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Determination of the Steady-State Intracellular Chloride Concentration in Capacitated Human Spermatozoa

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ABSTRACT: Chloride channels participate in the mammalian sperm acrosome reaction (AR). However, the mechanism by which sperm regulate intracellular Cl^- concentration ($[Cl^-]_i$) is not known. The steady-state $[Cl^-]_i$ has also never been reported for mammalian spermatozoa. Therefore, using chloride-sensitive fluorescent dyes, we sought to determine the steady-state $[Cl^-]_i$ of capacitated human spermatozoa by a null-point measurement technique. We found that the $[Cl^-]_i$ was sufficiently elevated (a conservative estimate of ≥41 mM) such that the opening of chloride channels should result in a Cl^- efflux and, hence, in depolarization. Moreover, the $[Cl^-]_i$ does not remain constant under varying extracellular Cl^- concentrations ($[Cl^-]_o$).

Key words: Methoxy-*N*-ethylquinolonium, *N*-(6-methoxyquinolyl) acetoethyl ester, progesterone, acrosome reaction.

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In most animal cells, Cl^- is predominantly passively distributed across the plasma membrane, and the opening of Cl^- channels generally has a stabilizing effect on the membrane potential, an effect that is similar to that obtained with K^+ channels (Jentsch and Günther, 1997). This occurs because the Cl^- electrochemical gradient across the plasma membrane is generally not far from equilibrium. Consequently, at the resting membrane potential of cells, the opening of passive conductance Cl^- channels (i.e., those not directly dependent on adenosine triphosphate transport mechanisms) will not result in large excursions of the membrane potential or in large changes of the intracellular Cl^- concentration ($[Cl^-]_i$). In certain cells, however, $[Cl^-]_i$ can deviate from the equilibrium potential by tens of millivolts (Jentsch and Günther, 1997). If this $[Cl^-]_i$ is above the electrochemical equilibrium, then the opening of Cl^- channels will result in a net Cl^- efflux and depolarization, as demonstrated by the seemingly paradoxical excitation elicited by gamma-aminobutyric acid (GABA) in some neurons (Smith et al, 1995). Similarly, there is evidence to suggest that this mechanism of Cl^- efflux through a $GABA_A$ receptor-like Cl^- channel is involved in the progesterone-initiated acrosome reaction (AR) in human spermatozoa (Meizel, 1997).

Sperm amino acid neurotransmitter receptor/Cl^- channels play an essential role in the AR (Meizel, 1997). Physiological agonists of the AR, progesterone (P) and the zona pellucida, initiate this modified exocytotic event at least in part through pathways involving a $GABA_A$ receptor-like Cl^- channel and a glycine receptor/Cl^- channel, respectively (Wistrom and Meizel, 1993; Melendrez et al, 1994; Melendrez and Meizel, 1995; Shi and Roldan, 1995; Shi et al, 1997). The exact role that Cl^- fluxes play in regulating the AR is not yet resolved; however, recent evidence suggests that in the case of the P-initiated AR, Cl^- efflux may modulate the spatial–temporal characteristic of the P-activated Ca^{2+} influx (Meizel et al, 1997).

Very little is known about the sperm $GABA_A$ receptor-like Cl^- channel. Similarities in subunit composition to the neuronal protein have been demonstrated by immunoblot analysis (Wistrom and Meizel, 1993). Additionally, tyrosine kinase–dependent regulation of channel function may also be shared with its neuronal counterpart (Meizel and Turner, 1996). In contrast, differences in agonist sensitivity and selectivity between the two channels are implied from AR results with P, P metabolites, synthetic progestins, and GABA (Meizel, 1997). However, studies using the determination of the AR, an endpoint assay, are of limited use in elucidating the mechanistic details by which sperm amino acid neurotransmitter receptor Cl^- channels regulate this important event in fertilization. A more direct approach to determining these details is to directly measure $[Cl^-]_i$ under various conditions.

Mammalian spermatozoa, with few exceptions (Wey-

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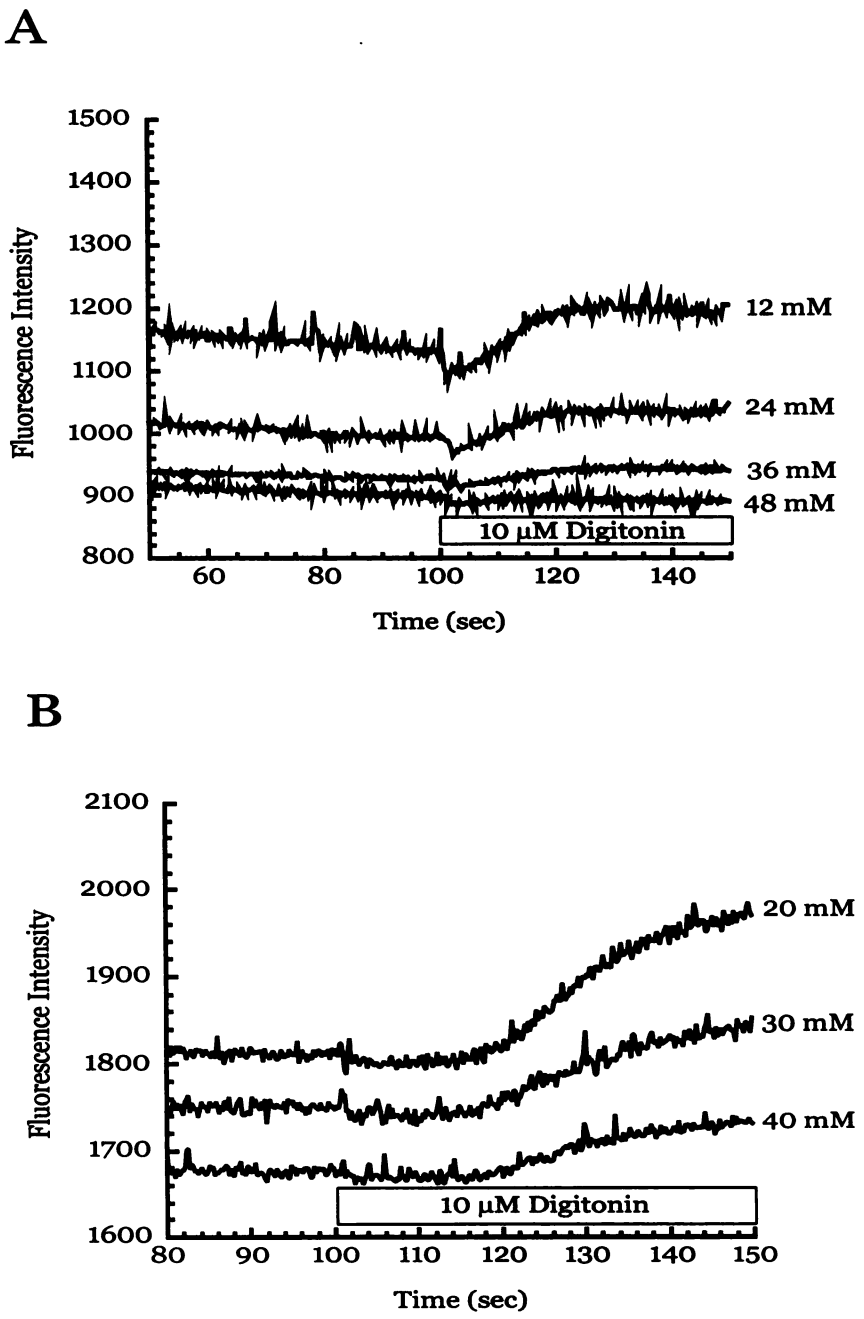

FIG. 1. Null-point determination of intracellular Cl^- concentration ($[Cl^-]_i$) using the chloride-sensitive dyes *N*-(6-methoxyquinolyl) acetoethyl ester **(A)** and methoxy-*N*-ethylquinolonium **(B)**. Capacitated human spermatozoa were resuspended in a HEPES-buffered medium of varying $[Cl^-]$, which is indicated next to each curve. At the 100-second time point, the sperm suspension was treated with 10 μM digitonin. The data in this figure are from a single donor but represent results similar to those obtained from a total of four experiments with different donors.

land et al, 1994; Espinosa et al, 1998), are generally considered to be unsuitable candidates for electrophysiological experimentation because of their compact shape and small size (35 fl cytoplasmic volume; Kleinhans et al, 1992). Consequently, other methods employing artificial planar lipid bilayers (Cox et al, 1991; Labarca et al, 1995) and voltage-sensitive fluorescent probes (Espinosa and Darszon, 1995; Zeng et al, 1995) have been used to study membrane potential and ion channels in mammalian spermatozoa. Here we report on the use of Cl^--sensitive fluorescent probes (Biwersi and Verkman, 1991; West and Molloy, 1996) to determine steady-state $[Cl^-]_i$ in human spermatozoa so as to support the hypothesis that in capacitated human spermatozoa, the $[Cl^-]_i$ is sufficiently el-

evated (compared to somatic cells) such that the opening of Cl^- channels results in a Cl^- efflux.

Methods

Chloride-Sensitive Probe Preparation

Several chloride-sensitive probes (quinoline derivatives) were purchased from Molecular Probes (Eugene, Oregon). The emission fluorescence intensity (FI) of these probes changes with changing $[Cl^-]_i$ as a result of collisional quenching by halide ions. In our investigations, we used both 6-methoxy-*N*-ethylquinolonium (MEQ) and *N*-(6-methoxyquinolyl) acetoethyl ester (MQAE). Both dyes can be passively loaded into sperm (MEQ in the reduced dihydro form, DiH-MEQ) and are relatively insensitive to alterations in pH, NO_3^-, SO_4^{2-}, and PO_4^-. Intracellular DiH-MEQ is rapidly oxidized to the cell-impermeant, Cl^--sensitive MEQ, resulting in cytosolic trapping.

The preparation of DiH-MEQ consists of a simple reduction with $NaBH_4$ (Sigma Chemical Co., St. Louis, Missouri) following a protocol set forth by the manufacturer (Molecular Probes, MP 06886). The important aspects of this protocol dictate that the technician must maintain a 21 molar ratio of sodium borohydrideMEQ, must test the diethyl ether solvent for traces of peroxides, and must thoroughly remove the last traces of solvent (ether) after extraction. In contrast to the MP 06886 protocol, a single ether extraction was found sufficient. After the ether solvent had been evaporated under continual argon purging, the weight of the DiH-MEQ residue was determined. From this weight, the volume of DMSO needed to produce a 50–100 mM stock solution of DiH-MEQ was calculated. For determination of successful DiH-MEQ preparation, an aliquot of the stock solution was diluted 1:1,000 into ddH_2O and the absorbance at 344 nm determined before and following the addition of H_2O_2, which oxidizes the DiH-MEQ to MEQ. From this absorbance data, the ratio of MEQ to DiH-MEQ was calculated to confirm the successful preparation of the reduced form and to verify the concentration of the stock solution. The DiH-MEQ was made fresh on the day of each experiment.

Sperm Preparation

Human semen was obtained from healthy donors by masturbation, and the spermatozoa were separated from the seminal fluid by Percoll (Sigma) gradient centrifugation, as previously described. These specimens (after Percoll gradient centrifugation) contained $\geq 50 \times 10^6$ sperm/ml with $<0.5 \times 10^6$ non-sperm cells/ml and very few sperm with abnormal morphology. The washed sperm suspensions were incubated overnight (22–26 hours at 37°C, 5% CO_2 in air) in bicarbonate-buffered human sperm medium (HSM) containing 26 mg/ml bovine serum albumin to promote *in vitro* capacitation (Suarez et al, 1986; Garcia and Meizel, 1996). Capacitated sperm suspensions in HSM were pooled, centrifuged ($300 \times g$ for 10 minutes), and the sperm pellets were resuspended in HEPES-buffered medium (FM, Thomas and Meizel, 1988). The sperm suspensions in FM were maintained in tightly capped 15-ml polypropylene centrifuge tubes at 37°C (during dye-loading incubation) or at room temperature (during centrifugation steps). Dye loading was accomplished by the adding a small aliquot (2 µl) of a 1000-fold concentrated DiH-MEQ stock solution (DMSO solvent) or by diluting the sperm suspensions with an MQAE solution (6 mM in FM). The final dye concentrations were 50–100 µM for DiH-MEQ and 5 mM for MQAE. The final sperm concentrations were 18×10^6 sperm/ml (MQAE) or 6×10^6 sperm/ml (MEQ) in 2-ml aliquots. Sperm suspensions were incubated with their respective dyes for 1 hour at 37°C, after which they were centrifuged ($300 \times g$ for 20 minutes) through a 40% Percoll solution to separate the spermatozoa from any free dye in the medium. The Percoll solution was prepared from a stock solution of 95% Percoll, 150 mM NaCl, and 10 mM HEPES (pH = 7.5) diluted with FM. Following centrifugation, the sperm pellets were resuspended in FM (14 ml/tube) and recentrifuged ($300 \times g$ for 10 minutes). The resulting sperm pellets were pooled, and the sperm concentration of the pooled sample was determined. Aliquots of this concentrated sperm suspension were diluted with FM and the Cl^--deficient equivalent (FM-Cl in mM; 118 $NaCH_3SO_4$, 8.6 K-gluconate, 0.36 KH_2PO_4, 25 HEPES, 2.5 Ca-gluconate, and 0.5 Mg-gluconate) to produce the desired $[Cl^-]$ in a 1-ml sperm suspension containing 6×10^6 sperm/ml.

Measurement of $[Cl^-]_i$

Fluorescence measurements were made with a Hitachi F-2000 spectrofluorometer equipped with a heated cuvette holder set to maintain a temperature of 37°C. Fluorescence excitation wavelengths of 344 and 355 nm and emission wavelengths of 440 and 460 nm were used for MEQ and MQAE, respectively. Emission fluorescence intensity (FI) data from sperm suspensions were collected for 100 sec before the addition of 10 µM digitonin (Sigma) and for 100 seconds afterward. The digitonin treatment equilibrates the intracellular and extracellular $[Cl^-]$ by permeabilizing the sperm plasma membrane (Garcia and Meizel, 1996). The mean FI (5–10 seconds or 25–50 data points) after (≥30 seconds, steady state) the addition of digitonin was subtracted from the mean (5–10 seconds or 25–50 data points) FI just prior to the addition of digitonin. These differences were plotted against the extracellular Cl^- concentration ($[Cl^-]_o$) to calculate the null-point $[Cl^-]_i$ using simple regression analysis. Utilizing the calculated $[Cl^-]_i$, the equilibrium potential for Cl^- (E_{Cl}) was determined according to the Nernst equation ($E_{Cl} = 61$ mV $\times \log[Cl^-]_o \div [Cl^-]_i$).

Results

Successful dye labeling (≥80%) was verified before each experiment using epifluorescence microscopy (data not shown). Both fluorescent dyes were predominantly localized in the area of sperm head and midpiece. Additionally, all labeled sperm populations were ≥70% motile, which indicates that dye loading was not detrimental to the spermatozoa. When MQAE-labeled spermatazoa were resuspended in media with an $[Cl^-]_o$ between 12-48 mM, the FI was diminished as the $[Cl^-]_o$ increased (Fig. 1A). A similar relationship between the FI and $[Cl^-]_o$ was also

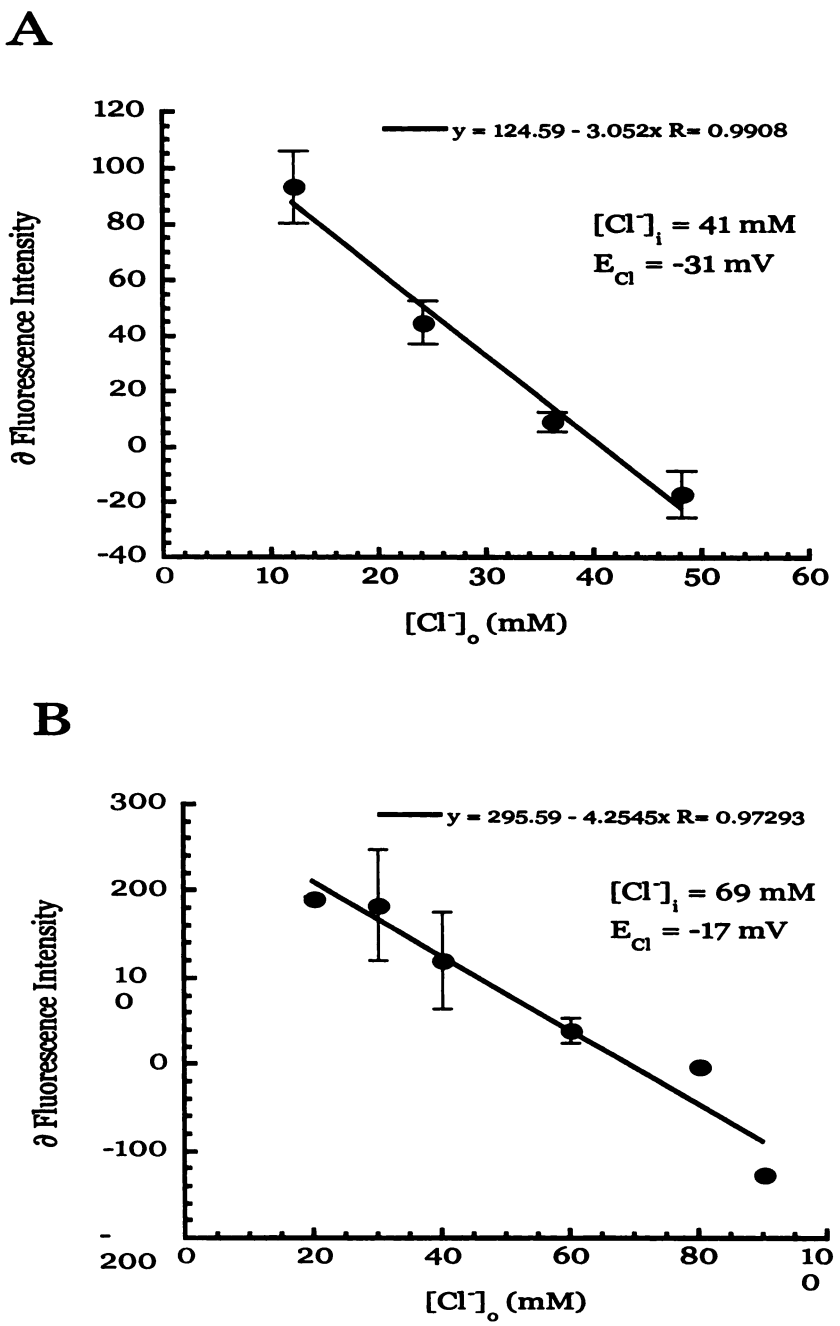

FIG. 2. Linear regression analysis of the difference (∂) in *N*-(6-methoxyquinolyl) acetoethyl ester **(A)** and methoxy-*N*-ethylquinolonium **(B)** fluorescence intensity following digitonin (10 μM) permeabilization of capacitated spermatozoa in HEPES-buffered media of varying Cl^- concentrations. The data represent four experiments with different donors. The intracellular Cl^- concentration ($[Cl^-]_i$) was determined from the regression formula. The Cl^- equilibrium potential (E_{Cl}) was determined using the Nernst equation, assuming an extracellular Cl^- concentration ($[Cl^-]_o$) of 133 mM.

identified for MEQ-labeled spermatazoa (Fig. 1B). Therefore, the $[Cl^-]_i$ does not remain constant with varying $[Cl^-]_o$.

Low dose (10-μM) digitonin treatment rapidly (≤45 seconds) permeabilizes the human sperm plasma membrane (Garcia and Meizel, 1996). The addition of 10 μM digitonin to sperm suspensions in the present study caused a change in the FI of sperm suspensions (Fig. 1). The amplitude and direction of that change was dependent on the $[Cl^-]_o$. When the difference in FI was plotted against $[Cl^-]_o$ (Fig. 2), we calculated an $[Cl^-]_i$ of 41 mM (MQAE) and of 69 mM (MEQ). Based on these values, the corresponding E_{Cl} is −31 or −17 mV for MQAE- or MEQ-labeled spermatozoa, respectively.

Discussion

$GABA_A$ receptor-like Cl^- channels have been identified in spermatozoa by immunoblot analysis and by indirect immunofluorescence (Wistrom and Meizel, 1993). Moreover, pharmacological studies have demonstrated that these Cl^- channels are involved in the P-initiated AR (Wistrom and Meizel, 1993; Shi and Roldan, 1995; Shi et al, 1997; Espinosa et al, 1998). We have previously suggested that a Cl^- efflux through such a channel may modulate the P-initiated AR (Turner and Meizel, 1995). Here we have report the E_{Cl} of capacitated human spermatozoa using Cl^--sensitive fluorescent probes (MQAE and MEQ). If we compare these values (−30 mV, MQAE; −17 mV, MEQ) to the estimated values of the equilibrium membrane potential (V_{eq}) of capacitated mouse (−50 mV; Espinosa and Darszon, 1995; Zeng et al, 1995) and human spermatozoa (−69 to −75 mV; Calzada et al, 1991; Calzada and Tellez, 1997), also determined with voltage-sensitive fluorescent dyes, we see that E_{Cl} is greater than V_{eq}. This condition predicts that the opening of passive conductance sperm Cl^- channels, such as $GABA_A$ receptor/Cl^- or glycine receptor/Cl^- channels, would result in a net Cl^- efflux and depolarization.

It is conceivable that the treatment of human spermatozoa with digitonin permeabilized the cell membrane, resulting in the release of cytosolic dye. Since the sensitivity of MEQ, and likely that of MQAE, to Cl^- is lower in the cytosol than in aqueous buffers (Biwersi and Verkman, 1991), released dye would be more readily quenched (i.e., FI decreased) in external medium of lower (≈8×) $[Cl]^-$. However, in our results, we demonstrate that digitonin treatment results in an increased FI ($[Cl^-]_o <$ 36 mM, MQAE). Still, our results may have underestimated the $[Cl^-]_i$ and could actually be considered conservative values. Whether or not any dye was released because of treatment with digitonin, our present calculations demonstrate that the $[Cl^-]_i$ would be sufficiently high enough such that the opening of a Cl^- channel would result in a net Cl^- efflux. An alternative method for equilibration of external and internal Cl^-, which involved the use of the ionophores nigericin and tributyltin (Krapf et al, 1988), was attempted, but it was found to also cause the release of cytosolic dye from the area of the anterior sperm head (data not shown).

In conclusion, activation of $GABA_A$ receptor-like Cl^- channels by P, under the physiological conditions described above, should result in a net Cl^- efflux and membrane depolarization. This Cl^- conductance pathway may in turn modulate the P-activated Ca^{2+} influx that triggers the human sperm AR, as we have previously hypothesized, if in fact a voltage-dependent Ca^{2+} channel is involved in the P-initiated AR (reviewed in Shi and Roldan, 1995; Meizel, 1997). However, it should be noted that Cl^- efflux is not the only mechanism by which P induces a membrane depolarization in human spermatozoa. Foresta and coworkers (Foresta et al, 1993) have demonstrated that P causes a membrane depolarization by triggering a cation influx through a nonselective cation channel.

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