Mouse sperm patch-clamp recordings reveal single Cl^- channels sensitive to niflumic acid, a blocker of the sperm acrosome reaction

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Abstract Ion channels lie at the heart of gamete signaling. Understanding their regulation will improve our knowledge of sperm physiology, and may lead to novel contraceptive strategies. Sperm are tiny (~ 3 μm diameter) and, until now, direct evidence of ion channel activity in these cells was lacking. Using patch-clamp recording we document here, for the first time, the presence of cationic and anionic channels in mouse sperm. Anion selective channels were blocked by niflumic acid (NA) (IC_{50} = 11 μM). The blocker was effective also in inhibiting the acrosome reaction induced by the zona pellucida, GABA or progesterone. These observations suggest that Cl^- channels participate in the sperm acrosome reaction in mammals.

Key words: Acrosome reaction; Ion channel; Niflumic acid; Sperm patch-clamp recording

1. Introduction

Ion channels are deeply involved in the egg-sperm dialogue. Components from the outer layer of the egg induce changes in sperm permeability to ions which, depending on the species, regulate sperm motility, chemotaxis and the acrosome reaction (AR). This reaction is required for sperm to fertilize the egg in many species, including man. The egg's extracellular matrix, the zona pellucida (ZP), induces the AR in mammals [1]. Nonetheless, other agents like γ-aminobutiric acid (GABA) and progesterone can induce this reaction [2–6]. The sperm AR is inhibited by ion channel blockers, evidencing their predominant role in this process, and their suitability as targets for contraception [7].

Due to the small size of sperm thus far only planar bilayer techniques have yielded direct information about their ion channels [8–12]. To explore how ion channels participate in the AR we have recorded single channel currents directly from mouse sperm. Here, using patch-clamp recording we document, for the first time, the presence of cation and anion channels in mammalian sperm head plasma membrane. Anion selective channels were blocked by niflumic acid (NA) (IC_{50} = 11 μM). The blocker inhibits also the acrosome reaction induced by the zona pellucida, GABA or progesterone, suggesting that Cl^- channels participate in the sperm AR in mammals. Voltage-gated Cl^- currents, blocked by μM NA (IC_{50} = 100 μM), were monitored also in pachytene spermatocytes [13–15], indicating that NA sensitive Cl^- channels are expressed early in spermatocyte differentiation.

2. Materials and methods

2.1. Sperm collection and AR induction

Sperm were obtained from CD-1 mice $>3\frac{1}{2}$ months killed by cervical dislocation. The cells were collected from excised epididymides as described [16]. Cells (3.6×10^6/ml) were suspended in M-199 media (from Sigma) supplemented with 25 mM $NaHCO_3$, 1 mM sodium pyruvate and 0.4% glucose, and were capacitated at 37°C for 30 min in 5% CO_2 atmosphere. Thereafter the agents (heat-solubilized ZP, progesterone or GABA) were added and cells were left to acrosome react for 30 min. Inhibition was studied with the same procedure except that the blocker was added 5 min prior to the agonist. At the end of the experiment AR was determined by established procedures [17,18]. A minimum of 100 sperm were counted under light field microscopy and expressed as the fraction of reacted sperm induced by the agonist vs. a maximum of reacted sperm obtained with the Ca^{2+} ionophore A-23187 (AR/ARA-23) at 15 μM.

2.2. Electrophysiology

2.2.1. Sperm. Seals were obtained using Kimax or 7052 glass pipettes having >5 megaohms resistance and <1 μm of tip diameter. Sperm were rinsed for 10 min in (mM): 60 NaCl, 150 sucrose, 20 sodium lactate, 1 sodium pyruvate, 5.6 glucose, 1 EDTA, 10 benzamidine and 20 μg/ml aprotinin; pH 8.4, pelleted at $325\times g$ for 10 min at room temperature in cylindrical 2 ml Eppendorf tubes before patch-clamping them. The pelleted cells were resuspended, left for 10 min at 20–23°C and then placed on ice. An aliquot was allowed to settle for 5–10 min on a coverslip and placed in a recording chamber on the stage of an inverted microscope (Nikon Diaphot TMD, Nikon Corp.) endowed with Hoffman Optics. Experiments were done at 20–23°C and currents monitored with and Axoclamp 1-B amplifier (Axon instruments, Foster City, CA), sampling at 10–25 kHz, and filtering at 2–5 kHz. Data analysis was performed with Pclamp6 routines. Patch-clamping the sperm head is laborious due to its small size and flatness. A perpendicular approach between pipette and cell surface is necessary to achieve high resistance seals (>1 gigaohms, Fig. 1A). This occurred only in $\sim 7\%$ of trials, out of which $\sim 80\%$ displayed single channel activity. In 25% of the high resistance seals spontaneous excised patches were obtained. In this cases the polarity of the membrane patch was not known. Excised patches were lost very rapidly, therefore, ion substitution experiments were not possible and data were acquired only with the solution used during sealing. Analysis was done only on records with >5 megaohms of seal resistance. Apparent permeability on excised patches were calculated according to [19]. The solutions in pipette and in bath employed in sperm patch-clamp experiments are indicated in the figure legends.

2.2.2. Spermatogenic cells. Testis from adult CD-1 mice were used as a source of germ cells. Spermatogenic cells were obtained as described [13,14]. The dissociated cells were stored at 4–10°C and remained healthy for at least 12 h. An aliquot of the cell suspension was placed in a recording chamber superfused with recording medium (in mM: 130 NaCl, 3 KCl, 10 $CaCl_2$, 2 $MgCl_2$, 1 $NaHCO_3$, 0.5 NaH_2PO_4, 5 Na-HEPES, 5 glucose, 0.16 amiloride, pH 7.35) at 20–

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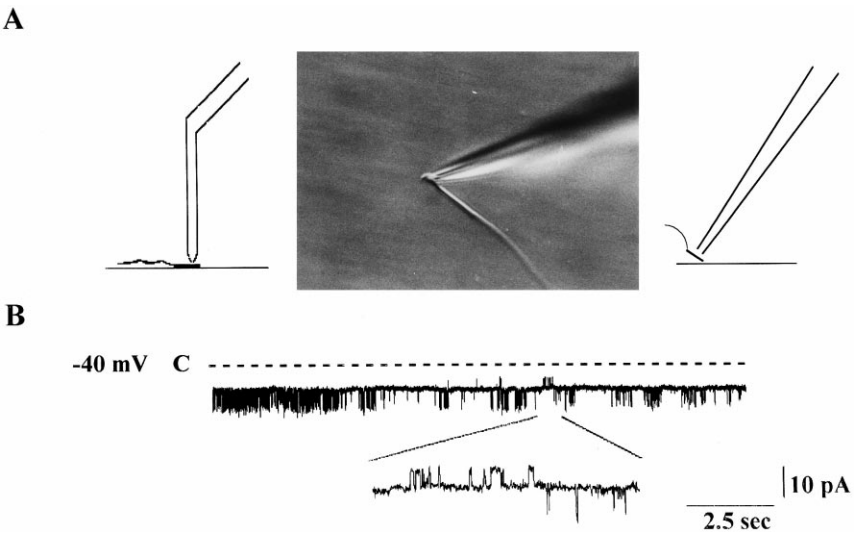

Fig. 1. Direct single channel recording in mouse sperm. A: The center shows a micro-photograph of a patch-clamped mouse sperm (head sperm width = ~3 μM). Schematic representations of perpendicular approaches to sperm are illustrated on both sides of the photo. B: Single channel activity in an excised patch. The pipette was filled with (in mM): 30 NF, 120 NaCl, 0.1 GABA, 10 HEPES, pH 7.2. The bath solution was (in mM): 145 NaCl, 10 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES, pH 7.4. The trace obtained at −40 mV shows the presence of mainly two types of transitions (see text). The discontinuous line indicates zero current (C, closed state).

23°C. The solution in the pipette was (in mM): 110 Cs-Methanesulfonate, 10 CsF, 15 CsCl, 2 Cs-EGTA, 4 ATP-Mg, 10 phosphocreatine, 5 Cs-HEPES, pH 7.35. Glass borosilicate pipettes were pulled to tip diameters of about 1.5 μm, having resistances between 2 and 5 megaohms when filled with pipette solution. Records were low-pass filtered at 2 kHz (4-pole Bessel filter). A p/4 pulse protocol was used routinely to minimize leak and capacitive currents from current records [20].

2.3. Membrane potential measurements

In cell attached experiments (Fig. 3) real ionic cytoplasmic concentrations are not known for the patched cell. Membrane potential (V_m)

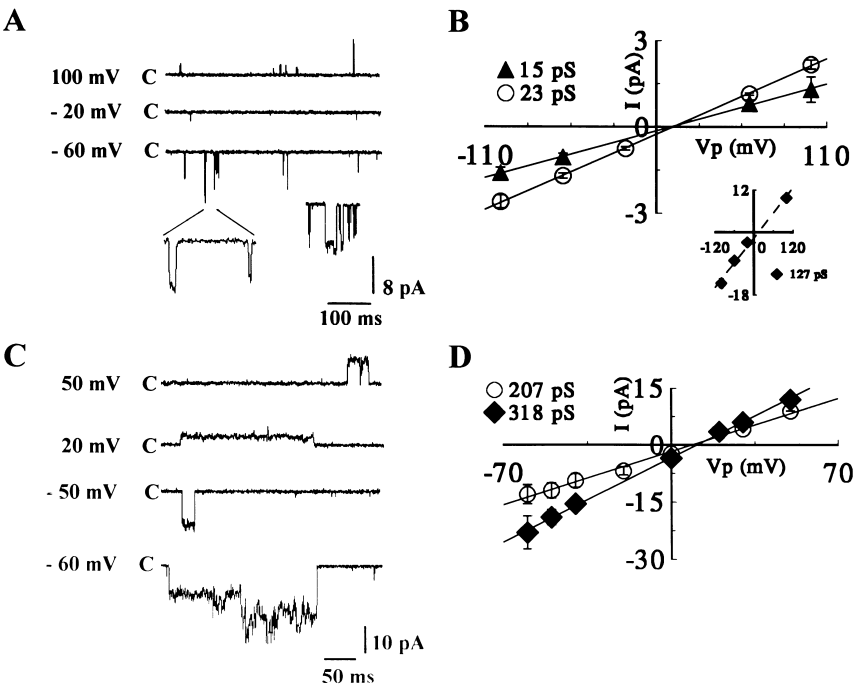

Fig. 2. Cationic channels. The traces shown in this figure were recorded on spontaneous excised patches at the indicated voltages. A fast mouse sperm cation channel displaying several conductance levels. Kinetic analysis yielded basically a monoexponential closing time constant of 0.82 ± 0.27 ms at −60 mV, and opening time constants of 4.3 ± 1 ms and 53 ± 12 ms. Left inset shows a 20-fold time expansion of the indicated area of the burst at −60 mV. Right inset shows a burst from the same recording at −60 mV (identical scales). Solutions were (in mM): 145 NaCl, 2.5 $CaCl_2$, 10 $MgCl_2$, 10 HEPES-Na, pH 7.4 (in bath); and, 120 CsCl, 0.05 $CaCl_2$, 10 EGTA-Cs, 10 HEPES-Cs, pH 7.2 (in pipette). Similar transitions were observed in 12 experiments. B: I–V relations of the channel in A, E_{rev} = 10 mV. The inset shows the I–V relation of the largest conductance. C: Representative traces of a slow high-conducting channel showing two main conductance levels. Similar transitions were observed in 11 experiments. D: I–V relations of the channel in C. Solutions were (in mM): bath as in A; and in the pipette; 30 KF, 90 KCl, 0.05 $CaCl_2$, 10 EGTA-K and 10 HEPES-K, pH 7.2. Smaller fast cationic transitions similar to those in A are also present.

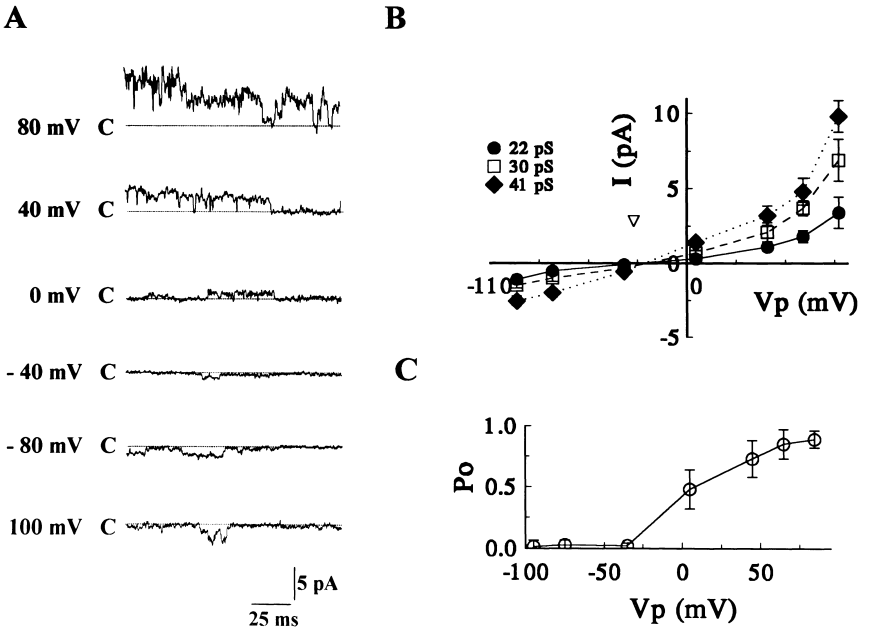

Fig. 3. Mouse sperm anion channels. A: Anion single channel transitions at the indicated pipette potentials recorded in the cell attached configuration. The pipette contained (mM): 120 NaCl, 30 NaF, 10 HEPES-Na, pH 7.6. B: I–V relations from A showing outward rectification at high positive applied potentials. Slope conductances of 22, 30 and 41 pS were measured in the linear region. E_{rev} averaged -30 ± 5 mV ($n = 3$), which considering the resting potential of sperm under equivalent conditions (−85 mV, see Section 2) and 10 mM Cl^- in the sperm cytoplasm [22], indicates anion selectivity. C: Open probability (P_o) of the anion channel measured during 6.4 s at each pipette potential (V_p).

determinations in sperm suspensions were done as in [21] using DiS-$C_3(5)$, a V_m sensitive fluorophore. The resting potential of sperm suspended in the external solution (TEA-Cl 60, TEA-$MeSO_4$ 70, 10 $CaCl_2$, pH 7.4) used for cell attached experiments was estimated to be -85 ± 7 mV ($n = 8$). The internal K^+ concentration used for this calculations was 120 mM [22].

3. Results and discussion

To explore the in situ characteristics of sperm ion channels, we recorded single channel currents directly from mouse sperm. Single channel currents could be recorded in 42

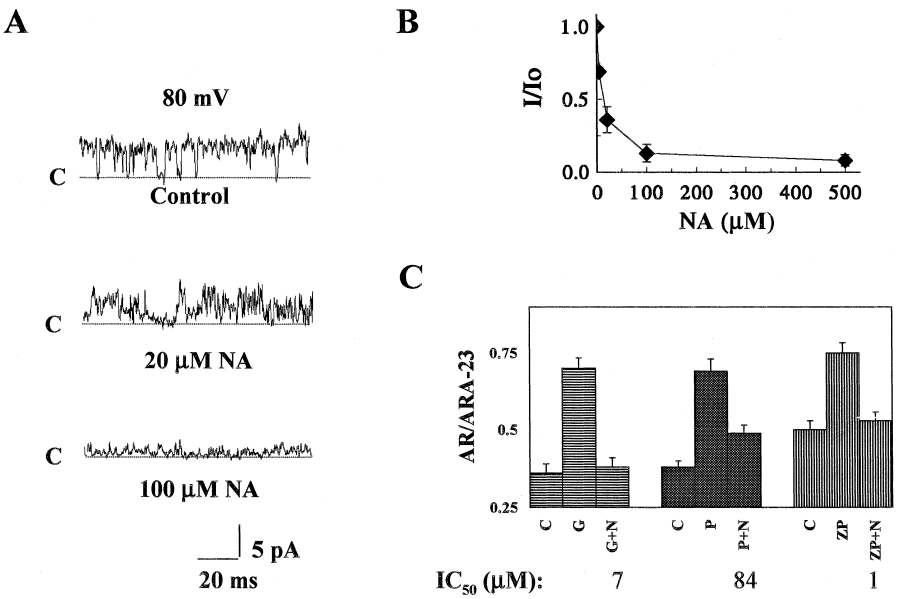

Fig. 4. Niflumic acid (NA) blocks a mouse sperm Cl^- channel and the AR induced by ZP3, GABA and progesterone. A: Conditions were as in Fig. 3. Voltage in pipette was 80 mV in all cell attached records shown. The control trace is at the top, the following traces were taken 10 min apart, after perfusing the external chamber with control solution plus 20 μM (middle trace) or 100 μM (lower trace) NA. B: Dose dependence of channel blockade by NA (average ± S.E.M., $n = 3$). C: NA inhibits the mouse sperm acrosome reaction (AR) induced by GABA (0.1 μM), progesterone (45 μM) and solubilized zona pellucida (5 ZP/μl). AR and its inhibition were determined as indicated in Section 2. The bars represent the average ± S.E.M. ($n = 5$) of the ratio % AR under a certain condition (C, control; G, GABA; N, NA; P, progesterone; ZP, zona pellucida) and the AR achieved with the Ca^{2+} ionophore A-23187 (ARA-23). The numbers under the bars are the IC_{50} determined in five concentration dependence experiments.

patches, 14 of which were excised patches, the remaining ones corresponding to cell attached patches. Fig. 1B shows single channel activity in an excised patch at −40 mV. Mainly, two types of current transitions can be observed: (1) fast transitions that occur in bursts; and (2) smaller upward transitions (inset below). In addition, a slow high-conducting channel similar to the one of Fig. 2C, was also observed during this recording (not shown).

Single channel currents shown in Fig. 2A were monitored in an excised patch at different applied voltages. These transitions have fast kinetics with spike shaped transitions being the most common, although square shaped transitions are also present (see record at higher resolution and the bursting period in Fig. 2A). From the I–V plots, which have an $E_{rev} = 10$ mV, a PNa^+/PCs^+ of 1.5 (150 Na^+_{bath}/145 $Cs^+_{pipette}$) was calculated. In a separate experiment PNa^+/PK^+ was 0.71, indicating a permeability sequence $K^+ > Na^+ > Cs^+$, analogous to that seen in bilayers ($K^+ > Rb^+ > Na^+ > Cs^+$) for a cAMP-activated sea urchin sperm cationic channel [12]. Similar single channel currents were recorded in four excised patches and eight cell attached ones. In three experiments where transitions were recorded at various voltages, P_o did not vary significantly (0.012 ± 0.022 in the ± 100 mV range). In ~25% of the experiments where fast cationic transitions were seen, burst periods with $P_o > 0.3$ were observed between voltages $\geq$+40 mV or $\leq$−40 mV (inset in Fig. 2A). The pattern of the transitions is also reminiscent of a mouse sperm cationic channel detected in planar bilayers [11]. Moreover, the poor selectivity of this channel suggests it could participate in the ZP induced depolarization during mouse and bull sperm AR [23].

Fig. 2C shows a record of an excised patch displaying slower cationic transitions with main conductance substrates of 207 and 318 pS ($E_{rev} = 11$ mV; $PCa^{2+}/PNa^+ = 6$; assuming $PNa^+/PK^+ = 1$). Similar transitions were recorded in 11 experiments (eight cell attached and three excised patches). Planar bilayer studies indicated the presence of a similar high conductance, poorly selective cationic channel that allows divalents through, in sea urchin [24] and mouse sperm plasma membranes [10,11]. Patch-clamp recording shows that this cation selective channel displays slow kinetics with long open times (tenths of milliseconds to seconds), resembling the behavior of the Ca^{2+} selective channel reconstituted in planar lipid bilayers.

In three experiments it was possible to record channel currents that were blocked by NA (a Cl^- channel blocker, Figs. 3 and 4). Fig. 3A shows single channel currents monitored at different applied voltages. Inspection of the records revealed the presence of open substrates with conductances of 22, 30 and 41 pS, measured in the ohmic region of the I–V relation. The reversal potential, −30 mV, is consistent with that predicted for an anion selective channel, assuming a resting potential of −85 mV (see Section 2, Fig. 3B). At more positive pipette potentials, the channel rectifies, probably due to the presence of 30 mM F^- in the pipette. Some Cl^- channels are known to have a low F^- permeability ($PCl^-/PF^- = 50$; [25]). P_o of the channel increases as the applied potential becomes more positive (Fig. 3C). Blockade by NA was dose-dependent ($IC_{50} = 11$ μM, Fig. 4A, B). Lipid bilayer studies had also shown the presence of anion channels in sea urchin [26] and mouse sperm plasma membranes [11,27].

Several reports indicate the participation of Cl^- channels in sperm physiology. NA was reported to inhibit a Ca^{2+} induced

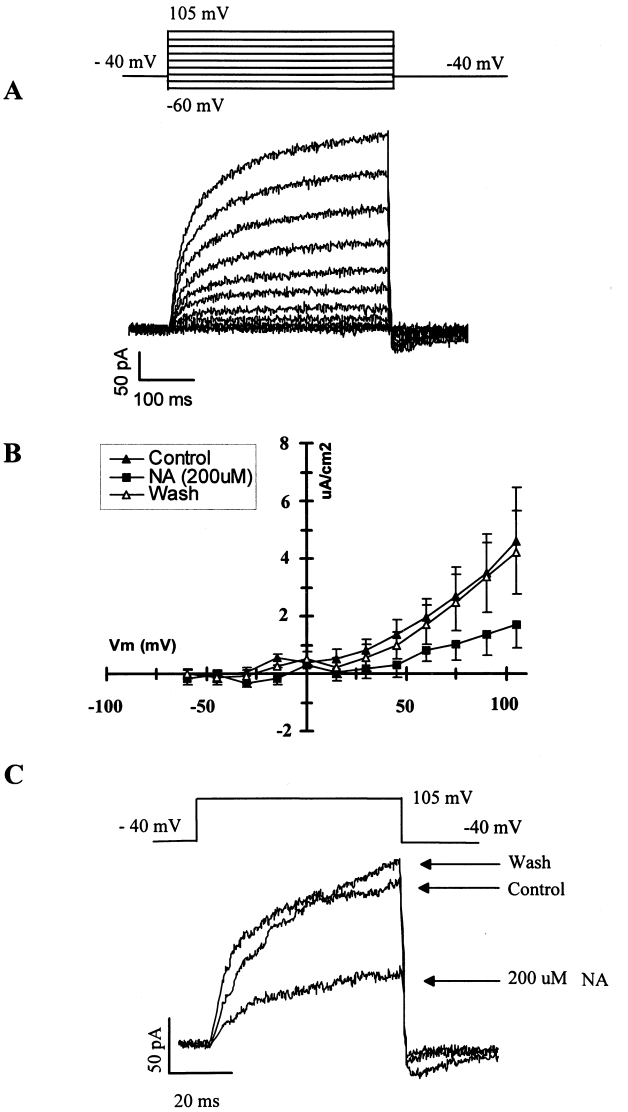

Fig. 5. NA blocks outward whole cell currents resulting from Cl^- influx in pachytene spermatocytes. Spermatogenic cells and whole-cell recordings were obtained as described in Section 2. Patch pipettes were filled with (in mM): 110 Cs-Methanesulfonate, 10 CsF, 15 CsCl, 2 Cs-EGTA, 4 ATP-Mg, 10 phosphocreatine, 5 Cs-HEPES, pH 7.35; the bath solution contained (in mM): 130 NaCl, 3 KCl, 2 $MgCl_2$, 1 $NaHCO_3$, 0.5 NaH_2PO_4, 5 Na-HEPES, 5 glucose, 10 $CaCl_2$, 0.16 amiloride, pH 7.35. Currents were triggered by depolarizations from −40 mV holding potential up to 100 mV in 15 mV steps. A: Cl^- currents elicited at 40 mV (top shows voltage protocol) and their block by 200 μM NA. B: I–V relation of the Cl^- currents in mouse pachytene spermatocytes (filled triangles), and their blockade (squares) and recovery (empty triangles) after exposure to 200 μM NA. The points represent the average of three experiments, the standard deviation was smaller than the symbols. The estimated IC_{50} was 100 μM.

hyperpolarization which is partially driven by Cl^- in mouse sperm [21]. External Cl^-, and putative $GABA_A$-R channels have been implicated in the GABA and progesterone induced AR in human and mouse sperm [4,5,28]. In porcine and human sperm, Glycine receptor activation was reported to elicit AR [29] and Cl^- efflux [30]. In other cell systems, NA and other fenamates (non-steroidal anti-inflammatory drugs) have been shown to block Ca^{2+}-activated Cl^- channels [31,32], as well as an heterologously expressed $GABA_A$-R [33]. More-

over, Sigel et al. (1989), reported the expression of a rat β homopentamer $GABA_A$-R which opens in the absence of GABA [34]. Future experiments will have to be done to determine if the sperm Cl^- channel reported here is of the $GABA_A$-R type.

Since NA blocks Cl^- channels in mouse sperm and in other systems, the effect of this compound was tested on the AR. NA blocked the AR induced by GABA (0.125 μM), progesterone (45 μM) or solubilized zona pellucida (5 ZP/μl) (Fig. 4C). The ZP3 and GABA induced AR was more sensitive to NA (IC_{50} of 1 and 7 μM, respectively) than that induced by progesterone (84 μM). This was unexpected since it has been proposed that progesterone triggers the AR by potentiating the $GABA_A$-R channel [2,4,5]. This result may indicate that distinct Cl^- channels participate in AR induced by the different agonists. Alternatively, progesterone could modulate two different surface sperm receptors one coupled to a Ca^{2+} channel [2,35,36] and the other, a $GABA_A$-R type, which upon progestin binding decreases its affinity for NA [29,37].

We investigated further whether spermatogenic cells are endowed with NA sensitive Cl^- channels. Cl^- currents, activated by depolarization, and blocked by NA (IC_{50} 100 μM), could be monitored in pachytene spermatocytes (Fig. 5). These results show that NA sensitive Cl^- channels are expressed early in spermatocyte differentiation. However, at difference with anion channels present in the sperm, Cl^- channels in spermatocytes display a lower affinity for this blocker.

At the least, our results lead to the conclusion that mouse sperm, and spermatocytes, as early as pachytene, are endowed with Cl^- channels blocked by NA. Furthermore, the cumulated experimental evidence suggests that mouse sperm Cl^- channels may participate in the AR induced by ZP3 and GABA. It is possible that one of these Cl^- channels is an isoform of the $GABA_A$-R, and that mammalian sperm posses more than one mechanism capable of triggering the events leading to the fusion of the acrosomal vesicle.

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