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A high-conductance voltage-dependent multistate Ca^{2+} channel found in sea urchin and mouse spermatozoa

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Abstract

Ion fluxes through poorly understood channel-mediated mechanisms participate in the interaction between spermatozoa and egg. Previously, we reported the characterization in planar bilayers of a high conductance Ca^{2+}-selective, voltage-dependent multistate channel from *S. purpuratus* sea urchin sperm plasma membranes [14]. Here we show that this ion channel can be directly transferred to planar lipid bilayers upon sperm addition, from sea urchin (*S. purpuratus* and *L. pictus*) and from mouse. We found that spermatozoa from these species posses a conspicuous Ca^{2+}-selective, high conductance, multi-state, voltage-dependent channel, which displays similar voltage dependence and equal PBa^{2+}/P_K^+ ~4 in the three species. The presence of this Ca^{2+} channel in such diverse species suggests it plays a relevant role in sperm physiology. The high sensitivity of planar bilayers to detect single ion channels can now be used to study ion channel regulation and gamete interaction.

Key words: Spermatozoa; Sperm ion channel; Ca^{2+} channel

1. Introduction

Ion fluxes are fundamental in the response of spermatozoa to the egg [1,2]. It is now well established for many species, particularly sea urchins [3,4] and mammals [2,5], that an elevation of intracellular $[Ca^{2+}]_i$, dependent on external Ca^{2+}, is required for the sperm acrosome reaction (AR) to occur. This process is necessary so that spermatozoa can fuse and activate the egg in many species [1,2,6]. Pharmacological evidence indicates the participation of voltage-dependent Ca^{2+} channels in the response of spermatozoa to agonists that induce the AR [3,5,7,8]. Recently, we obtained evidence also that Ca^{2+} channels participate in the response of sea urchin sperm to speract, an egg peptide that induces changes in $[Ca^{2+}]_i$, membrane potential, and intracellular pH (pH_i) [9].

Characterization of the electrophysiological properties of spermatozoa has been precluded by their small size [10]. Even though sea urchin sperm can now be swollen and more easily patch clamped [11] than the normal cells [10], thus far whole cell or excised patch recordings have not been reported. Due to their importance in sperm physiology we have focused our attention on Ca^{2+} selective channels, studied by the planar bilayer technique, which although indirect, is endowed with an exquisite sensitivity for single or multichannel detection, and allows continuous access to both membrane faces [12,13].

We have previously characterized a high conductance Ca^{2+}-selective, voltage-dependent, multi-state channel from isolated *S. purpuratus* sea urchin sperm plasma membranes fused to planar bilayers [14]. Here we show that an analogous Ca^{2+} channel can be incorporated in planar bilayers directly using spermatozoa from two sea urchin species *S. purpuratus* and *L. pictus*. A similar channel was observed either using isolated membranes or whole spermatozoa from mouse. The occurrence of this Ca^{2+}-selective channel in spermatozoa from widely diverse species indicates that it could play an important role in sperm physiology.

2. Materials and methods

Artificial sea water (ASW) contained in mM: 486 NaCl, 10 KCl, 26 $MgCl_2$, 30 $MgSO_4$, 10 $CaCl_2$, 2.4 $NaHCO_3$, 0.1 EDTA, HEPES 10, pH 8.0. Spawning was induced in adult *S. purpuratus* and *L. pictus* by intracoelomic injection of 0.5 M KCl, and semen was collected undiluted with a Pasteur pipette. The AR was assayed using phase contrast microscopy, and egg jelly or the AR-inducing factor were obtained as previously described [15].

Sea urchin plasma membranes were isolated [16] and preloaded with 0.4 M sucrose by overnight incubation at 4°C to provide the osmotic gradient which favors fusion with planar bilayers [17]. Preloaded vesicles were stored in small aliquots at −70°C until use.

Mouse sperm membrane isolation, based on Gillis et al. [18], was as

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follows: CD-1 mouse sperm were collected at 36°C by incubating (20 min) the incised caudae epididymides from 50 adults in 25 ml of buffer A (in mM: 4 KCl, 140 NaCl, 10 glucose, 25 HEPES, 4 EGTA pH 7.0). Spermatozoa were sieved through a nylon mesh, pelleted (5 min at 1,000 × *g*, 22°C) and resuspended in buffer A (1 ml) to overlay them on a 25 ml pre-formed (10 min at 12,000 × *g*) 50% Percoll gradient in buffer A. The gradient was centrifuged (5 min at 7,750 × *g*, 22°C) and the purified sperm collected in the upper band and washed in buffer A (20 ml) by centrifugation (5 min at 1,000 × *g*, 4°C). The pelleted cells were resuspended in buffer A (20 ml, plus 1 mg/ml soybean trypsin inhibitor and 0.1 mM PMSF) and subjected to nitrogen cavitation (4°C) at 500 psi (10 min) in a Parr bomb (Parr Instrument Co., Moline, IL). The cavitated suspension was pelleted (10 min at 7,750 × *g*) and plasma membranes collected from the supernatant (35 min at 200,000 × *g*). The membrane pellet was resuspended in 200 μl of buffer B (in mM: 150 NaCl, 50 Tris-HCl, pH 7.0) and overlayed on a discontinuous density gradient (4.5 ml of 0.4, 0.8, 1.2, 1.6 and 2.0 M sucrose, 0.1 mM PMSF, 25 mM HEPES, pH 7.0). The gradient was centrifuged (SW-50.1 rotor, 4 h at 135,000 × *g*, 4°C). The two membrane bands were washed separately in buffer B (10 ml), recovered by centrifugation (30 min at 200,000 × *g*, 4°C), preloaded with 0.4 M sucrose, 5 mM HEPES pH 7.0 and stored as described for sea urchin sperm plasma membranes.

Black lipid membranes were formed according to Müeller and Rudin [19], from synthetic diphytanoylphosphatidylcholine (DPPC) or PE/PS 1:1 (Avanti Polar Lipids, Birmingham, AL) dispersed in n- decane (20 mg/ml) on a 200 μm diameter hole in a plastic partition as previously described [14]. The membrane potential (E_m) was applied at the *cis* side of the chamber, where spermatozoa or membrane vesicles were added, while the other side (*trans*) was held at virtual ground by a Dagan 3900 patch clamp (Minneapolis, MN) in the mixed RC mode. Ag/AgCl electrodes were connected trough 3 M KCl agar bridges to the voltage source (*cis*) and to the *I–V* transducer (*trans*). The ionic currents were filtered by the *I–V* transducer's internal four pole Bessel filter, at a −3 db cutoff frequency of 200 Hz, and were digitized at 1 kHz with the Axotape software (Axon, Foster city, CA). After formation, membrane vesicles (30–50 μl, ~17 μg protein/ml final) or a fifty-fold dilution of normal or swollen spermatozoa (50–100 μl, ~100 μg protein/ml final) were added to the *cis* chamber. Channel insertion was usually detected within 5 minutes by a sudden change in the conductance level and/or by the appearance of single channel transitions. The solutions used are indicated in the figure legends.

S. purpuratus sperm were added to the *cis* side of preformed planar lipid bilayers in ASW *cis* and HEPES-K 0.5 M *trans*, and *L. pictus* sperm were swollen in 25 mM $BaCl_2$, 125 mM KCl, 5mM HEPES pH 8.0 and added to the *cis* chamber of a planar bilayer made in 25 mM $BaCl_2$, 125 mM KCl, 5 mM HEPES pH 8.0 *cis*, and 25 mM $BaCl_2$, HEPES 5 mM pH 8.0 *trans*. Under phase contrast microscopy they appeared as spheres similar to those found by diluting sea water tenfold [11].

3. Results and discussion

Direct addition of *S. purpuratus* spermatozoa to planar lipid bilayers only occasionally yielded Ca^{2+} channel activity (1/18 experiments). We have shown that during the AR two pharmacologically distinguishable Ca^{2+} channels are activated [8]. Therefore, to improve the frequency of incorporation of Ca^{2+}-selective channels to the planar bilayer, sperm cells were exposed to homologous egg-jelly or the isolated factor which induce the AR. Addition of egg-jelly exposed spermatozoa to 47 bilayers yielded single channel activity of various types in 17 cases, from these 4 were Ca^{2+} channels. Insertion of an identical channel into planar bilayers was described previously by our group [14] upon fusing purified plasma membrane fragments from this same sea urchin into lipid bilayers. Current traces of this channel obtained at different E_m are shown in Fig. 1. The only obvious difference between channels incorporated from the cells and from isolated plasma membranes [14] is an inversion in the sidedness indicated by their opposite voltage-dependence. The channels incorporated from cells are fully-open at negative voltages.

Since it has been shown that osmotic gradients enhance fusion (for review see [13]), we also explored whether osmotically swollen sperm from another sea urchin species could also transfer ion channels to the planar bilayer. *L. pictus* spermatozoa swollen by resuspension in 25 mM $BaCl_2$, 125 mM KCl, 5 mM HEPES, pH 8.0 (see Section 2) and exposed to the egg jelly, transferred large, perfectly cation-selective channels to the bilayer. The channels exhibit properties similar to those of the large Ca^{2+} channel from *S. purpuratus*, namely: selectivity for divalents, voltage-dependence, multiple open conductance substates and blocking by Co^{2+} ions. These characteristics are summarized in Figs. 2 and 3. The left side of Fig. 2 shows current records from a bilayer with two channels in 125 mM KCl, 25 mM $BaCl_2$, 5 mM HEPES, pH 8.0 *cis*, and 25 mM $BaCl_2$, 5 mM HEPES, pH 8.0. The *L. pictus* channel spends most of the time in a fully open main state, with occasional closings to less conducting states at negative applied voltages. At positive voltages, the channel exhibits several open substates. Under the experimental conditions used the *I–V* relation (Fig.2 right side), built with the amplitudes corresponding to the fully-open, main conducting state, yields a slope conductance of 230 pS, and a reversal potential of −10 ± 2 mV (n = 3), from which a permeability ratio P^{2+}_{Ba}/P^{+}_{K} = 3.5 could be estimated [13,14]. This permeability ratio indicates that the channel selects for

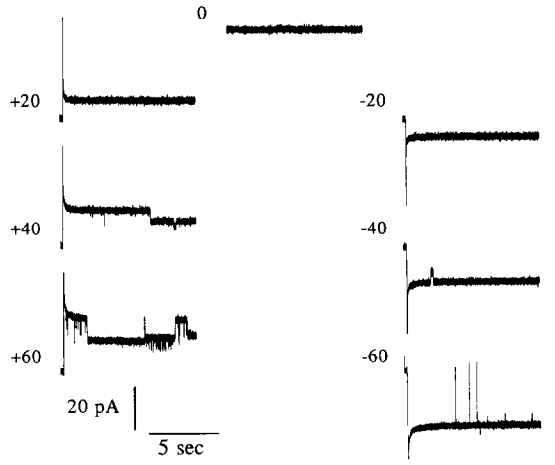

Fig. 1. Current records of Ca^{2+} channels transferred to lipid bilayers directly using *S. purpuratus* sperm (see Section 2). The channel was incorporated into a DPPC bilayer in ASW in the *cis* side and 500 mM HEPES-K in *trans*, pH 8.0. Numerical values at the left side on each record are the applied E_m in mV. Zero current level is indicated by a dotted line in each record and the current and time scales are at the bottom.

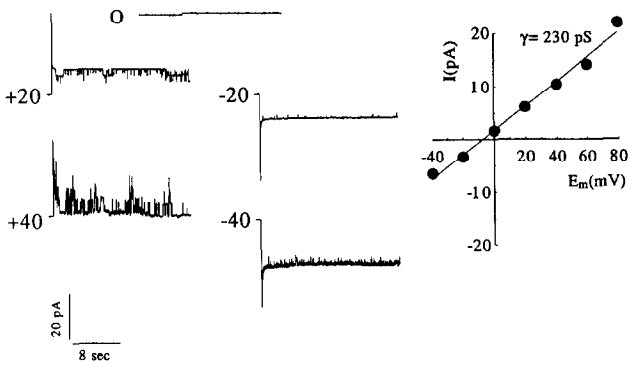

Fig. 2. Current records (left) and *I–V* curve (right) of Ca^{2+} channels transferred by swollen *L. pictus* sperm to a PE/PS bilayer. Current traces correspond to a two channel bilayer in KCl 100 mM *cis*, $BaCl_2$ 25 mM *trans*, pH 8.0. The *I–V* curve is from the fully open main conducting state of the channel.

divalent cations, similar to the Ca^{2+}-selective channel found in membranes from *S. purpuratus* [14].

As the Ca^{2+} channel from *S. purpuratus*, the Ca^{2+} channel from *L. pictus* is also blocked by mM Co^{2+} [14]. Fig. 3 documents that Co^{2+} added to the *cis* bilayer chamber both reduces the channel conductance of the substates and increases the residence time in the open states. The left panel shows current records and the right panel, the relative current of the most frequent substate conductance as a function of $[Co^{2+}]_{cis}$. As illustrated in the right panel, the Co^{2+} block of the sperm Ca^{2+} channel is weakly voltage dependent since its effect is only slightly higher at positive than at zero voltages. A full evaluation of the effect of Co^{2+} is currently under way in our laboratory.

The presence in the sperm membrane of two different sea urchin species of Ca^{2+}-selective channels, exhibiting very similar properties, suggests an important physiological role for these channels. In view of this, it was of interest to carry on studies addressed at determining whether the presence of such conspicuous channels is restricted to echinoderm spermatozoa. For this purpose, we proceeded to fuse plasma membrane fragments purified from mouse sperm to planar lipid bilayers (Fig. 4). We found that fusion inserts to the planar lipid bilayer Ca^{2+}-selective channels exhibiting properties that closely resemble those of sea urchin sperm. The channel displays similar voltage-dependence, conductance substates, and a linear *I–V* relationship for the main conducting fully open channel, giving a slope conductance of 381 pS in 200 mM KCl, 5 mM HEPES pH 8.0 *cis*, 25 mM $BaCl_2$, 5 mM HEPES, pH 7.6, with a reversal potential $E_r = -9$ mV, indicating a $P^{2+}_{Ba}/P^{+}_{K} = 4$. The channel inserts also into planar lipid bilayers by direct exposure to mouse spermatozoa (not shown).

The high conductance (235 pS in 25 mM $BaCl_2$), low selectivity for Ca^{2+} ($P^{2+}_{Ba}/P^{+}_{K} \sim 4$), and substate complexity of the channel we have recorded from sea urchin and mouse spermatozoa, are properties closer to those displayed by the ryanodine receptor [20], than L, N, T or P Ca^{2+} channels [21]. A similar Ca^{2+} channel has been reported in plasma membranes from boar sperm incorporated to planar bilayers [22]. The presence of the aforementioned Ca^{2+} channel in spermatozoa from phylogenetically distant species may argue that it plays an important role in the physiology of this specialized cell.

The fact that egg jelly increases the probability of detecting the sea urchin Ca^{2+} channel is consistent with findings with Fura-2 loaded spermatozoa indicating that Ca^{2+} channels are activated during the AR. This activation may involve an increase in pH_i, second messengers [8] and/or covalent modifications (proteolysis, phosphorylation, dephosphorylation, etc.). The requirement for activation suggests that ion channels are being transferred directly from the cell to the bilayer. Possibly the Ca^{2+} channel from mouse spermatozoa could be recorded from untreated cells since spontaneous AR is higher in mammalian sperm [5]. Preliminary results using nystatin (Liévano, Labarca and Darszon, unpublished), an antibiotic which forms unselective channels only in membranes containing sterols, as a monitor for cell plasma membrane fusion [23], point out that the channels are derived from the plasma membrane of spermatozoa. In addition, controls with supernatants from spermatozoa used under various conditions to incorporate channels did not significantly yield channel activity. Therefore, our results indicate that it is possible to transfer functional ion channels directly from sea urchin and mouse spermatozoa to planar bilayers.

Planar bilayers are sensitive detectors of single channel molecules. Thus, their incorporation from spermatozoa provides an interesting and informative model for gam-

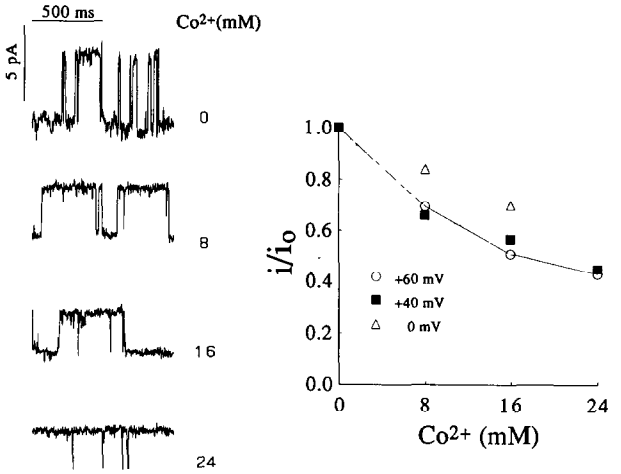

Fig. 3. Effect of increasing amounts of Co^{2+} added to the *cis* chamber on the most prominent substate appearing at positive E_m values. Numbers on the right side of each current record (left side) indicate the $[Co^{2+}]_{cis}$. The right side shows the relative current as a function of increasing $[Co^{2+}]$ added to the *cis* chamber at +60 mV (open circles) +40 mV (closed boxes) and 0 mV (open triangles) applied E_m.

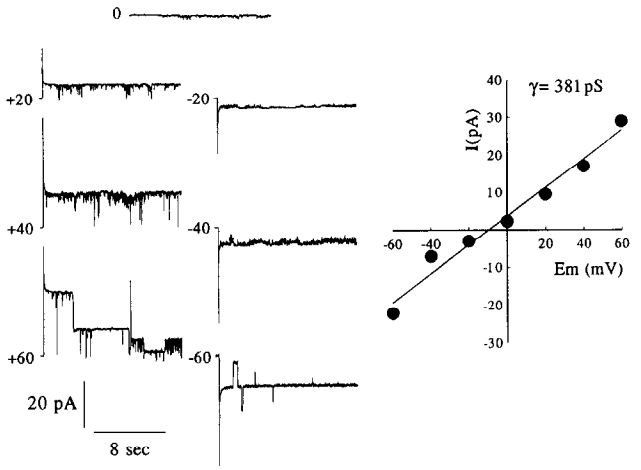

Fig. 4. Current records (left) and *I–V* curve (right) of Ca^{2+} channels from mouse spermatozoa plasma membranes fused to a lipid bilayer. Records obtained from the channel in KCl 200 mM *cis*, $BaCl_2$ 25 mM *trans*, 5 mM HEPES, pH 7.6. *I–V* curve was constructed as in Fig. 2.

ete interaction and perhaps fusion, a key event in fertilization and in many other fundamental cell processes [24]. The assay will be advantageous also in monitoring changes in ion channel activity following sperm activation and in channel studies in other small cells.

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