Removal of external calcium with EGTA (from 2.5 mm to nanomolar levels) caused a remarkable depolarization in human sperm. This depolarization was initially fast. It was followed by a slow phase that brought the V_m to values of over 0 mV in 1–2 min. The slow and sustained phase correlated with a sustained decrease in intracellular calcium. However, calcium removal still induced depolarization in sperm with enhanced intracellular calcium (induced by progesterone), indicating that the sustained depolarization was not caused by a sustained intracellular calcium decrease. The depolarization was reduced as the external sodium content was substituted with choline, indicating that it was due to a sodium current, and was observed in lithium but not in tetramethylammonium-containing medium. In low sodium medium, the decrease of sodium after calcium removal induced depolarization to the extent of which slightly increased in 2 min. The depolarization was completely inhibited by external magnesium (K_i = 1.16 mM). The addition of calcium or magnesium to calcium removal-induced depolarized sperm induced hyperpolarization that was inhibited by ouabain and was also prevented in medium without potassium, suggesting that the activity of the electronegative Na⁺,K⁺-ATPase was involved. The conductance activated by calcium removal might unveil the presence of a calcium channel that in the absence of external calcium allows sodium permeation and that in normal conditions might contribute to the resting intracellular calcium concentration.

Sperm of mammalian species present physiological changes that involve modulation of calcium entry mechanisms. In the female reproductive tract or in vitro in defined media, sperm undergoes in hours a series of complex biochemical changes known as “capacitation.” Capacitation leads to changes in sperm motility patterns that requires calcium influx, resulting in an increment of ~100 nM in resting intracellular calcium ([Ca²⁺]i) (1–3). Only capacitated sperm bind to the zona pellucida and undergo the acrosome reaction (4), an exocytotic process necessary for egg-sperm fusion. The acrosome reaction is physiologically induced by the egg zona pellucida glycoprotein ZP3, which triggers a rapid increase in [Ca²⁺]i, that may involve the gating of voltage-dependent (VDCC)³ and activation of store-operated (SOC) calcium channels (5, 6). Hence, it is evident that the study of the ion transport systems that set and regulate the membrane potential and the [Ca²⁺]i, is necessary to fully understand the molecular basis of capacitation and acrosome reaction in human sperm.

Unfortunately, the head of mature sperm is not suitable for patch clamp methods because of its particular shape and small size (7). Alternatively, the use of optical methods to detect membrane potential and intracellular ions has provided valuable information regarding the ion transport mechanism in mammalian sperm populations. The resting membrane potential values obtained with fluorescent probes in mouse and human sperm populations range between −60 and −20 mV (8–11). Incidentally, it has been observed that in medium prepared without calcium, the resting membrane potential of human sperm populations have depolarized values as compared with those incubated in calcium-containing media (12, 13). In mouse sperm, the addition of calcium to sperm incubated in medium without calcium produces a transient plasma membrane hyperpolarization that is in part sensitive to the Na⁺,K⁺-ATPase inhibitor ouabain (10). This evidence has led to the notion that calcium may regulate the resting membrane potential in mammalian sperm.

In this paper, I report the effect of removing calcium from the medium and the subsequent calcium readdition on membrane potential and intracellular calcium in human sperm. It was possible to precisely compare membrane potential and internal calcium changes in human sperm population by using a simultaneous recording system (13, 14). It was found that removing calcium from the medium with EGTA produces a magnesium-sensitive, sodium-dependent depolarization in human sperm. This evidence suggests that sperm is endowed with calcium channels that in the absence of calcium allow sodium permeation.

**EXPERIMENTAL PROCEDURES**

**Materials and Media—**Disopropylthiodicarbocyanine iodide diSC3(5) was obtained from Molecular Probes. Fura-2/AM (acetoxyethyl ester), ionomycin, and valinomycin were from Sigma. The other reagents were obtained from Sigma, Merck, or Baker. Hepes-buffered human sperm medium (H-HSM), originally proposed by Suarez et al. (15), had the following composition (in mM): 117.5 NaCl, 8.6 KCl, 2.5 CaCl₂, 0.49 NaH₂PO₄, 0.49 MgCl₂, 0.3 sodium pyruvate, 19 sodium lactate, 2 glucose, and 25 HEPES-Na (pH 7.6). H-HSM was modified so that NaCl was substituted with choline-Cl (lowNaH-HSM): This medium still contained ~32 mM sodium (Na-Hepes, sodium lactate, sodium pyruvate, and NaH₂PO₄).

Sperm Purification and Fura-2/AM Loading—Human semen was obtained from a panel of eight healthy 18–24-year-old donors. Normal

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³ The abbreviations used are: VDCC, voltage-dependent calcium channels; SOC, store-operated calcium channels; diSC3(5), diisopropylthiodicarbocyanine iodide; H-HSM, Hepes-buffered human sperm medium; PTI, Photon Technology International; TMA, tetramethylammonium.
samples were selected according to the World Health Organization protocol as reported previously (14). Sperm purification was performed using Percoll gradients according to Suarez et al. (15) with minor modifications (14). Purified sperm (0.6–1.5 \times 10^8 cells) were loaded with 2 µM Fura-2/AM in 2 ml of H-HSM medium at 36 °C for 40 min. Once washed, cells were incubated in 25 ml of H-HSM medium at 36 °C and immediately used for intracellular calcium and membrane potential measurements.

**Simultaneous Measurement and Calibration of \([Ca^{2+}]_i\) and Membrane Potential**—Membrane potential and \([Ca^{2+}]_i\) were simultaneously detected with the fluorescence probes diSC\(_3\)(5) and Fura-2/AM, respectively, with a PTI fluorometer (Photon Technology International) as reported previously (14). 2–5 ml of Fura-2/AM-loaded sperm (corresponding to \(\sim 1 \times 10^8\) cells) was centrifuged at 300 \(\times \) g for 5 min. The pellet (~100 µl) was immediately added to the fluorescence cuvette containing 2.5 ml of H-HSM (or other) + 0.5 µM diSC\(_3\)(5), previously thermostatted at 36 °C and under constant magnetic stirring. The fluorometer has two photomultipliers placed at 90° with respect to the Xenon lamp source. One photomultiplier detected the Fura-2/AM signal with a 488-nm filter (Andover). exciting at 600 nm with the excitation monochromator of the PTI system. The other photomultiplier detected the diSC\(_3\)(5) fluorescence at 670 nm, exciting at 500 nm with a halide lamp placed in front of the xenon source. Both excitation and emission wavelengths for diSC\(_3\)(5) measurements were achieved with optical interference filters (Andover). Data was acquired and digitalized at 0.83 Hz with the PTI interface system.

**Calibrations**—The diSC\(_3\)(5) fluorescence was calibrated in Fura-2/AM-loaded sperm suspensions as reported previously (14) with some modifications. At the end of each trace, 1.5 µl of the potassium ionophore valinomycin was added to bring the membrane potential to the Nernst potential value for potassium distribution (16), with a slope that in the sperm ranged between 0.018 and 0.025 (fractional change of fluorescence per millivolt). Taking the calibration parameters into account, the actual values for fluorescence were transformed into membrane potential values (\(V_{m}^i\)) according to Equation 1,

\[
V_m = \frac{F_m - F_b}{m} \times \log \left( \frac{[K]_{ext}}{[K]_{int}} \right)
\]

(Eq. 1)

where \(m\) and \(b\) are the parameters of the linear calibration curve, i.e., the slope and the \(y\) axis value (fractional change of fluorescence) at 0 mV, respectively. These parameters were obtained by fitting linear the calibration data with a Microlab Origin, version 6.0 software. A calibration curve was made for each single trace using the step calibration described above with a maximal calibration value of ~15 mV. However, the linear relationship is maintained up to 5 mV range (tested, data not shown). It should be noted that mitochondrial potential does not contribute to diSC\(_3\)(5) signal in human sperm (13), which is consistent with a minor role of this organelle in supporting human sperm motility (17, 18). Thus, simultaneous measurements of intracellular calcium and membrane potential were performed in the absence of mitochondrial inhibitors or uncouplers. The Fura-2/AM values were calibrated in H-HSM containing 500 nM diSC\(_3\)(5) as reported previously (14).

**Experimental Assays**—The effect of calcium removal from the medium with the calcium chelator EGTA on the intracellular calcium and membrane potential in human sperm was investigated. To conduct this test, 3 mM EGTA was added to the medium to decrease the 2.5 mM calcium present in H-HSM to nanomolar levels (see below). Because calcium chelation with EGTA produces proton release at an extent that can acidify the Hepes-buffered H-HSM medium, the concentrated EGTA solution (500 mM) was prepared in 3.2 M NaOH. In this condition, the addition of 3 mM EGTA to H-HSM did not produce any change in H-HSM pH (measured with a pH electrode). To estimate the calcium concentration in mixtures of EGTA-Ca in H-HSM and in H-HSM supplemented with magnesium, the calcium concentration calculator program Maxchelator (V.2.1) written by Chris Patton from the Hopkins Marine Station (Stanford University) was used.

**Statistical Analysis**—Numeric results are expressed as the means ± S.E. \(n\) means number of individuals tested and analyzed with Student's t test. Two-tailed \(p\) values < 0.05 were considered statistically significant.

**RESULTS**

Fig. 1A shows the effect of 3 mM EGTA on the membrane potential and intracellular calcium detected simultaneously in human sperm. At this concentration, EGTA diminishes the extracellular calcium from 2.5 mM to ~129 nM (see “Experimental Procedures”). The addition of EGTA caused a decrease in \([Ca^{2+}]_i\) from an average of 220 ± 22 nM to 61 ± 6 nM with a 50% decrease time of 8.7 ± 1.9 s (\(n = 8\) individuals ± S.E.). Concomitantly, a remarkable depolarization of the plasma membrane was induced. The depolarization was initially fast and was followed by a large and slow phase that brought the mem-
brane potential from resting $-46 \pm 3$ to $+18 \pm 3.4$ mV ($n = 8$, mean $\pm$ S.E.) in 1–2 min. This value remained nearly constant after a 10-min incubation in the fluorescence cell (data not shown). Once the membrane potential reached a constant value, $3 \text{mM CaCl}_2$ was added to restore the original calcium concentration. This addition produced an overshoot in $[\text{Ca}^{2+}]_i$, i.e. a fast increase in $[\text{Ca}^{2+}]_i$ that reached a peak in seconds at values above the resting internal calcium ($495 \pm 32 \text{nM}$) and then was followed by a slow decrease that brought it in $-2$ min the $[\text{Ca}^{2+}]_i$, near to resting values. Concomitantly, calcium restoration produced a slow hyperpolarization that reached constant values in 2–3 min. In most cases, the membrane potential reached values more negative than resting ($-66 \pm 3.9$ mV, $n = 8$, after 3 min of calcium addition), indicating that the membrane potential was not reversed to resting conditions. However, the mechanism involved in the depolarization induced by calcium removal was reversible. As shown in Fig. 1A, calcium removal induced by a further addition of 3.5 mM EGTA still induced depolarization that was again reversed by calcium readdition.

The effect of EGTA was studied at different concentrations (Fig. 1B). The addition of 4.0, 3.0, and 2.75 mM EGTA that lowers the H-HSM external calcium concentration to approximately 41, 129, and 261 nM, respectively (see “Experimental Procedures”), caused in all of the cases sustained intracellular calcium decreases and sustained depolarization. These changes reached similar values, indicating that the effect of EGTA was saturating in both parameters. The addition of 2.0 and 2.25 mM EGTA that leaves $-500$ and $250 \mu \text{M}$ calcium in the medium produced small and fast transient depolarization with almost no change in internal calcium (Fig. 1B). The transient peaks slightly increased as the external calcium concentration decreased. The addition of 2.5 mM EGTA (that diminished calcium to 8 $\mu \text{M}$) produced in this case a large but transient depolarization, which was accompanied with a transient $[\text{Ca}^{2+}]_i$ decrease, the depolarization phase was related to the decrease in $[\text{Ca}^{2+}]_i$, and the hyperpolarization phase to $[\text{Ca}^{2+}]_i$ recovery to resting values. This external calcium concentration (8 $\mu \text{M}$) seemed to be close to a critical concentration, which could lead to either a sustained or transient depolarization, as suggested by the fact that in some experiments 2.5 mM EGTA induced sustained depolarization. In these cases, the sustained depolarization was always accompanied by sustained decreases in $[\text{Ca}^{2+}]_i$ (traces not shown).

To explore whether the sustained depolarization was caused by the sustained $[\text{Ca}^{2+}]_i$ decrease, the effect of calcium removal was studied in cells with a high $[\text{Ca}^{2+}]_i$, content. To do this, a rise in $[\text{Ca}^{2+}]_i$, was induced by progesterone (2, 8), and at the moment that $[\text{Ca}^{2+}]_i$, reached enhanced values, calcium removal was induced. As shown in figure 2, calcium removal triggered depolarization at $[\text{Ca}^{2+}]_i$, values as high as 400 nM that was reversed by calcium readdition. The depolarization progressed and reached sustained values as the $[\text{Ca}^{2+}]_i$, decrease occurred even above resting values having 80% of the total depolarization when the decreasing $[\text{Ca}^{2+}]_i$, reached resting values. This finding indicated that the sustained decrease in $[\text{Ca}^{2+}]_i$, did not provoke by itself the sustained depolarization induced by calcium removal and supported the hypothesis that the whole depolarization is caused by calcium removal from external binding sites.

The ion transport mechanism involved in the depolarization induced by calcium removal was studied by modifying the ionic composition of H-HSM. The total NaCl content was substituted with choline-Cl in H-HSM, a medium that still contains $-51 \text{mM}$ sodium from 19 mM sodium lactate $+ -12.5$ mM Na-Hepes $+ 0.3$ mM NaHPO$_4$ $+ 0.3$ sodium pyruvate and $-19 \text{mM NaOH}$ when the mixture EGTA-NaOH is added to the fluorescence cell (see “Experimental Procedures”). As shown in Fig. 3, A and B, in a wide range of sodium concentrations from 51 to 169 mM, the resting intracellular calcium and resting membrane potential values remained closed. In contrast, the EGTA-induced depolarization was remarkably diminished: the lower the external sodium, the lower the depolarization with no depolarization or slight hyperpolarization at 51 mM external sodium. This result indicated that the depolarization induced by calcium removal was attributed to a sodium current. In these experiments, the effect of calcium readdition is also shown. The smaller the sodium-dependent depolarization, the smaller the hyperpolarization induced by calcium restoration. It was noticeable that at low external sodium concentrations, calcium readdition produced a small transient depolarization. At high external [Na] (>70 mM), the hyperpolarization induced by calcium readdition seemed to hide the transient depolarization observed at low external [Na]. The calcium peak induced by calcium readdition in EGTA-treated sperm was smaller in low sodium medium as compared with normal H-HSM medium.

To explore whether the sodium-dependent depolarization triggered by calcium removal inactivated or has inactivating components, the effect of sodium addition after calcium removal on the membrane potential was evaluated in lowNa-H-HSM. Fig. 4 shows that 60 mM NaCl that was added 1 and 2 min after calcium removal with EGTA produced a depolarization that was similar in shape and in extent slightly bigger (120 $\pm$ 10%, $n = 5$, $p < 0.05$, after 2 min) than when the depolarization was triggered by EGTA in lowNa-H-HSM medium supplemented with 60 mM NaCl. These experiments indicated that the sodium-dependent depolarization induced by calcium removal did not inactivate in at least 2 min in lowNa-H-HSM but, conversely, a slight activation occurred.

The selectivity of the sodium-dependent depolarization was studied. The effect of calcium removal induced by 3 mM EGTA was analyzed in lowNaH-H-HSM supplemented with 60 mM NaCl, LiCl, or TMA-Cl (tetramethylammonium chloride). As shown in Fig. 5, no depolarization was observed in medium.
with TMA. In contrast, in lithium containing medium, the depolarization was significantly (p < 0.05) larger (47 ± 4.0 mV, n = 4) than in sodium-containing medium (30 ± 3.8 mV). It was not possible to extend these studies to potassium, cesium, and rubidium since these cations caused depolarization of the membrane potential to values close to the $E_K$ = −15 mV.

It has been established that some calcium channels allow sodium influx in the absence of calcium that is blocked by magnesium (19, 20). Thus, it was pertinent to explore whether the sodium-dependent depolarization induced by calcium removal was affected by magnesium. In the range of magnesium tested (up to 3 mM), the resting membrane potential, the resting $\left[Ca^{2+}\right]_i$, and particularly, the $\left[Ca^{2+}\right]_i$ decrease induced by calcium removal were almost unaffected (Fig. 6A). However, increasing amounts of MgCl$_2$ in the H-HSM medium caused a remarkable inhibition of the sodium-dependent depolarization induced by calcium removal with EGTA (Fig. 6A) with a $K_i$ of 1.16 ± 0.12 mM (n = 5, mean ± S.E.) (Fig. 6B). Expectedly, magnesium was also able to reverse the depolarization induced by calcium removal (Fig. 6C). The hyperpolarization was dependent on the magnesium concentration and reached saturation at values close to those that produced blockade of the depolarization induced by calcium removal. Interestingly, the hyperpolarization was not related to any change in intracellular calcium. This suggested that at least a considerable extent of the hyperpolarization did not depend on the calcium influx or on the intracellular calcium increase that is induced by calcium addition in EGTA-treated sperm.

The hyperpolarization induced by calcium and magnesium on calcium removal-induced depolarized sperm was studied. In mouse sperm, it has been observed that in calcium-deprived medium, calcium addition produce a transient hyperpolarization.
Fura-2/AM-loaded sperm was added to the fluorescence cell containing the following free calcium concentration upon 3 mM EGTA addition in magnesium are drawn in condition that stops the 3Na\(^+\) ion was prevented on H-HSM prepared without potassium, a hyperpolarization (Fig. 7). Consistently, the hyperpolarization induced by calcium removal as a function of the external magnesium concentration.

These amounts of magnesium gave the following free calcium concentration upon 3 mM EGTA addition in nanomolars (the external magnesium are written in parentheses): 109 (0 mM), 129 (0.5 mM), 149 (1.0 mM), 200 (2.0 mM), and 245 (3.0 mM). In normal H-HSM (Fig. 7).

The sodium-dependent depolarization values induced by calcium removal are far from the Nernst potential for sodium distribution (\(E_{Na}\)). For instance, taking into consideration that \([Na]\)\(_{in}\) = 18 mM in human sperm (22) at 121 mM external sodium, the \(E_{Na} = +51\) mV and the membrane potential reached upon calcium removal is \(-3\) mV (Fig. 4). This indicates that the sodium-sensitive membrane potential value should result from the activation of the putative sodium current plus preexisting conductance that set the membrane potential in normal conditions and/or other conductance that is induced to buffer the depolarizing membrane potential. Potassium efflux though the activated permeability pathway could also explain why the sodium-dependent depolarization does not reach the \(E_{Na}\), even though potassium permeability should be considerably lower than that of sodium since the \(E_{K} = -71\) mV.

What kind of electrogenic sodium transporter may cause the depolarization induced by external calcium removal? Activation of an electrogenic 3Na\(^+\),1Ca\(^{2+}\) exchange should be discarded since experiments made in low sodium medium show that (a) the depolarization induced by sodium or lithium can be triggered in the absence of any change in [Ca\(^{2+}\)], indicating that the depolarization occurs independently of changes in [Ca\(^{2+}\)], and (b) calcium removal decreases [Ca\(^{2+}\)], without affecting the membrane potential, indicating that the [Ca\(^{2+}\)] decrease may occur independently of the sodium-dependent depolarization.

The most coherent hypothesis is that the sodium-dependent depolarization is caused by opening of sodium channels that are normally blocked by calcium ions. The simultaneous detection of intracellular calcium indicates that the initial fast component of the depolarization may occur before the [Ca\(^{2+}\)] starts to decrease. Indeed, this fast component may be triggered transiently when external calcium is suddenly lowered to the hundreds of micromolar range with EGTA at [Ca\(^{2+}\)], values still close to resting. The slow, large, and sustained depolarization is always accompanied by a slow [Ca\(^{2+}\)] decrease to levels below 100 nM and is induced when the external calcium concentration is lowered to the units of micromolar range being reproducibly observed in the hundreds of nanomolar range. In principle, the fact that a sustained [Ca\(^{2+}\)] decrease was always related to the sustained sodium-dependent depolarization suggested that there was an intracellular control site at [Ca\(^{2+}\)], with a \(K_d\) below 100 nM. However, the depolarization induced by calcium removal was observed even when the [Ca\(^{2+}\)] was enhanced with progesterone, indicating that the induction of depolarization is related to calcium removal from external binding sites. The inhibitory effect of magnesium on the depolarization induced by calcium removal and its ability to reverse the depolarization support the notion that the putative channel is controlled by divalent cation. Magnesium may act at the same level than calcium but with a much lower affinity (\(K_d = 1.16\) mM).

A sodium channel blocked in normal media seems to play no role in sperm physiology. Alternatively, the putative sodium
channel may reveal the presence of a calcium channel in the plasma membrane that allows sodium permeation in the absence of external calcium. l-Type VDCC and transient receptor potential channels such as the SOCs allow large sodium currents when external calcium is brought to nanomolar and micromolar levels, respectively (19, 20). In mature human sperm, there is a nifedipine-insensitive voltage-dependent calcium influx system, suggesting that these cells are endowed with VDCC (8, 14). Molecular biology studies indicate that human sperm have a peculiar form of VDCC called cat sperm (23, 24).

The acrosome vesicle is an internal store of calcium (25, 26) that could activate a SOC channel (5), which has been identified as a C2-type TRP protein with the use of antibodies in mouse sperm (27). In cells suitable for patch clamp recordings, the sodium currents observed in the absence of external calcium could share some properties with the putative channel reported here. Indeed, in rat basophilic leukemia cells, SOC channels are sensitive to external magnesium in the millimolar range, allowing lithium permeation, but show similar selectivity for potassium and sodium (28, 29). In this study, SOC channel activation and contribution to the depolarization would be expected as a result of an intra-acrosomal calcium decrease imposed by calcium removal from external medium.

The fact that the hyperpolarization is sensitive to ouabain and prevented in medium without potassium suggests that the activity of the 3Na+/2K+-ATPase present in the plasma membrane that diminishes its own activity as [Ca2+]i decreases (30). When calcium is restored, the channel would switch its selectivity for calcium and allow calcium to pass through, increasing the [Ca2+]i. Because the calcium conductance through this channel would be much lower than sodium, no contribution to membrane potential would be expected. As for the effects of magnesium, the possibility that the putative channel allows magnesium permeation remains to be established. In any case, the hyperpolarization induced by calcium or by magnesium would be observed as a lack of contribution of the depolarizing sodium conductance to the membrane potential.

The hypothesis that the sodium-dependent depolarization and prevented in medium without potassium suggests that the activity of the 3Na+/2K+-ATPase present in the plasma membrane that diminishes its own activity as [Ca2+]i decreases (30). When calcium is restored, the channel would switch its selectivity for calcium and allow calcium to pass through, increasing the [Ca2+]i. Because the calcium conductance through this channel would be much lower than sodium, no contribution to membrane potential would be expected. As for the effects of magnesium, the possibility that the putative channel allows magnesium permeation remains to be established. In any case, the hyperpolarization induced by calcium or by magnesium would be observed as a lack of contribution of the depolarizing sodium conductance to the membrane potential.
that is partially blocked by nimodipine, trifluoperazine, and magnesium, suggesting that this depolarization because of calcium influx through calcium channels (10).

In summary, this work shows evidence that suggests that human sperm is endowed with a magnesium-sensitive sodium conductance that is activated when external calcium is removed from the medium. This conductance may unveil the presence of calcium channels that might contribute to resting intracellular calcium. The physiological role of these putative channels, especially in sperm capacitation where regulation of the resting intracellular calcium is crucial, remains to be explored.

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Induction of a Sodium-dependent Depolarization by External Calcium Removal in Human Sperm
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