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IDENTIFICATION OF CALCIUM CONDUCTING CHANNELS IN ISOLATED BOAR SPERM PLASMA MEMBRANES

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Ion channel recordings were obtained from liposomes containing purified boar sperm plasma membrane proteins using a tip-dip method. Liposomes prepared in HEPES-TRIS and clamped by electrodes containing Ba-HEPES displayed channel activity that was partially inhibited by verapamil or nitrendipine and completely inhibited by La^{3+}. Reversal of current at pipette negative voltages was observed only when Ba^{2+} ions were also present in the bath solution. These data indicate that channels capable of carrying calcium currents are prominent components of the plasma membrane of mammalian sperm.

While the existence of ion-selective channels in the plasma membrane of invertebrate sperm appears to have been firmly established (1-6), much less is known about such channels in mammalian sperm. However, recent reports from several laboratories indicate that ion-selective channels may play a key role in mammalian gamete interactions. These studies have relied on the use of fluorescence probes to measure changes in internal Ca^{2+} and H^{+} concentrations during capacitation and after alteration of the composition of buffers in which sperm were suspended (7-9). In this communication we describe experiments, using patch clamp techniques, which demonstrate directly the presence of channels in the plasma membrane of boar spermatozoa that are capable of conducting calcium ions.

METHODS AND MATERIALS

Boar sperm plasma membranes were isolated by nitrogen cavitation of cauda epididymal sperm, obtained fresh from epididymides after slaughter, and purified by ultracentrifugation on sucrose gradients as we have previously

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described (10-11). Plasma membranes originate from all regions of the sperm surface at the cavitation pressure used (650PSI).

A simple procedure (12) was used to insert plasma membranes into liposomes. Phosphatidylethanolamine (PE) and phosphatidylserine (PS) from bovine brain (Sigma) were mixed (3:1) in 20 mM HEPES buffer containing 0.1 mM $CaCl_2$, pH 7.0 and 6-40 µg plasma membrane protein. This mixture was sonicated three times (15 second intervals) with a Bronson Sonifier at maximum power (4C°). The sonicate was frozen (-70C°) and thawed to room temperature at least three times before use. Some clamps were prepared without added inorganic ions (other than $CaCl_2$ need to induce proteoliposome formation) and were buffered with 20 mM HEPES-Tris only.

A Yale style patch clamp was used in conjunction with a Bessel filter. Data were filtered at 1kHz and sampled at 3kHz. Data were collected at either steady-state holding voltages or with a voltage ramp (± 100 mv, 200 mSec duration). An upward deflection represents current flow into the pipette. The voltage reported is the inside of the pipette with reference to the bath.

RESULTS

In our first series of experiments, we used liposomes prepared in the presence of 400 mM NaCl-HEPES, pH 7.0 and electrode filling solutions containing 0.2 M NaCl and 0.1 M $BaCl_2$. Typical conductance patterns obtained with these preparations are shown in Fig. 1.

Channel current increased in amplitude as the holding voltage was increased in either the positive or negative direction (Fig. 1a). The variation in the frequency of channel closings and current amplitude changes at each holding voltage indicated that more than one channel and probably more than one channel type was present in this clamp. The I-V plot shown in Fig. 1b describes more completely the conductance changes between +100 mV and -100 mV; the reversal potential is near 0 mV. It was not possible to determine precisely from this plot to what extent specific ions were contributing to the conductance pattern at all holding voltages.

Since our primary interest was to determine whether channels capable of conducting calcium ions were present in these membranes, we prepared liposomes in the absence of inorganic ions (except for the small amount of $CaCl_2$ needed to prepare them). Bath solutions were buffered with HEPES-TRIS and electrode filling solutions contained 0.1 M Ba-HEPES also buffered with HEPES-TRIS, pH 7.0. Channel activity was easily detected in these preparations with many displaying apparent single channel openings (Fig. 2). Conductance calculated

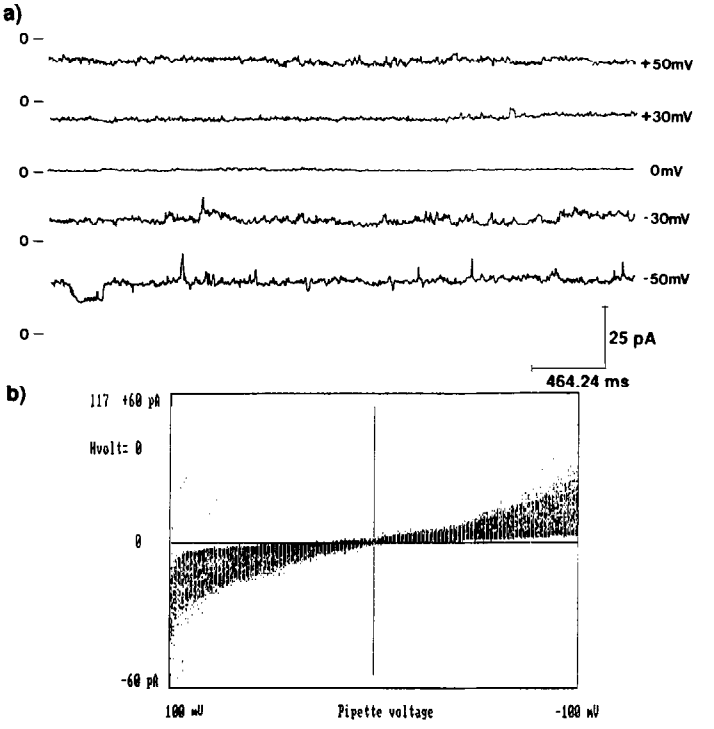

FIGURE 1: Channel conductances in proteoliposomes prepared in HEPES-buffered NaCl. Patch pipettes contained 0.2 M NaCl, 0.1 M $BaCl_2$ and 20 mM Na-HEPES, pH 7.0. The bath solution (liposomes) contained 400 mM NaCl, 20 mM HEPES-TRIS and 20 µg/ml plasma membrane protein. Tracings in a) show conductances at different holding voltages. Zero channel current positions for each trace are indicated along the left-hand margin. An I-V plot for these data is shown in the lower panel. All of the data points from 20 200 mSec ramps are plotted.

as the current amplitude divided by the holding voltage was in the range of 10-20 pS. Other patches examined showed these characteristic small openings as well as larger transitions (Fig. 3a). Estimates of conductance here were

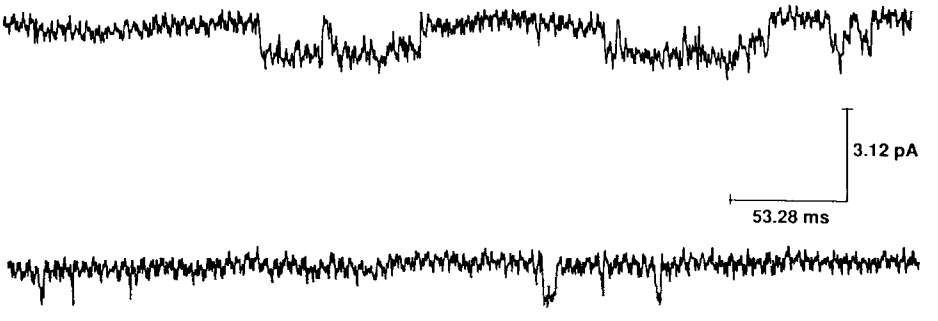

FIGURE 2: Channel conductances in proteoliposomes prepared in the absence of monovalent inorganic cations. Sample tracings are shown at +100 mV (inside) holding voltage. Patch pipettes contained 0.1 M Ba-HEPES and 20 mM HEPES-TRIS, pH 7.0. Proteoliposomes were buffered in 20 mM HEPES-TRIS.

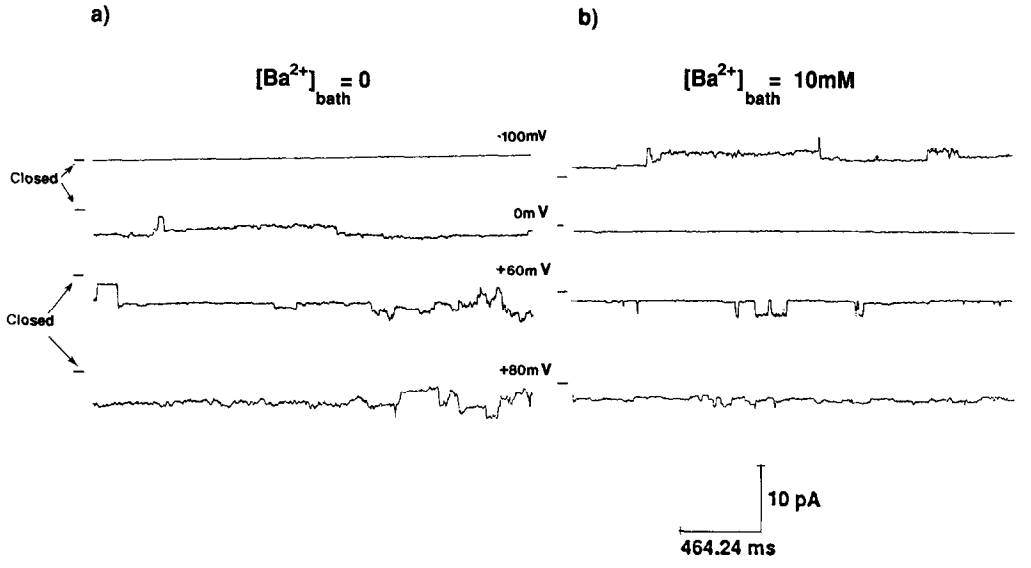

FIGURE 3: Ba^{2+} conductances at various holding voltages in the absence (Panel a) and presence (10 mM, Panel b) of Ba^{2+} in the bath. The composition of the electrode filling solution is the same as in Figure 2.

in the 50-60 pS range. Patches typically contained one to two larger channels and several smaller ones. When Ba^{2+} was present only in the electrode solution, openings were observed only at positive pipette holding voltages; no current was observed at voltages as high as -100 mV (Fig. 3a). When Ba^{2+} (10 mM) was added to the bath, current reversal was observed at negative potentials (Fig. 3b); I-V plots indicated a reversal potential near-25 mV. This indicated that the channels observed were conducting only Ba^{2+} (i.e. capable of conducting divalents). When Ca-HEPES was used in place of Ba^{2+}, similar conductance patterns were obtained.

The phenylalkylamine calcium channel blocker verapamil, and the dihydropyridine, nitrendipine, inhibited conductance of Ba^{2+} ions (Fig. 4). Verapamil induced rapid channel closings when first added to the bath (Panel a, trace b), but this effect was short-lived, and conductances often returned to near control levels (trace c). Nitrendipine reduced channel conductances

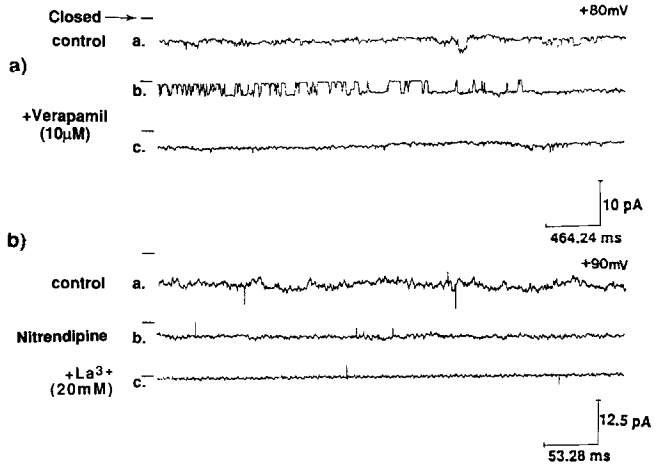

FIGURE 4: Effect of various Ca^{2+} channel antagonists on Ba^{2+} conductances.

a) Trace a: control; traces b and c: after verapamil addition. Note that while verapamil decreased channel conductance, complete closings were short-lived. Holding voltage was +80 mV.

b) Trace a: control; trace b: after addition of nitrendipine (11 μM). Trace c: after La^{3+}. Nitrendipine inhibits conductance but La^{3+} abolishes it. Solutions were prepared as in Figure 2. The spikes are an artifact. Holding voltage was +90 mV.

but did not abolish them in some patches (Panel b, trace b). In others nitrendipine had no apparent effect; La^{3+} ions at concentrations above 0.5 mM always abolished channel activity (Panel b, trace c).

DISCUSSION

The experiments described in this report provide the first direct evidence that ion channels are present in the plasma membrane of mammalian spermatozoa. Although sodium, potassium, and chloride channels may be present, we focused on the characterization of divalent-conducting channels. The channels we found: 1) conduct both barium and calcium, 2) reverse only when barium is present on both sides, 3) have conductances in the range of 10-20 pS and larger, 4) are partially blocked by verapamil and nitrendipine and completely blocked by La^{3+}. These characteristics meet many of the criteria for defining a calcium channel (13). These studies are complicated

in that we generally get more than one channel per patch and often more than one channel type.

Blocker studies have provided some insight into the nature of these channels. La^{3+} blocked all activity as observed for most types of calcium channels. Nitrendipine reduced current levels in some but not all preparations. The frequency of closings was increased by verapamil, but this effect was short-lived. This general behavior suggests that several types of channels very likely exist in boar sperm plasma membranes. One type resembles L-channels found in abundance in skeletal muscle (sensitive to nitrendipine). Others may represent a larger calcium conductance described in sea urchin sperm (15). This diversity is very likely a reflection of the regional heterogeneity of the sperm surface and the different functions of its several domains (14).

Patch clamp techniques have been used to detect K^+ and Cl^- channels in plasma membranes from sea urchin sperm (2) and recently a Ca^{2+}-conducting channel has also been detected in this species (15). Moreover, phenylalkylamine and nitrendipine receptors have been detected in the head and flagellar membranes of sea urchin sperm (1) and organic channel antagonists have been shown to block the acrosome reaction (5,6). In mammalian sperm far less is known about ion channels, but recent studies by Lee and Storey (16) indicate that the last phase in the sequence of events that lead to the acrosome reaction in the mouse appears to be a rapid influx of Ca^{2+}. Based on the effects of H^+,K^+ and Ca^{2+} channel blockers on internal calcium levels, Babcock and colleagues have suggested that selective increases in Ca^{2+} uptake during capacitation in bull sperm involves activation of voltage-dependent Ca^{2+} channels possibly under the control of a G protein (7,8,17). Solubilized zonae induce the acrosome reaction in bull sperm (8) and pertussis toxin blocks the rises of internal Ca^{2+} and pH that are a part of this signal pathway (9). It is therefore tempting to speculate that zonae-gated (organic channel antagonist-insensitive) Ca^{2+} channels may also be part of this

pathway. While this speculation may be premature, this report does provide the first direct evidence that Ca^{2+} channels are prominent components of the mammalian sperm plasma membrane and presents the opportunity to begin a description of the biochemical mechanisms involved in their control.

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