Mouse sperm membrane potential: changes induced by Ca^{2+}

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Abstract Mouse sperm resting membrane potential (E_r) (−42 ± 8.8 mV), determined with a potential sensitive dye, depended on extracellular K^+ and, in the absence of extracellular Ca^{2+} ($[Ca^{2+}]_e$), on external Na^+ ($[Na^+]_e$). Ca^{2+} addition (>5 μM) to sperm in Ca-free media induced a transient hyperpolarization (Ca-ith) which strongly depended on $[Na^+]_e$ and less on external Cl^- ($[Cl^-]_e$). Cd^{2+} and Mn^{2+} (μM) mimicked the Ca^{2+} effect, but not Ba^{2+}. The Ca-ith was partially inhibited by ouabain (74%, IC_{50} = 5.8 μM) and niflumic acid (38%, IC_{50} = 240 μM), indicating the participation of the Na-K ATPase and Cl^- channels. In Ca-free low-Na^+ media, Ca^{2+} addition caused a depolarization sensitive to: nimodipine (25 μM), trifluoperazine (12.5 μM) and Mg^{2+} (1.2 mM), suggesting the participation of Ca^{2+} channels. Since some inhibitors of the sperm Ca-ith block the acrosome reaction (AR), both processes may share transport systems.

Key words: Sperm membrane potential; Ion channel; Na-K ATPase; Acrosome reaction

1. Introduction

Mammalian sperm are exposed to important alterations in their ionic milieu, and their plasma membrane permeability, as they progress in their journey towards the egg. Changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) and intracellular pH (pH_i) are fundamental for mammalian sperm motility [1,2], and for the AR induced with the zona pellucida glycoproteins [3] or with progesterone [4,5]. Some of the possible protagonists responsible for these ionic permeability changes are: ion channels for Ca^{2+} [6–8]; K^+ [1,9], Cl^- [10] and one for cations gated by cyclic nucleotides [11]. The sperm Na-K ATPase has also been implicated in capacitation [12] and in the AR [13].

Several of the transport systems mentioned above are voltage-dependent [14]. However, little is known about the ion permeabilities that determine the resting membrane potential (E_r) in mammalian sperm and how it changes during capacitation and the AR [9,15]. Besides the well-known $[Ca^{2+}]_i$ and pH_i changes that occur during the AR [3,9], there is only one report showing sperm membrane potential changes correlated with ligand-induced $[Ca^+]_i$ increases [16].

To further understand the ionic basis of the E_r, mouse spermatozoa were exposed to different ionic conditions and membrane potential (E_m) was evaluated using the potential sensitive dye dipropylthiodicarbocyanine (Dis-C_3-(5)) [9,17,18]. We also characterized E_m changes induced by Ca^{2+} additions to spermatozoa suspended in different Ca-free media. The results indicate that cation and anion channels, and the Na-K ATPase participate in the Ca-induced E_m changes. Some of the inhibitors of these E_m changes also block the AR, suggesting that certain transport systems are involved in both processes.

2. Materials and methods

Valinomycin, ouabain, niflumic acid, methane-sulfonic acid and choline chloride were from Sigma (St Louis, MO). Dis-C_3-(5) was from Molecular Probes (Eugene, OR). Nimodipine (NDP) and trifluoperazine (TFP) were generously donated by Arturo Hernández.

CD-1 mice older than 3.5 months were sacrificed by cervical dislocation and sperm cells collected from excised epididymides as described [19]. Each mouse yielded 15–30 × 10^6 spermatozoa. The incubation media was a modified Krebs-Ringer bicarbonate buffer (HMB media) containing 110 mM NaCl, 10 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM HEPES, 20 μM EGTA, 20 mM Na-lactate, 1 mM Na-Pyruvate and 1 mg/ml glucose, pH 7.5–7.6, adjusted with NaOH. Ca^{2+} (1.7 mM) was added or not (Ca-free media) depending on the experiment. The Ca-free media had ~nM Ca^{2+}. Sperm were stored at 23 ± 3°C in HMB media without Ca^{2+}, 20–60-μl aliquots were diluted with the required experimental media to a final volume of 800 μl at 37°C (see below).

Na^+ and/or K^+ were substituted with isoosmotic concentrations of choline$^+$ or *N*-methyglucamine. Cl^- was substituted by methane-sulfonic acid titrated with the hydroxide of the required cation. For experiments in low-Na media (~10 mM), except HEPES, other buffers, Mg^{2+} and organic compounds were omitted.

E_m was measured exposing sperm (2.5 ± 0.5 × 10^6/ml) to 1 μM Dis-C_3-(5) in a gently stirred cuvette at 37°C and the fluorescence (620/670 nm excitation/emission wavelength pair) recorded continuously in a Hansatech MkII fluorometer (Norfolk, UK), as described [18]. After reaching a steady fluorescence (1–3 min), E_r and E_m changes were recorded. Thereafter, the K^+ ionophore valinomycin was added and the external K^+ concentration ($[K^+]_e$) increased stepwise to calibrate the fluorescence changes in mV (Fig. 1A) [18]. K^+ equilibrium potentials were calculated with the Nernst equation [14], considering 120 mM as the $[K^+]_i$ [20].

To study the reversibility of the Ca^{2+}-induced transient hyperpolarization, sperm were washed in Ca-washing media (CWM; the same as Ca-free media + 2.5 mM EGTA, pH 7.8). When required, spermatozoa were washed by centrifugation 10 min at 20°C and 300 × *g*.

3. Results

3.1. Membrane potential under different ionic conditions

In HMB media + 1.7 mM Ca^{2+} at 37°C, mouse sperm E_r was estimated to be −42 ± 8.8 mV (n = 16) (Fig. 1A). The dependence of E_r as a function of the external concentration of Na^+ ($[Na^+]_e$), K^+ and Cl^- and how Ca^{2+} modulates this potential is shown in Fig. 1B–D. The experiments in Ca-free media were done with (X) or without (solid symbols) preincubating sperm for 20 min at 37°C. The behavior of E_r was basically the same in both conditions, except for 156 mM Na^+ (see below). In the experiments in 1.7 mM Ca^{2+} sperm were not preincubated.

In sperm suspended in Ca-free media, E_r depended on $[Na^+]_e$. In non-preincubation experiments, when $[Na^+]_e$ changed from 110 to 156 mM, approximating its value in Ca^{2+} media, E_r hyperpolarized (Fig. 1B). This change in E_r may involve a

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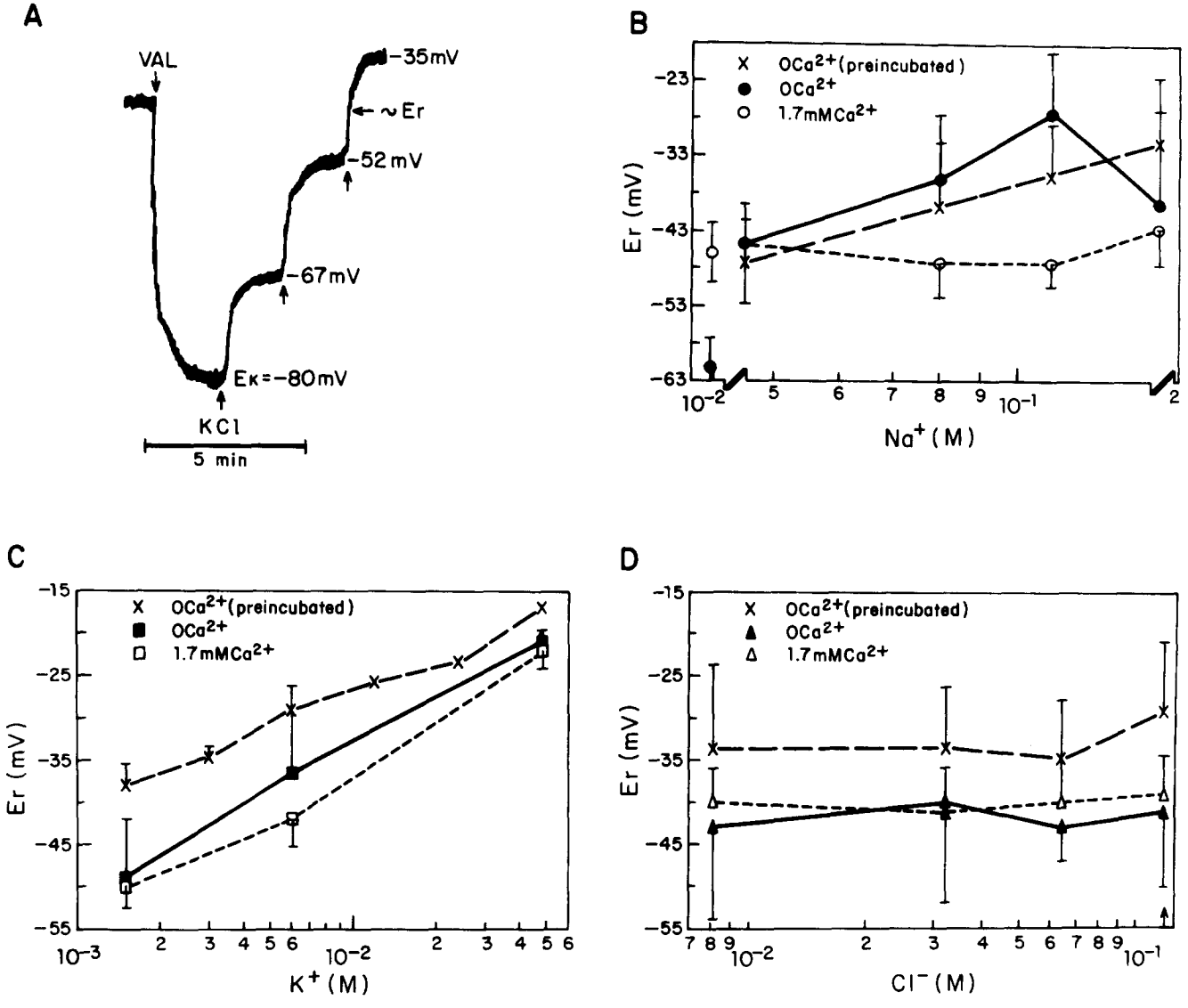

Fig. 1. Influence of external Na^+, K^+ and Cl^-, on resting membrane potential (E_r) of mouse spermatozoa. (A) Sperm suspended in HMB hyperpolarized (downward deflection) after adding 625 nM of valinomycin (VAL). Thereafter, additions of 3.75, 7.5 and 15 mM KCl allowed membrane potential calibration (Nernst potentials on the right, see Section 2), and E_r determination (horizontal arrow). The time scale is in the bottom. The external ionic dependence of the sperm E_r and its modulation by Ca^{2+} is illustrated in parts B for $[Na^+]_e$, C for $[K^+]_e$ and D for $[Cl^-]_e$ in 1.7 mM Ca^{2+} (empty symbols) or in Ca-free media (solid symbols). The crosses correspond to experiments where sperm were preincubated for 20 min at 37°C in that condition. E_r varied according to $[K^+]_e$ in 1.7 mM Ca^{2+} as well as in Ca-free media. However, $[Na^+]_e$ changed E_r only in Ca-free media and $[Cl^-]_e$ seemed not to affect it. Each point is the average of at least four experiments (± S.D.). In Fig. D, the arrow on the x axis corresponds to 120 mM Cl^-.

voltage-dependent channel or transport system. It is not seen after a preincubation possibly because some ionic equilibration has occurred. When physiological concentrations of external Ca^{2+} ($[Ca^{2+}]_e$) (1.7 mM) were used, $[Na^+]_e$ no longer affected E_r, which was −45.2 ± 5.4 mV (n = 33) in the 10–156-mM range of $[Na^+]_e$.

$[K^+]_e$ influenced E_r both, in presence or absence of external Ca^{2+} (Fig. 1C). Increasing $[K^+]_e$ from 1.5 to 48 mM made E_r ~29 mV more positive. Since in Ca^{2+}-free media $[Na^+]_e$ affected E_r, these experiments were done maintaining $[Na^+]_e$ at 110 mM and having 46 mM choline$^+$, which was then substituted by K^+ as required. Contrary to $[Na^+]_e$ and $[K^+]_e$, substituting $[Cl^-]_e$ for methane-sulfonate did not affected E_r neither in the presence nor absence of $[Ca^{2+}]_e$ (Fig. 1D).

3.2. Ca-induced transient hyperpolarization (Ca-ith), analogs and inhibitors

Sperm suspended in a Ca-free media (+ 20 μM EGTA) transiently hyperpolarized upon Ca^{2+} addition (>5 μM). Fig. 2A,C shows that the amplitude of the hyperpolarization depended on the concentration of Ca^{2+} added. This response was maximal at ~500 μM $[Ca^{2+}]_e$ and displayed an IC_{50} of 56 μM. This phenomenon was mimicked by other divalent cations, such as Cd^{2+} and Mn^{2+}, suggesting a common target. Moreover, adding 500 μM Cd^{2+} or 1.25 mM Mn^{2+} before Ca^{2+}, completely inhibited the Ca-ith (Fig. 2C). Other divalent cations that showed some effect at higher concentrations (mM range) were Zn^{2+}, Ni^{2+}, Sr^{2+} and Mn^{2+} and the trivalent cation La^{3+}. In contrast, Ba^{2+} induced a small depolarization of 5.2 ± 2.2 mV (n = 5) and did

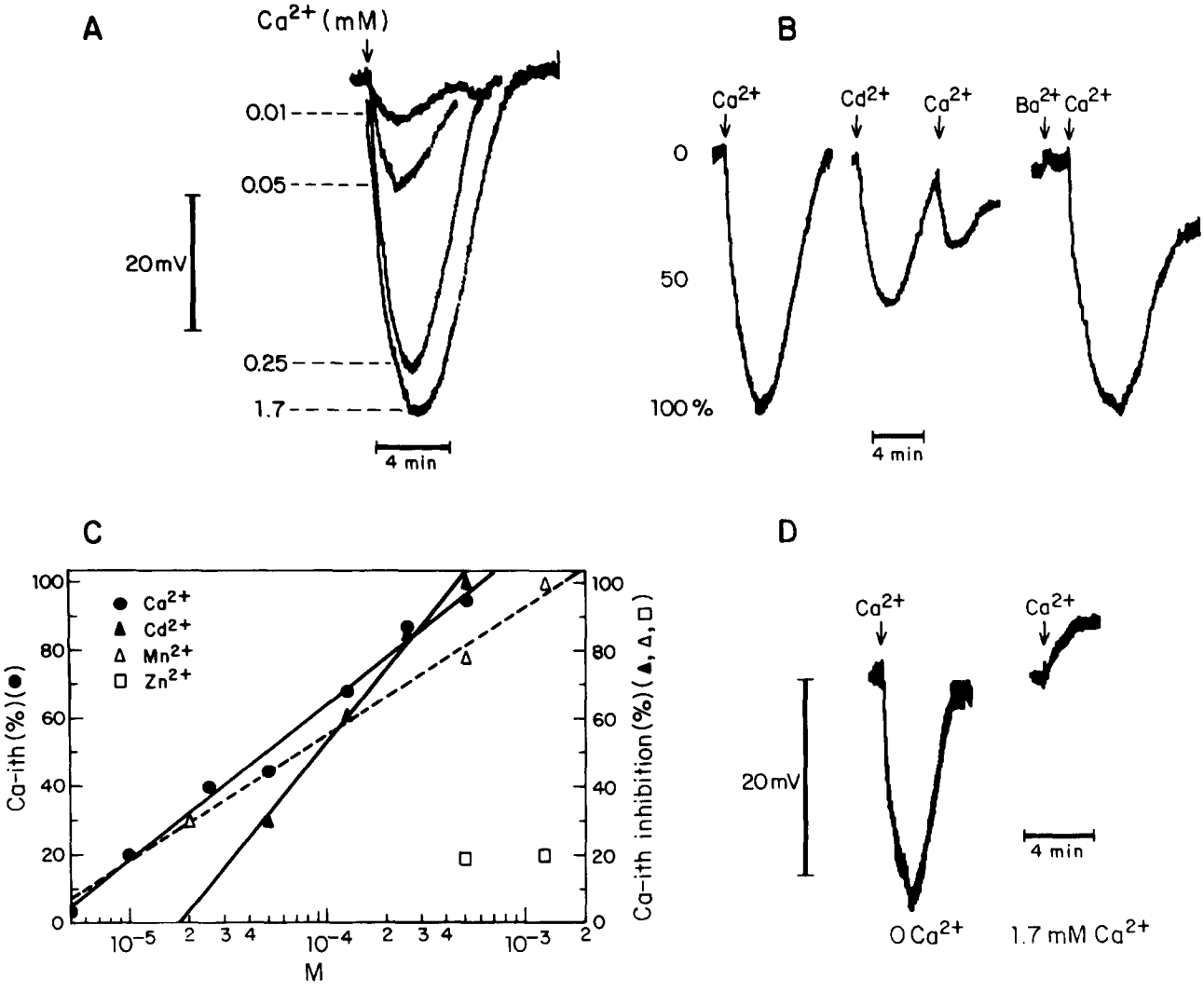

Fig. 2. Ca-ith and analogs. (A) Representative membrane potential responses to Ca^{2+} addition of sperm in Ca-free media upon (≥5 independent experiments/concentration). Numbers on the left of the traces indicate the amount of Ca^{2+} (mM) added. (B) Other divalent cations, such as Cd^{2+} (middle trace), induce an analogous hyperpolarization and inhibit the Ca-ith. Ba^{2+} instead induces a small depolarization (5.2 ± 2.2 mV, right trace) and does not inhibit the Ca-ith. Additions in these traces were: 1.7 mM for Ca^{2+} and Ba^{2+} and 0.125 mM for Cd^{2+}. Since these traces were from different sperm populations, the Ca-ith was normalized (% at the right). (C) Dose dependence of the Ca-ith (●, IC_{50} 56 μM) and its inhibition by Cd^{2+} (▲), Mn^{2+}(△) and Zn^{2+} (□) (y axis on the right). Inhibition of the Ca-ith was determined adding 1.7 mM Ca^{2+} after exposure to the indicated concentration of divalent cation. In all cases, S.D. were < ± 10% (n = 4, except for Mn^{2+} where n = 3) and were not included. (D) Ca-ith reversibility. Sperm cells were exposed to 1.7 mM Ca^{2+} for 10 min at room temperature, washed in CWM and the Ca-ith experiment done. Practically 100% of the Ca-ith was recovered (left trace). Sperm in 1.7 mM Ca^{2+} depolarized (right trace) upon a further Ca^{2+} addition. Arrows indicate Ca^{2+} addition (1.7 mM). Time scales are under the traces

not inhibit the Ca-ith (Fig. 2B). Sperm exposed 10 min to 1.7 mM Ca^{2+} in HMB media and washed by centrifugation in CWM, not containing Ca^{2+}, recovered their response (Fig. 2D).

To determine the ionic basis of Ca-ith we measured its amplitude varying $[Na^+]_e$ and $[K^+]_e$ by replacing them with choline$^+$ and $[Cl^-]_e$ with methane-sulfonate. The hyperpolarization clearly depended on $[Na^+]_e$ (Fig. 3A,B). It was maximal at 156 mM and practically disappeared below 46 mM $[Na^+]_e$, with a ~50 mV difference in hyperpolarization between these two Na^+ concentrations. Lowering $[Cl^-]_e$ from 118 to 11 mM diminished the Ca-ith by ~22 mV, <50%. In contrast, $[K^+]_e$ did not affect the Ca-ith, even though in these experiments E_r changed from −63 ± 8.7 to −30 ± 3 mV between the lowest and highest $[K^+]_e$, respectively (Fig. 1C). These results indicate that sperm suspended in Ca-free media undergo Na^+ and Cl^- permeability changes upon Ca^{2+} addition.

To assess which transport systems participate in the Ca-ith, several inhibitors and blockers were tested (20 mM TEA, 3 μM apamin, 5 μM TTX, amiloride 10–250 μM, >1.5 μM ouabain and >20 μM niflumic acid). From those examined, ouabain and niflumic acid had a clear inhibitory effect (Fig. 3C,D). Ouabain inhibited ~70 ± 10% (n = 21) of Ca-ith with an IC_{50} of 5.8 mM and did not require preincubation to have an effect. Even after a 10–20-s incubation with saturating ouabain concentrations, a fraction of the hyperpolarization remained (Fig. 3A, Table 1). Addition of ouabain itself slowly hyperpolarized spermatozoa suspended in Ca-free media. We believe this is not the cause of

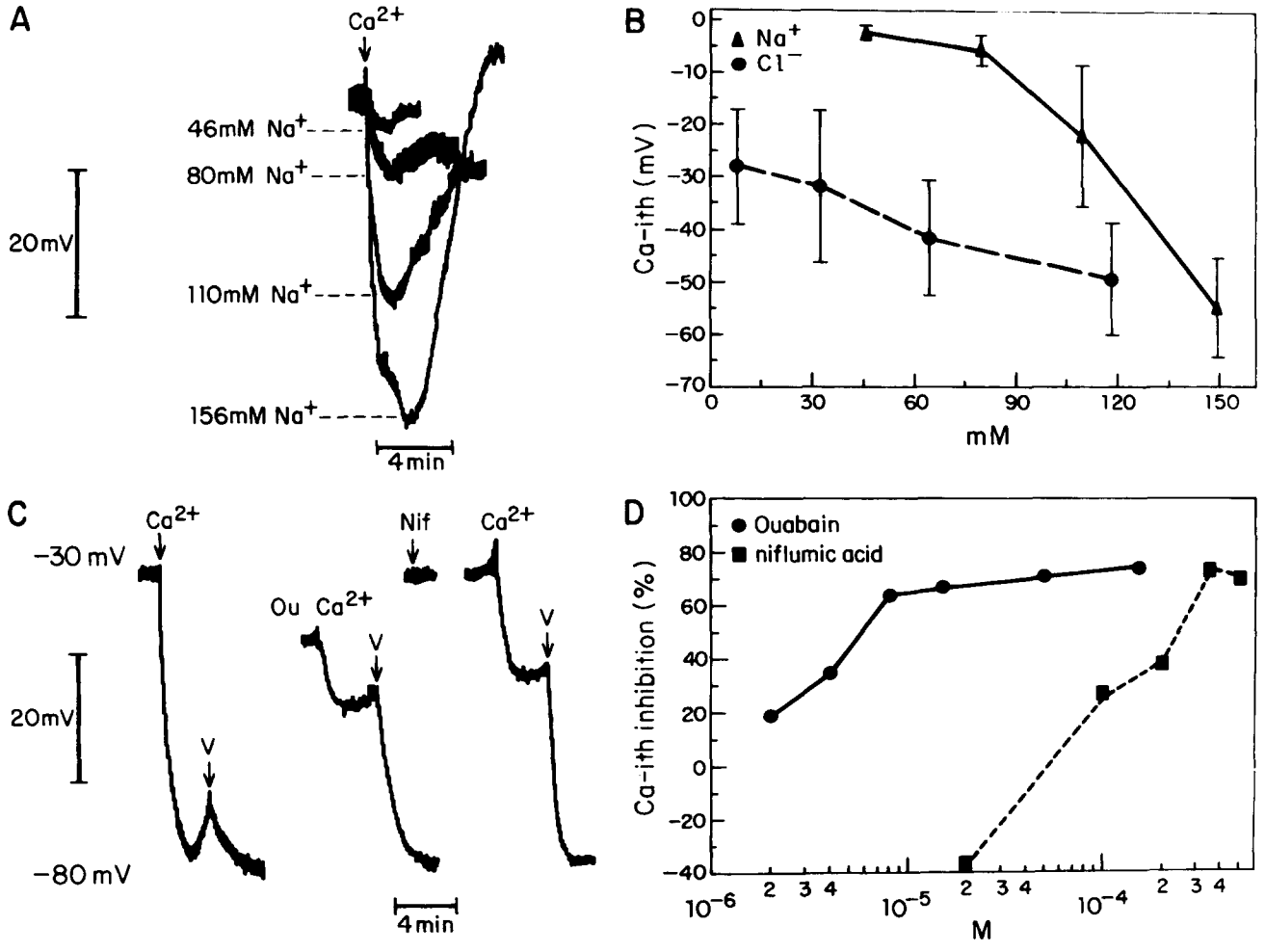

Fig. 3. Ionic dependence and pharmacology of the Ca-ith. (A) Ca-ith at different $[Na^+]_e$ (numbers on the left), Na^+ was substituted with $choline^+$. (B) $[Na^+]_e$ and $[Cl^-]_e$ dependence of the Ca-ith ($n = 6$). (C,D) Ca-ith inhibition by ouabain and niflumic acid (Nif). Cells were preincubated 2–6 min in Nif (interruption on the 3rd trace in Fig. 3C), the fluorescence artifact caused by the blocker was corrected. V indicates valinomycin addition for E_r and slope determinations. In Fig. 3D, S.D. were < ± 10% ($n = 4$). The lower doses of Nif tested (20 μM) stimulated the Ca-ith causing a 'negative inhibition' (see text for a possible explanation).

the ouabain-induced reduction in the Ca-ith, since in low $[K^+]_e$ experiments, where E_r is more hyperpolarized, Ca-ith amplitude is normal (not shown).

Niflumic acid, a Cl^- channel blocker [21], required a 6-min incubation to fully cause its effect (~40% inhibition of the Ca-ith), at concentrations commonly used in other cells (<200 μM) [22]. Higher concentrations (>350 μM) inhibited up to 73% (Fig. 3D, Table 1) however, they themselves hyperpolarized. This indicates that niflumic acid may be acting elsewhere besides the putative Cl^- channel (i.e. K^+_{Ca} channels activated by fenamates [22]). On the other hand, 20 μM of this compound potentiated the Ca-ith (37%), as has been observed under certain conditions for GABA-induced Cl^- currents from a rat brain GABA-receptor expressed in *Xenopus* oocytes [23].

3.3. Ca-induced depolarization (Ca-id)

Addition of Ca^{2+} to sperm suspended in a Ca-free low-Na media (~10 mM) did not result in a hyperpolarization, but in a sustained depolarization (Ca-id). Mg^{2+} was omitted from the assay in some experiments because, at the concentrations normally present in Ca-free media (1.2 mM), it blocked >50% of the depolarization (Fig. 4B). Residual Mg^{2+} contributed by the cell aliquot stored in Ca-free media was ~30 μM. As in the case

Table 1
Effect of ouabain or niflumic acid on E_r and the Ca-ith

Ouabain (μM)	n	E_r (mV)	Ca-ith (mV)	inhibition %
0	9	−43 ± 12	−39 ± 13	0
1.5	8	−39 ± 10	−32 ± 9	20
15	8	−46 ± 5	−13 ± 2	67
150	8	−54 ± 6	−10 ± 4	74
Niflumic Ac. (μM)				
0	15	−35 ± 12	−29 ± 10	0
20	4	−30 ± 10	−40 ± 10	−37
200	12	−34 ± 8	−18 ± 6	38
350	10	−49 ± 6	−8 ± 3	73
500	5	−54 ± 11	−9 ± 2	70

The negative inhibition means that at 20 μM niflumic acid a potentiation of Ca-ith is seen (for details, see Section 3). All experiments were done in HMB Ca-free media.

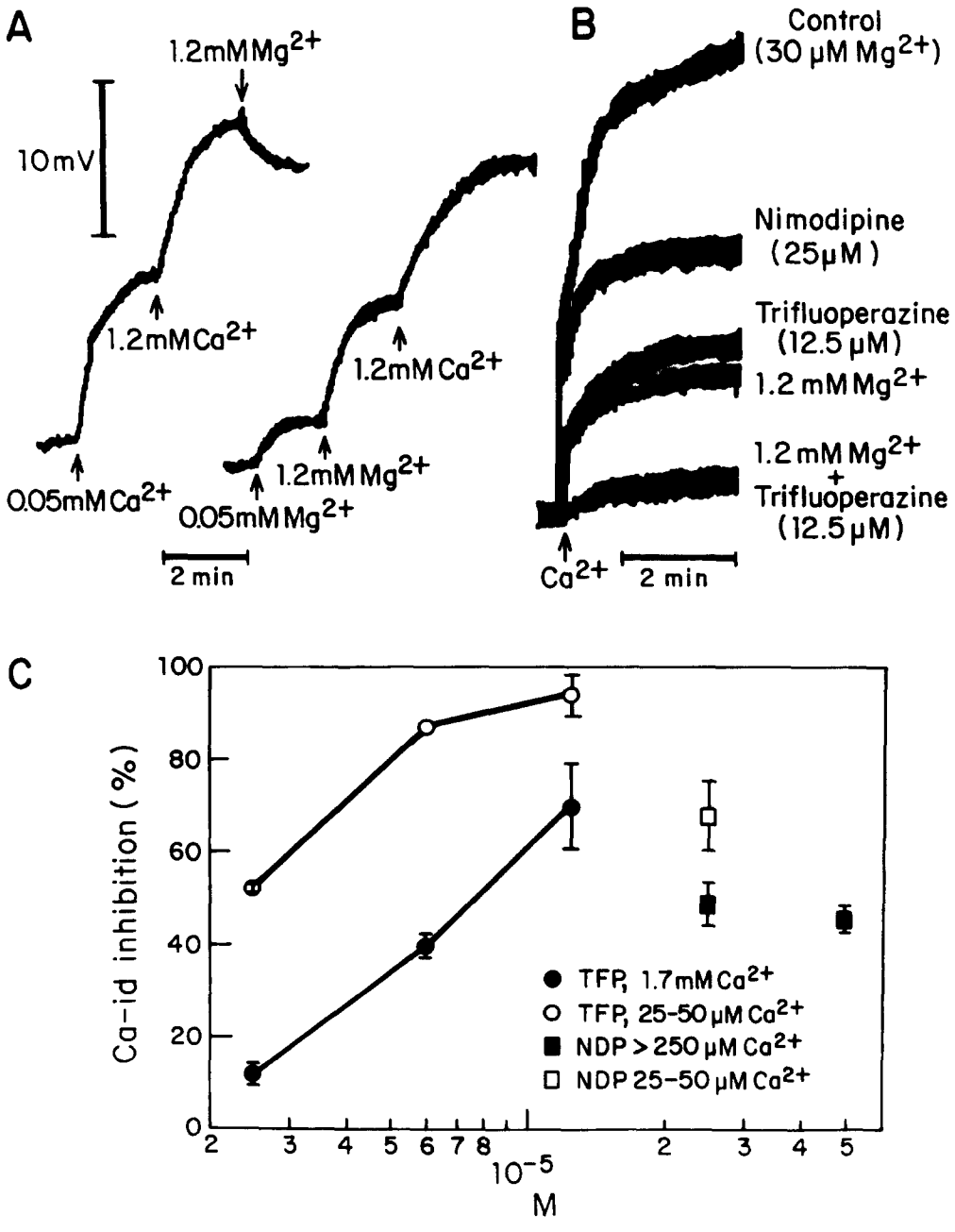

Fig. 4. Ca-id and its blockers. Ca-id experiments were done in low-Na^+ media (<10 mM Na^+, 140 mM choline$^+$, 6 mM K^+, 30 μM Mg^{2+}, 0 Ca^{2+} and 150 mM Cl^-). (A) E_m depolarization induced by 0.05 and 1.2 mM Ca^{2+} or Mg^{2+}. Residual Mg^{2+} in these experiments was quelated with 12–25 μM EDTA. Addition of Mg^{2+} blocked the Ca-id (first and third experiments) and by itself induced a smaller depolarization than Ca^{2+}. The Ca-id remained even after adding 1.25 mM Mg^{2+}. (B) TFP (12.5 μM), NDP (25 μM) and Mg^{2+} (1.2 mM) blocked the Ca-id (1.7 mM Ca^{2+}). Time scales are under the traces. (C) TFP and NDP dose-dependent inhibition of the Ca-id caused by low (<50 μM) or high (>250 μM) Ca^{2+} concentrations.

of the hyperpolarization in normal Na^+ media, the Ca-id was dependent on the amount of Ca^{2+} added, displaying a similar saturation and IC_{50} (not shown). Fig. 4A illustrates the depolarization induced by Ca^{2+} at 0.05 and 1.2 mM Ca^{2+} (left trace). The magnitude of maximal depolarization was 21.4 ± 3.2 mV (n = 6) and half-maximal depolarization was reached at ~50 μM Ca^{2+}. The trace on the right of Fig. 4A shows how Mg^{2+} inhibited the depolarization. In 0Ca-0Mg media, Mg^{2+} itself depolarized but with lower potency than Ca^{2+} (right trace). Thus, Fig. 4A suggests that both divalent ions permeate through the same channel, Ca^{2+} passes more efficiently and Mg^{2+} blocks. This is consistent with the properties of other Ca^{2+} channels [14,25].

The Ca-id was blocked with TFP and NDP (Fig. 4B,C). TFP (12.5 μM) blocked 70 ± 9.2% (n = 6) of Ca-id when 1.7 mM Ca^{2+} was added and 94 ± 4.5% (n = 5) with 0.05 mM Ca^{2+} additions. NDP (>25 μM) blocked the Ca-id 48 ± 3.8% (n = 7) for Ca^{2+} additions >0.25 mM and 68 ± 7.5% (n = 4) for 0.05 mM Ca^{2+} additions. This indicates that Ca^{2+} may be permeating through an L-type Ca^{2+} channel (see Section 4). Other blockers tested showed no apparent effect at concentrations higher than

those reported to block their respective channels (2.5 μM ω-conotoxin for Ca^{2+} channels; 70 nM TiTxγ toxin and 6 μM TTX for Na^+ channels; 10 mM TEA^+, 50 nM β-dendrotoxin and 20 μM charybdotoxin for K^+ channels).

4. Discussion

The resting membrane potential of mouse spermatozoa in HMB media, estimated with a membrane potential sensitive cyanine dye, was -42 ± 8.8 mV. This value is close to the one determined in bull spermatozoa under similar conditions [9]. This potential was sensitive to extracellular K^+ and not to Cl^-. In Ca-free media (~nM $[Ca^{2+}]_e$), ion substitution experiments indicated that mouse sperm have a substantial permeability to Na^+. E_r hyperpolarized in this media as $[Na^+]_e$ was lowered from 110 to 10 mM (Fig. 1B), but not in the presence of normal Ca^{2+} concentrations.

Addition of Ca^{2+} (> 5 μM) to sperm in Ca-free media induced a transient hyperpolarization. This Ca-ith strongly depended on $[Na^+]_e$. When $[Na^+]_e$ was replaced by a non-permeant monovalent cation, the Ca-ith decreased and completely disappeared at around 40 mM $[Na^+]_e$. These results suggest that in the absence of Ca^{2+}, Na^+ can permeate through Ca^{2+} channels, as is the case in other systems [14,25]. On the other hand, ouabain, a specific inhibitor of the Na-K ATPase, inhibited 70% of the Ca-ith, indicating the participation of this pump in the response. In red blood cells, squid axon [26] and in skeletal myoballs [27], the Na-K ATPase contribution to the resting membrane potential were calculated to be −0.3, 5 and −5.7 mV, respectively. However, under non-stationary conditions, the contribution of this pump to E_m could be much larger [28]. Ouabain (mM) has been shown to inhibit the AR in mouse spermatozoa [13]. Amiloride (10–250 μM), a poorly specific Na-Ca exchanger inhibitor [29], failed to affect Ca-ith hinting it does not contribute to this response.

Replacing $[Cl^-]_e$ for a non-permeant anion decreased the Ca-ith; and niflumic acid, a blocker of some Cl^- channels, significantly inhibited this response. This result suggests the participation of a Ca^{2+}-modulated Cl^- channel, which would open as Ca^{2+} enters the cell [21]. However, > 50% of the Ca-ith remains in low $[Cl^-]_e$, arguing, that other ion permeabilities are involved in this response. In the human sperm AR, a progesterone potentiated GABA-Cl channel has been implicated in the AR induced by this hormone; in mouse sperm GABA and progesterone can act sinergistically to induce the AR [30]. Interestingly, the rat brain GABA receptor expressed in *Xenopus* oocytes is blocked (60%) by niflumic acid [23]. Although there is no evidence that $[Ca^{2+}]_i$ may modulate the sperm GABA receptor as for other systems [31], it is worth considering its possible participation in the Ca-ith.

Ca^{2+} influx is essential for the sperm AR, a crucial event in fertilization in mammals and in many other species [2,15]. Dihydropyridines, Co^{2+} and Ni^{2+}, known blockers of voltage-dependent Ca^{2+} channels, inhibit the AR of ram, bull and mouse sperm, suggesting their participation in this reaction [9]. Ca^{2+} additions in low Na^+ media resulted in a sperm depolarization (Fig. 4). This Ca-id was sensitive to relatively high NDP concentrations (25 μM) that block L-type [32] or even T-type Ca^{2+} channels [33], consistent with the presence of voltage-dependent Ca^{2+} channels in mammalian sperm [9]. T-type Ca^{2+} channels have been described in spermatogenic cells [34].

TFP (12.5 μM), which can block Ca^{2+} channels directly [35] and inhibits the AR in sea urchin sperm [36, 37], also inhibited the Ca-id. This compound, a known calmodulin inhibitor [38], may be blocking the Ca-id in mouse sperm acting directly on Ca^{2+} channels and/or via calmodulin. On the other hand, toxins that block Na^+ channels (TTX and TiTxγ) or K^+ channels (TEA^+, apamin, charibdotoxin and β-dendrotoxin) did not inhibit neither the Ca-ith nor the Ca-id.

Taken altogether, these observations can be considered in the following working scheme to explain the Ca-ith. Na^+ can permeate through Ca^{2+} channels in the absence of external Ca^{2+} increasing $[Na^+]_i$ and stimulating the Na-K ATPase [26]. Addition of Ca^{2+} blocks Na^+ influx through the channel and increases the influence of the Na-K ATPase on E_m, both effects would hyperpolarize sperm. As Ca^{2+} enters the cell through the channel (and/or other membrane transporters), it activates a Ca^{2+}-modulated Cl^- channel, which accounts for part of the Ca-ith explaining its sensitivity to niflumic acid. Mn^{2+} and Cd^{2+} and at higher concentrations Ni^{2+}, Zn^{2+} and Co^{2+}, mimic the Ca-ith, they can apparently block Na^+ uptake through the channel and compete for the Ca^{2+}-binding site(s), since they inhibit the response induced by a subsequent addition of Ca^{2+}. In contrast, Ba^{2+} which permeates well through Ca^{2+} channels and has less affinity for channel Ca^{2+}-binding sites [39], at mM concentrations did not induce a hyperpolarization but a depolarization. Since certain inhibitors used here to block the Ca-ith and the Ca-id also block the AR, some of the transport systems involved in these mouse sperm responses to Ca^{2+} may participate in the AR

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