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Ion channel activity of membrane vesicles released from sea urchin sperm during the acrosome reaction ☆

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Abstract

The sperm acrosome reaction (AR) involves ion channel activation. In sea urchin sperm, the AR requires Ca^{2+} and Na^+ influx and K^+ and H^+ efflux. During the AR, the plasma membrane fuses with the acrosomal vesicle membrane forming hybrid membrane vesicles that are released from sperm into the medium. This paper reports the isolation and preliminary characterization of these acrosome reaction vesicles (ARVs), using synaptosome-associated protein of 25 kDa (SNAP-25) as a marker. Isolated ARVs have a unique protein composition. The exocytosis regulatory proteins vesicle-associated membrane protein and SNAP-25 are inside ARVs, as judged by protease protection experiments, and membrane associated based on Triton X-114 partitioning. ARVs fused with planar bilayers display three main types of single channel activity. The most frequently recorded channel is cationic, weakly voltage dependent and has a low open probability that increases with negative potentials. This channel is activated by cAMP, blocked by Ba^{2+}, and has a PK^+/PNa^+ selectivity of 4.5. ARVs represent a novel membrane preparation suitable to deepen our understanding of ion channel activity in the AR and during fertilization.

Keywords: Acrosome reaction; Acrosome reaction vesicles; Exocytosis; Fertilization; Planar bilayers; Sea urchin sperm; Sperm ion channels

The acrosome reaction (AR) of animal sperm is a ligand-mediated, ion channel regulated, and exocytosis of a single secretory vesicle. This vesicle, named the acrosomal vesicle (AV), is located in the head of the sperm. The contents released by the AR allow the sperm to penetrate the egg's extracellular matrices and fuse with its plasma membrane [1–4]. Acrosomal exocytosis is unique in that the plasma and vesicle membranes fuse together at multiple points to form hybrid membrane vesicles that are released from the sperm [5,6]. Ultrastructural studies have shown that the released vesicles are composed of both plasma membrane and acrosomal vesicle membranes [7–9]. Until now, a detailed characterization of the protein components in these vesicles from the sperm of a free-spawning marine invertebrate has been lacking.

We previously isolated the sea urchin sperm homologues of the exocytotic regulatory proteins syntaxin, vesicle-associated membrane protein (VAMP), and synaptosome-associated protein of 25 kDa (SNAP-25) [10]. These three proteins are shed from the sperm in the

☆ *Abbreviations:* AR, acrosome reaction; AV, acrosomal vesicle; ARVs, acrosome reaction vesicles; SNAP-25, synaptosome-associated protein of 25 kDa; VAMP, vesicle-associated membrane protein; DPPC, 1,2-diphytanoyl-sn-glycero-3-phosphocholine.

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acrosome reaction vesicles (ARVs) resulting from the AR [11,12]. ARVs represent a unique population of vesicles containing the regulatory proteins of the AR derived from both the plasma and AV membranes. Unlike other well-studied exocytotic vesicles such as synaptosomes, the release of ARVs from the sperm precludes their retrieval by the cells. They are the product of a unidirectional exocytotic system in which protein interactions before and after AR exocytosis can be directly compared [12].

We describe the isolation of ARVs from ionophore activated isolated flagella-less sea urchin sperm heads. The large quantity of sea urchin sperm that can be obtained makes this an ideal system for identification and isolation of ARV proteins regulating the AR. The preliminary characterization of an ARV cation-selective channel is reported to demonstrate the utility of this novel membrane vesicle system to the deeper understanding of AR induction.

Materials and methods

Isolation of ARVs. Sea urchins (*Strongylocentrotus purpuratus*) were spawned into 30 mM Mes-buffered and filtered seawater, pH 6.0. Pigmented coelomocytes were sedimented by centrifugation at 800*g* for 15 min (4 °C) when large quantities of sperm were used. For diluted sperm volumes up to 25 ml samples, centrifugation at 200*g* for 5 min is enough. The supernatant sperm suspension was blended in a Warring Blender for 10 s or passed 20 times (twice) through a 21-gauge syringe [13] at 4 °C. Sperm heads, free of fragmented flagella, were isolated by sedimentation at 1500*g* for 20 min and resuspension in fresh Mes-buffered seawater. This wash was repeated until no flagellar fragments were visible among the isolated sperm heads by phase contrast microscopy. Flagella-less sperm heads were washed twice in 30 mM Hepes-buffered seawater, pH 8.0, and then acrosome reacted by addition of 50 μM nigericin (Sigma) and 10 μM A23187 (Calbiochem). The ionophores were dissolved in DMSO, the final concentration of which was 1%. By 30 min after ionophore addition 60–80% of the flagella-less sperm heads had acrosome reacted. Sperm heads were sedimented by centrifugation at 7000*g* for 20 min. The supernatant was removed and the centrifugation was repeated. The final supernatant was centrifuged at 200,000*g* for 1 h (4 °C) to sediment the ARVs.

Immunoblots and protein samples. Qualitative and quantitative immunoblots with antibodies to syntaxin, VAMP, and SNAP-25 were performed as described [11,12]. ARVs were banded in a 0.4–2.0 M sucrose gradient in 10 mM Hepes, pH 7.4, 10 mM EDTA at 25,000*g* for 6 h (4 °C). Peak fractions of ARVs were determined by turbidity at $A_{280\,nm}$. Trypsin treatment of 10 μg ARV protein was in a final volume of 25 μl containing 100 ng pancreatic trypsin. Digestions were incubated 15 min at 37 °C in the presence or absence of 2% Nonidet-P40 (NP40). Triton X-114 phase separation of the ARV proteins was performed as described [14]. Protein was determined by the BCA reaction with bovine serum albumin as a standard (Bio-Rad) and silver staining of SDS–PAGE gels was as described [15]. To obtain solubilized plasma membrane proteins in the absence of nuclear, mitochondrial, and axonemal proteins, intact sperm and sperm heads were homogenized and extracted for 30 min in 1% NP40 (4 °C) and the 30,000*g* supernatants were collected.

Planar bilayers. ARVs at ~0.5–2 mg/ml in 10 mM Hepes, pH 7.0, were preloaded with 0.5 M sucrose overnight on ice. Some preparations were further sonicated (2 min) in a water bath sonicator (4 °C). The vesicles were then aliquoted and stored at −70 °C until used. Planar bilayers were formed with the synthetic lipid, 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPPC; Avanti Polar Lipids), and fused with ARVs (1–25 μg protein) as described [16]. Single channel currents were recorded unfiltered at 2–5 kHz using either a modified VCR or

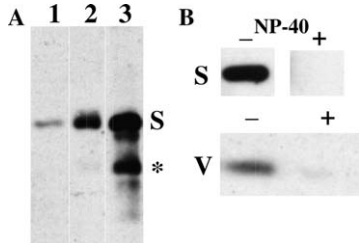

Fig. 1. (A) Immunoblots of 15% SDS–PAGE (2 μg protein per lane) showing the enrichment of SNAP-25 (S) in ARVs (lane 3) as compared to: NP40 solubilized proteins from intact sperm (lane 1), or the isolated heads (lane 2). Sea urchin SNAP-25 migrates at 32 kDa. The 28 kDa band (*) is known by sequence to be a proteolytic fragment of SNAP-25 [12]. (B) SNARE proteins in ARVs are protected from trypsin digestion, showing that ARVs are tightly sealed. Ten micrograms of ARV protein per sample was digested with 100 ng pancreatic trypsin in the absence (−), or presence (+), of 2% non-ionic detergent NP-40. The digests were separated by 15% SDS–PAGE, and immunoblots probed with antibodies to SNAP-25 (S, 32 kDa) and VAMP (V, 17 kDa).

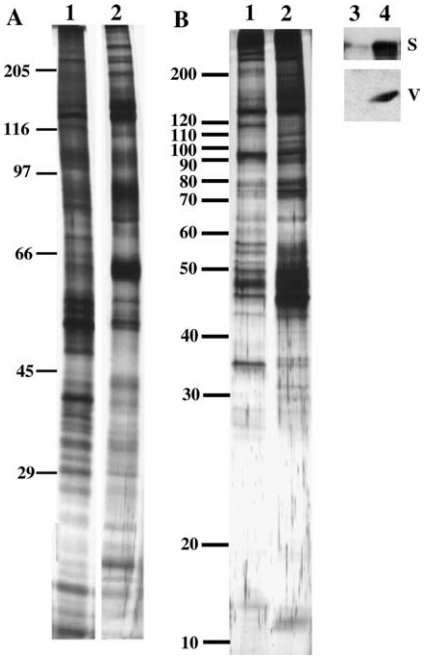

Fig. 2. (A) Comparison of the protein compositions of ARVs (lane 1) with respect to total sperm membrane (lane 2), as shown on a silver-stained 15% SDS–PAGE. Four microgram of protein were loaded per lane. Molecular mass standards in kDa are shown on left. (B) The partitioning of ARV proteins by Triton X-114 phase separation. Silver-stained 15% SDS–PAGE separation of equal volumes of ARV proteins partitioned in the aqueous phase (lane 1) and Triton X-114 (lane 2) detergent phase. Samples were immunoblotted with antibodies to SNAP-25 (S) and VAMP (V). These two SNARE proteins partition into the detergent phase (lane 4), consistent with their association mainly with the membranes and their low abundance in the aqueous phase (lane 3).

directly on hard disc. Single channel current analysis was performed after filtering the recordings at 500 Hz (using a four-pole Bessel filter) using the pCLAMP 6.0 software package (Axon Instruments). The bilayer was formed having 100 mM KCl both in *cis* (the bilayer chamber side where the ARVs are added) and in *trans*. After adding ARVs in *cis*, KCl was raised to 300 mM to favour fusion.

The K^+/Na^+ selectivity was determined as follows: after detecting single channel activity under the KCl gradient described above, the *cis* side was perfused with >10 chamber volumes (1 ml) of 300 mM NaCl. Reversal potentials (E_{rev}) were estimated from the *I–V* curves of single channel transitions [16].

Results

Isolation of ARVs

Silver-stained SDS–PAGE analysis and Western blotting had shown that flagellar membrane proteins contaminated ARV preparations if intact sperm were acrosome reacted by ionophore treatment. To avoid this problem, flagella were removed from sperm prior to ionophore treatment of the flagella-less sperm heads. Phase contrast microscopy at approximately 1200× magnification was used to confirm that the isolated sperm heads had intact acrosomal vesicles before treatment with a combination of ionophores nigericin and A23187. Treatment with ionophore for 30 min resulted in induction of the AR in 60–80% of the flagella-less sperm heads.

SNAP-25 is released from sperm with the ARVs [12]. This protein is more abundant in ARVs released from flagella-less sperm heads than it is in total sperm membranes, or total membranes from flagella-less sperm heads. Using SNAP-25 as an ARV marker protein, and quantitative immunoblotting, the ARVs were found to be 13.8-fold enriched for SNAP-25 over total sperm membranes, and 4.6-fold enriched over sperm head membranes (Fig. 1A). Identical results were obtained using two different sets of standards to calculate the fold enrichment (see Materials and methods).

ARVs have a unique protein composition when compared to total sperm membrane protein. For example, they have a greater abundance of proteins in the 29–45 kDa range as well as different bands throughout the

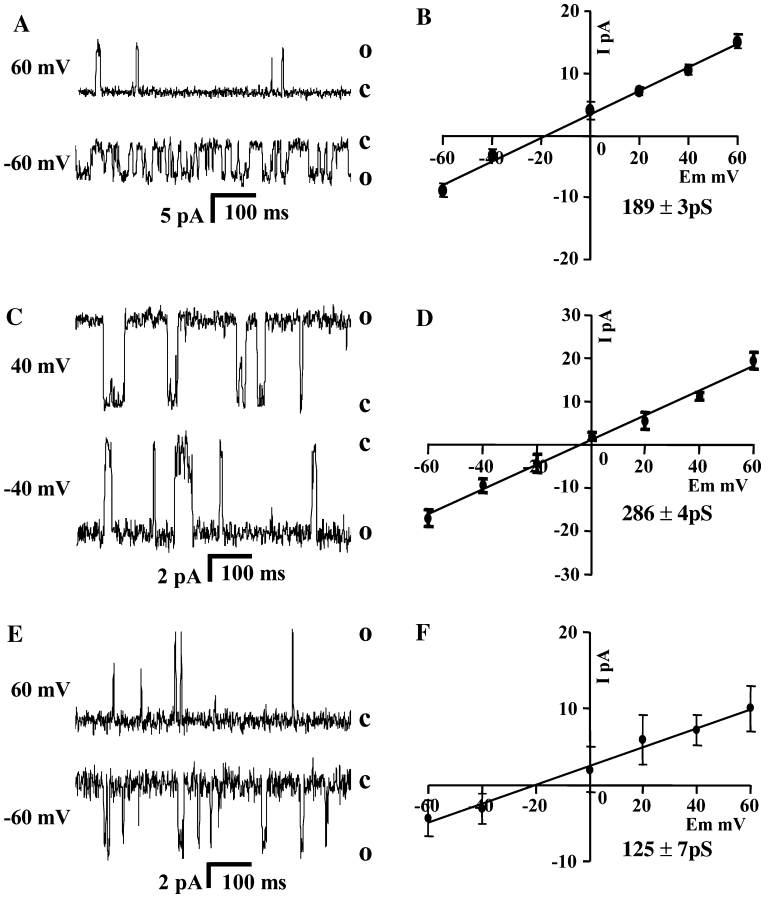

Fig. 3. Single channel currents from ARVs fused with planar bilayers. Illustrative recordings from 3 different planar bilayers displaying distinct channels are shown in (A,C,E). Open (o) and closed (c) states are indicated. The recordings obtained as discussed in Materials and methods were low pass filtered at 500 Hz. In each case recordings for two applied voltages and the corresponding single channel conductance and E_{rev} obtained from the *I* (current)–*V* (voltage) plot are shown in (B) ($E_{rev} = -18$ mV), (D) ($E_{rev} = -4$ mV), and (F) ($E_{rev} = -19$ mV). The *cis* chamber contained 300 mM KCl and the *trans* chamber contained 100 mM KCl.

two lanes (Fig. 2A). SNAP-25, at 32 kDa [12], is a visible silver-staining band showing that it is a prominent component of ARVs. The diameter of ARVs as determined by transmission electron microscopy of spread, negatively stained preparations varied from 70 to 300 nm (data not shown).

Characterization of ARVs

The exocytotic R-SNARE protein VAMP, and the Q-SNARE proteins SNAP-25 and syntaxin [10] are found in ARVs [12]. All three proteins are present in a complex associated with ARVs [12]. If exocytotic membrane fusion occurs between the plasma and acrosomal membranes by the hypothesized mechanism [10], then both Q- and R-SNAREs should be associated within the ARVs. To test this, ARVs were treated with trypsin in the presence and absence of the non-ionic detergent NP40. Immunoblots showed that both VAMP and syntaxin were completely degraded in the presence of detergent, whereas in the absence of detergent, both proteins were protected from protease digestion. These data indicate that ARVs are tightly sealed membrane vesicles containing these SNARE proteins (Fig. 1B).

To determine if Q- and R-SNARE proteins are released from, or remain associated with, the ARV membrane, ARV proteins were partitioned by Triton X-114 detergent extraction. An approximately equal number of proteins appear to partition between the aqueous and detergent phases (Fig. 2B). This result suggests that the aqueous-partitioning proteins are either bound to the ARVs or are captured inside ARVs during their formation. Both VAMP and SNAP-25 partition mainly in the detergent phase, consistent with the idea that both proteins remain membrane associated within ARVs.

Single channel activity in planar bilayers containing ARVs

The regulation of ion plasma membrane permeability is critical to sperm physiology; in particular for motility and for the triggering of acrosomal vesicle exocytosis [1,2]. Identification and characterization of ion channel activities present in sea urchin sperm membrane fractions is important to gain deeper insight into the mechanism of the AR. The small size of sea urchin sperm makes it difficult to study their electrophysiology [17,18]. An appealing alternative, especially for ion channels found in the acrosomal membrane, is to fuse ARVs with planar bilayers in the hope of discovering novel ion channel activities [1,16]. ARVs contain components from a specific area of the head plasma membrane and from the acrosomal membrane. Planar bilayers with fused ARVs display various types of single channel activity (Fig. 3). A 189 pS cationic channel was only recorded in 3 out of 65 experiments (E_{rev} = −18 mV). A larger conductance, non-selective channel of 286 pS was seen in 7 out of 65 experiments (E_{rev} = −4 mV). The most frequently detected channel (52/65 experiments) has a unitary main conductance of ~125 pS in bilayers bathed by 300 mM KCl in *cis* and 100 mM KCl in *trans*. This channel has a reversal potential of −19 mV, which is close to the Nernst potential predicted for a cation-selective pore. This ARV channel is weakly voltage dependent, displaying a very low open probability at positive potentials that mildly increases as the applied potential becomes more negative (Figs. 4A and B). At least two distinct conductance states were observed in steady-state recordings (Fig. 4C). It is uncertain from these data whether this is due to multiple

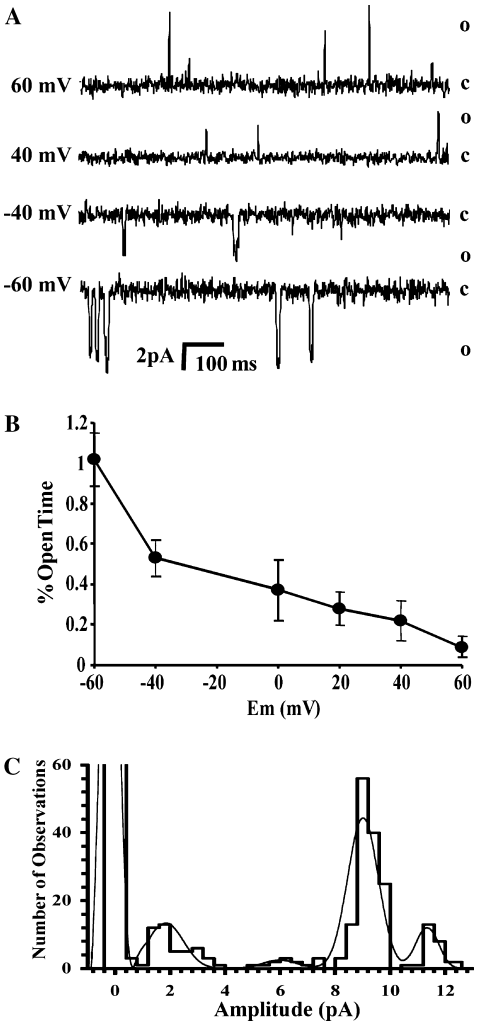

Fig. 4. The most frequent ARV channel is cation-selective and has a low open probability. (A) Steady-state single channel records at the holding potentials indicated with 300 mM KCl in *cis* and 100 mM KCl in *trans*. Open (o) and closed (c) states are indicated. (B) Open probability as a function of applied voltage. The percent open time was obtained by computer analysis (see Materials and methods). (C) Amplitude distribution of a typical bilayer containing the cation-selective channel recorded at +60 mV. The current peaks had values of 2.3 ± 0.23, 6.35 ± 0.66, 9.17 ± 0.2, and 11.5 ± 0.13, respectively.

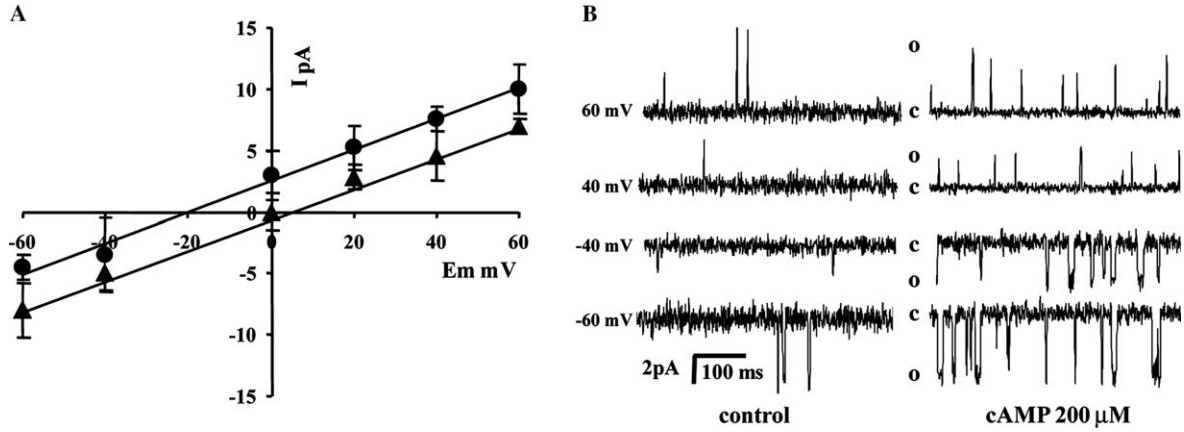

Fig. 5. K^+/Na^+ permeability ratio and regulation by cAMP of the cation-selective channel conductance present in ARVs. (A) *I–V* relationships of conductance states present in the ARVs fused to planar bilayers with 300 mM KCl in *cis* and 100 mM KCl in *trans* (circles; $E_{rev} = -20$ mV) and after perfusion in *cis* with 300 mM NaCl (triangles; $E_{rev} = 1.5$ mV). Single channel transition values are the average ± SD, $n = 3$. The results yield a K^+/Na^+ permeability ratio of 4.5. (B) Recordings of the most frequent ARV channel in the absence (control) and presence of 200 μM cAMP in the *cis* chamber in a bilayer bathed by 300 mM KCl in *cis* and 100 mM KCl in *trans*. Open (o) and closed (c) states are indicated.

conductance states of one channel or from the presence of two different channels. No transitional states between these conductances were observed, suggesting that they may represent distinct channels. However, in all the experiments where this channel activity was recorded (52 trials), both conductances were present.

Experiments performed to determine the K^+/Na^+ selectivity of the most frequent ARV channel revealed that the permeability ratio for these cations is 4.5 (Fig. 5A). This value and the kinetics, and voltage dependence of this channel are reminiscent of those displayed by a flagellar channel regulated by cAMP studied in planar bilayers [16]. Thus, we tested if the ARV channel is also regulated by this nucleotide. Addition of 200 μM cAMP to the *cis* side of the bilayer resulted in an increase in the number of channel openings at all potentials tested, without significantly affecting the mean open time (Fig. 5B). This results in an 8 ± 0.6 ($n = 3$) fold increase in the open time. The modulation of this channel by cAMP suggests once again the importance of this cyclic nucleotide as a regulator of sperm ion channels [2,19,20]. In addition, as with the flagellar channel, this ARV channel is blocked by 10 mM Ba^{2+} in *trans* (data not shown).

Discussion

The sperm AR is a unique exocytosis that is a key step in animal fertilization. Studies of the signal transduction events underlying AR regulation are important to a molecular description of sexual reproduction. We have isolated a novel preparation of membrane vesicles shed from sea urchin sperm as a result of the fusion of plasma and AV membranes during the AR. Sea urchin sperm are ideal cells for such isolations because of the vast number and amount of membrane proteins that can be easily obtained at low cost. We have begun ARV preparations with as much as 100 ml of undiluted *S. purpuratus* semen, representing 4×10^{12} individual sperm cells and 10 g of sperm protein. Enrichments for adenylate cyclase had indicated that the sperm membrane fraction is approximately 10% of the total sperm protein [21]. Isolated ARVs are just a small fraction of the total sperm membrane protein. Sea urchins and other marine invertebrates may be the only animals where the vast quantity of sperm obtainable permits the isolation and biochemical analysis of a substantial amount of ARVs.

ARVs are enriched for SNAP-25 as compared to total sperm membranes (13.8-fold) and sperm head membranes (4.6-fold). Protease protection of SNARE proteins indicated that ARVs are tightly sealed vesicles. Compared to total sperm membrane protein, ARVs contain a unique profile of protein components (Fig. 2A). Further analysis of these proteins could be extremely interesting. ARVs could prove valuable for identifying other acrosome reaction regulatory proteins, similar to the isolation and study of proteins in the synaptosomes of neuronal tissue. The previous identification of a complex of VAMP, SNAP-25, and syntaxin in ARVs demonstrates this point [12]. ARVs may also contain signal transducing proteins that are coupled to the exocytotic machinery. Such proteins could be ion channels, Ca^{2+} sensors, and ligand-activated receptors. The presence of the cation-selective channel in ARVs fused with planar bilayers illustrates this point.

This is the first demonstration of the presence of ion channels in vesicles naturally shed from sperm as a result of the acrosome reaction. The presence of these channels in ARVs is not proof that they are involved in triggering the sperm AR. It also does not prove that any of them is specific to the acrosomal area of the sperm membrane.

Their presence in ARVs only shows that they are contained in the membrane region that participates in the AR. Isolation, cloning, expression, and antibody localization of these channels might yield new information as to the likelihood of their participation in the AR.

The most frequently recorded ARV channel (Figs. 3–5) has properties in common with a mildly K^+ selective channel present in flagellar membrane vesicles that has also been studied in planar bilayers [16]. These two channels have low open probabilities that increase with more negatively applied potentials; both have similar conductances, are activated by cytosolic cAMP, and have a PK^+/PNa^+ selectivity 4.5. It will be important to determine the relation between these mildly selective K^+ channels and the recently described and cloned hyperpolarization-activated SpHCN channel from sperm of this species of sea urchin [20]. Single channel recordings of the SpHCN channel will clarify this possibility. Since both the flagellar membrane vesicle channel and the SpHCN channel are positively modulated by cAMP it is plausible to postulate that they could participate in the regulation of sperm motility.

Induction of the AR involves increases in cAMP and a transient membrane hyperpolarization [19,22]. These changes could activate the ARV channel, resulting in membrane depolarization in a manner described for the SpHCN channel [16,20,23]. The presence of a family of different hyperpolarization-activated channels in mammals [23] suggests that several channel isoforms may also be present in sea urchins and localized to different parts of the sperm. Unpublished experiments are consistent with the findings presented here indicating that ARVs contain several types of ion channels. ARVs provide an excellent novel material for characterizing these ion channel activities.

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References

[1] A. Darszon, C. Beltrán, R. Felix, T. Nishigaki, C.L. Trevino, Ion transport in sperm signaling, Dev. Biol. 240 (2001) 1–14.
[2] D.L. Garbers, Molecular basis of fertilization, Annu. Rev. Biochem. 58 (1989) 719–742.
[3] V.D. Vacquier, Evolution of gamete recognition proteins, Science 281 (1998) 1995–1999.
[4] P.M. Wassarman, Mammalian fertilization: molecular aspects of gamete adhesion, exocytosis, and fusion, Cell 96 (1999) 175–183.
[5] C. Barros, J.M. Bedford, L.E. Franklin, C.R. Austin, Membrane vesiculation as a feature of the mammalian acrosome reaction, J. Cell Biol. 34 (1967) C1–C5.
[6] P. Primakoff, D.G. Myles, A.R. Bellvé, Biochemical analysis of the released products of the mammalian acrosome reaction, Dev. Biol. 80 (1980) 324–331.
[7] L. Russell, R. Peterson, M. Freund, Direct evidence for formation of hybrid vesicles by fusion of plasma and outer acrosomal membranes during the acrosome reaction in boar spermatozoa, J. Exp. Zool. 208 (1979) 41–56.
[8] B.L. Hylander, R.G. Summers, An ultrastructural investigation of the spermatozoa of two ophiuroids, *Ophiocoma echinata* and *Ophiocoma wendti*: acrosomal morphology and reaction, Cell Tissue Res. 158 (1975) 151–168.
[9] A.I. Yudin, W. Gottlieb, S. Meizel, Ultrastructural studies of the early events of the human sperm acrosome reaction as initiated by human follicular fluid, Gamete Res. 20 (1988) 11–24.
[10] R.C. Lin, R.H. Scheller, Mechanisms of synaptic vesicle exocytosis, Annu. Rev. Cell Dev. Biol. 16 (2000) 19–49.
[11] J.R. Schulz, G.M. Wessel, V.D. Vacquier, The exocytosis regulatory proteins syntaxin and VAMP are shed from sea urchin sperm during the acrosome reaction, Dev. Biol. 191 (1997) 80–87.
[12] J.R. Schulz, J.D. Sasaki, V.D. Vacquier, Increased association of synaptosome-associated protein of 25 kDa with syntaxin and vesicle-associated membrane protein following acrosomal exocytosis of sea urchin sperm, J. Biol. Chem. 273 (1998) 24355–24359.
[13] C. Beltrán, A. Darszon, P. Labarca, A. Liévano, A high-conductance voltage-dependent multistate Ca^{2+} channel found in sea urchin and mouse spermatozoa, FEBS Lett. 338 (1994) 23–26.
[14] J.S. Brusca, J.D. Radolf, Isolation of integral membrane proteins by phase partitioning with Triton X-114, Methods Enzymol. 228 (1994) 182–193.
[15] J. Morrisey, Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity, Anal. Biochem. 117 (1981) 307–310.
[16] P. Labarca, C. Santi, O. Zapata, E. Morales, C. Beltrán, A. Liévano, A. Darszon, A cAMP regulated K^+-selective channel from the sea urchin sperm plasma membrane, Dev. Biol. 174 (1996) 271–280.
[17] D. Sanchez, P. Labarca, A. Darszon, Sea urchin sperm cation-selective channels directly modulated by cAMP, FEBS Lett. 503 (2001) 111–115.
[18] J. Gorelik, Y. Gu, H.A. Spohr, A.I. Shevchuk, M.J. Lab, S.E. Harding, C.R. Edwards, M. Whitaker, G.W. Moss, D.C. Benton, D. Sanchez, A. Darszon, I. Vodyanoy, D. Klenerman, Y.E. Korchev, Ion channels in small cells and subcellular structures can be studied with a smart patch-clamp system, Biophys. J. 83 (2002) 3296–3303.
[19] A. Darszon, P. Labarca, T. Nishigaki, F. Espinosa, Ion channels in sperm physiology, Physiol. Rev. 79 (1999) 481–510.
[20] R. Gauss, R. Seifert, U.B. Kaupp, Molecular identification of a hyperpolarization-activated channel in sea urchin sperm, Nature 393 (1998) 583–587.
[21] S.B. Podell, G.W. Moy, V.D. Vacquier, Isolation and characterization of a plasma membrane fraction of sea urchin sperm exhibiting species-specific recognition of the egg surface, Biochim. Biophys. Acta 778 (1984) 25–37.
[22] M. Gonzalez-Martinez, A. Darszon, A fast transient hyperpolarization occurs during the sea urchin sperm acrosome reaction induced by egg jelly, FEBS Lett. 218 (1987) 247–250.
[23] A. Ludwig, X. Zong, M. Jeglitsch, F. Hofmann, M. Biel, A family of hyperpolarization-activated mammalian cation channels, Nature 393 (1998) 587–591.