the intracellular calcium was sustained to a value higher than before potassium addition. Similar changes were found in separate assays (not shown), indicating that measurements made with Fura-2 and DiSC3(5) can be performed simultaneously.

Since the result described in Fig. 1 suggested that the increase in $[Ca^{2+}]_i$ was due to calcium influx through voltage-dependent calcium channels, we investigated the voltage dependence of calcium increase by varying the amount of KCl in HSMm containing valinomycin. As shown in Fig. 2A, the increase in $[Ca^{2+}]_i$ depended on the amount of potassium added. The upper part of the curve shows the depolarization reached at the indicated amounts of KCl added and the lower part, the simultaneous intracellular calcium recordings. Even at the lowest potassium concentration added (3.5 mM), we observed a slight increase in $[Ca^{2+}]_i$. At 7.5, 15, 30 and 60 mM KCl addition, the internal calcium increased clear and steeply. Fig. 2B shows the increase in $[Ca^{2+}]_i$ triggered by depolarization as a function of the theoretical E_k. The curve showed that the increase in $[Ca^{2+}]_i$ was induced even at the lowest voltage tested (-60 mV) and increased steeply until -18 mV. In inset, it is shown the calibration curve of our $DiSC_3(5)$ fluorescence measurements (see Section 2). We found a linear relationship between -60 and -14 mV, that is, in the range of voltages shown in Fig. 2B.

To evaluate the activity of these putative voltage-dependent calcium channels under physiological resting conditions, we compared the effect of KCl addition on the simultaneous recording of intracellular calcium and membrane potential in incapacitated sperm not hyperpolarized with valinomycin. The resting membrane potential obtained within 2 h after Fura-2 loading was -40 ± 16 mV (S.D., $n = 7$). Fig. 3 shows that, under resting conditions, 60 mM KCl induces an increase in internal calcium. The simultaneous membrane potential trace showed that at rest, 60 mM KCl depolarized the membrane to a similar value close to that obtained with valinomycin. However, the increase in internal calcium induced by KCl was markedly enhanced (3.9 times ± 0.93 S.D., $n =$

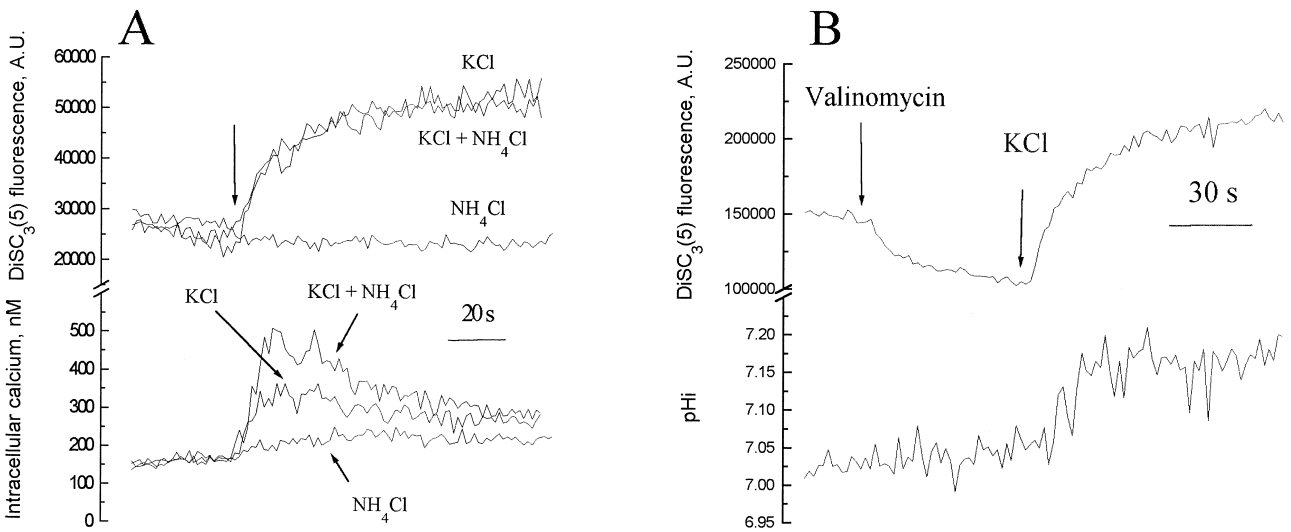

Fig. 5. Effect of pHi on the depolarization-induced calcium influx. Human incapacitated sperm was loaded with BCECF-AM as indicated in Section 2 and added to the fluorescence cuvette containing HSMm with 500 nM $DiSC_3(5)$. Simultaneous recordings were performed at the ratiometric 500/439–550 nm for BCECF and 600–670 for $DiSC_3(5)$. In (A) is shown the effect of NH_4Cl 10 mM, 60 mM KCl and 60 mM KCl + 10 mM NH_4Cl (simultaneous addition) on the simultaneous recordings of intracellular calcium and membrane potential, added as indicated by the arrow. (B) shows a simultaneous recording of pHi and membrane potential (see Section 2). Valinomycin 1 μM and KCl 60 mM were added as indicated. In all traces, 1 μM valinomycin was added 1 min before the mentioned additives. These traces are representative of five experiments. Other experimental conditions are as described in Fig. 1.

5) when the membrane was previously hyperpolarized with valinomycin. These results stressed the importance of valinomycin to be able to trigger an enhanced increase in $[Ca^{2+}]_i$ induced by depolarization, and suggested that a hyperpolarization would remove the presumptive voltage-dependent calcium channels from an inactivated state. Consequently, at resting membrane potential (~ -40 mV), these channels would be in an inactivated state (see Section 4).

Experiments made without external calcium are shown in Fig. 4. We studied the effect of membrane potential on $[Ca^{2+}]_i$ (simultaneous recordings) in sperm incubated in HSMm without calcium $+0.5$ mM EGTA (0CaHSMm). In these traces, the resting membrane potential were more negative than in normal medium; they were closer or equal to the E_k. Fig. 4A shows the effect of adding 2.5 mM calcium to sperm incubated in 0CaHSMm, at 4 different membrane potentials (clamped with valinomycin and the indicated concentration of potassium). In medium without calcium, valinomycin addition did not produce the slight increase in internal calcium observed in normal, calcium containing medium (see also Figs. 1 and 3), indicating that in calcium containing media, valinomycin induced calcium influx. Upon depolarization, as expected, there was no change in intracellular calcium in sperm incubated in medium without calcium. This result supported the hypothesis that the depolarization-induced calcium increase was entirely due to calcium influx from the medium. When calcium was restored, a transient calcium influx that depended on membrane potential was observed; the more depolarized sperm, the more calcium influx occurred. It is important to note that calcium influx through the voltage-dependent pathway did not further depolarize the membrane, indicating that 1 μM valinomycin effectively clamped the membrane potential at the indicated values. Fig. 4B shows the effect of calcium restoration at 10, 60, 90, 120, 180 and 240 s after sperm depolarization with 60 mM KCl, that is, to -17 mV. It was observed a similar peak at 10 and 60 s followed by a decrease that reached control values (that is, calcium restoration with no depolarization, trace g) in 120 s.

Unlike the results presented here in human sperm, the increase in calcium induced by high potassium in other mammalian sperm species has been observed only in experimental conditions that increase internal pH (with NH_4Cl) [10] or external pH (medium at pH 8.5) [12]. This calcium influx is blocked by micromo-

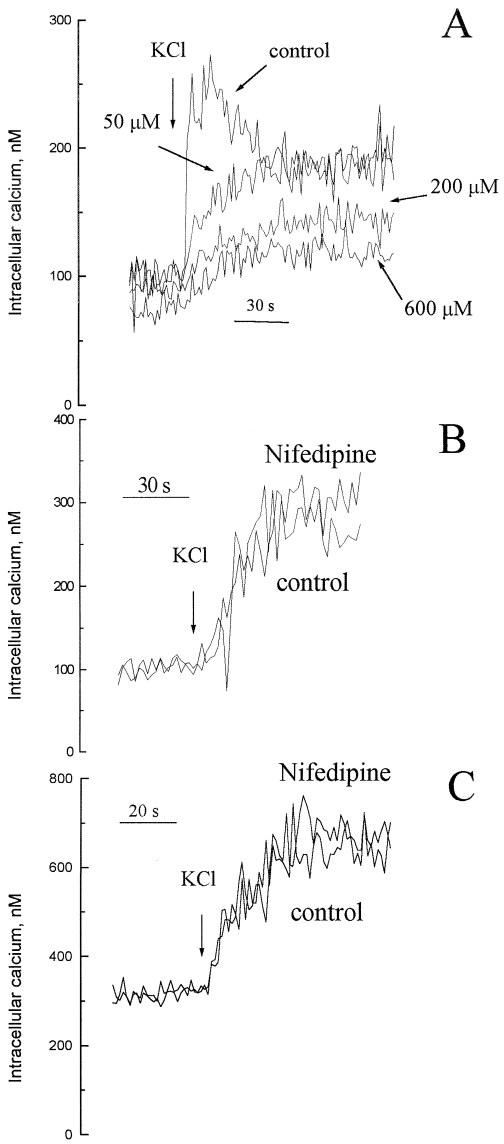

Fig. 6. Effect of calcium channel blockers on the depolarization induced calcium influx. In all experiments, human incapacitated sperm was treated with 1 μM valinomycin (not shown in the figures) and 1 min later was depolarized with 60 mM KCl. In (A), sperm was treated with the indicated amounts of $NiCl_2$, (added 1 min before valinomycin). In B and C, sperm was treated with 50 μM nifedipine during 5 min, under darkness in the same fluorescence cuvette, then, recording was started and KCl were added as indicated. In (B), experiment was done in normal HSMm and in (C), HSMm was buffered at pH 6.4. In this latter case, HEPES was replaced by 10 mM PIPES. Other experimental conditions are as described in Fig. 1.

lar concentrations of nifedipine when sperm is incubated with this dihydropyridine at pH 6.5 [12]. Furthermore, in ram sperm, internal pH is possibly regulated by membrane potential, so that a depolarization induces an alkalinization. To test the possible contribution of pHi on the observed voltage-dependent calcium influx, we investigated the role of membrane potential in internal pH, and the effect of NH_4Cl addition on the voltage-dependent increase in $[Ca^{2+}]_i$. Fig. 5A shows that 10 mM NH_4Cl by itself, increased intracellular calcium in a very small extent. Ammonia added simultaneously with 60 mM KCl enhanced intracellular calcium 92% $\pm$ 41 (S.D. $n = 5$) as compared with 60 mM KCl alone. Fig. 5B shows simultaneous recordings of intracellular pH and membrane potential. Intracellular pH was not affected by hyperpolarization; however, a slow and slight alkalinization was observed upon depolarization.

The effect of calcium channel blockers on the voltage-dependent calcium influx is shown in Fig. 6. An inhibitory effect of nickel on the transient increase in internal calcium was observed. Nifedipine, even at 50 μM, had no effect in normal HSMm. We did not observe any detectable effect in experiments done in HSMm buffered at pH 6.5 (Fig. 5C), nor at -30 mV resting membrane potential (not shown), that is, at a membrane potential at which dihydropyridines have higher affinity for calcium channels [26].

4. Discussion

In this paper, we describe the effect of membrane potential on intracellular calcium in incapacitated human sperm. To improve the interpretation of our experiments, we set-up an experimental approach that allows measuring these parameters simultaneously (see Section 2).

Simultaneous detection showed that a KCl-induced depolarization in the range of -60 to -15 mV (the range tested) from resting -70 mV, caused a rapid calcium transient increment that reached a peak in seconds and was followed by a sustained value higher than resting. The more sperm depolarization, the more internal calcium increased, indicating that a voltage-dependent calcium transport system was present in sperm. Experiments performed in zero calcium indicated that indeed, the voltage-dependent calcium increment was due to calcium influx from the external medium, and that this permeability pathway remained open 1 min in human incapacitated sperm.

At the resting membrane potential, ~ -40 mV, valinomycin hyperpolarized the incapacitated sperm (-71 mV) and enhanced 4 times the calcium increase induced by depolarization with KCl (Fig. 3), suggesting that hyperpolarization removed voltage-dependent calcium channels from an inactivated state. However, it should be noted that valinomycin, by virtue of its K ionophore activity, can also uncouple mitochondria. So valinomycin, as uncoupler, could contribute to the observed enhancement of the calcium increment induced by KCl depolarization by means of a mitochondrial collapse that should disable mitochondria to act as calcium buffer. However, as described in Section 2, the fact that cyanide or CCCP did not alter DiSC3(5) fluorescence indicates that uncapacitated sperm mitochondria should have a potential close to zero, suggesting that the observed calcium increments induced by KCl when preceded by valinomycin, were not due to a valinomycin-induced mitochondrial collapse. So, the most plausible explanation for the valinomycin effect was that hyperpolarization removed the putative voltage-dependent calcium channels from their inactivated state.

In mouse spermatocytes, the only detectable voltage-dependent calcium current is a T-type that inactivates in milliseconds and is blocked by nifedipine and nickel [6–8]. Since mouse mature sperm is not able to synthesize proteins, and the acrosome reaction is blocked by the channel blockers nickel and nifedipine, it has been proposed that the physiological inductor of the AR, the zona pellucida protein ZP3, should open this channel by depolarization. This has lead to the hypothesis that the T-type calcium channels found in mouse spermatocytes could increase a sustained calcium influx if they activate between -50 (pulse) and -40 mV (prepulse) [6,7]. This is the range of voltages that comprises the area under the intersection of the activation and steady-state inactivation curves; at these voltages, there are tiny currents with no complete inactivation [6,7]. In this regard, the small increase in intracellular calcium induced by hyperpolarization in human sperm (this paper), might be explained if hyperpolarization removed inactiva-

tion and at this voltage (−70 mV) there was a voltage-dependent calcium influx.

Although the voltage dependence of the calcium influx in human sperm might correspond to a T-type channel, it is difficult to explain our observations exclusively in terms of this calcium channel regulation. First, the human sperm voltage-dependent calcium influx was blocked by nickel, so does happen in T-channels [7]. However, unlike the T-current, nifedipine, even at 50 μM, (or 20 μM verapamil) did not inhibit the voltage dependent calcium influx in human sperm. Secondly, the continuous increase in calcium (during seconds, immediately after potassium addition) observed at depolarizations of −30 and −15 mV suggests that the voltage-dependent calcium pathway does not inactivate in milliseconds as it would be expected for the T-type channel. What we observed, in experiments made in medium without calcium, was that the voltage-dependent calcium influx remained open around 1 min and declined to zero in 2 min. This suggests that these putative voltage-dependent calcium channels have a very slow inactivation. In this regard, Hockberger and Nam [27] have reported a very slow inactivation (inactivation constant of minutes) in high voltage-activated calcium channels present in developing neurons. Interestingly, this slow inactivating current, like the voltage-dependent calcium influx that we report in this paper, is also nifedipine insensitive.

In mouse and bovine sperm, it has been observed that potassium induces an increase in internal calcium only at external pH 8.5 or at normal pH but the presence of NH_4Cl increases internal pH [10,12,28]. In addition, in ram sperm, there is a presumptive voltage-dependent pHi control system that alkalinizes the cell upon depolarization and acidifies upon hyperpolarization [12]. In uncapacitated human sperm, we observed that the voltage-dependent calcium influx did not require an increase of internal or external pH. Furthermore, even in HSMm medium buffered at pH 6.4 the voltage-dependent calcium influx was still observed. Nevertheless, NH_4Cl enhanced the voltage-dependent calcium influx. On the other hand, depolarization also induced a small increase in pHi; hence, the voltage dependent calcium influx triggered by potassium could be enhanced by intracellular pHi increase. It should be mentioned that, even though in human sperm, an increase in external or internal pH is not required to observe voltage-dependent calcium influx, the acrosome reaction requires it, since experiments reported by Brandelli et al. [15] indicate that the AR can be induced by high K only in medium at pH 8.5.

It is possible that more than one voltage-dependent calcium channel might be involved in the observed responses. In this paper, we show that, nickel blocked the transient peak of the depolarization-induced calcium influx, but the sustained response was less affected, suggesting that there are two different sensitivities to the blocker. Furthermore, it has been suggested that a non-voltage-dependent calcium influx should contribute to the voltage and pH-dependent calcium influx observed in bovine and mouse sperm [8,29]. These authors have suggested that an initial calcium influx through the T-type calcium channel would increase internal calcium which, in turn, would open calcium-sensitive calcium channels. As for invertebrate sperm, Guerrero and Darszon [24] have found in sea urchin sperm a calcium permeability pathway that depends on calcium and pHi. In addition, in sea urchin and mouse sperm plasma membrane preparations incorporated in planar lipid bilayers, it has been reported a multisate, low selective calcium channel [30,31] that is insensitive to dihydropyridines. Therefore, human mature sperm could have other type of channels, whose opening might contribute to the observed sustained voltage-dependent increase in calcium.

Zeng et al. [14] have reported that bovine and mouse sperm hyperpolarize during capacitation, and that this plays a physiological role, since the T-type calcium channel would be removed from an inactivated state and ready to be opened by physiological inductors. The zona pellucida inductor of the acrosome reaction, ZP3, induces in capacitated sperm a slow depolarization that would open voltage-dependent calcium channels [10]. Traces presented in Fig. 3 support this hypothesis. In human incapacitated sperm, at the resting membrane potential (around −40 mV), depolarization induces a small increase in intracellular calcium, whereas if the sample is hyperpolarized to −70 mV, depolarization induces an enhanced increase in internal calcium. In agreement with this, we have observed that human sperm membrane potential tends to close EK (−70 mV) in hours, that is, during capacitation (unpublished obser-

vations). In fact, the high standard deviation obtained in resting membrane potential estimations within 2 h after Fura-2 loading (-40 mV $\pm$ 16 mV) could be a reflection of the tendency of the membrane potential to hyperpolarize. All these observations suggest that in human sperm, a major physiological role of capacitation could be membrane potential hyperpolarization. In this regard, voltage-dependent calcium influx described in this paper might play a crucial role in the progesterone-induced acrosome reaction, since this hormone induces in human sperm a depolarization [32,33] and triggers calcium influx [34]. Experiments are currently conducted in our laboratory to study the role of membrane potential calcium influx in specific physiological conditions, such as the capacitation process and the acrosome reaction in human sperm.

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