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Glucose induces a Na^+,K^+-ATPase-dependent transient hyperpolarization in human sperm. I. Induction of changes in plasma membrane potential by the proton ionophore CCCP

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Abstract

When human sperm was incubated in medium deprived of glucose, glucose restoration caused a transient hyperpolarization of the plasma membrane. This hyperpolarization was also induced by fructose but not by 2-deoxyglucose, a substrate that cannot be metabolized. The hyperpolarization was inhibited by NaF, a glycolysis inhibitor, but not by mitochondrial inhibitors (cyanide, rotenone and antimycin), suggesting that it depended on glycolysis. Furthermore, the hyperpolarization was still induced in medium containing a high concentration of KCl and was insensitive to the K^+ channel blocker TEA and the Cl^- channel blocker niflumic acid, but it was blocked by ouabain. This suggested that upon glucose addition, there was an increase in the concentration of ATP, that in turns increased the Na^+,K^+-ATPase activity. Since this pump is electrogenic ($2K^+/3Na^+$) the plasma membrane hyperpolarized. On the other hand, CCCP, a proton ionophore, inhibited the hyperpolarization induced by glucose. When CCCP was added to glucose-treated hyperpolarized sperm, it caused a depolarization that triggered a Ca^{2+} influx sensitive to nickel, an inhibitor of voltage-dependent calcium channels. Moreover, CCCP caused hyperpolarization in sperm incubated in medium without calcium, a known condition that depolarizes sperm. This indicated that CCCP induced proton permeability in the plasma membrane that was able to change the membrane potential to a value corresponding to the E_H and that was also able to clamp it, so that it prevented the hyperpolarization induced by glucose.

Keywords: Sperm; Glucose metabolism; Na^+,K^+-ATPase; Membrane potential; Carbonyl cyanide *m*-chlorophenyl hydrazone; Diisopropylthiodicarbocyanine iodide

1. Introduction

In mammalian sperm, modulation of ion transport systems plays a fundamental role in fertilization. Indeed, during capacitation, a spontaneous Ca^{2+}-dependent process that makes sperm acquire a high motility and enables them to undergo the acrosomal

Abbreviations: $diSC_3(5)$, diisopropylthiodicarbocyanine iodide; HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]); CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; HSM, human sperm medium; H-HSM, HEPES buffered HSM; 0glu-H-HSM, H-HSM without glucose

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exocytosis (AE), there is a slow Ca^{2+} influx. The AE, that is induced by the egg zona pellucida glycoprotein ZP3 (reviewed in [1,2]) is also related to a remarkable Ca^{2+} influx, – probably mediated by voltage dependent calcium channels (VDCC) (reviewed in [1]), and an increase in internal pH. The plasma membrane potential seems to play an important role since, besides VDCC, it may also modulate intracellular pH (reviewed in [1]). Thus, the ion transport mechanisms involved in membrane potential regulation deserves to be explored in sperm physiology, especially in light of the proposed mechanisms of AE induction.

In sperm cells, the ion transport systems cannot be easily studied with voltage clamp methods [1]. Alternatively, fusion of purified sperm plasma membrane to artificial planar lipid bilayers has allowed detection of unitary ion channels. Mammalian sperm is endowed with K^+ channels modulated by cAMP and niflumic acid-sensitive Cl^- channels [1]. On the other hand, optical recordings of membrane potential indicate that a K^+ [3,4], and perhaps a Na^+ [5] conductance contribute in part to the membrane potential. Thus, a complex pattern of ion conductances could set the membrane potential whose value in mouse, bovine and human sperm ranges between −30 and −60 mV [3,5,6].

Besides ion channels, electrogenic transport systems may also contribute to the plasma membrane potential. In skeletal muscle, the Na^+,K^+-ATPase, can contribute in 6 mV to the membrane potential [7]. On the other hand, in tumor ascites cells, in which ion permeability through channels is poor, a Na^+,K^+-ATPase has been found to contribute to membrane potential in about −70 mV [8]. In mouse sperm, a contribution of the Na^+,K^+-ATPase on membrane potential that is observed when Ca^{2+} is added to sperm incubated in medium without Ca^{2+} has been reported [5].

In this paper, we report a contribution of the Na^+,K^+-ATPase to the plasma membrane potential of human sperm that was evident in a particular experimental condition. When human sperm was incubated in a medium deprived of glucose, glucose restoration caused an ouabain sensitive hyperpolarization that was followed by a slow depolarization to resting values. We show experiments that indicate that an increase in ATP synthesis, generated by glycolysis, stimulated the Na^+,K^+-ATPase that resulted in a hyperpolarization of the plasma membrane. On the other hand, evidence is also presented indicating that CCCP, a proton ionophore that collapses the mitochondrial potential, may also change the plasma membrane potential in human sperm.

2. Materials and methods

2.1. Materials and media

Acetoxymethyl ester (fura 2-AM) and diisopropylthiodicarbocyanine iodide ($diSC_3(5)$) were obtained from Molecular Probes; ionomycin and valinomycin from Sigma. The other reagents were from Sigma, Merck and Baker. Human sperm medium deprived of glucose (0glu-HSM) had the following composition [9] (in mM): NaCl, 117.5; KCl, 8.6; $CaCl_2$, 2.5; $MgCl_2$, 0.49; Na^+-pyruvate, 0.3; Na^+-lactate, 19; $NaHCO_3$, 25; and 3 mg/ml BSA (bovine serum albumin) (pH 7.6–7.7). Fluorescence recordings were done in medium with the same composition except that $NaHCO_3$ was replaced by 25 mM HEPES (pH 7.6) and BSA was removed (0glu-H-HSM).

2.2. Sperm purification

Human semen was obtained from 10 healthy donors (from 18 to 22 years old). These samples were normal according to the World Health Organization protocol [10]. Sperm purification was performed using percoll gradients according to Suarez et al. [9] except that glucose-free solutions were used. Liquefied semen was layered on a discontinuous percoll gradient (75 and 50% in 150 mM NaCl, HEPES 10 mM, pH 7.6) and centrifuged at $300 \times g$ for 20 min. Sperm pellet was washed in 0glu-H-HSM and immediately loaded with fura 2-AM as described below. Membrane potential and $[Ca2+]_i$ were detected simultaneous and constantly monitored. The resting $[Ca^{2+}]_i$ found in different batches of sperm in 0glu-H-HSM (see below) ranged between 135 and 230 nM, indicating that sperm samples were viable. Sperm motility was evaluated by phase-contrast microscopy. In 0glu-H-HSM sperm motility was low and 2 mM glucose addition stimulated sperm motil-

ity to normal values. Sperm motility, assessed in capacitating medium (with glucose), was not appreciably affected by 0.5 μM $diSC_3(5)$, 0.4 μM valinomycin, or 0.4 μM CCCP (not shown). Mitochondrial inhibitors, such as antimycin, rotenone and cyanide, did not modify sperm motility either, confirming a previous study [11].

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

Membrane potential ($diSC_3(5)$) and $[Ca^{2+}]_i$ (fura 2) were detected simultaneously with a PTI spectrofluorometer (Photon Technology), according to Linares-Hernández et al. [3]. Fura 2 was excited alternatively at 340 and 380 nm with the automatic excitation monochromator of the PTI system and $diSC_3(5)$ was excited at 600 nm with an additional halide lamp placed in front of the xenon source. Fura 2 and $diSC_3(5)$ fluorescences were detected with two photomultipliers, located at 90° with respect to respect the excitation source, and optical filters of 488 and 670 nm, respectively (Andover). The ratiometric readings of fura 2 and the single reading of $diSC_3(5)$ were acquired at 0.85 Hz and the data were collected and analyzed with the PTI computer interface.

2.4. Measurement and calibration of $[Ca^{2+}]_i$ and membrane potential

Cells ($1–2\times10^8$) were loaded in 1 ml 0glu-H-HSM with 3 μM fura 2-AM and incubated 40 min at 37°C. Cells were washed with 0glu-H-HSM and maintained in 25 ml 0glu-HSM, at 37°C and an atmosphere of 5% CO_2–95% air (100% humidity). To do fluorescence recordings, 2–3 ml of fura 2-loaded sperm were centrifuged at $300\times g$ for 5 min and the pellet (30–50 μl) was added into the fluorometer cuvette containing 2.5 ml 0glu-H-HSM+0.5 μM $diSC_3(5)$, the membrane potential sensitive dye. The experiments were performed at 36–37°C under constant magnetic stirring (400 rpm). Ratios of fura 2 fluorescence (see above) were calibrated by using $K_d = 224$ nM as described by Linares-Hernández et al. [3].

The membrane potential of fura 2-loaded sperm was calibrated by inducing an electrochemical K^+ potential with 0.4 μM valinomycin at different K^+ concentrations [3]. We plotted $F_x - F_{8.6}/F_{8.6}\times100$ vs. E_k, where $F_{8.6}$ is the fluorescence value induced with valinomycin in 0glu-H-HSM (that is, at $[K]_{ext} = 8.6$ mM), and F_x, is the fluorescence obtained with different external K^+ concentrations. The Nernst electrochemical potential for K^+ distribution (E_K), at 37°C, is, $E_K = -61.54$ mV log $[K^+]_i/[K^+]_{ext}$, where $[K^+]_i = 120$ mM. This internal $[K^+]$ was reported in human sperm incubated in glucose containing medium [3]. The same internal K^+ concentration was also found in sperm incubated in 0glu-H-HSM (not shown). The calibration curve is linear in the range of 8.6 to 68.6 mM external K^+ [3]; in experiments performed in 1 mM external K^+ (Fig. 6), the $diSC_3(5)$ fluorescence obtained upon valinomycin addition was far from the linear relationship, that is, less negative than that corresponding to a the theoretical $E_k = -120$ mV [3].

2.5. Measurement of sperm ATP

Purified human sperm ($1–2\times10^8$ cells) were prepared and loaded with fura 2-AM (as described above) either with glucose or not glucose containing media. Thereafter, sperm was incubated 30 min in 25 ml of either 0glu-HSM or HSM (glucose containing medium) at 37°C and an atmosphere of 5% CO_2–95% air (100% humidity). Five milliliters of this suspension was centrifuged at $300\times g$, 10 min and resuspended in 2 ml of either 0glu-H-HSM or H-HSM, and incubated 5 min at 37°C. Aliquots of 1 ml sperm suspensions were treated with 1 ml 10% trichloroacetic acid (TCA). The remaining 1 ml of sperm incubated in 0glu-H-HSM was supplemented with 2 mM glucose, incubated 5 min at 37°C, and treated with 1 ml 10% TCA. These TCA-precipitated samples were centrifuged and the supernatant used for ATP determination. The ATP concentration was measured according to the luciferin-luciferase method [12] with a Bioorbit 1250 luminometer.

3. Results

3.1. Glucose induces a transient hyperpolarization in human sperm deprived of glucose

Fig. 1 shows the effect of glucose addition on

$[Ca^{2+}]_i$ and membrane potential of human sperm deprived of glucose. After a few seconds of addition, glucose caused a $diSC_3(5)$ fluorescence decrease, suggesting a hyperpolarization, that was followed by a slow fluorescence recovery. These fluorescence changes were accompanied by small changes in $[Ca^{2+}]_i$ (detected simultaneously). The fluorescence quenching was related to a decrease in the concentration of $[Ca^{2+}]_i$, whereas the fluorescence recovery was related to an increase.

An important consideration regarding the effect of glucose is that in spite of the presence of lactate and pyruvate in the medium, glycolysis products could still induce an increase in mitochondrial activity, and thus mitochondrial hyperpolarization. Since $diSC_3(5)$ has a positive charge and permeates the membranes, it could also detect mitochondrial potential. Indeed, according to the sensing mechanism of $diSC_3(5)$, a mitochondrial membrane hyperpolarization (mitochondrial activation) should cause an accumulation and consequently a quenching of $diSC_3(5)$ fluorescence; conversely, a collapse of mitochondrial membrane potential should cause an increase of fluorescence [13,14]. It was then necessary to assess if the glucose-induced fluorescence quenching was contributed by mitochondrial hyperpolarization. To do this, we studied the effect of glucose in the presence of the mitochondrial inhibitors rotenone, antimycin and cyanide, that inhibit mitochondrial activity at three different sites and thus should inhibit mitochondrial hyperpolarization [15]. We also assessed the effect of CCCP, a proton ionophore that collapses the proton gradient and thus mitochondrial potential. As previously reported in sperm incubated in glucose-containing medium [3], rotenone, antimycin and cyanide did not affect the resting fluorescence recording, indicating no detectable $diSC_3(5)$ fluorescence contributed by mitochondria (Fig. 2A). When glucose was added, it still induced fluorescence quenching although it was slightly smaller than control (Fig. 2A). This indicated that most of the signal (about 75%) was due to plasma membrane potential.

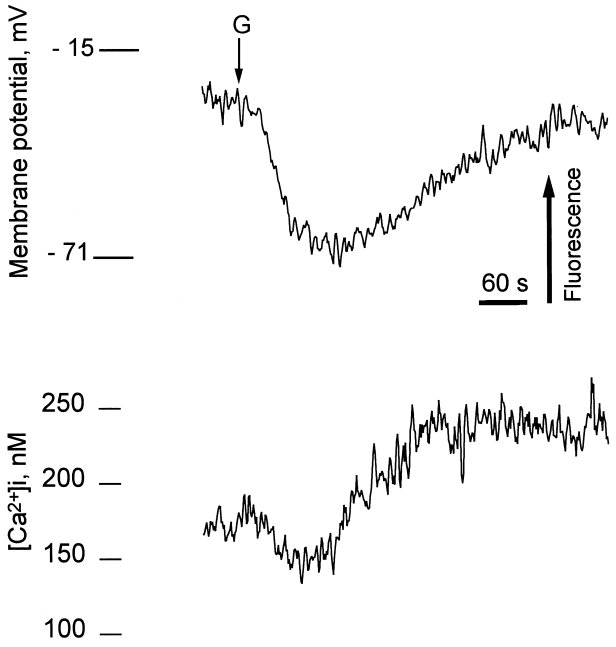

Fig. 1. Effect of glucose on $[Ca^{2+}]_i$ (lower trace) and the fluorescence of the membrane potential sensitive dye $diSC_3(5)$ (upper trace) in human sperm incubated in H-HSM medium without glucose (0glu-H-HSM). Fura 2-loaded human sperm (around 10^7 cells) were added to a fluorescence cuvette containing 2.5 ml 0glu-H-HSM+0.5 μM $diSC_3(5)$ and both fluorescences were detected simultaneously at 36°C and under constant magnetic stirring, as described in Section 2. After 4 min, 2 mM glucose was added. The calibration values shown at the left of the trace of $diSC_3(5)$ fluorescence indicate the values reached upon a further addition of 0.4 μM valinomycin (−71 mV) and, 1 min later, 60 mM KCl (− 15 mV). The rest of the experimental conditions are described in Section 2. The traces are representative of five experiments.

When mitochondria collapse was induced with CCCP, a very small increment in basal $diSC_3(5)$ fluorescence was observed indicating a corresponding small contribution of mitochondrial potential to the overall signal. However, unexpectedly, CCCP blocked the glucose-induced fluorescence quenching (Fig. 2B) suggesting that this quenching could be due to activation and consequently hyperpolarization of mitochondria. This result clearly contradicted experiments performed with mitochondrial inhibitors. To solve this apparent contradiction we explored the effect of CCCP on membrane potential.

CCCP addition to sperm hyperpolarized with glucose caused a rapid increase in $diSC_3(5)$ fluorescence (Fig. 3A) that could indicate either mitochondria membrane potential collapse (see above) or/and plasma membrane depolarization. To determine the origin of the CCCP-induced increase in fluorescence, we examined if CCCP-gated voltage-dependent calcium channels (VDCC) by simultaneous detection of $[Ca^{2+}]_i$. If the plasma membrane was depolarized by CCCP, gating of VDCC would be expected,

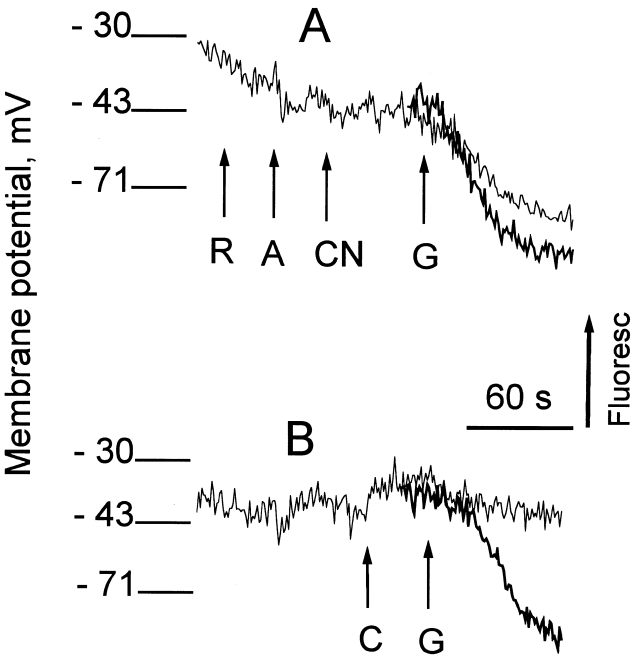

Fig. 2. Effect of mitochondrial inhibitors and the proton ionophore CCCP on the diSC3(5) fluorescence quenching induced by glucose in fura 2-loaded human sperm incubated in 0glu-H-HSM. In A, sperm were treated with 0.4 μM rotenone (R), 2 μM antimycin (A), and 1 mM NaCN (CN), and then 2 mM glucose was added as indicated. In B, 0.4 μM CCCP (C) was added 30 s before the addition of 2 mM glucose. In both panels, the control traces (no CCCP or mitochondrial inhibitor addition) are indicated in bold. Membrane potential calibrations are indicated at the left of the traces. The rest of the experimental conditions are described in Fig. 1. The traces are representative of five experiments.

which are present at the plasma membrane. Consistently, an increment of $[Ca^{2+}]_i$ was also induced by CCCP at an extent similar to that obtained with a comparable depolarization by K^+ (first calibration step) (Fig. 3A). Hyperpolarization with valinomycin caused a decrease in $[Ca^{2+}]_i$ to resting levels that could be a reflection of VDCC deactivation. The Ca^{2+} influx induced by CCCP was sensitive to nickel, a VDCC blocker [16] that also inhibited the voltage-dependent Ca^{2+} influx (calibration of Fig. 3A and [3]). This result indicated that indeed, CCCP depolarized the plasma membrane to ~ -36 mV, that is, a value close to the Nernst electrochemical potential for proton distribution ($E_H = -40$ mV, by taking pH_i 6.9 [3] and an average of extracellular pH 7.55). Furthermore, in experiments carried out in medium with low calcium (0glu-H-HSM+5 mM EGTA), the fluorescence value was higher as compared with Ca^{2+}-containing medium, suggesting that resting membrane potential was depolarized (Fig. 3B, traces a and b). Foresta et al. have also reported that human sperm depolarizes its membrane potential in medium without Ca^{2+} [17]. This suggests that membrane potential depends on $[Ca^{2+}]_i$. In medium without Ca^{2+}, if the membrane potential were less negative than E_H, CCCP should hyperpolarize. Fig. 3B shows that this was, indeed, the case: CCCP induced a hyperpolarization to the E_H with no change in $[Ca^{2+}]_i$. Glucose addition to sperm incubated in 0glu-H-HSM without calcium caused hyperpolarization to values more negatives than E_H; in this case CCCP induced the expected depolarization to a value close to E_H (Fig. 3B, traces b and b′). Furthermore, at resting membrane potential values close to −40 mV, CCCP caused only small changes in $diSC_3(5)$ fluorescence, suggesting that in this case, the membrane potential almost equaled E_H (Fig. 2B and [3]). These results indicated that CCCP was able to induce proton permeability through the plasma membrane in human sperm leading to change of its membrane potential to the E_H. Consequently, the inhibitory effect of CCCP on the glucose-induced hyperpolarization (Fig. 2B) could be due to a tendency of this proton ionophore to maintain (clamp) the membrane potential at the E_H. As shown in Fig. 3A,B, further addition of valinomycin overcame the weaker clamping effect of CCCP and changed the fluorescence to the E_K.

Taking all these lines of evidence into consideration, the transient quenching of $diSC_3(5)$ fluorescence induced by glucose was almost entirely due to a plasma membrane hyperpolarization. According to the calibration, the glucose-induced hyperpolarization was of around 30 mV (from a resting membrane potential of -44 ± 3.4 mV in the absence of glucose to -77 ± 5 mV; mean ± S.E., $n = 5$); values beyond the E_K were frequently found.

3.2. Mechanism of the hyperpolarization induced by glucose

Since glucose is the main energy source of sperm (in terms of cell motility) [18], we examined the relationship between hyperpolarization and carbohydrate catabolism. Fig. 4A, shows that the hyperpola-

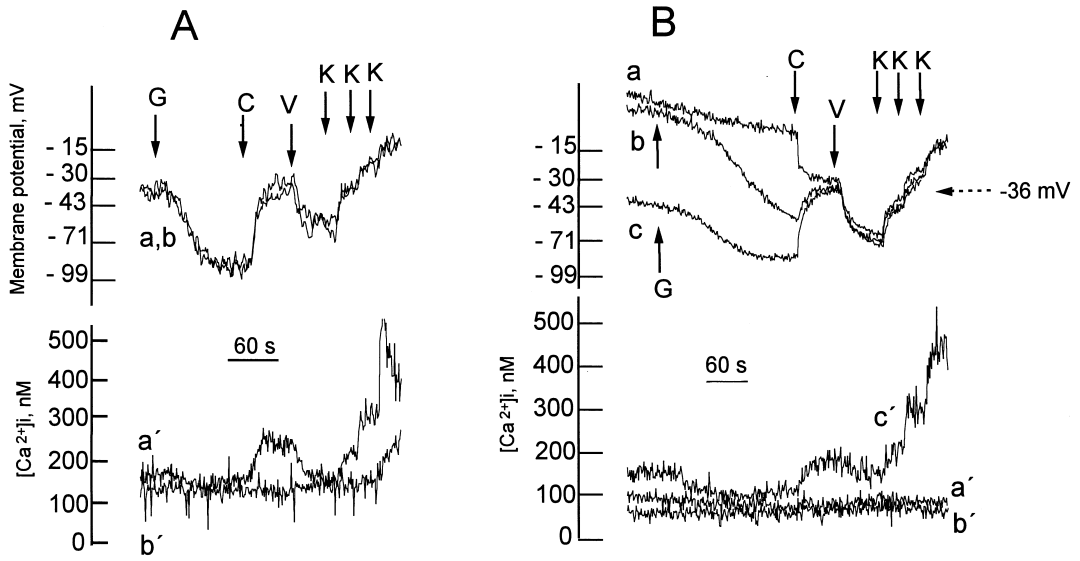

Fig. 3. Opposite effects of the proton ionophore CCCP on human sperm plasma membrane potential. (A) Effect of 0.4 μM CCCP (C) on diSC$_3$(5) fluorescence (trace a) and $[Ca^{2+}]_i$ (trace a′) detected simultaneously in fura 2-loaded sperm in which a hyperpolarization (diSC$_3$(5) quenching) has been induced with the addition of 2 mM glucose (G). Once the glucose-induced quenching of diSC$_3$(5) fluorescence reached a minimum and stable value, CCCP was added. Traces b and b′ are identical as described for traces a and a′, except that the experiment was done in the presence of 600 μM $NiCl_2$ (added at the beginning of the experiment, 7 min before CCCP addition). The traces are representative of five experiments. (B) Effect of 0.4 μM CCCP on diSC$_3$(5) fluorescence (a) and $[Ca^{2+}]_i$ (a′) detected simultaneously in fura 2-loaded human sperm incubated in 0glu-H-HSM+5 mM EGTA ('zero calcium' medium at pH 7.56). Traces b and b′ show the effect of CCCP on sperm incubated in zero calcium medium and hyperpolarized with 2 mM glucose, as indicated. Traces c and c′ show experiments in which 0.4 μM CCCP was added to glucose-treated sperm in normal (Ca^{2+}-containing) 0glu-H-HSM medium. In traces a, b and c, 0.4 μM valinomycin (V) and 15, 15 and 30 mM KCl (K) were added as indicated (in traces b and b′, the effect of 30 mM KCl is not shown). The calibrated membrane potential values are shown at the left of the diSC$_3$(5) traces. The dotted arrow indicating −36 mV is the membrane potential value reached upon CCCP addition. The traces are representative of five experiments.

rization was also triggered by another metabolizable sugar, fructose, but not by 2-deoxy glucose, a sugar that is transported into the cell and phosphorylated by hexokinase, but does not pass on the next catalytic step [19]. We also assessed the effect of NaF, an inhibitor of enolase [19], the enzyme that catalyzes the reaction from 2-phosphoglycerate to phosphoenolpyruvate, with the production of two ATP molecules. In the concentration range that inhibited sperm motility (around 0.5 mM), NaF inhibited the glucose-induced hyperpolarization (Fig. 4B). The NaF concentration that inhibited 50% of the glucose-induced hyperpolarization was 1.3 ± 0.4 mM (mean ± S.E., $n = 4$). These results suggested that the glucose-induced hyperpolarization was related to mechanisms involving glucose catabolism. However, it should be noted that fluoride, at concentrations used to inhibit glycolysis, is also able to inhibit the Na^+,K^+-ATPase [20]. The inhibitory effect of fluoride on this ATPase could contribute to the inhibition of the hyperpolarization induced by glucose (see below).

The ion transport mechanisms involved in the hyperpolarization induced by glucose were investigated. The hyperpolarization was not affected in medium containing either high KCl, or the K^+ channel blocker TEA [16], or niflumic acid, a Cl^- channel blocker [21] (traces not shown). The hyperpolarization induced by glucose in the presence of 50 μM niflumic acid, 68.6 mM KCl and 10 mM TEA were 28 ± 5, 29 ± 3 and 28 ± 5 mV (mean ± S.E., $n = 4$) respectively, which were statistically indistinct of control experiments (31 ± 4 mV, mean ± S.E., $n = 4$). Thus, Cl^- or K^+ channel opening did not contribute to a detectable extent to the glucose-induced hyperpolarization. However, ouabain, a Na^+,K^+-ATPase blocker, inhibited the hyperpolarization ($IC_{50} = 700$ nM) indicating that this pump was involved in the hyper-

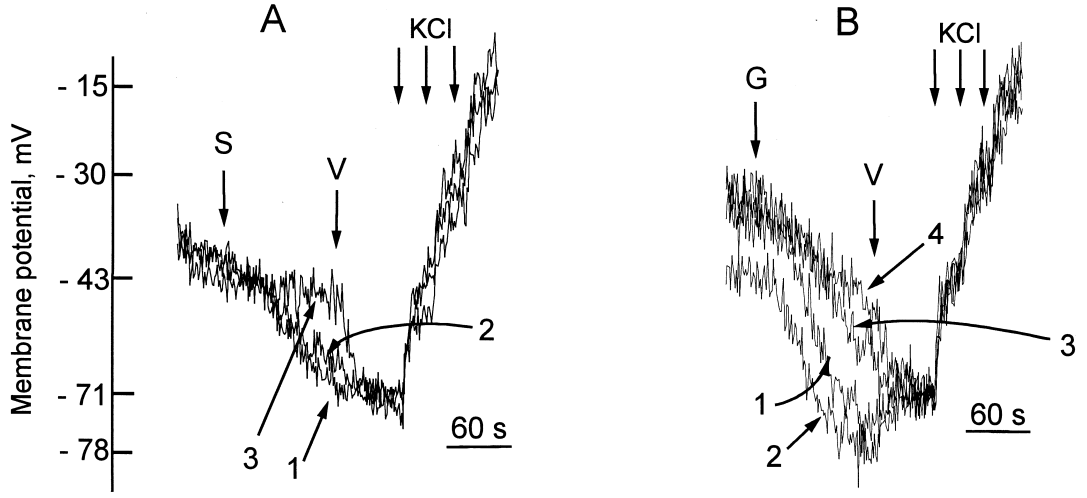

Fig. 4. The hyperpolarization induced by glucose is related to glucose metabolism in human sperm. In A, it is shown the effect of 2 mM glucose (trace 1), 2 mM fructose (trace 2) and 2 mM 2-deoxyglucose (trace 3) on membrane potential (detected with $diSC_3(5)$) in fura 2-loaded sperm incubated in 0glu-H-HSM. The sugars (S) were added as indicated. The traces are representative of five experiments. In B, it is shown the effect of different concentration of NaF on the glucose (G) induced hyperpolarization of fura 2-loaded human sperm incubated in 0glu-H-HSM. The NaF concentrations were (in mM): 1, 0; 2, 0.4; 3, 1.0; 4, 2.0. NaF was added 4 min before 2 mM glucose addition, that is, at the beginning of the fluorescence recording. The calibration with 0.4 μM valinomycin (V) and 15, 15, and 30 mM KCl (K) is also shown. The rest of the experimental conditions are described in Fig. 1. The traces are representative of four experiments.

polarization (Fig. 5A,B). To further assess the role of Na^+,K^+-ATPase in the hyperpolarization induced by glucose, the effect of glucose addition on membrane potential was tested in sperm incubated in medium

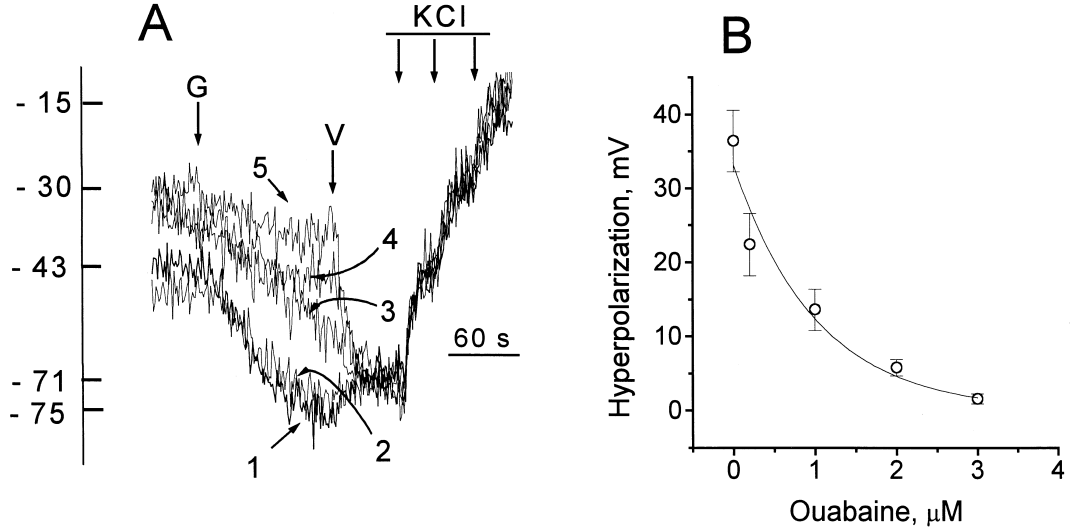

Fig. 5. Effect of ouabain on the glucose-induced hyperpolarization detected with $diSC_3(5)$ in fura 2-loaded human sperm. In A, it is shown the effect of different concentrations of ouabain on the glucose-induced hyperpolarization. Ouabain was added at the beginning of the trace, that is, 4 min before 2 mM glucose addition, at the following concentrations (in μM): 1, 0; 2, 0.2; 3, 1.0; 4, 2.0; 5, 3.0. Valinomycin (0.4 μM) and 15, 15 and 30 mM KCl were added as indicated. In B, it is shown the glucose-induced hyperpolarization as a function of ouabain concentration obtained from the experiments shown in (A). The bars are S.E. with $n=5$. The rest of the experimental conditions are described in Fig. 1.

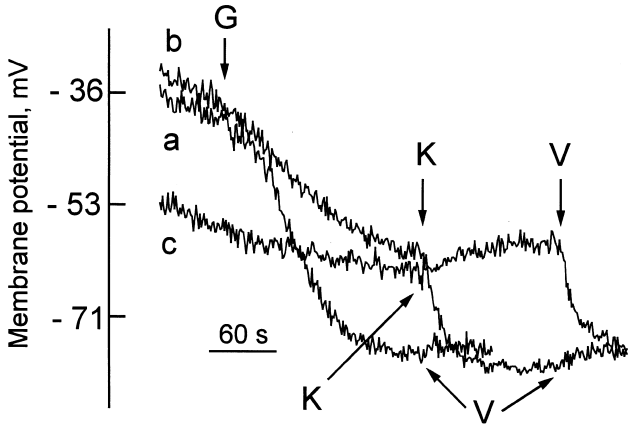

Fig. 6. Effect of 2 mM glucose (G) on membrane potential detected with $diSC_3(5)$ in fura 2-loaded human sperm incubated in 0glu-H-HSM without K^+. Trace a is a control experiment in 0glu-H-HSM containing 1 mM KCl. Trace b shows the effect of glucose on sperm incubated in 0glu-H-HSM without KCl. Then, 1 mM KCl (K) was added. Trace c is the same as b, but in the presence of 3 μM ouabain; 0.4 μM valinomycin (V) was added as indicated. The traces are representative of five experiments.

without K^+, a condition that stops this ATPase activity. As expected, in medium without K^+, the hyperpolarization induced by glucose was remarkably inhibited (Fig. 6, trace b); the small hyperpolarization triggered by glucose could be related to the presence of micromolar amounts of KCl present in the fluorescence cell that were added with sperm (which was in 0glu-HSM). When 1 mM KCl was added (a K^+ concentration that saturates the pump [22]), a hyperpolarization took place the extent of which reached control values. This suggested that indeed K^+ addition triggered Na^+,K^+-ATPase activity and hence hyperpolarized sperm. Consistently, the K^+-induced hyperpolarization in glucose-treated sperm was inhibited by ouabain (Fig. 6, trace c); conversely, a small depolarization was induced by K^+ in the presence of this inhibitor. It should be noted that the resting membrane potential measured in 0glu-H-HSM without potassium was rather unstable. Thus, the more negative membrane potential observed in sperm incubated in 0glu-H-HSM without potassium and the presence of ouabain (Fig. 6, trace c) was related to this lack of stability.

The amount of sperm ATP was measured under the experimental conditions used in this study. In fura 2-loaded sperm incubated 30 min in capacitating medium, the amount of ATP was 170 ± 19 pmol/1×10^6 cells (mean ± S.E., $n = 3$). In capacitating medium without glucose, the ATP content decreased to 122 ± 21 pmol/1×10^6 cells (mean ± S.E., $n = 3$) and increased to 155 ± 18 pmol/1×10^6 cells (mean ± S.E., $n = 3$) upon glucose addition (2 mM) and 5 min incubation. The increment in ATP concentration was consistent with the hypothesis that the glucose-induced hyperpolarization was related to activation of Na^+,K^+-ATPase by an increase in ATP production by glycolysis.

Results presented so far indicated that the hyperpolarization induced by glucose was due to a glycolysis-dependent ATP production that in turns stimulated a Na^+,K^+-ATPase. Since this pump is electrogenic ($3Na^+/2K^+$), its activation would be responsible for the observed hyperpolarization the extent of which was around −30 mV. However, in non-capacitated human sperm isolated and incubated in glucose containing media, the contribution of this pump, assessed by the effect of ouabain on resting membrane potential, was very small. In glucose-containing H-HSM medium, 3 μM ouabain during 4 min incubation (as in experiments depicted in Figs. 5 and 6) caused a depolarization of 6.5 ± 1.2 mV (mean ± S.D., $n = 4$).

4. Discussion

In this paper, we show that glucose induced a transient hyperpolarization in sperm incubated in medium deprived of glucose. The hyperpolarization occurred only with metabolizable carbohydrate, either fructose or glucose, but not by the non-metabolizable sugar 2-deoxyglucose, and was blocked by the glycolysis inhibitor sodium fluoride. These lines of evidence suggested that ATP, that increased by 27% upon glucose induction, was involved in the hyperpolarization. No evidence of K^+ or Cl^- channel activation was found. The hyperpolarization was sensitive to ouabain, suggesting that a Na^+,K^+-ATPase activated by ATP generated by glycolysis was involved; additionally, the inhibitory effect of fluoride, the inhibitor of glycolysis, on the hyperpolarization induced by glucose could also be due to a direct blocking action on the Na^+,K^+-ATPase [20].

Consistently, the glucose-induced hyperpolarization did not occur in medium without K^+, that stops this ATPase activity, and the further addition of KCl (1 mM) caused a ouabain-sensitive hyperpolarization, indicating that the pump was started by K^+. From the experimental evidence shown in this paper, the ion transport mechanism involved in the effect of glucose could be the following. Glucose or other metabolizable sugars are transported into the cell and enter to the glycolytic pathway. The ATP generated starts or increases a Na^+,K^+-ATPase, and causes a hyperpolarization due to its electrogenic exchange ($3Na[Ca^{2+}]_i/2K^+$). This process is accompanied by a decrease in $[Ca^{2+}]_i$ possibly due to closing of VDCC and/or an increase in a Ca^{2+} pumping activity (due to the increase in ATP production). In medium without glucose, the cells were able to maintain low values of $[Ca^{2+}]_i$ (135–230 nM) and a slow motility. Thus, the ATP produced by endogenous metabolism (in medium without glucose) was mainly limiting for Na^+,K^+-ATPase activity, but sufficient to sustain other processes, such as calcium pumping activity or a slow motility. It is important to note that glycolysis is the main metabolic pathway for ATP production in human sperm. Mitochondrial activity seems to have a minor role since sperm motility is not affected by mitochondrial inhibitors [11], nor in the absence of oxygen [23]. Consistently, the human sperm membrane potential detected with diS-$C_3(5)$ is insensitive to cyanide and the glucose-induced hyperpolarization was only slightly affected by mitochondrial inhibitors ([3] and Fig. 2A of this paper).

Even though changes in glucose concentration from zero to millimolar does not reflect any physiological condition, the observed phenomena can provide interesting information about the ion transport mechanisms that set and regulate the membrane potential in human sperm. In excitable cells, that have a low electrical resistance membrane, the Na^+,K^+-ATPase can still contribute to membrane potential in about 6 mV [7]. In contrast, ascites tumor cells are able to set a −70-mV membrane potential that can be collapsed by ouabain, suggesting that the Na^+,K^+-ATPase entirely sets this potential [8]. In human sperm, the Na^+,K^+-ATPase contributed in as much as −30 mV only when glucose was added to sperm incubated in medium without glucose. However, ouabain depolarized only 6–7 mV the plasma membrane potential of sperm incubated in normal medium with glucose. This may indicate that in normal conditions, the putative ion fluxes through ion channels that set the membrane potential (usually around −40 mV) could diminish the contribution of the Na^+,K^+-ATPase to the plasma membrane potential. These ion channels could open and cause the depolarization phase of the transient hyperpolarization induced by glucose in sperm deprived of glucose. This hypothesis implies that in medium without glucose and consequently in conditions in which ATP synthesis is relatively low, a fraction of the ion channels close. In this case, the membrane potential has a similar value as in glucose-containing medium, but would have an increased plasma membrane electrical resistance. Accordingly, in mouse sperm incubated in medium without Ca^{2+}, it has been found that Ca^{2+} induces a hyperpolarization that is contributed in a large extent by Na^+,K^+-ATPase activity [5]. This contribution has been explained as an increased influence of this pump on membrane potential due to a rise in electrical resistance caused by Ca^{2+} blockade of Na^+ influx. This influx would occur through a putative Ca^{2+} channel, that in medium without Ca^{2+} would allow Na^+ permeation [5].

In this work we also showed that electrogenic proton transport by CCCP was able to change the membrane potential to values close to E_H (Nernst potential for proton distribution between the cytoplasm and the medium). CCCP depolarized the plasma membrane in sperm hyperpolarized with glucose. This depolarization was confirmed by the fact that CCCP triggered an expected Ca^{2+} influx (triggered by voltage) which was sensitive to nickel, a VDCC blocker. The effect of CCCP on plasma membrane potential was further confirmed in experiments made in low Ca^{2+} medium, a known condition that depolarizes human sperm membrane potential [17]. In this condition, CCCP hyperpolarized the plasma membrane. Moreover, in sperm hyperpolarized by glucose in low calcium medium, CCCP induced a depolarization. In these cases, that is, in the depolarizing or the hyperpolarizing direction, the membrane potential changes induced by CCCP moved to $\sim$−36 mV, that is, to a value near to the theoretical E_H (−40 mV). Accordingly, CCCP induced minor changes at a membrane potential (V_m) of around −40 mV, that

is, when $V_m \sim E_H$. These results suggested that the inhibitory effect of CCCP on the glucose induced hyperpolarization (Fig. 2B) could be due to membrane potential clamping. This means that the proton permeability mediated by CCCP that contributed to the membrane potential was sufficiently high to maintain the membrane potential to a value close to E_H so that the hyperpolarization generated by Na^+,K^+-ATPase activation was prevented. Therefore, in sperm hyperpolarized with glucose, CCCP brought the membrane potential to the E_H, overcoming the negative potential set by the Na^+,K^+-ATPase. On the other hand, we should mention that in a previous paper, we reported a lack of effect of CCCP on $diSC_3(5)$ fluorescence in human sperm that was interpreted as an indication of no contribution of mitochondrial potential [3]. Actually, this lack of effect was probably due to the fact that the $E_H \sim -40$ mV, that was the resting membrane potential most frequently found [3].

Another interesting finding was that in the time course of the glucose-induced hyperpolarization, the membrane potential reached a minimal value and then started to depolarize to resting levels. This might be a compensating mechanism that tends to maintain the membrane potential at ~ -40 mV, that is in fact the membrane potential normally found in non-capacitated human sperm incubated in glucose-containing medium [3]. Since this depolarization was triggered in response to a hyperpolarization, that in turn was associated with a $[Ca^{2+}]_i$ decrease and a glucose-generated ATP increment, it would be possible that the ion(s) conductance responsible of the depolarization phase slowly responds to low Ca^{2+} and/or ATP concentrations. Gating of VDCC could also contribute to the depolarization. Current research in our laboratory is devoted to exploring these hypotheses.

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