Sea urchin sperm cation-selective channels directly modulated by cAMP

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Abstract Components of the sea urchin outer egg jelly layer such as speract drastically change second messenger levels and membrane permeability in sperm. Ion channels are deeply involved in the sperm–egg dialogue in sea urchin and other species. Yet, due to the small size of sperm, studies of ion channels and their modulation by second messengers in sperm are scarce. In this report we offer the first direct evidence that cation-selective channels upwardly regulated by cAMP operate in sea urchin sperm. Due to their poor selectivity among monovalent cations, channel activation in seawater could contribute to sperm membrane repolarization during the speract response. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Speract; cAMP; Hyperpolarization-activated cyclic nucleotide-gated channel; Sperm; Sea urchin

1. Introduction

The dialogue between sea urchin gametes is initiated by small peptides from the egg jelly layer. Speract, a decapeptide (Gly-Phe-Asp-Leu-Asn-Gly-Gly-Gly-Val-Gly) isolated from the *Hemicentrotus pulcherrimus* [1], *Strongylocentrotus purpuratus* and *Lytechinus pictus* [2] egg jelly, induces complex sperm plasma membrane ion permeability changes. Upon binding to its receptor, speract transiently activates a membrane guanylate cyclase increasing cGMP [3,4] and temporarily hyperpolarizes sperm. K^+ channels have been proposed to mediate this hyperpolarization [5–7], which stimulates Na^+/H^+ exchange [5] increasing intracellular pH (pH_i). Thereafter, the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is also transiently elevated causing sperm to depolarize [8]. Speract also stimulates a sea urchin sperm membrane adenylate cyclase that is modulated by pH_i, $[Ca^{2+}]_i$ and membrane potential [9–12].

Sea urchin sperm are quite small ($\sim$2 μm head diameter) and thus very difficult to patch clamp [13]. Swelling them in diluted seawater facilitates patching and sperm ion channel study. Swollen sperm preserve their $[Ca^{2+}]_i$, pH_i and E_m regulation and their response to speract [6].

Previous evidence suggested the presence of cation-selective channels activated directly by cAMP in sea urchin sperm [14,15]. Here, we report the first direct recording of cation-selective channels upwardly regulated by cAMP in swollen sperm of *S. purpuratus* and *L. pictus* sea urchins.

Using the cell-attached configuration of the patch-clamp technique, we recorded two different single channel conductances of 31 and 43 pS. Both conductances are weakly voltage dependent, monovalent cation permeable and modulated by membrane permeable analogues of cAMP. Possibly one channel with two states may explain the two main conductances observed. The protein kinase inhibitor H7 does not inhibit channel activation by db-cAMP, suggesting a direct modulation by the cyclic nucleotide. The channel is only about 3 times more permeable to K^+ than to Na^+. As a consequence of its selectivity, activation of this channel under physiological conditions would lead to sperm depolarization due to Na^+ influx. The potential role for this depolarization in the speract response is discussed.

2. Materials and methods

2.1. Biochemical and biological material

Inorganic salts, db-cAMP, 8Br-cAMP, 8Br-cGMP and IBMX, were from Sigma (St. Louis, MO, USA). H7 was from Calbiochem (La Jolla, CA, USA) and synthetic speract was from Peninsula Laboratories (Belmont, CA, USA). *S. purpuratus* and *L. pictus* sea urchins were from Marinus (Long Beach, CA, USA). Sperm were obtained as previously described [5]. The sperm suspension was diluted 1/60 in low Ca^{2+} artificial seawater (ASW) containing in mM: 486 NaCl, 10 KCl, 26 $MgCl_2$, 30 mM $MgSO_4$, 1 $CaCl_2$, 0.1 EDTA, 2.5 $NaHCO_3$, 10 HEPES-Na, pH 7.0 (955 mOsm).

2.2. Patch-clamp experiments

Sperm were swollen as described [6], using modified 10-fold diluted ASW containing in mM: 49 NaCl, 1 KCl, 25 $MgCl_2$, 1 $CaCl_2$, 0.1 EDTA, 10 HEPES-Na, pH 7.0 (155 mOsm). This solution was used in the bath chamber in all experiments. Patch pipettes of 25–65 MΩ resistance were filled with a solution containing 100 mM KCl, 50 mM NaCl, 5 mM EGTA, 10 mM HEPES-Na, pH 7.0. Sperm were visualized under phase contrast microscopy and patch clamped in the cell-attached configuration with an Axopatch-200A amplifier (Axon Instruments, Foster City, CA, USA). Single channel currents were digitized at 5 kHz and filtered on line at 2 kHz. The inhibitors and cyclic nucleotides were perfused changing the whole bath solution. All experiments were performed at 20°C.

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Abbreviations: AR, acrosome reaction; ASW, artificial sea water; 8Br-cAMP, 8-bromoadenosine 3′:5′-cyclic monophosphate; 8Br-cGMP, 8-bromoguanosine 3′:5′-cyclic monophosphate; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; db-cAMP, N^2,2′-*O*-dibutyryladenosine 3′:5′-cyclic monophosphate; V_m, transmembrane potential; V_c, command potential; V_r, resting membrane potential; V_{rev}, reversal potential; HCN, hyperpolarization-activated cyclic nucleotide-gated; H7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; IBMX, 3-isobutyl-1-methylxanthine; pH_i, intracellular pH; SPIH, hyperpolarization- and nucleotide-activated channel from sea urchin sperm

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2.3. Data analysis

Single channel currents were filtered at 500 kHz and analyzed using pClamp v.6.0. The applied potential was transformed to a calculated membrane potential according to the cell-attached configuration convention. The relation between command potential (V_c) and transmembrane potential (V_m) is:

$$V_m = V_r - V_c$$

where V_r is the resting membrane potential [16]. A value of −50 mV was used for V_r in the calculations, as reported from membrane potential measurements with fluorescent probes in swollen sperm populations [17].

Relative ion permeability (P_{K^+}/P_{Na^+}) was calculated with the Goldman–Hodgkin–Katz equation [18]:

$$\frac{P_{K^+}}{P_{Na^+}} = \frac{[Na^+]_i \exp^{(zFV_{rev}/RT)} - [Na^+]_o}{[K^+]_o - [K^+]_i \exp^{(zFV_{rev}/RT)}}$$

where z is the valence of the ions, F is the Faraday constant, R is the gas constant, T is the temperature in Kelvin degrees and V_{rev} is the reversal potential. V_{rev} was estimated from the average current–voltage (I–V) curve of the single channel transitions from five experiments.

3. Results

3.1. 'Cell-attached' recording of cation-selective channels in swollen sea urchin sperm

Sea urchin sperm have a collection of ionic channels that participate in their physiological responses to egg jelly components. Mostly these ion channels have been studied with fluorescent dyes in sperm and in membrane vesicles or incorporating sperm plasma membranes into planar lipid bilayers [8]. To overcome the difficulties of patch clamping sea urchin sperm (~3% success rate) [13], the cells can be swollen in diluted ASW. This strategy allowed the detection of a 5 pS K^+ channel activated by speract [6].

Here we have further documented that in swollen sperm (~4 μm diameter) the success rate for obtaining high resistance seals increases to 33.5% (789 patches from 2356 attempts). Single channel activity was detected in 37% (292) of the high resistance seals.

3.2. Channel activation by db-cAMP

Failure to obtain whole cell or excised patch recordings in swollen sperm forced us to use membrane permeable cAMP analogues, like db-cAMP and 8Br-cAMP, to test the influence of cyclic nucleotides on the ion channels we record on cell-attached patches. Fig. 1A illustrates a channel that is modulated by db-cAMP. The channel's open probability (P_o) increased after perfusion of 300 μm of db-cAMP. Fig. 1B summarizes the external cyclic nucleotide dose dependence of channel activity. At 300 μm db-cAMP, the P_o of the channel, estimated at zero applied voltage, increased 5.1-fold on average, from 0.3 ± 0.08% to 1.53 ± 0.47% ($n = 6$). At 1 mM db-cAMP, P_o increased 11.6-fold, to 3.5 ± 0.9% ($n = 2$) (Fig. 1B). The dotted line is the best fit of the function:

$$f/f_0 = 1 + (f_{max}([\text{db-cAMP}]/[\text{db-cAMP}] + K_d))$$

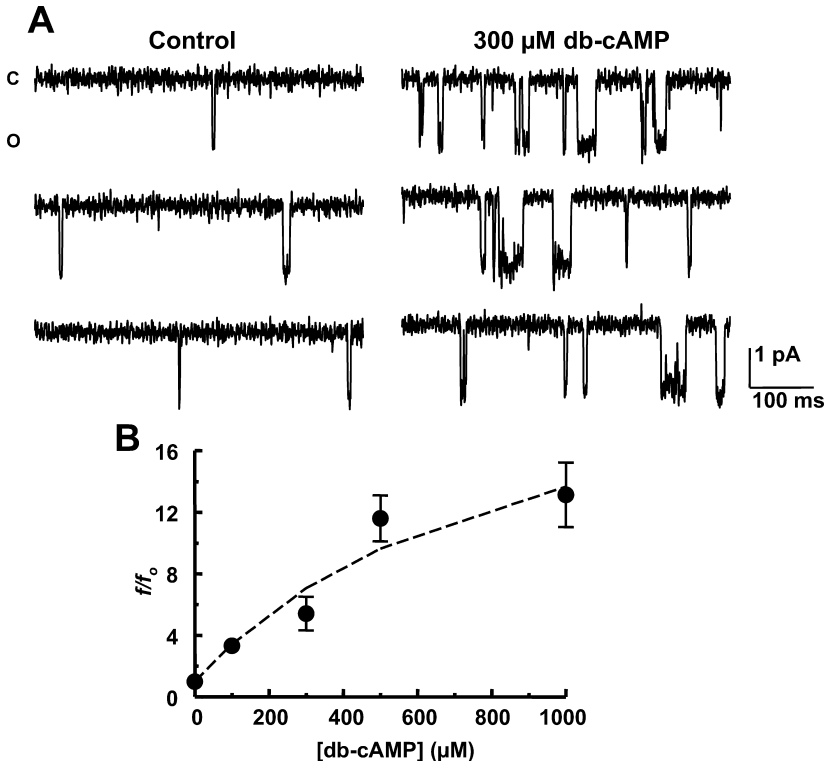

Fig. 1. db-cAMP upward modulation of a cationic channel from swollen sea urchin sperm. A: Representative traces of steady state single channel activity are shown at control conditions (left) and db-cAMP (right). Single channel currents were recorded at zero applied voltage. Downward deflections indicate channel openings (O) from the closed state (C). The applied potential was transformed to the membrane potential as indicated in Section 2. The bath and pipette solutions are mentioned in Section 2 (B). Dose dependence study of channel P_o as a function of [db-cAMP]. Filled circles represent the ratio f/f_0 where f is the fraction of open time at any [db-cAMP] and f_0 the fraction of open time without db-cAMP, that was used as a normalized parameter to assay the effect of nucleotide on open probability. Each point represents the average open probability ± S.E.M. obtained from 2–6 experiments.

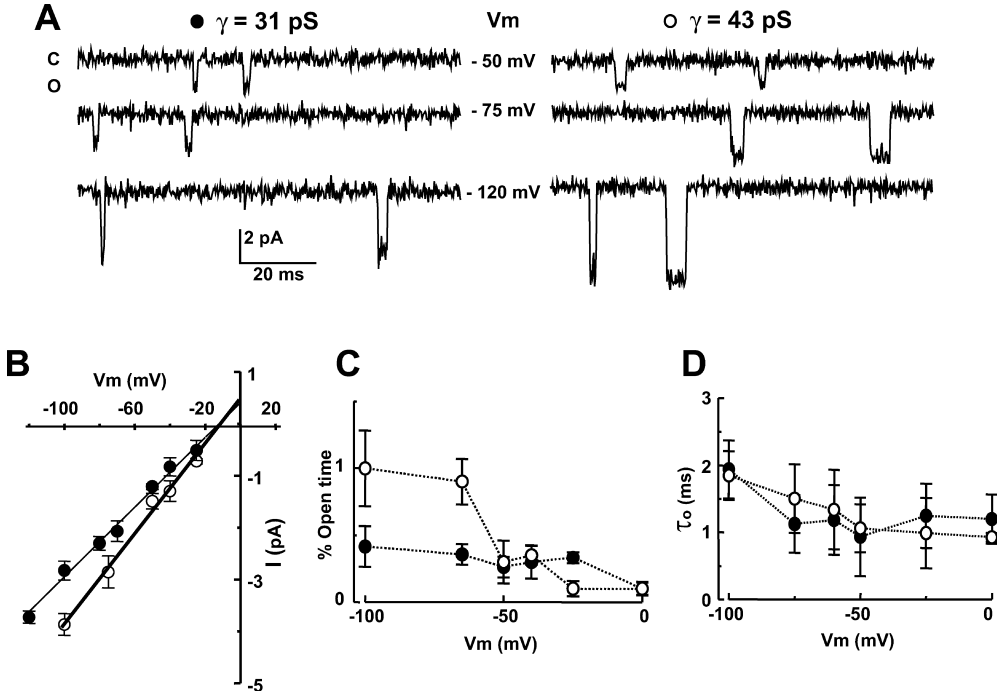

Fig. 2. Thirty-one and 43 pS cationic conductances in cell-attached patches from swollen sea urchin sperm. A: Representative single channel recordings at different membrane potentials under the same conditions as in Fig. 1. The left panel corresponds to the 31 pS conductance (filled circles in B,C,D) and the right panel to the 43 pS conductance (open circles in B,C,D). B: Current–voltage relationship for both conductances obtained from amplitude histograms at various applied voltages that were fitted with Gaussian distributions. The slope conductance and V_r were estimated from the linear fit to Ohm's equation (solid lines). C: P_o as a function of membrane potential, the fraction of open time (% open time) was calculated from the event list at each voltage. D: Open time duration (τ_o) as function of membrane potential. The mean open time was estimated from the distribution of open times at different potentials. Each point in B, C and D is the average from five different experiments and the bars are ±S.E.M. The lines joining experimental points C and D were drawn by eye.

to the experimental data. In this equation, f is the fraction of open time at any db-cAMP concentration, f_0 is the fraction of open time in the absence of the nucleotide, f_{max} is the value of f/f_0 at very large [db-cAMP] and K_d is the apparent dissociation constant. Under these conditions K_d (876 μm) is overestimated since the intracellular concentration of db-cAMP is much lower than that added externally. 8Br-cAMP increased the activity of these channels in the same way as db-cAMP (not shown). In contrast, 8Br-cGMP, an analogue for cGMP, at concentrations >200 μm did not influence the activity of the channels (not shown).

3.3. Characterization of the ionic conductances activated by db-cAMP

Fig. 2A shows steady state single channel recordings at different membrane potentials of the two main conductances modulated by db-cAMP in the absence of the analogue. Excursions between closed and open states of both conductances have fast kinetics; spike shaped transitions are frequently observed.

In our conditions, one of the main conductances (Fig. 2A right) was of 43 ± 1.7 pS, calculated from the linear fit of the I–V relation from five different experiments (Fig. 2B). The potential at which the current inverts (reversal potential) was −11.7 ± 1.7 mV, indicating cation selectivity. The channel responsible for these transitions displays a very low open probability (P_o) at the tested applied voltages ($P_o < 1\%$) and is only weakly voltage dependent (Fig. 2C). The fraction of time the channel spends in the open state depends slightly on the membrane potential. On the other hand the mean open time (τ_o), estimated from the distribution of the open times, was essentially independent of membrane potential in the physiological range (Fig. 2D). The other main conductance found was of 31 ± 1.2 pS (Fig. 2A left). The linear fit of the I–V curve yields a reversal potential of −12.8 ± 2.1 mV, again indicating cation selectivity (Fig. 2B). The open probability of this transition is also very low ($P_o < 1\%$) (Fig. 2C) and shows only a small voltage dependence in the 0 to −100 mV range (Fig. 2D).

The permeability ratio, P_{K^+}/P_{Na^+}, estimated as explained in Section 2, was low for both the 31 and the 43 pS conductances (3.5 and 2.8, respectively) indicating that they only modestly select between K^+ and Na^+.

As we can see, the two conductances reported here are very similar; they are cation selective, have a low open probability and are only slightly voltage dependent. Though we rarely found both conductances in the same recording, their shared properties could suggest that one channel with two sub-conductance states is responsible for them. On these grounds, in further analysis results from either conductance were pooled as if they belonged to the same channel protein.

3.4. Channel activation by cAMP

The intracellular concentration of cAMP, at any moment, is a balance between its synthesis by adenylate cyclase and its degradation by phosphodiesterases. IBMX, a phosphodiesterase inhibitor, elevates the intracellular concentration of cAMP in swollen sea urchin sperm [11,12]. Consistent with the idea that cAMP regulates the sea urchin sperm channel recorded here, perfusion of 100 μm IBMX significantly increased P_o

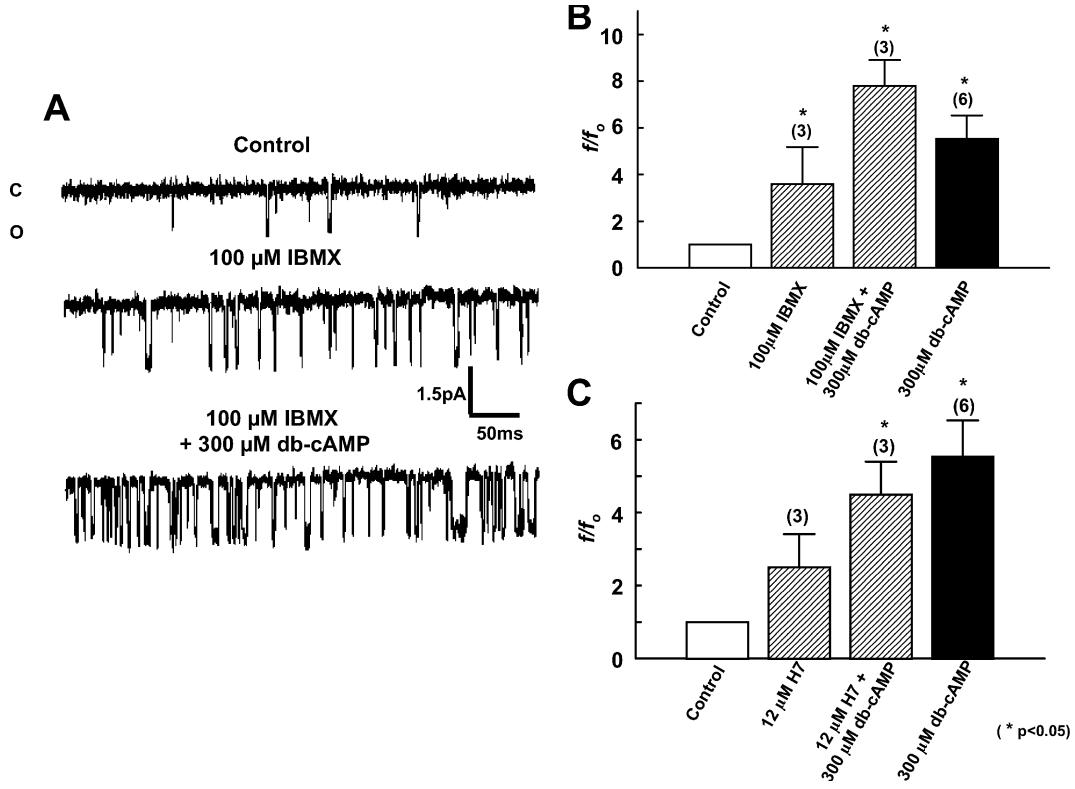

Fig. 3. Modulation of cationic channels from swollen sea urchin sperm by IBMX and H7. A: Single channel currents recorded as in Fig. 1. Representative traces of steady state single channel activity in control, IBMX or IBMX plus db-cAMP perfusion are shown. B: IBMX, db-cAMP and IBMX+db-cAMP increase P_o. C: H7 causes a non-significant increment in P_o and does not affect the P_o increase induced by db-cAMP. Each bar represents the ratio f/f_0 where f is the fraction of open time in the presence of the tested compound and f_0, the fraction of open time without any treatment. f/f_0 was used as a normalized parameter to assay the effect of different compounds on P_o. The bars represent ± S.E.M. The numbers in parentheses above the bars indicate the number of experiments.

(Fig. 3A). In these experiments the effects of IBMX and db-cAMP on both conductances were pooled. The P_o increment induced by this IBMX concentration was equivalent to that caused by 300 μm db-cAMP alone (Fig. 3B). As expected, perfusion of 300 μm db-cAMP in the presence of 100 μm IBMX caused a more important increase in P_o (Fig. 3B).

3.5. H7, a protein kinase inhibitor, does not inhibit db-cAMP channel activation

Channel activation by cAMP could be due to direct binding of the nucleotide to the channel or indirectly by inducing a change in its phosphorylation state. We used a high concentration of H7, which inhibits protein kinases A, C and G, to study their participation in the cAMP-induced activation of the channels. The increase in channel P_o caused by db-cAMP was not influenced by H7 (Fig. 3C). Addition of 12 μm H7 alone caused a small and not statistically significant increase in channel P_o (Fig. 3C), which could be explained if the channel is also modulated by its phosphorylation state [21].

4. Discussion

Sperm are very small yet by swelling them we have been able to characterize cation channels using the on-cell patch-clamp technique. We found evidence for two main single channel conductances of 31 and 43 pS activated by cAMP permeable analogues and by IBMX. Both conductances are cation selective, with a $P_{K^+}/P_{Na^+} \sim 3$, have a low open probability and are only slightly voltage dependent. Though rarely present in the same recording, their biophysical properties suggest that one channel with two sub-conductance states is responsible for them. H7, an antagonist of several kinases did not inhibit channel activation by db-cAMP (Fig. 3C). Although this fact is consistent with a direct activation of channels by the cyclic nucleotide, it will be necessary to demonstrate this in isolated sperm membrane patches.

The channel characterized here has similarities with a K^+ channel upwardly regulated by cAMP incorporated to planar bilayers by fusing purified flagellar membrane fragments obtained from *S. purpuratus* [14]. The kinetics and selectivity, $P_{K^+}/P_{Na^+} \sim 4$, of both channels are practically indistinguishable. However, the cationic single channel conductances reported here are significantly smaller than the 103 pS conductance of the channel described in planar bilayers. The different conditions used to study channels in bilayers and on cell-attached patches may explain, at least in part, why their conductances do not match.

In 1998 Gauss et al. cloned the first cyclic nucleotide-gated channel from the sea urchin sperm [15]. When expressed in a heterologous system, this channel is weakly K^+ selective, activated by a hyperpolarization and by the direct action of cAMP. The channel, called 'hyperpolarization- and nucleotide-activated channel from sea urchin sperm' or simply SPIH, is mainly localized on the sperm flagellum [15] and was classified as a member of the family of HCN-gated channels [19]. Antibodies to SPIH also stain the flagella of *L. pictus* sperm (data not shown).

The SPIH channel shares multiple characteristics with the

planar bilayer channel and those characterized in this work. It is only about 4 times more permeable to K^+ than to Na^+ and its open probability is directly increased by cAMP, and only weakly by cGMP. In the absence of cAMP, SPIH macroscopic currents inactivate, while in its presence they do not. The cation channels we record on cell-attached patches do not inactivate; it is likely that in our experiments there is a basal cAMP concentration in the cytoplasm. To the best of our knowledge this is the first direct recording of cAMP-regulated channels in sea urchin sperm.

The only HCN-gated channel that has been studied at the single channel level is the HvHCN channel [19,20]. It was cloned from the silkmoth antenna and is activated by cAMP and hyperpolarization. The HvHCN channel has a single channel conductance of 30 pS, is cationic and displays a $P_{K^+}/P_{Na^+} \sim 3$ [20]. Its properties match markedly well those of the channel characterized here.

The SPIH channel is blocked by external μm Cs^+ [15] while the K^+ channel modulated by cAMP in bilayers is blocked by external mM TEA^+ and Ba^{2+} [14]. Though the cell-attached configuration is not ideal for ion channel pharmacology using non-permeable compounds, 10 mM Cs^+ in the recording pipet failed to block the cation channels (data not shown). This finding suggests that the cation channels recorded in the present work may not be due to SPIH. However, it is possible that when expressed in heterologous systems some properties of SPIH channels may be different than in the sea urchin sperm. In addition, the ionic conditions are very different in the two systems. Conclusions about the identity, regulation and pharmacology of these channels require developing strategies that allow the whole cell and excised patch configurations. Spermatogenic cells may be an alternative, as it has for mammalian sperm [22,23].

Speract increases cGMP levels [9] and activates K^+-selective channels that hyperpolarize sperm [5,7]. The hyperpolarization is followed by a Na^+ dependent repolarization and Ca^{2+} dependent depolarization [8,15]. In addition, the hyperpolarization stimulates a pH_i increase and adenylate cyclase is activated, elevating the levels of cAMP [11,12]. The cation channels described here would be activated by the hyperpolarization and by the increase in cAMP levels induced by speract. Considering that this channel displays a $P_{K^+}/P_{Na^+} \sim 3$, its opening under physiological conditions ($\sim$0.5 M external NaCl) would allow Na^+ influx and sperm depolarization [14]. This channel could therefore contribute to repolarize sperm after the hyperpolarization induced by speract and explain the Na^+ dependence of this event [15].

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