Mouse sperm membrane potential hyperpolarization is necessary and sufficient to prepare sperm for the acrosome reaction.

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Running Title: *Hyperpolarization and acrosome reaction*

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Background: Sperm capacitation, a process associated with phosphorylation and membrane potential changes, is required for acrosome reaction and fertilization.
Results: Inducing hyperpolarization in non-capacitated sperm does not result in protein tyrosine phosphorylation but allows physiologically-induced $[Ca^{2+}]_i$ increases and acrosome reaction.
Conclusion: Sperm hyperpolarization appears to be necessary and sufficient for acrosome reaction.
Significance: Advancing our understanding of capacitation, the acrosome reaction and fertilization.

SUMMARY

Mammalian sperm are unable to fertilize the egg immediately after ejaculation; they acquire this capacity during migration in the female reproductive tract. This maturational process is called capacitation and in mouse sperm it involves a plasma membrane reorganization, extensive changes in the state of protein phosphorylation, increases in intracellular pH (pH_i) and Ca^{2+} ($[Ca^{2+}]_i$), and the appearance of hyperactivated motility. In addition, mouse sperm capacitation is associated with the hyperpolarization of the cell membrane potential. However, the functional role of this process is not known. In this work, to dissect the role of this membrane potential change, hyperpolarization was induced in non-capacitated sperm using either the ENaC inhibitor amiloride, the CFTR agonist genistein or the K^+ ionophore valinomycin. In this experimental setting, other capacitation-associated processes such as activation of a cAMP-dependent pathway and the consequent increase in protein tyrosine phosphorylation were not observed. However, hyperpolarization was sufficient to prepare sperm for the acrosome reaction induced either by depolarization with high K^+ or by addition of solubilized *zona pellucidae* (sZP). Moreover, K^+ and sZP were also able to increase $[Ca^{2+}]_i$ in non-capacitated sperm treated with these hyperpolarizing agents but not in untreated cells. On the other hand, in conditions that support capacitation-associated processes blocking hyperpolarization by adding valinomycin and increasing K^+ concentrations inhibited the agonist-induced acrosome reaction as well as the increase in $[Ca^{2+}]_i$. Altogether, these results suggest that sperm hyperpolarization by itself is key to enabling mice sperm to undergo the acrosome reaction.

In the female tract, mammalian sperm undergo capacitation, a functional maturational process that prepares them for fertilization. Although the acquisition of fertilizing capacity is still considered the endpoint of capacitation, various

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indications suggest that the physiological changes occurring to the sperm during capacitation are not a single event, but a series of sequential and concomitant processes (1). Capacitation is correlated with functional changes in sperm parameters such as the activation of sperm motility known as hyperactivation (2) and the preparation to undergo a physiologically induced acrosome reaction (3). Taking into consideration the highly differentiated and compartmentalized nature of sperm, it can be postulated that the molecular basis of capacitation should account for independent changes occurring in different sperm compartments such as the flagellum (e.g. hyperactivation) and the head (e.g. preparation for the acrosome reaction) (3). Biochemically, capacitation is associated with: 1) changes in the sperm plasma membrane (e.g. decrease in cholesterol, asymmetry of phospholipids) (4-6), 2) activation of protein phosphorylation (e.g. increase in PKA activity; increase in tyrosine phosphorylation) (4,5); and 3) changes in ion homeostasis (e.g. increase in pHi and K^+ permeability, elevation of $[Ca^{2+}]i$, and decrease in the intracellular concentration of Na^+ ($[Na^+]i$) demonstrated in mouse sperm (6,7), that lead to a sperm plasma membrane potential (Em) hyperpolarization in mouse, rabbit, bovine and horse (6-9). The consequences of these molecular changes are not well understood. However, it has been shown in multiple species that activation of PKA is needed for hyperactivation and to prepare the sperm for the acrosome reaction (3). On the other hand, the role of the capacitation-associated Em hyperpolarization has been hypothesized to be related with the ability of sperm to generate a transient Ca^{2+} elevation during the acrosome reaction induced by physiological agonists (e.g. solubilized zona pellucida (sZP)) (10-12).

This hypothesis was consistent with measurements of voltage dependent Ca^{2+} channels (Ca_Vs) activated by low voltages (T or Ca_V3) in spermatogenic cells and in testicular sperm (11,13-15). However, this possibility has been recently challenged by two main observations: 1) $Ca_V3.1$ and 3.2 mice knock out genetic models are fertile (16); and 2) patch clamp measurements in epididymal sperm failed to detect Ca_Vs (17,18). These findings suggest that these channels are not essential for fertilization. Nevertheless, these reports are silent in relation to the role of the capacitation-associated Em hyperpolarization. Regarding this process, mice genetic models lacking the sperm-specific K^+ channel SLO3 are basically sterile (19,20). Sperm from these mice do not undergo Em hyperpolarization during capacitation. In addition, these sperm are not able to acrosome react even when exposed to the Ca^{2+} ionophore A23187. Interestingly, if these sperm are hyperpolarized with the K^+ ionophore valinomycin, they become responsive to A23187 (19). These observations suggest that hyperpolarization is necessary for the acrosome reaction.

In the present work, we evaluated the hypothesis that hyperpolarization is necessary and sufficient for sperm to undergo $[Ca^{2+}]_i$ increases and the acrosome reaction in response to sZP. To test this hypothesis, Em hyperpolarization was induced with either valinomycin, amiloride (7) or genistein (21) in sperm-incubated under conditions that do not support capacitation (absence of HCO_3^- and BSA). Under these conditions, the cAMP/PKA pathway and the consequent increase in tyrosine phosphorylation did not occur. However, sperm underwent hyperpolarization and acquired the ability to acrosome react in response to a K^+ induced depolarization. Moreover, once hyperpolarized, addition of sZP induced an increase in $[Ca^{2+}]_i$ and the acrosome reaction, changes that were not observed when the hyperpolarizing agents were omitted.

On the other hand, to test whether hyperpolarization was needed for the preparation to undergo the ZP-induced acrosome reaction, sperm were incubated under conditions that support capacitation but in the presence of valinomycin and increasing concentrations of K^+ to block the capacitation-associated hyperpolarization. Under these conditions, sperm underwent activation of PKA and tyrosine phosphorylation. However, neither the ZP-induced acrosome reaction nor the increase in $[Ca^{2+}]_i$ were observed. Our findings are consistent with the proposal that sperm hyperpolarization by itself is key to enabling mice sperm to undergo the acrosome reaction.

MATERIALS AND METHODS

Materials. Amiloride, genistein, carbonyl cyanide m-chlorophenylhydrazone (CCCP) and valinomycin were purchased from Sigma (St Louis, MO). Fluo-4AM, BCECFAM, 3'-dipropylthiadicarbocyanine iodide ($DiSC_3(5)$) and natural mouse laminin were purchased from Life Technologies (Invitrogen) (Grand Island, NY). Anti-phosphotyrosine (pY) monoclonal antibody (clone 4G10) was from Upstate Biotechnology (Lake Placid, NY) and rabbit monoclonal anti-phosphoPKA substrates (clone 100G7E) was purchased from Cell Signaling (Danvers, MA). Stock solutions (1 mM final) of $DiSC_3(5)$, CCCP, amiloride, genistein and valinomycin were prepared in dimethyl sulfoxide (DMSO) and stored at –20 ℃ except when otherwise stated.

Sperm Preparation. Experimental protocols were approved by the University of Massachusetts and by the Instituto de Biotecnología/UNAM Animal Care Committees. In all the experiments, cauda epididymal mouse sperm were collected from CD1 retired male breeders by placing minced cauda epididymis in a modified Krebs-Ringer medium (Whitten's-HEPES-buffered (WH) medium) (22). This medium, which does not support capacitation, was prepared without bovine serum albumin (BSA) and $NaHCO_3$. After 5 min, sperm in suspension were washed in 10 ml of the same medium by centrifugation at 800 x g for 10 min at room temperature (RT) (24°C). Sperm were then resuspended to a final concentration of 2 X 10^7 cells/ml and diluted ten times in the appropriate medium depending on the experiment performed. In experiments where capacitation was investigated, 5 mg/ml of BSA and 24 mM of $NaHCO_3$ were added. The pH was maintained at 7.4.

Membrane Potential Assay in Sperm Populations. Em was measured as previously described (23). Briefly, sperm were collected as indicated above and diluted in media supporting or not capacitation in the absence or in the presence of amiloride (1 µM), genistein (10 µM) or valinomycin (1 µM), as indicated in the Results section. Eight min before measurement, 1 µM $DiSC_3(5)$ (final concentration) was added to the sperm suspension and further incubated

for 5 min at 37°C. CCCP (0.5 µM final) was then added to collapse mitochondrial potential and sperm incubated for 2 additional min. After this period, 0.8 ml of the suspension was transferred to a gently stirred cuvette at 37°C and the fluorescence monitored with a Hansatech MkII fluorometer (Norfolk, UK) at a 620/670-nm excitation/emission wavelength pair. Calibration was performed as described before (23) by adding 1 µM valinomycin (except in the experiments already containing this antibiotic) and sequential additions of KCl (24). The equilibrium potential for K^+ (E_k) was calculated with the Nernst equation considering intracellular mouse sperm K^+ is 120 mM (23).

SDS-PAGE and Immunoblotting. After treatments, sperm were collected by centrifugation, washed in 1 ml of PBS, resuspended in Laemmli sample buffer without β-mercaptoethanol, and boiled for 5 min. After centrifugation, 5% β-mercaptoethanol was added to the supernatants and boiled again for 5 min. Protein extracts equivalent to 1-2 $\times$ 10^6 sperm per lane were subjected to SDS-PAGE and electro-transferred to PVDF membranes (Bio-Rad) at 250 mA for 60 min at 0°C. Membranes were blocked with 2% fish skin gelatin (Sigma) in TBS containing 0.1% Tween-20 (T-TBS). Antibodies dilution in T-TBS were as follows: 1/10,000 for anti-PY (clone 4G10) and 1/5,000 for anti-pPKAs (clone 100G7E). Secondary antibodies were used at 1/10,000 in T-TBS. Films were developed using an enhanced chemiluminescence detection kit (Amersham Corp.) according to the manufacturer's instructions. If needed, PVDF membranes were stripped at 60°C for 15 min in 2% SDS, 0.74% µ-mercaptoethanol, 62.5 mM Tris, pH 6.5, and washed 6 x 5 min in T-TBS. In every case, molecular masses were expressed in kDa.

Assay for Acrosome Reaction. Zona pellucidae were prepared from homogenized ovaries of virgin female 60-day-old outbred CD1 mice (Charles River Laboratories) as described (24,25) and solubilized for all experiments by the procedures outlined previously (4). The percentage of acrosome reaction was measured using Coomassie Blue G-250 staining as described (26). Briefly, sperm were incubated at 37°C for 45 min under the conditions mentioned

for each experiment followed by the addition of 5 zona pellucidae equivalents/ul. After an additional 30 min of incubation at 37°C, fixative solution (5% final concentration of formaldehyde in phosphate-buffered saline) was added to each tube. Following fixation, 10 µl aliquots of suspension were spread onto glass slides and air-dried. The slides were then stained with 0.22% Coomassie Blue G-250 in 50% methanol and 10% glacial acetic acid for 3–5 min, gently rinsed with deionized H_2O, air-dried, and mounted with 50% (v/v) glycerol in phosphate-buffered saline. To calculate the percentage of acrosome reaction, at least 100 sperm were assayed per experimental condition for the presence or absence of the characteristic dark blue acrosomal crescent. The percentage of acrosome-reacted spermatozoa was calculated for each experimental condition dividing the number of acrosome-reacted spermatozoa by the total number of spermatozoa scored (sum of acrosome-reacted and non acrosome-reacted) and multiplying this ratio by 100.

Intracellular Ca^{2+} imaging. Epididymal motile mice sperm were collected by swim-up in WH medium at 37°C for 15 min. The motile cells were incubated with 2 µM Fluo-4 AM and 0.05% pluronic acid in WH medium supplemented or not with BSA and $NaHCO_3$ according to experimental capacitating conditions. Once loaded, sperm were immobilized on mouse laminin (100 mg/ml) coated cover slips to allow recordings. Sperm were incubated with WH medium alone for control experiments or with amiloride (1 µM), genistein (10 µM) or valinomycin (1 µM) during 15-30 minutes. Ca^{2+} imaging in a system which consisted of a Nikon Diaphot 300 inverted microscope with a Plan Apo 60X/1.40 oil Nikon objective was performed before, during and after K^+ or sZP stimulation; as a vitality control 10 µM ionomicyn was added at the end of each experiment. Fluo-4 loaded sperm were excited with a stroboscopic LED-based fluorescence illumination system as previously described (Nishigaki et al 2004), with 4 ms light excitation pulses. Fluorescence was captured with a Cool Snap (Photometrics USA) at 0.5 Hz or 1 Hz. Image sequences (movies) were obtained from a given field of view and saved in tif format. Movies were processed and analysed with macros written -in Image J (Version 1.38, National Institutes of Health). Regions of interest (ROIs) were drawn on each sperm and then transferred to the movie for quantification. A plot was generated in Origin 6.0 (Microcal Software, Northampton, Mass, USA). Fluorescence is expressed as F-F0/F0.

RESULTS

1. Sperm Em hyperpolarization is not sufficient to induce the capacitation-associated increase in phosphorylation. To examine the role of sperm Em independently of other capacitation-associated events, three compounds known to hyperpolarize sperm in the absence of capacitating conditions (i.e. capacitating media minus BSA and HCO_3^-) were tested. Our group has shown previously that both amiloride (7) and genistein (21) induce Em hyperpolarization in sperm incubated under non capacitating conditions. It is also well established that valinomycin hyperpolarizes sperm to the K^+ equilibrium potential (E_K) (23,27). These results were confirmed in figure 1A. However, the consequences of these additions in other capacitation-associated parameters such as the increase in phosphorylation of PKA substrates and the increase in tyrosine phosphorylation have not been evaluated. To test whether hyperpolarization is sufficient to induce these phosphorylation events, sperm were incubated in the absence of HCO_3^- and BSA for 1.5 hours and in the presence of increasing concentrations of amiloride (Fig. 1B), genistein (Fig. 1C) or valinomycin (Fig. 1D). Western blots using anti phosphorylated-PKA substrates (anti pPKAs) and anti phosphotyrosine (anti PY) antibodies indicate that these compounds are not able to induce capacitation-associated changes in phosphorylation. Altogether, these results point out that these conditions can be used to investigate the role of Em hyperpolarization independently of other capacitation-associated events.

2. Addition of K^+ increased $[Ca^{2+}]_i$ and the percentage of acrosome reaction only in hyperpolarized sperm populations. To examine the role of the hyperpolarization we asked if this membrane potential change was able to prepare sperm to respond to a K^+ induced depolarization. Addition of high K^+ concentrations (50-100

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mM) to capacitated mouse sperm has been shown to induce the acrosome reaction (11,28). Ca^{2+} changes were investigated using single cell analysis in sperm incubated in conditions that support or not capacitation or hyperpolarized with amiloride, genistein or valinomycin.

Addition of 50 mM K^+ to capacitated sperm increased $[Ca^{2+}]_i$ (Fig. 2A & B) and the percentage of acrosome reaction (Fig. 2C). On the other hand, the same K^+ addition did not induce the acrosome reaction in non capacitated sperm (Fig. 2C). Interestingly, K^+ was able to increase the percentage of acrosome reacted sperm in non capacitated sperm if these sperm were previously incubated in the presence of either amiloride, genistein or valinomycin (Fig. 2C). These results suggest that only hyperpolarized sperm are capable of responding to a K^+ depolarization.

The K^+-induced $[Ca^{2+}]_i$ increase was not observed in Fluo-4 loaded sperm that had not been hyperpolarized (Fig. 3A). In contrast, pharmacologically hyperpolarized sperm underwent an increase in $[Ca^{2+}]_i$ when depolarized by a K^+ addition (Fig. 3B, C and D and supplemental movies 1-5). Similar to the case of capacitated sperm, as described before, the sperm population is heterogeneous and not all cells respond in the same manner (10). Analysis of the data revealed that 25 % or more of the cells subjected to the various hyperpolarizing conditions underwent $[Ca^{2+}]_i$ elevations (Fig. 3E). Furthermore, the responding cells increased their $[Ca^{2+}]_i$ when depolarized by K^+ by at least 40% in all hyperpolarizing conditions (Fig. 3F).

3. Sperm plasma membrane hyperpolarization allows sZP-induced increases in $[Ca^{2+}]_i$ and acrosome reaction. Results in the previous section suggested that hyperpolarization is sufficient to prepare sperm for a depolarization-induced increases in $[Ca^{2+}]_i$ and acrosome reaction. The question arises whether hyperpolarization is also capable of preparing non capacitated sperm to respond to a more physiological agonist such as the sZP. To this end acrosome reaction and $[Ca^{2+}]_i$ were measured in non capacitated sperm that had been incubated in the same conditions as in figures 2 and 3, but exposed to sZP instead of KCl. Sperm incubated in non capacitating conditions

increased neither the percentage of acrosome reaction (Fig. 4) nor $[Ca^{2+}]_i$ (Fig. 5A and supplemental movie 6). On the other hand, both capacitated and pharmacologically hyperpolarized sperm were able to respond to sZP by promoting their acrosome reaction (Fig. 4) and increasing their $[Ca^{2+}]_i$ (Fig. 5 B-E and supplemental films 7-9). Around 30 % or more of capacitated or pharmacologically hyperpolarized sperm responded to sZP with a $[Ca^{2+}]_i$ increase (Fig. 5 F, supplemental movie 10) that ranged from ~25% in genistein treated non capacitated sperm to >50% in capacitated sperm exposed to sZP (Fig. 5G).

4. Hyperpolarization is necessary to prepare the sperm for the sZP-induced acrosome reaction. Results in previous sections indicate that Em hyperpolarization in the absence of other capacitation-associated events such as the increase in the cAMP/PKA-induced tyrosine phosphorylation is sufficient to prepare sperm for an agonist-induced acrosome reaction. However, these experiments are silent with respect to the need of hyperpolarization for the agonist-induced acrosome reaction. If this is the case, not allowing the sperm Em to hyperpolarize should block the preparation for the acrosome reaction. To investigate this hypothesis, sperm were incubated in complete capacitation medium in the presence of 1 μM valinomycin and different external K^+ concentrations, and Em was evaluated as before (Fig. 6A). Noticeably, the capacitation-associated increase in phosphorylation of PKA substrates or tyrosine residues was not blocked even at external K^+ concentrations as high as 70 mM (Fig. 6 B). In contrast, sperm incubated in capacitating conditions but exposed to 70 mM external K^+ and valinomycin, which are at ~-10 mV, did not undergo an increase in either $[Ca^{2+}]_i$ (Fig. 6C) or the acrosome reaction (Fig. 6D) when challenged with sZP (see supplemental movie 11).

DISCUSSION

Although Em hyperpolarization during capacitation in sperm from mouse and other species has been well documented, little is known regarding its physiological role (3). One of the functions of capacitation is to prepare sperm for an agonist-induced acrosome reaction.

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To separate the changes in Em from other aspects of capacitation such as the activation of phosphorylation, in this work, hyperpolarization was induced in non capacitated sperm (*i.e.* in the absence of HCO_3^- and BSA) using three different compounds: amiloride, genistein and valinomycin. In these conditions, sperm did not undergo other capacitation-associated events such as the increase in PKA activity or the consequent increase in protein tyrosine phosphorylation. However, these apparently non capacitated but hyperpolarized sperm were able to undergo a $[Ca^{2+}]_i$ rise and the acrosome reaction when challenged with either high K^+ or sZP. Though these three compounds cause hyperpolarization, they do it through different mechanisms. Genistein, directly activates CFTR but it is also a more promiscuous agent than valinomycin or amiloride. Genistein at higher concentrations is able to inhibit tyrosine kinases (29) which can also regulate voltage dependent Ca^{2+} channels (Cavs) (30,31). Considering that in addition to its effect on hyperpolarization, genistein can have other possible targets it is not unexpected it has some differential effects when compared with valinomycin and amiloride.

Cauda epididymal mouse sperm have a resting Em of around -40 mV. Ion substitution experiments revealed that this relatively depolarized Em is mainly due to a combination of K^+ and Na^+ electrogenic permeabilities (8,23,32-34). During capacitation, the sperm resting Em becomes hyperpolarized due to an increase of K^+ conductance and a reduction of Na^+ conductance (7,19,20,35). However, how changes in Em are regulated during capacitation is not well understood. Two complementary processes might explain this phenomenon: the first involves activation of a cAMP/PKA pathway that induces the opening of CFTR channels (36,37). In sperm, similar to other systems, activation of CFTR either by a physical interaction or through Cl^- movement, inhibits ENaCs with the consequent reduction in Na^+ permeability and hyperpolarization (6,21). Consistent with this hypothesis, ENaC and CFTR channels are present in sperm and hyperpolarization can be induced with the ENaC blocker amiloride, and with the CFTR activator genistein (7,21,37-39). In addition, we have recently shown that there is a cAMP/PKA-dependent intracellular Na^+ reduction during sperm capacitation (6). The second process encompasses well documented changes in pHi during capacitation that might activate K^+ rectifier channels (33,40) and Slo3 (19,20) with the consequent hyperpolarization of the sperm Em. This hypothesis is supported by the phenotype of the Slo3 KO whose sperm do not hyperpolarize during capacitation (19). There is a complex but crucial interplay between Em and pHi in mammalian sperm. It is worth noting that CFTR's conductance is also pHi dependent (41). Therefore, it is also possible that the regulation of other transporters like ENaCs by CFTR activation is also pHi dependent.

Two lines of evidence suggest that sperm must be sufficiently hyperpolarized to undergo the physiologically relevant acrosome reaction. First, as mentioned earlier, sperm from Slo3 null mice do not hyperpolarize during capacitation; in addition, these sperm fail to undergo the acrosome reaction when challenged with A23187. Interestingly, when these sperm are hyperpolarized with valinomycin they become responsive to A23187 (19). Second, in the present work, when hyperpolarization was blocked by incubating sperm in media that support capacitation but supplemented with valinomycin and 70 mM K^+, activation of PKA as well as the consequent increase in protein tyrosine phosphorylation were not impaired. However, sperm incubated in high K^+ and valinomycin did not undergo an increase in $[Ca^{2+}]_i$ or the acrosome reaction when exposed to sZP. It is worth noting that Xia and Ren (42) reported that adding valinomycin and 58 mM KCl after 65 min of capacitation does not inhibit the $[Ca^{2+}]_i$ response induced by sZP. In these conditions, the capacitation-associated hyperpolarization has already taken place and these findings indicate that the changes caused by the hyperpolarization are not reverted by a posterior depolarization. Using a similar experimental setting, we have observed that addition of valinomycin and 58 mM KCl after capacitation is sufficient to induce the acrosome reaction (~22 % increase over spontaneous acrosome reaction, n=4, not shown). Similarly to the Xia and Ren (42) report, in sperm treated with high K^+ and valinomycin after capacitation, a $[Ca^{2+}]_i$ increase was observed in 25 % of the

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sperm population when challenged with sZP (not shown).

Our observations reveal a connection between hyperpolarization and the preparation for the acrosome reaction. However, the experiments in this manuscript do not rule out the possibility that the induction of the acrosome reaction requires other steps in addition to the hyperpolarization. In this respect, the work by Mayorga's group has revealed intermediate stages in which vesicles are visualized attached to the sperm plasma membrane (43). Recently Rab3A, RIM and Munc13 located in the human sperm acrosomal region have been shown to participate in a pre-fusion step before Ca^{2+} efflux from the acrosome. RIM and Rab3A are involved in docking of the acrosomal membrane to the plasma membrane during the Ca^{2+} induced acrosome reaction (44,45). In mouse sperm, hyperpolarization could for instance facilitate vesicle attachment.

Although it has been proposed that hyperpolarization of the sperm Em drives Ca_V3 channels from an inactive state to a closed state that can be activated by agonists, this hypothesis has not been fully demonstrated. Varied evidence suggests that Ca_V3 channels are present in sperm: 1) Patch clamp measurements demonstrated the presence of Ca_V3 channels in their precursor spermatogenic cells (34,46); 2) whole cell patch clamp recordings at the cytoplasmic droplet documented Ca_V3 channel currents in testicular sperm (15); 3) genetic approaches using mice in which two of the three Ca_V3 isoforms, 1 & 2, were independently eliminated by homologous recombination indicated that $Ca_V3.2$ appears to be the main contributor to the Ca_V currents observed in spermatogenic cells; 4) the presence of the $Ca_V3.2$ alpha subunit in the postacrosomal region of mouse sperm (47) was verified by immunofluorescence using $Ca_V3.2$ null sperm as a negative control (16) and 5) sperm from $Ca_V3.2$ null mice displayed a diminished increase in $[Ca^{2+}]_i$ induced by a K^+ depolarization. Although these data suggest the presence of functional $Ca_V3.2$ in mouse sperm (16), patch clamp experiments using corpus epididymal sperm instead of testicular sperm did not resolve Ca_V3 or other Ca_V currents (42). As $Ca_V3.2$ mice are fertile and their sperm are able

to generate a sZP-induced $[Ca^{2+}]_i$ increase, other Ca_Vs (16) or Ca^{2+} transporters may participate in this fundamental process. In this regard, several Ca_Vs of the high-voltage-activated (HVA) family have been found in spermatogenic cells and in mature mouse and human sperm, such as $Ca_V1.2$, $Ca_V2.1$, and $Ca_V2.3$ (reviewed in (11)).

As an alternative to the Ca_V3 channel hypothesis, Xia and Ren (48) have recently proposed that CatSper participates in the sZP-induced increase in $[Ca^{2+}]_i$. Consistent with this hypothesis, CatSper null sperm loose the initial $[Ca^{2+}]_i$ response to sZP. Interestingly, they retain a delayed $[Ca^{2+}]_i$ rise and they undergo the acrosome reaction upon sZP stimulation. The sZP induced increase in $[Ca^{2+}]_i$ related to CatSper appears to originate in the sperm principal piece where this channel is localized (48). These data indicate that other channels, ion-transporters and/or the regulation of intracellular Ca^{2+} stores can mediate the sZP-induced $[Ca^{2+}]_i$ increase (48).

The aforementioned experiments were done in conditions that support capacitation and the capacitation-associated hyperpolarization of the sperm Em, and thus, are silent regarding the sole role of the capacitation-associated hyperpolarization. In the present manuscript, to investigate the necessity of hyperpolarization for the sZP response, cauda epididymal sperm were incubated in high K^+ and valinomycin during the whole capacitation period to block the capacitation-associated hyperpolarization without inhibiting other capacitation-associated pathways (e.g. PKA activation and the increase in protein tyrosine phosphorylation). Using this experimental design, sZP was unable to induce a $[Ca^{2+}]_i$ increase or the acrosome reaction.

As mentioned in the introduction, capacitation induces changes in signaling pathways both in the sperm tail and in the head. While changes in the flagellum are usually thought to be involved in the regulation of sperm motility, most of the preparation for the acrosome reaction is believed to take part in the sperm head. In this respect, several lines of evidence suggest that regulation of the sperm Em during capacitation occurs mainly in the flagella. First, functional and/or immunofluorescence experiments suggest that Slo3 (20,49), ENaC (7) and CFTR (21,50), three channels postulated to mediate the capacitation-

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associated hyperpolarization, are present in the sperm flagellum. Second, sustained hyperpolarization appears to be downstream of the activation of the HCO_3^-/SACY/PKA pathway. Both SACY (51) and the catalytic subunit of PKA (Wertheimer and Visconti, unpublished) are only present in the sperm flagellum. Finally, the aforementioned work by Xia and Ren (48) suggests that the sZP-induced $[Ca^{2+}]_i$ increase associated to CatSper starts in the flagellum. If Em changes in sperm affect all compartments, and considering the results from this work indicating that hyperpolarization is necessary and sufficient to prepare the sperm for a sZP-induced acrosome reaction, changes in the Em can be postulated as a mechanism to synchronize flagellar and head signaling. However, it is worth taking into account that as in neurons, it is possible that sperm are not isopotential (Em may not be homogeneous in all sperm compartments) (52), which would complicate the proposed synchronizing role of the hyperpolarization.

The notion that Ca_V channels are apparently no longer functional in epididymal sperm questions the long proposed role of hyperpolarization as a required step to remove channel inactivation during the ZP-induced acrosome reaction. All things considered, our working model for why Em hyperpolarization is needed for mouse sperm physiology can be summarized as follows:

After leaving the epididymis, sperm encounter a 9 to 10 fold increase of external HCO_3^- concentration, leading to an intracellular increase of this anion. This could occur through HCO_3^-/Cl^- exchangers (53) and/or Cl^- channels such as CFTR (21), resulting in a transient Em hyperpolarization which is enhanced and maintained by the closure of ENaCs (7,21). This hyperpolarization is believed to activate the sperm specific Na^+/H^+ exchanger (sNHE) (3,11), elevate pHi and the HCO_{3^-} increase stimulate

SACY (54). Potentially sNHE is positively modulated by both a hyperpolarization and by cAMP (3,11). The resulting pHi increase would activate Slo3 and CatSper (19,54). Depending on the molar ratio of these channels, their conductances and the selectivity of Slo3, mouse sperm Em could further hyperpolarize. The alcalinization associated to capacitation could also stimulate K^+ inward rectifiers reported to be present in mouse sperm (33,40) which would contribute to a sustained hyperpolarization. The resulting cAMP elevation has several additional targets such as CNG channels (55) and PKA that would further activate CFTR (35,37). The mouse sperm hyperpolarization may have distinct kinetic components and multiple targets. Hyperpolarization increases the driving force for Ca^{2+} uptake through Ca^{2+} permeable channels such as CatSper and TRPs (17,56), a crucial event known to occur during capacitation (reviewed in (3)). As discussed earlier, it would affect voltage dependent transporters such as Ca_Vs, if functionally available in mature capacitated sperm. Other ion transporters present in sperm are sensitive to membrane potential such as the Ca^{2+} ATPase (57,58) whose absence renders sperm infertile (59). In conclusion, sperm membrane potential arises as a possible event that orchestrates and synchronizes changes occuring in the flagella with those needed in the head to trigger the acrosome reaction.

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FIGURE LEGENDS

Figure 1. Hyperpolarization is not sufficient to induce phosphorylation events. A. Em hyperpolarization was measured using the fluorescence dye Dis-C_3(5) after incubating sperm for 40 min under non capacitating conditions with 1 µM amiloride (AML), 10 µM genistein (GEN) and 1 µM valinomycin (VAL) (see Materials and Methods). **B, C and D. Sperm were incubated in non capacitating (NC) conditions for 90 min (media devoid of HCO_3^- and BSA),** containing either increasing concentrations of amiloride (**B**) or genistein (**C**) and 1 µM valinomycin (**D**). Normal phosphorylation upon capacitacion (C) is also shown for comparison. Samples were processed for western blot analyses with anti-pPKAs (clone 100G7E) and anti-pY antibodies (clone 4G10) as described in Materials and Methods. These hyperpolarization treatments were not able to induce capacitation-associated changes in phosphorylation of sperm proteins. n=4 independent experiments (*p <0.5, **p < 0.01, *** p < 0.001).

Figure 2. K^+ addition induces $[Ca^{2+}]_i$ increases and the acrosome reaction in hyperpolarized sperm. A. Pseudocolored fluorescence images illustrating $[Ca^{2+}]_i$ levels before (CTRL) and after addition of 50 mM K^+ (K^+) to capacitated mouse sperm. The $[Ca^{2+}]_i$ increase induced subsequently by 10 µM ionomycin (IONO) is also shown as a positive control. **B.** Representative $[Ca^{2+}]_i$ trace of an individual sperm subjected to the conditions described above. Arrows indicate agonist application. A 54 ± 8 % of the sperm display this $[Ca^{2+}]_i$ response to the K^+ addition (n= 4 independent experiments, 247 cells analyzed). The white scale bar corresponds to 10 µm. **C.** Addition of 50 mM K^+ induced acrosome reaction in capacitated (C) but not in non capacitated sperm (NC). The K^+ induced depolarization was able to induced acrosome reaction in non capacitated sperm previously hyperpolarized after incubation for 40 min with amiloride (AML), genistein (GEN) or valinomycin (VAL) as indicated in Fig. 1. (*p <0.5, **p < 0.01, *** p < 0.001 and n=4).

Figure 3. K^+ addition promotes $[Ca^{2+}]_i$ increases only in hyperpolarized non capacitated mouse sperm. Fluorescence images corresponding to $[Ca^{2+}]_i$ responses obtained before (left panels) and during K^+ (50 mM) (middle panels) and ionomycin (10 µM) (IONO) additions (right panels) under several incubation conditions: **A.** Sperm incubated in non capacitating medium (CTRL). **B. C.** and **D.** Sperm incubated during 30 mins with hyperpolarizing agents: 1 µM amiloride (AML) **(B)**, 10 µM genistein (GEN) **(C)** and 1 µM valinomycin (VAL). **(D).** Right traces show representative single cell $[Ca^{2+}]_i$ recordings obtained during each experiment. Arrows indicate additions of K^+ and ionomycin (IONO). White scale bars correspond to 10 µm. **E.** Summary of the percentage of sperm displaying $[Ca^{2+}]_i$ increases in response to K^+ application under each incubating condition. **F.** Percent $[Ca^{2+}]_i$ increase induced by K^+ under the indicated condition with respect to the increase induced by ionomycin (100%). Data represent the average $\pm$ SEM, n=5 independent experiments, asterisks indicate p values (*p <0.5, **p < 0.01) the number of cells analyzed for each condition are: CTRL=142, C =247, AML=129, GEN= 123 and VAL =107).

Figure 4. Plasma membrane hyperpolarization prepares sperm for the sZP-induced acrosome reaction. Capacitated (C) sperm undergo the sZP induced acrosome reaction, while non-capacitated sperm (NC) (incubated in the absence de HCO_3^- and BSA) do not. In contrast, non-capacitated sperm hyperpolarized with either amiloride (AML), genistein (GN) or valinomycin (VAL), as described in Fig.

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2, undergo the sZP induced acrosome reaction. Asterisks indicate p values (*p <0.5, **p < 0.01, *** p < 0.001), n=4.

Figure 5. Plasma membrane hyperpolarization prepares sperm to undergo the sZP-induced increase in [Ca^{2+}]$_i$. Fluorescence images showing sperm [Ca^{2+}]$_i$ changes induced by sZP and ionomycin (IONO) in control non-capacitating medium (CTRL) (**A**), in capacitating medium (C) (**B**) and after incubation in hyperpolarizing conditions with amiloride (AML) (**C**), genistein (GEN) (**D**) and valinomycin (VAL) (**E**) as in Fig. 3. Right panels illustrate representative single sperm [Ca^{2+}]$_i$ recordings corresponding to each experiment. **F.** Summary of the percentage of sperm displaying [Ca^{2+}]$_i$ increases in response to sZP application under each incubation condition. **G.** Percent [Ca^{2+}]$_i$ increase induced by sZP under the indicated condition with respect to the one induced by ionomycin. Data represent the average ± SEM, n=5 independent experiments, asterisks indicate p values (*p <0.5, **p < 0.01 and ***p < 0.00) the number of cells analyzed for each condition are: CTRL=104, C=142, AML=64, GEN=74 and VAL=67). White scale bars correspond to 10 μm.

Figure 6. Hyperpolarization is necessary for sperm to undergo the sZP-induced acrosome reaction A. Sperm capacitated (with 1 μM valinomycin) in the absence (C) or in the presence of the indicated K$^+$ concentrations display the Ems shown, determined as indicated in Materials and Methods, the Em of non capacitated sperm also treated with 1 μM valinomycin is also shown for comparison (NC). The Em values in parenthesis indicate the calculated Nernst potentials at each external K$^+$ concentration. Data represent the average ± SEM of n=3 independent experiments, asterisks indicate p values (*p <0.05, **p < 0.01 and ***p < 0.001). **B.** Sperm were incubated in capacitating medium containing 1 μM valinomycin (VAL) and different K$^+$ concentrations. Samples were processed for western blot analyses with anti-pPKAs and anti-pY antibodies, as described in Experimental Procedures. The capacitation-associated increase in phosphorylation of PKA substrates or tyrosine residues was not blocked even at concentrations of external K$^+$ as high as 70 mM. Controls of non capacitated (NC) and capacitated sperm (C) are also shown for comparison. The intensity of the anti-pPKAs blot in the lane corresponding to 5.6 mM KCl is lower than the others possibly due to a small loading difference. Several independent experiments indicate this is not a significant difference. **C.** Representative images illustrating the [Ca^{2+}]$_i$ changes of mouse sperm incubated in the presence of 50 mM K$^+$ and valinomycin before (K$^+$+ VAL) and during sZP and ionomycin (IONO) additions (upper panels). The lower panel shows a representative recording of the indicated sperm illustrating the inability to manifest [Ca^{2+}]$_i$ increases induced by sZP when sperm are exposed to high K$^+$ and valinomycin, while the ionomycin response is preserved (87 ± 5 % of the sperm present this behavior in 4 independent experiments with 42 cells analyzed). White scale bar corresponds to 10 μm. **D.** sZP cannot induce the acrosome reaction in sperm capacitated in the presence of 70 mM external K$^+$ with or without valinomycin (VAL). Data represent the average ± SEM of n=5 independent experiments.
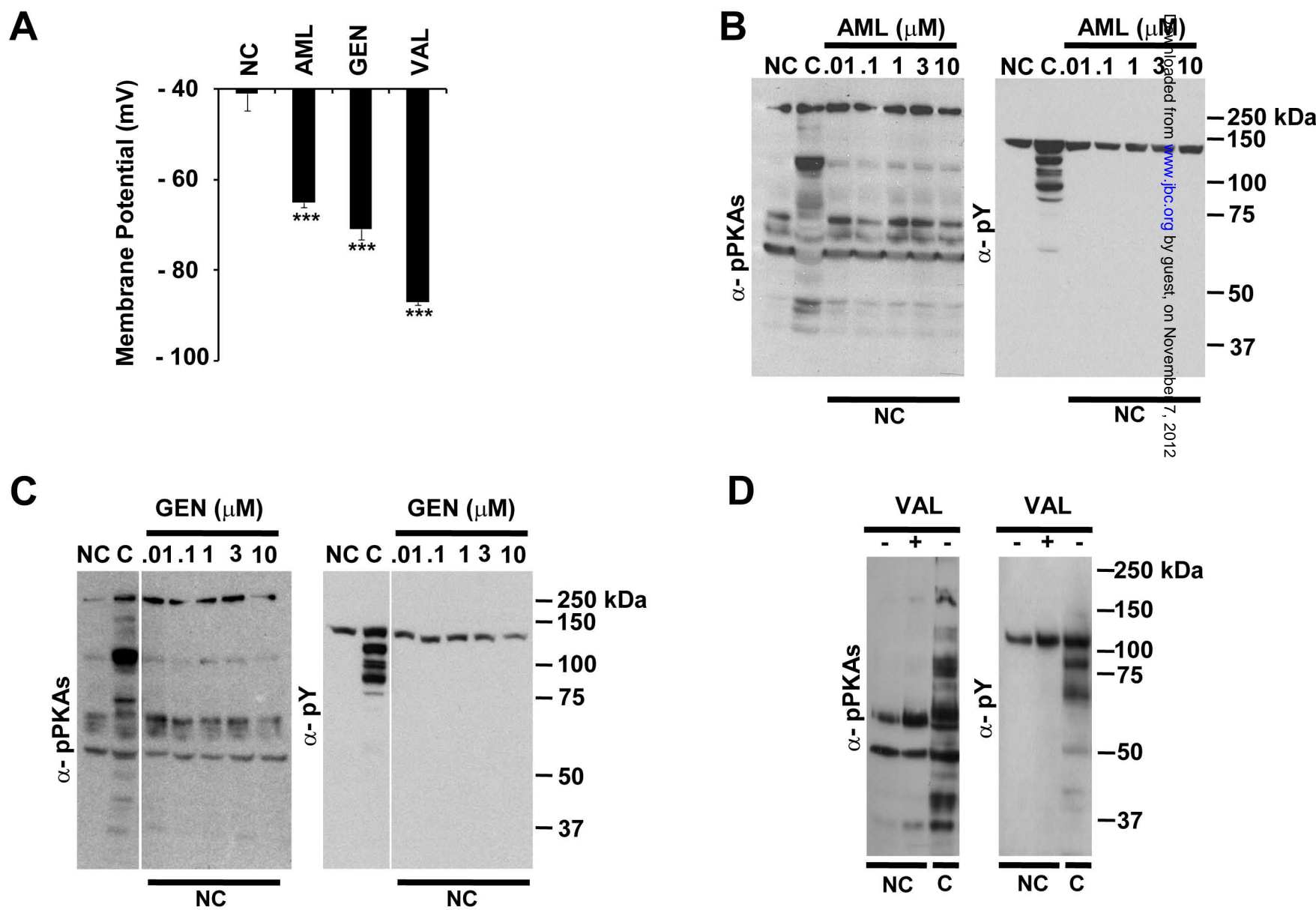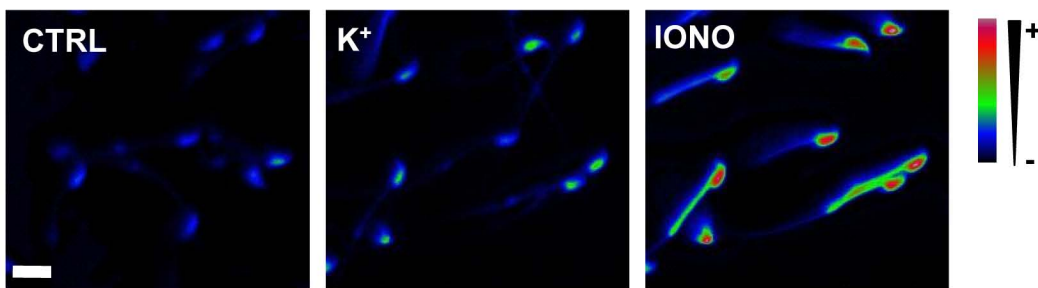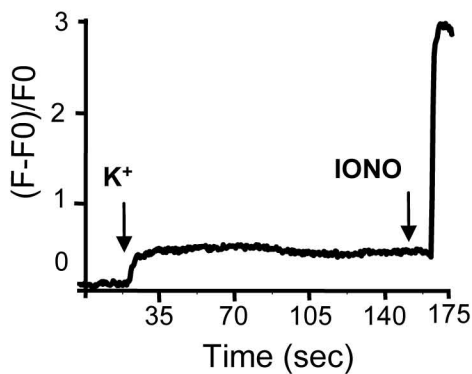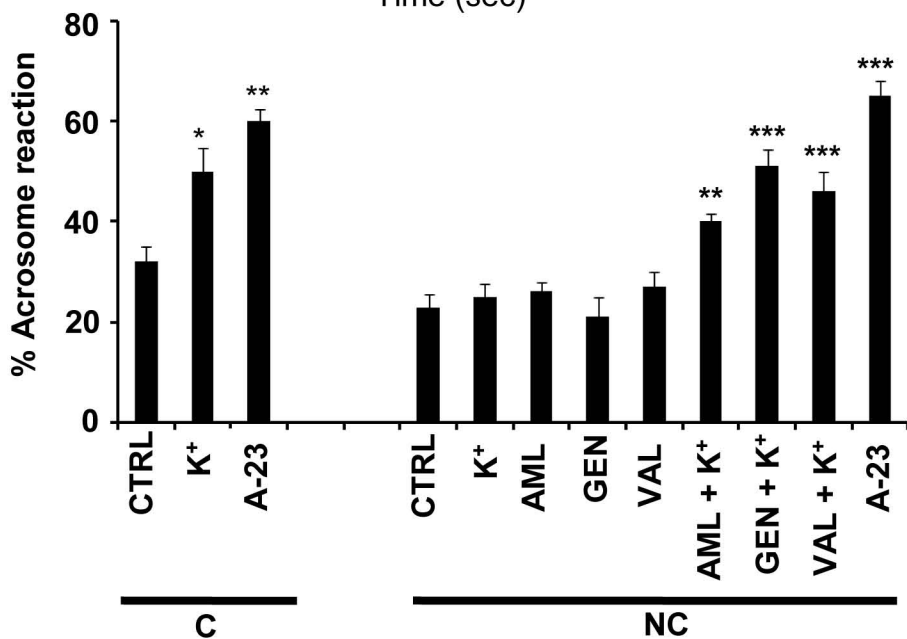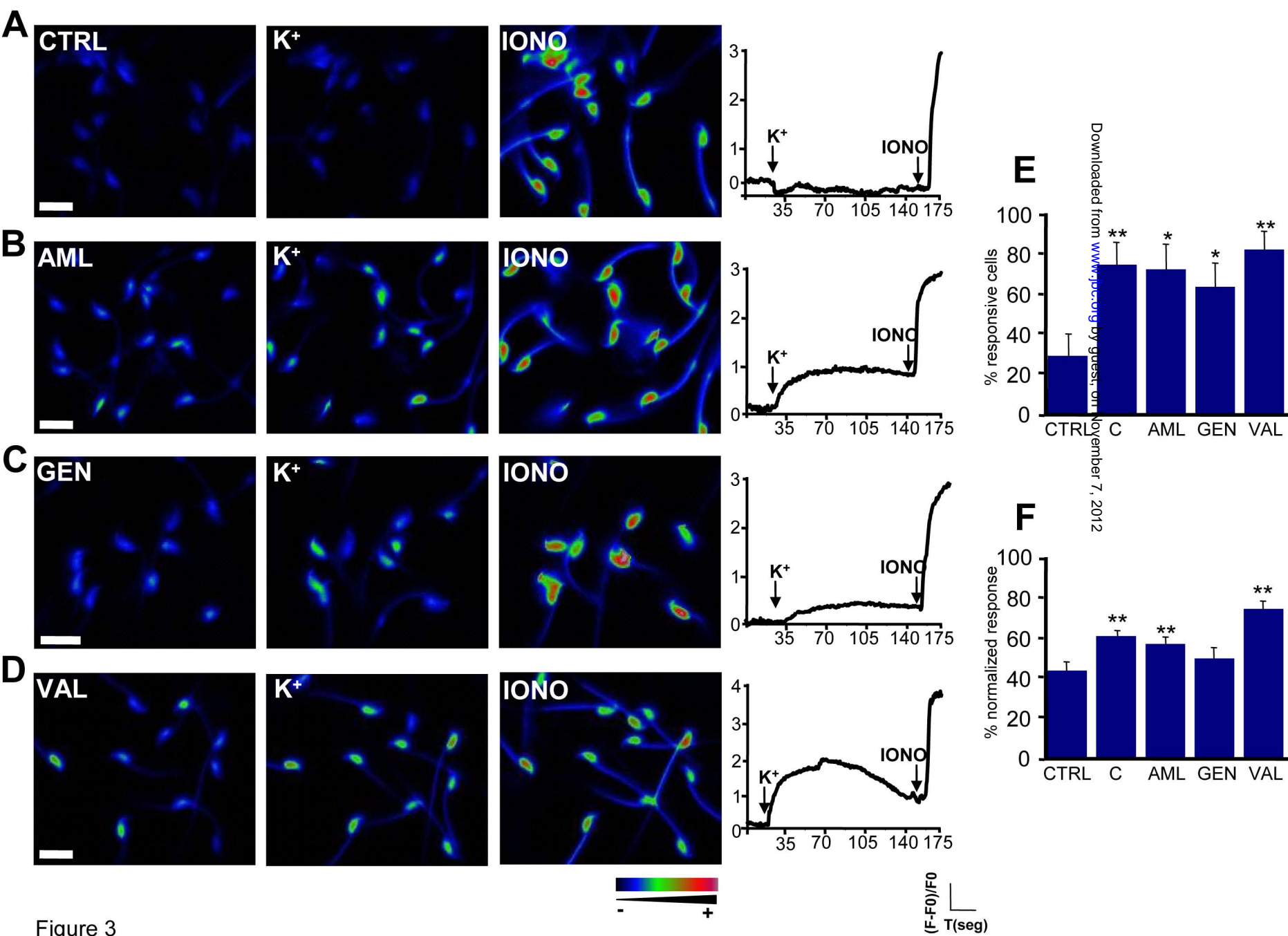
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Figure 1

15

A

CTRL K+ IONO

B

C

Figure 2

Figure 3

17

Figure 4

Figure 5

19

A

Membrane Potential (mV)

NC (−87)
C (−87)
20 mM K⁺ (−48) **
30 mM K⁺ (−37) **
40 mM K⁺ (−29) ***
50 mM K⁺ (−23) ***
60 mM K⁺ (−19) ***
70 mM K⁺ (−14) ***
80 mM K⁺ (−11) ***

B

$K^+[mM] + VAL$

α-pPKAs
NC C 5.6 30 50 70

α-pY
NC C 5.6 30 50 70

— 250
— 150
— 100
— 75
— 50
— 37

C

K⁺+VAL   sZP   IONO

+
−

$(F-F0)/F0$

sZP   IONO

Time (sec)
35 70 105 140 175

D

% Acrosome Reaction

C
sZP ***
C
sZP
VAL
VAL+sZP

70 mM K⁺

Figure 6

20