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Intracellular Sodium Increase Induced by External Calcium Removal in Human Sperm

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ABSTRACT: In human sperm, removal of external calcium produces a fast Na^+-dependent depolarization that is presumably due to sodium permeation through calcium channels. Calcium restoration produces a ouabain-sensitive hyperpolarization that brings the membrane potential to values frequently more negative than resting. In this work, we show evidence indicating that external calcium removal induces an increase in the intracellular sodium ($[Na^+]_i$) and that this phenomenon is related to the Na^+-dependent depolarization. Calcium restoration blocked the $[Na^+]_i$ increase and then produced a slow decrease that was inhibited by ouabain. The $[Na^+]_i$ increase was inhibited by nanomolar–micromolar calcium or by millimolar magnesium, which has been previously shown to inhibit the Na^+-dependent depolarization. This evidence supports the hypothesis that, in zero-calcium medium, a calcium channel that would contribute to resting intracellular calcium levels allows sodium permeation, producing depolarization and a significant $[Na^+]_i$ increase. Sodium loading would stimulate the Na^+,K^+-ATPase, the activity of which contributes to the sperm hyperpolarization observed upon calcium restoration.

Key words: Membrane potential, intracellular calcium, SBFI, $diSC_3(5)$.

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In mammals, a successful fertilization requires that sperm accomplish specialized functions that involve intracellular calcium ($[Ca^{2+}]_i$) changes. Indeed, processes such as the hyperactivation of motility, the so-called "capacitation" and the acrosome reaction (AR), require concerted changes in ion permeability leading to intracellular calcium increases (Darszon et al, 2005). In mouse sperm, it has been postulated that the AR induced by ZP3 involves the sequential activation of T-type voltage-dependent calcium channels (VDCCs) and store-operated calcium channels (Darszon et al, 2005). Likewise, a peculiar form of VDCC present in the sperm flagella, catsper, is required for the hyperactivation of motility (Ren et al, 2001; Carlson et al, 2003; Kirichok et al, 2006). Hence, it is evident that the molecular basis underlying sperm function requires a full comprehension of the ion transport system that sets and regulates $[Ca^{2+}]_i$.

In mouse (Espinosa and Darszon, 1995) and human (Foresta et al, 1993; Guzmán-Grenfell et al, 2000) sperm, the studied species, calcium removal from the medium depolarizes sperm. In human sperm, the depolarization is large, approximately 50 mV, and Na^+ dependent; it is related to calcium removal at nanomolar–micromolar levels in the external medium, and it is always accompanied by a decrease in $[Ca^{2+}]_i$. Despite the clear positive relationship between $[Ca^{2+}]_i$ decrease and Na^+-dependent depolarization, evidence is strong that depolarization is not controlled by $[Ca^{2+}]_i$; instead, calcium removal from a putative external site triggers it. Calcium restoration produces a rapid $[Ca^{2+}]_i$ transient increase that peaks above resting and then decreases to basal values (González-Martínez, 2003). Concomitantly, Na^+-dependent depolarization is detained and hyperpolarization occurs, inhibited by ouabain or by the absence of potassium in the medium, suggesting that this hyperpolarization is produced by stimulated Na^+,K^+-ATPase activity (González-Martínez, 2003). This later effect of calcium occurs at the nanomolar–micromolar range and can be induced by magnesium in the millimolar range. Altogether, this evidence suggests that a putative calcium channel, which would contribute to resting $[Ca^{2+}]_i$, would be able to conduct sodium in the absence of calcium in human sperm. A corollary of this hypothesis is that Na^+-dependent depolarization would increase $[Na^+]_i$ so that, when calcium is restored and the depolarization stopped, the prevailing conductance would be set by Na^+,K^+-ATPase, the activity of which would be stimulated by the increased content of $[Na^+]_i$, causing hyperpolarization.

In this context, we studied whether Na^+-dependent depolarization affected $[Na^+]_i$ with the use of the sodium

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fluorescence probe SBFI. We provide evidence that approximately 50 mV Na^+-dependent depolarization induced by external calcium removal produces a slow increase in $[Na^+]_i$ content and that this increase could support a high Na,K^+-ATPase activity that hyperpolarizes the plasma membrane upon calcium restoration.

Methods

Materials and Media

The reagents used for media composition and Fura 2-AM were from Sigma Chemical Co (St Louis, Mo). SBFI-AM and $diSC_3(5)$ were from Molecular Probes (Invitrogen, Carlsbad, Calif). HEPES-buffered human sperm medium (HHSM) contained (mM, Suarez et al, 1986): 117.5 NaCl, 8.6 KCl, 2.5 $CaCl_2$, 0.49 $MgCl_2$, 0.3 $NaHPO_4$, 19 Na-lactate, 0.25 Na-pyruvate, 2.0 glucose, and 25 HEPES, adjusted to pH 7.6 with NaOH. The sodium content of this medium was modified, replacing NaCl by the same amount of cholineCl. The Intracellular medium (ic-HHSM) in which SBFI calibrations were performed contained (mM) 120 KCl, 0.49 $MgCl_2$, 0.3 NaH_2PO_4, 0.2 Na-pyruvate, 25 HEPES, 2 glucose, and different amounts of NaCl (0.5–100.5). The ionic force was maintained with cholineCl so that [NaCl] + [cholineCl] was 100.5 mM. The pH was brought to 6.7 with TRIZMA base. Medium zero-Na ic-HHSM was prepared without NaH_2PO_4 and Na-pyruvate.

Sperm Isolation and Dye Loading

Human semen was obtained from a panel of eight 19–33-year-old healthy donors. For intracellular sodium recordings, we selected semen samples that yielded 1–2 $\times$ 10^8 purified sperm. Sperm cells were isolated by percoll gradients as described (Suarez et al, 1986). The pellet was washed and resuspended in 1 mL HHSM containing 25 μM SBFI-AM (Molecular Probes) + 0.6% pluronic acid (Molecular Probes) and incubated for 90 min at 36°C, according to (Patrat et al 2000). In other experiments, sperm was loaded with 2 μM fura 2-AM (Sigma) as described in (González-Martínez, 2003). Once washed by centrifugation in the appropriate medium, the cells were used immediately for fluorescence recordings.

Detection and Calibration of Intracellular Sodium

Intracellular sodium was detected in SBFI-loaded sperm populations in a PTI spectrofluorometer (Photon Technology International, Birmingham, NJ). SBFI-loaded sperm (20–30 $\times$ 10^6 cells) were added to 2.5 mL of the appropriate medium kept at 36°C and under constant magnetic stirring. The sample was alternately excited at 340/380, and the fluorescence was detected with a visible long-wave pass filter of more than 495 nm (Andover Corp, Salem, NH) to optimize the signal to noise ratio. The 340/380 ratios were acquired and digitized at 0.83 Hz. A calibration curve was performed in ic-HHSM with different amounts of sodium (0.5–100.5 mM) and choline. Gramicidin was used to collapse the cationic gradients so that $[Na^+]_i$ nearly equaled external sodium. Hence, an approximately linear calibration curve in the range of 0–25 mM was achieved by comparing the ratios with the corresponding sodium concentrations. At $[Na] > 50$ mM, the ratios tended to saturate (Figure 1A). In this respect, the calibration data fitted the Hanes equation (Figure 1B), a linearized form of the Grynkiewicz equation (Diarra et al, 2001),

$$[Na^+]/(R - R_{min}) = \beta Kd/(R_{max} - R_{min}) + [Na^+]/(R_{max} - R_{min}) \quad (1)$$

where R is the fluorescence ratio excited at 340 and 380 nm (340/380), R_{min} is the R value experimentally determined in zero-sodium ic-HHSM (1.03 ± 0.002, n = 4, SE), R_{max} is the R value found at saturating sodium obtained from the slope of the curve $(1/(R_{max} - R_{min})$, and β is the fluorescence ratio excited at 380 nm, both in the absence and in the presence of saturating sodium (β = 1.6 ± 0.02, n = 4, SE). The slope and intercept $(\beta Kd/[R_{max} - R_{min}])$ of the curve were 1.43 ± 0.12 and 21.07 ± 0.88 (n = 4, SE), respectively. Given these values, the apparent dissociation constant of intracellular SBFI for intracellular sodium (Kd) in human sperm was 9.45 ± 0.70 mM (n = 4, SE), a value lower than that obtained in hippocampal neurons (15.7 mM) by Diarra et al (2001). Kd, R_{max}, R_{min}, and β were used to calibrate the R values in the Grynkiewicz equation (Diarra et al, 2001).

Detection and Calibration of $[Ca^{2+}]_i$ and Membrane Potential

The $[Ca^{2+}]_i$ and membrane potential were detected and calibrated in sperm populations, in some cases simultaneously, with fura 2 (excitation [exc] 340/380, emission [em] 488) and with $diSC_3(5)$ (exc 600, em 670) as described in González-Martínez, 2003.

Procedure to Remove Calcium From the Medium

Calcium was removed from the medium by the calcium chelator ethylene glycol tetraacetic acid (EGTA), as described in (González-Martínez, 2003). A stock solution containing 500 mM EGTA in 2 N NaOH was used. In this condition, calcium chelation with 3.5 mM EGTA did not modify the pH of the medium. The calcium

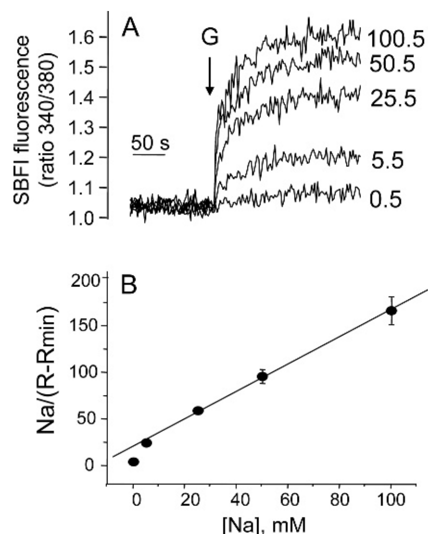

Figure 1. Detection and calibration of intracellular sodium ($[Na^+]_i$) in SBFI-loaded human sperm. A pellet with approximately 2×10^7 SBFI-loaded sperm was added to the fluorescence cell containing 2.5 mL of Intracellular HEPES-buffered human sperm medium (ic-HHSM) + different amounts of NaCl and cholineCl. The cell was kept at 36°C and under constant magnetic stirring. (A) Effect of gramicidin (1 μM) on the time course of 340/380 ratio fluorescence detected at more than 490 nm. The external sodium concentration (mM) is indicated at the right of the traces. (B) Hanes plot ($[Na^+]/(R - R_{min})$ vs $[Na^+]$). The R values at each [Na] (in the presence of gramicidin) were obtained from the traces. The continuous line corresponds to the equation $[Na^+]/(R - 1.03) = 20.5\ mM + 1.42[Na^+]$. The constants were used to calculate Kd by the Hanes equation (Equation 1), as described in Methods. (n = 4 individuals, $\bar{x} \pm$ SE).

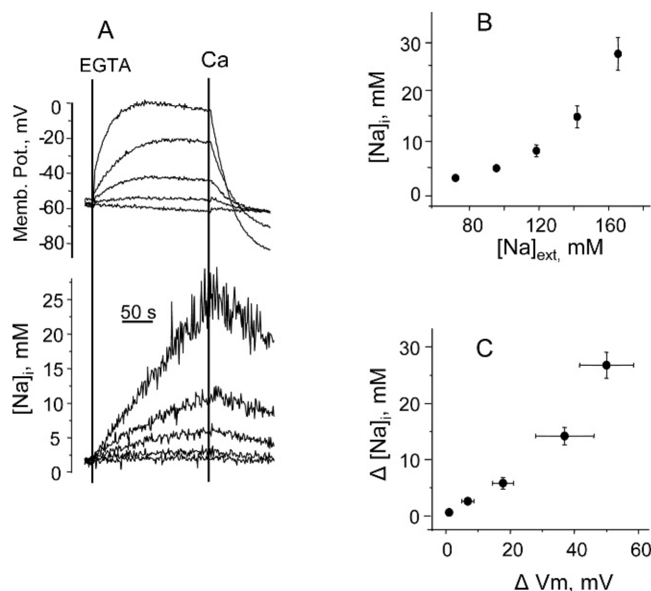

Figure 2. Intracellular sodium increase induced by calcium removal as a function of external sodium. SBFI-loaded sperm were poured into the fluorescence cells containing different sodium content. The final external sodium concentration, which includes the amount added during ethylene glycol tetraacetic acid (EGTA)–NaOH addition, is indicated in the abscissa of the curve (lower panel). **(A)** Representative traces of membrane potential (V_m) and intracellular sodium ($[Na^+]_i$) changes induced by 3.5 mM EGTA and subsequently by 3.5 mM $CaCl_2$ (Ca) additions. **(B)** $[Na^+]_i$, obtained 3 minutes after EGTA addition, as a function of external sodium ($[Na^+]_{ext}$). **(C)** $[Na^+]_i$ increase ($[Na^+]_i$ obtained 3 minutes after EGTA addition minus resting $[Na^+]_i$) as a function of the extent of membrane potential depolarization (V_m obtained 3 minutes after EGTA addition minus resting V_m; n = 5 individuals, $\bar{x} \pm$ SE).

concentration calculator program Maxchelator (V2.1), written by Chris Patton from Stanford University (http://www.stanford.edu/~cpatton/maxc.html) was used to estimate the calcium and magnesium concentrations in HHSM medium containing EGTA.

Results

We have previously shown that external calcium removal with EGTA produces depolarization that depends on the external sodium content. We investigated whether the Na^+-dependent depolarization reflected changes in $[Na^+]_i$. Figure 2 shows that the depolarization induced by external calcium removal with 3.5 mM EGTA, from 2.5 mM to 70 nM, was accompanied by a slow but consistently large increase in $[Na^+]_i$ that depended on the external sodium content. In HHSM medium (normal sodium content), the $[Na^+]_i$ content increased from resting 2.9 ± 0.3 mM to 28.3 ± 2.3 mM in 3 minutes (media ± SE, n = 7). The $[Na^+]_i$ increase reached nearly steady values close to 45 mM in about 4 more minutes (not shown). Calcium restoration, which produces a transient peak of calcium and a Na^+,K^+-ATPase–dependent hyperpolarization (González-Martínez, 2003), blocked the sodium influx and then produced a slow and small decrease in $[Na^+]_i$ (Figure 2A). The effect of external sodium on $[Na^+]_i$ did not reach saturation at the range of external sodium studied (Figure 2B). Both the extent of the Na^+-dependent depolarization (González-Martínez, 2003) and the $[Na^+]_i$ increase induced by calcium removal similarly depended on the external sodium concentration, indicating that both phenomena were related (Figure 2C). Interestingly, the sodium increase was inhibited at 70 mM external sodium (Figure 2A), a concentration that still favors sodium entry, as supported by the gramicidin-induced sodium influx in this condition (trace not shown).

In previous work, we showed that in calcium removal–induced depolarized sperm, calcium restoration or magnesium addition produce a Na^+,K^+-ATPase–dependent hyperpolarization (González-Martínez, 2003). As shown in Figure 3 (left panel, traces b and c), the $[Na^+]_i$ increase induced by calcium removal was blocked and then tended to reverse with the addition of calcium at concentrations ≥1 μM (1 μM and 2.5 mM). These amounts of calcium also produced calcium influx and

hyperpolarization (Figure 3, left panel). As for magnesium, the addition of 1.5 and 2.5 mM $MgCl_2$, which increases the external $[Mg^{2+}]$ to 1.99 and 2.99 mM, respectively (because HHSM contains 0.49 mM $MgCl_2$), blocked the sodium influx and subsequently produced a slight decrease in $[Na^+]_i$ (Figure 3, right panel, traces f and g). The effect of magnesium was not related to calcium release from EGTA because, according to the Maxchelator program (see Methods), the highest magnesium concentration used in this study (2.99 mM) barely increased the external free calcium, from 70 (in normal HHSM + 3.5 mM EGTA) to 120 nM. Consistently, as previously reported (González-Martínez, 2003), no effects were observed in $[Ca^{2+}]_i$, and a complete hyperpolarization was produced. The addition of 0.25 mM $MgCl_2$ (trace e) inhibited but did not reverse the $[Na^+]_i$ increase, an effect that correlated with a partial induction of the hyperpolarization, suggesting that a fraction of the sperm population could stop the Na^+-dependent depolarization. These effects were also observed when magnesium was added before EGTA; that is, the same amounts of magnesium similarly blocked the $[Na^+]_i$ increase induced by EGTA (traces not shown).

Because the hyperpolarization induced by calcium restoration or magnesium addition is sensitive to oubain (González-Martínez, 2003), we studied the effect of this inhibitor on $[Na^+]_i$ changes. In HHSM medium containing 142 mM sodium (including EGTA-NaOH), the calcium removal–induced $[Na^+]_i$ increase was remarkably enhanced by ouabain, and as expected for a extrusion mechanism involving the Na^+,K^+-ATPase, calcium restoration did not tend to reverse it (Figure 4A). It should be noted that ouabain barely affects that Na^+-dependent depolarization induced by calcium removal (González-Martínez, 2003), indicating that the observed enhanced increase in $[Na^+]_i$ was not related to an increase in sodium influx but to the inhibitory action on the Na^+,K^+-ATPase. Likewise, as expected, magnesium did not induce $[Na^+]_i$ decrease in calcium removal–induced depolarized sperm treated with ouabain (Figure 4B). These results indicated that the sodium increase induced by calcium removal resulted from a balance between sodium influx produced by the gating of the putative channel and sodium extrusion, activated by the increase in $[Na^+]_i$, catalyzed by the Na^+,K^+-ATPase.

Discussion

In this work, we show evidence that the Na^+-dependent depolarization induced by calcium removal produces $[Na^+]_i$ increase. This hypothesis is strongly supported by 1) the correlation of the extent of $[Na^+]_i$ increase with the extent Na^+-dependent depolarization and 2) the blocking of an increase in $[Na^+]_i$ by calcium and magnesium at micromolar and millimolar concentrations, respectively, which are conditions that also block the Na^+-dependent depolarization and then cause a Na^+,K^+-ATPase–dependent hyperpolarization (González-Martínez, 2003). Furthermore, the enhancing effect of ouabain, which blocks Na^+,K^+-ATPase, on the $[Na^+]_i$ increase induced by calcium removal and its blocking effect on the $[Na^+]_i$ decrease produced by calcium restoration or magnesium addition, indicates that the pump is rapidly activated as a consequence of the $[Na^+]_i$ increase.

Interestingly, the $[Na^+]_i$ increase induced by calcium removal is blocked in HHSM medium still containing 70 mM NaCl (Figure 2), an effect that is related to blocking of the Na^+-dependent depolarization induced under the same conditions (González-Martínez, 2003). Evidently, the sodium gradient still favors sodium entry at 70 mM external sodium, as supported by a fast sodium influx induced by gramicidin in this condition. Given that the Na^+-dependent depolarization does not reach the Nernst potential for sodium distribution (E_{Na}), the lack of depolarization might reasonably

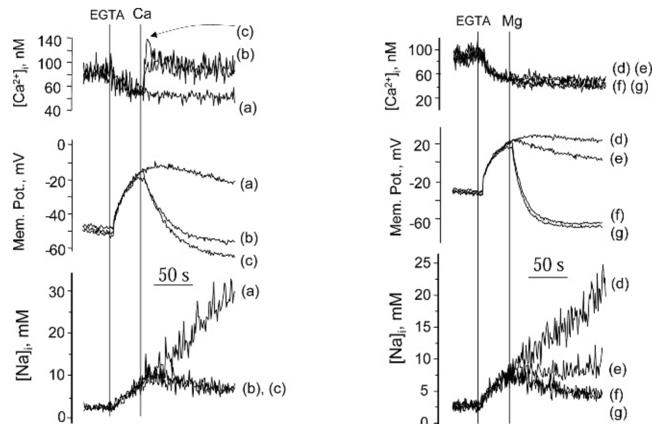

Figure 3. Effect of calcium and magnesium on the intracellular sodium ($[Na^+]_i$) influx induced by calcium removal with ethylene glycol tetraacetic acid (EGTA) in SBFI-loaded human sperm. Calcium was removed from the medium with 3.5 mM EGTA, and 40 seconds later, appropriate amounts of either calcium (left panel) or magnesium (right panel) were brought to different external concentrations. A sample of the same semen was also loaded with fura 2 to compare the effects with intracellular calcium ($[Ca^{2+}]_i$ and membrane potential (detected simultaneously). Left panel: Effect of calcium addition. The amounts of free $[Ca^{2+}]_i$ reached in the presence of 3.5 mM EGTA are indicated in parentheses: **(a)** no addition (70 nM), **(b)** 0.92 mM (1 μM), and **(c)** 3.5 mM (2.5 mM). Right panel: Effect of magnesium addition. The amount of free magnesium (0.49 mM of the HHSM plus the amounts added, in the presence of 3.5 mM EGTA) are indicated in parentheses: **(d)** no addition (0.37 mM), **(e)** 0.25 mM (0.56 mM), **(f)** 1.5 mM (1.61 mM), and **(g)** 2.5 mM (2.99 mM). The free divalent cations in the presence of EGTA were calculated by the Maxchelator (V2.1) program (see Methods).The traces are representatives of sperm samples obtained from 5 individuals.

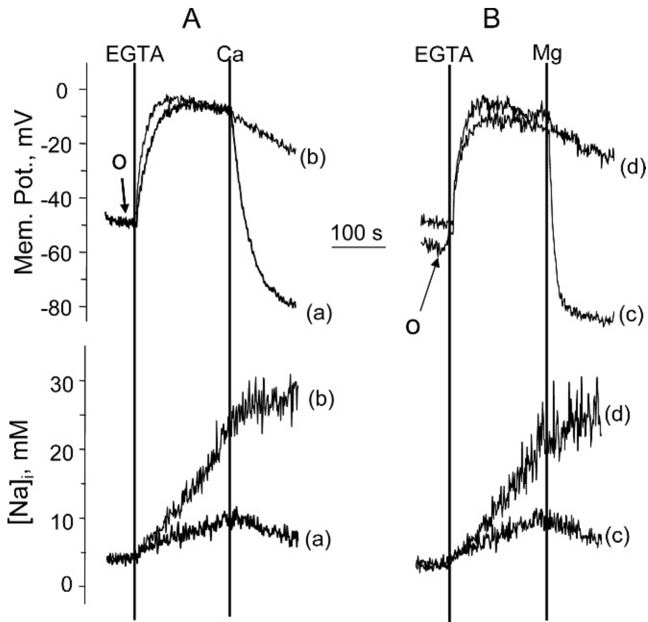

Figure 4. Effect of inhibition of the Na^{+},K^{+}-ATPase on calcium removal–induced sodium influx in human sperm. SBFI-loaded human sperm was poured in HEPES-buffered human sperm medium containing 128 mM sodium, and sodium influx was induced by calcium removal with 3.5 mM ethylene glycol tetraacetic acid (EGTA; total amount of sodium was 142 mM including EGTA-NaOH). Membrane potential (V_m) was recorded in sperm obtained from the same batch under identical conditions. Three minutes after EGTA, either 3.5 mM $CaCl_2$ (Ca) (that restores to 2.5 mM) **(panel A)** or 2.5 mM $MgCl_2$ (Mg) **(panel B)** were added as indicated. **(a, c)** control traces, **(b, d)** ouabain (O) was added 15 s before EGTA. Traces are representative of sperm samples obtained from 5 individuals.

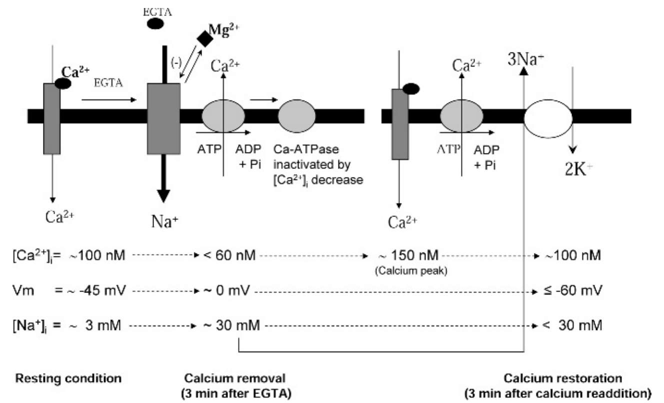

Figure 5. Hypothetical scheme of the ion transport systems activated by external calcium removal and by the subsequent calcium restoration in human sperm. The activation of these systems would lead to the indicated changes in intracellular calcium ($[Ca^{2+}]_i$, intracellular sodium ($[Na^{+}]_i$), and membrane potential (V_m) (see Discussion).

result from a contribution of potassium exiting through the same, or other, channels (González-Martínez, 2003). In this regard, at different external sodium concentrations, the depolarization induced by calcium removal reaches a constant value in about 1 minute, whereas that the $[Na^{+}]_i$ steadily increases for 3 minutes, suggesting that the sodium influx through the channel is perhaps opposed by potassium efflux. Thus, in medium containing 70 mM sodium, an $[Na^{+}]_i$ increase should have been detected. The lack of effect in low-sodium HHSM medium suggests that, besides the effect of gradient, external sodium could affect the opening of the channel. It should be additionally noted that in ic-HHSM, which has no calcium added, the $[Na^{+}]_i$ was unaffected in the range of external 05–100.5 mM sodium (Figure 1A). It is possible that contaminant calcium, the acidic pH of the medium (pH 6.7), or both prevented sodium influx. The effect of pH on the phenomena described here remains to be studied.

The results presented here are consistent with the hypothesis that, in resting conditions, there is a calcium channel the activity of which contributes to the resting intracellular calcium (González-Martínez, 2003). As schematized in Figure 5, the calcium selectivity of this channel would be conferred by the binding of calcium at an external site with a Kd in the hundreds of nanomolar units of micromolar range, a site that could be occupied by magnesium with a Kd in the millimolar range. Therefore, when calcium is removed from this site, the selectivity would be shifted to the large-conductance sodium channel. This would result in the observed depolarization from approximately −45 mV to values close to 0 mV (González-Martínez, 2003), the observed $[Ca^{2+}]_i$ decrease, because of the calcium-extruding activity present in the cell (reasonably the Ca^{2+}-ATPase) and the observed increase in $[Na^{+}]_i$ from approximately 3 mM to values close to 30 mM (this work). In this condition, the Na^{+},K^{+}-ATPase would be rapidly activated by the increase in $[Na^{+}]_i$, to oppose massive sodium entry. Upon external calcium restoration, the channel would recover its selectivity to a low-conductance calcium channel, producing a peak of calcium, and then a reactivation of the Ca^{2+}-ATPase, bringing the $[Ca^{2+}]_i$ levels to normal values. Consequently, the membrane potential would become mainly dependent on the highly active electrogenic Na^{+},K^{+}-ATPase hyperpolarizing the cell. Accordingly, the $[Na^{+}]_i$ would tend to decrease to resting values, although at a much slower rate than the hyperpolarization (this work). This finding suggests that the sustained hyperpolarization, which reaches values more negative than resting and frequently even more negative to the Nernst potential for potassium distribution (E_k) (González-Martínez, 2003), is supported by the enhanced $[Na^{+}]_i$. Interestingly, in a glucose-deprived medium, glucose induces a ouabain-sensitive hyperpolarization (Guzmán-Gren-

fell et al, 2000), indicating a relevant role of the enzyme in setting the membrane potential in human sperm subjected to these particular stressing conditions.

Patch clamp recordings performed with mouse sperm in cytoplasmic droplets show that the sperm flagellum contains an alkaline-activated, weakly voltage dependent calcium-selective channel named catsper (Kirichok et al, 2006). Sperm lacking this channel, which actually consists of 4 heterotetramers (catsper1–4; Jin et al, 2007; Qi et al, 2007) are unable to hyperactivate their motility; as a consequence, males are infertile (Ren et al, 2001; Carlson et al, 2003). In the absence of external calcium, these channels conduct sodium in a voltage-independent manner (Kirichok et al, 2006). In this regard, it is reasonable to assume that the Na^+-dependent depolarization induced by external calcium removal and the $[Na^+]_i$ increase reported here might be due to catsper opening in zero-calcium medium (Kirichok et al, 2006). Consequently, this channel (catsper) would contribute to set the resting $[Ca^{2+}]_i$. It is interesting to note that catsper null sperm incubated in the absence of external calcium maintain initial motility, whereas the wild-type sperm become motionless (Jin et al, 2007). This finding implies that internal calcium stores play a role in supplying calcium for sperm motility in zero-calcium medium and, according to the results presented here, it raises the possibility that an increase in intracellular sodium, a membrane potential depolarization (Espinosa and Darszon, 1995; González-Martínez, 2003), or both might inhibit calcium release from internal stores in wild-type sperm.

On the other hand, T-type VDCC detected in mouse (Arnoult et al, 1996; Santi et al, 1996) and man (Jagannathan et al, 2002) spermatocytes, which in GH3 pituitary cells also permit sodium permeation in the absence of external calcium (Suarez-Kurtz et al, 1987), have been immune detected in mature sperm of both species (Treviño et al, 2004). Thus, these channels could also be involved in the responses discussed here. The Na^+/Ca^{2+} has also been involved in setting the resting $[Ca^{2+}]_i$ in human sperm (Kraznai et al, 2006). In other cells, such as smooth muscle, there is evidence that VDCC and store-operated calcium channels, which also allow sodium permeation in the absence of external calcium (Minke and Cook, 2002), could contribute to resting $[Ca^{2+}]_i$ (Montano and Bazan-Perkins, 2005).

Acknowledgments

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