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Lithium and phorbol ester modify the activating capacity of ascidian spermatozoa

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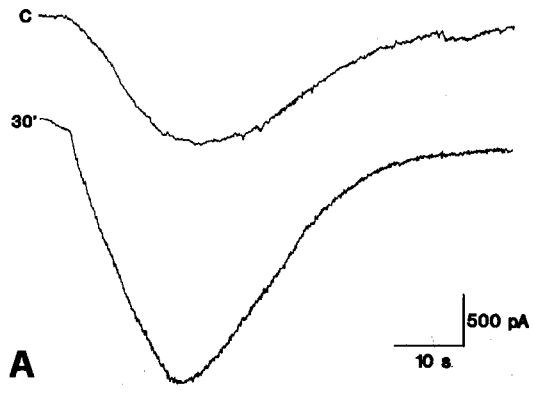
Abstract. In this paper we have shown, using the whole-cell voltage clamp technique, that two parameters of the fertilization current in ascidian eggs may be modified by exposing spermatozoa to lithium or to phorbol ester. When spermatozoa were pre-treated in 250 mM lithium sea water for up to 30 min there was a significant increase in the mean initial slope of the fertilization current, from 116 ± 90 to 169 ± 84 pA/s ($p < 0.05$). The peak current increased from 1371 ± 1079 to 1719 ± 1052 pA ($p > 0.05$). Pre-treatment in 200–600 nM phorbol 12-myristate 13-acetate also increased the activating capacity of ascidian sperm, as monitored by a significant increase in the initial slope current in control eggs; however, there was no increase in peak current. Furthermore, we have shown, using NH_4Cl, that an increase in intracellular pH alone is insufficient to change the activating capacity of spermatozoa. This is the first report showing that the kinetics of an egg activation event depend upon the physiological status of the spermatozoon.
Key words. Fertilization channels; ascidian eggs, sperm-borne factors.

Although progress has been made in elucidating the role of second messengers in the activation of eggs, particularly those of Ca, pH and inositol tri-phosphate ($InsP_3$) little is known about the primary trigger signal from the spermatozoon (see Dale[1], for general references). There are two current hypotheses. One school suggests that the interaction between an externally located receptor in the egg plasma membrane with a complementary molecule on the spermatozoon is the primary trigger, and that this signal is transduced to the egg cytoplasm by G-proteins[2]. The second hypothesis is that the spermatozoon contains a soluble activating factor that is released into the egg cytoplasm following gamete fusion[3–8].

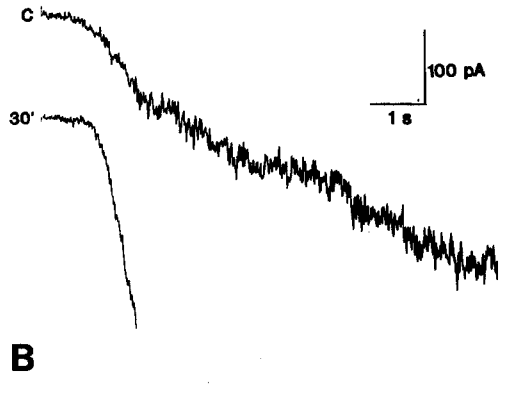
In *Ciona intestinalis* eggs, as in other eggs[9,10], the first indication of fertilization is a current that is inward at a holding voltage of − 80 mV. Single channel recordings have shown that the activation of large conductance, non-specific ion channels generate this current[11]. Although in ascidians the channel precursors giving rise to this current are homogeneously distributed over the egg surface, it appears that the spermatozoon only activates a limited number of channels close to the site of fusion[12]. Micro-injection of extracts of spermatozoa, $InsP_3$ or Ca^{2+} into both regulative and mosaic eggs induces several activation events[3–8,14]. Furthermore, $InsP_3$ increases in the spermatozoon during the acrosome reaction[15], a prerequisite for gamete fusion, and there is evidence that sea urchin spermatozoa contain enough $InsP_3$ to activate eggs[5]. In this study we have exposed ascidian spermatozoa to Li^+ and phorbol ester, agents known to alter the phosphoinositide cycle[16], in an attempt to modify their activating capacity. Since it has been shown in the sea urchin that the magnitude of the conductance change at activation is sperm-dependent[13], we have used the fertilization current in ascidian eggs to monitor sperm activating capacity.

Materials and methods

Gametes of the ascidian *Ciona intestinalis* in natural sea water at 22 °C were used. The chorion was removed manually using steel needles and recordings were carried out on glass slides. Micropipettes of 1–2 µm diameter, used to whole cell clamp the eggs (130 µm diameter), were filled with an intracellular-like medium (200 mM



A Fertilization currents from a holding potential of − 80 mV in eggs of the ascidian *Ciona intestinalis*. Eggs from a control batch, inseminated with control sperm(C), or spermatozoa pre-treated in Li^+ sea water (250 mM LiCl, 250 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 50 mM $MgCl_2$) for 30 min. Note the increase in peak amplitude and slope of the initial part of the current following exposure of the spermatozoa to Li^+.



B The leading edges of the fertilization currents in a second experiment using eggs and sperm from a different batch. C: control spermatozoa and eggs; 30': control eggs from the same batch inseminated with spermatozoa from the same batch that had been pre-treated for 30 min in Li^+ sea water.

K_2SO_4, 20 mM NaCl, 200 mM sucrose, 10 mM Hepes, 10 mM EGTA, pH 7.5). Giga ohm seals were obtained using conventional techniques. The pipette voltage was set to − 80 mV, the resting potential of these eggs, and the patch ruptured by negative pressure. Currents were measured with a List EPC-7 amplifier. Sperm were maintained dry at room temperature, until dilution in natural or artificial sea water.

Results

The fertilization current in control ascidian eggs, voltage clamped at − 80 mV, takes approximately 10–20 s to peak and then slowly inactivates over 1 min (fig A, top trace). In this paper we have studied in detail two parameters of this electrical response. Firstly, the time required for the current to reach 200 pA. (It is possible to fit a line manually to this first 200 pA of current, giving an initial slope rate). Secondly, the peak current.

In a study of 33 control eggs from 17 batches, carried out in February–March 1990, and inseminated with 25 batches of spermatozoa, the mean initial slope rate was 116 ± 90 pA/s (table). Peak currents for the same eggs ranged from 270 to 4060 pA, with a mean of 1371 ± 1079 pA.

Ascidian spermatozoa exposed to Li^+ sea water remained motile and did not show any alteration in behaviour. Such treated sperm were capable of fertilization and gave rise to normal embryos. Figure A shows a control fertilization current (C), and a current in an egg from the same batch, where the spermatozoa were pretreated for 30 min in 250 mM Li^+ sea water. Both the initial slope rate and peak current are seen to increase. Figure B shows the leading edges of the fertilization currents from a second batch of sperm and eggs, where (C) is the control experiment and (30') is from a control egg inseminated with Li^+ pre-treated spermatozoa. The mean initial slope current in 30 control eggs inseminated with Li^+ pre-treated spermatozoa was 169 ± 84 pA/s, while the mean current amplitude was 1719 ± 1052 pA (table), showing that Li^+ pre-treatment of spermatozoa significantly increased the initial slope in control eggs ($p < 0.05$) and possibly led to larger peak fertilization currents ($p > 0.05$). Pre-treating eggs in 250 mM Li^+ sea water had no effect on the slope or peak fertilization current.

Spermatozoa pre-treated in 200–600 nM phorbol 12-myristate 13-acetate (PMA, Sigma, St Louis), for 30–90 min, generated fertilization currents in control eggs with highly significantly increased initial slopes. In a series of experiments carried out in February 1991, the mean initital slope current in 33 control eggs inseminated with control spermatozoa from 11 different batches was 63 ± 35 pA/s, while in 29 control eggs fertilized with PMA-treated spermatozoa the mean initial slope current was 91 ± 47 pA/s ($p < 0.01$, table). There was no significant increase in the peak current in eggs when PMA treated spermatozoa were used (table).

Since exposing spermatozoa to both Li^+ sea water and PMA may lead to changes in intracellular pH, we carried

Fertilization current parameters in eggs of the ascidian *Ciona intestinalis* inseminated with control sperm and spermatozoa pre-treated in lithium, PMA or NH_4Cl

Initial slope (pA/s)			Peak (pA)	
Batches 1–17				
Control (n=33)	116±90	$p<0.05$	1371±1079	N.S.
Lithium (n=30)	169±84		1719±1052	
Batches 18–28				
Control (n=33)	63±35	$p<0.01$	1089±904	N.S.
PMA (n=29)	91±47		1684±992	
Batches 29–35				
Control (n=13)	133±134	N.S.	1435±707	N.S.
NH_4Cl (n=12)	123±94		1079+1300	

out a third series of experiments where sperm were exposed to 40–600 mM NH_4Cl sea water for 15–90 min. There was no significant difference in the initial slope rate or peak current in eggs inseminated with spermatozoa pretreated with NH_4Cl compared with those inseminated with control spermatozoa (table). The delay time from attachment of the spermatozoa to the egg surface to the generation of the fertilization current, however, was significantly increased from 111 ± 90 s to 431 ± 327 s ($n=12$, $p<0.01$).

Discussion

From measurements of single channel conductance, the probability of a channel being open and the total change in conductance at fertilization, we have previously estimated that 200–1000 fertilization channels are activated in ascidian eggs by the spermatozoon [11, 12]. The temporal linearity of the initial 200 pA of the fertilization current may be interpreted as being due to the progressive increase in the number of these channels being activated, with little channel modulation. If the channels are all activated more or less simultaneously the current may be described by the equation:

$$I = NiP = N\gamma V \left(\frac{\alpha}{\alpha + \beta}\right)(1 - e^{-t(\alpha + \beta)})$$

where α is the rate of opening, β is the rate of closing, N is the number of channels, γ the single channel conductance, V the holding voltage, i the single channel current and P the probability of a channel being open. If N, γ and V are constant then an increase in $\frac{\alpha}{\alpha + \beta}$ (the steady state probability that the channel will open) or a decrease in $\frac{1}{\alpha + \beta}$ (the time constant of the process) will both lead to an increase in the slope of I (the fertilization current). Thus I_{slope} is increased because the rate of opening of activated channels is increased. A similar increase in I_{slope} would be apparent if N or γ were increased, or alternatively, if the synchrony of channel activation was maximized.

It has been shown, using the fluorescent indicator Fura-2, that several seconds elapse following the initiation of the fertilization current before Ca is released from intracellular stores. This release of cytosolic Ca starts at the point of sperm-egg fusion, and then a wave of Ca release traverses the egg to the antipode [17]. Although a localized increase in cytosolic Ca may be undetectable using Fura-2, there are several lines of evidence suggesting that Ca released into the cytosol does not activate fertilization channels. First, buffering intracellular Ca at low levels by microinjection of EGTA into the egg prior to insemination results in a larger peak fertilization current, whereas raising the level of Ca itself does not activate these channels [18]. Second, exposing eggs to the Ca ionophore A 23 187 causes a cortical contraction without activating channels [14]. Rectification of the fertilization current after several seconds (fig.) implies regulation of channel behaviour, and this is perhaps due to the release of Ca or other second messengers from intracellular stores.

It is not clear how lithium affects the PI cycle of cells; it may lead to inositol depletion, by inhibiting enzymes which hydrolyze the inositol phosphates, thus reducing the production of second messengers [16]. Phorbol esters probably alter protein kinase C activity, and in fact it has been shown that PMA increases motility in human spermatozoa, possibly via protein kinase C [19]. The complex kinetics of the phosphoinositide cycle, together with the problems inherent in measuring the concentration of these low molecular weight second messengers in spermatozoa [5, 20], will make it difficult to identify the cellular targets of lithium and PMA. We are presently attempting to measure the concentration of $InsP_3$ in ascidian spermatozoa. Both agents may cause an increase in intracellular pH; however, the experiments with NH_4Cl indicate that intracellular pH alone does not alter sperm activating capacity. The increase in delay time following exposure to NH_4Cl is probably due to alterations to the sperm plasma membrane, impeding gamete fusion [1].

Ca has been suggested to be the activating sperm-borne factor [6, 21], and certainly intracellular Ca in the sperm will be regulated by PI metabolism. This hypothesis seems unlikely in ascidian eggs, since experimental manipulation of intracellular Ca levels in the egg does not affect fertilization channel activation or behaviour [14, 18]. In sea urchins and mammals, soluble activating factors extracted from spermatozoa appear to be proteins [3, 8]. The present investigation implies a role for the phosphoinositide cycle of the spermatozoon in regulating the activating factor. Although we have not excluded the hypothesis of an externally placed receptor activating mechanism [2], our experiments show for the first time that an egg activation event depends quantitatively upon the physiological status of the spermatozoon.

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In vitro stability and protein composition of thick filaments from insect flight muscles

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Abstract. Thick and thin filaments of synchronous and asynchronous insect flight muscles were separated by density gradient centrifugation. A good release of myofilaments from myofibrils was obtained by sonication of myofibrils in relaxing solution with pH 6.1 (locust), pH 6.4 (honeybee) and pH 6.6 (fleshfly), respectively. Thick filaments but not thin filaments were dissolved, if sucrose gradient centrifugation was used to separate the filaments. Thus, sucrose gradients are the medium of choice if actin filaments are to be purified. Glycerol-containing gradients selectively dissolved myosin filaments from fleshfly muscles. The stability of the myosin filaments of all muscles was sufficient in gradients with 10–30% formamide.
Key words. Insect muscle; myosin filaments; contractile proteins.

The contractile apparatus of insect flight muscles differs from that of vertebrate skeletal muscles in structure and in its regulatory mechanisms, and has a considerably higher ATP splitting rate during activity. The molecular basis of these differences, however, is not yet fully known. Careful studies on the protein pattern of actin filaments[1–3] and the Z-line[1] of insect flight muscles demonstrated the existence of insect-specific proteins. For insect myosin filaments, muscle-specific contents of paramyosin[4,5] and the existence of a high molecular weight protein connecting the myosin filaments to the Z-line[1,6,7] have been found, but M-line proteins could not yet be identified. One of the reasons for this information gap seems to be the instability of native thick filaments from insect muscles during density gradient centrifugation. The aim of this work, therefore, was to improve the purification procedures for these filaments to facilitate the analysis of their protein composition.

Flight muscles (0.5 to 1 g) of 6 locusts (*Locusta migratoria*) or whole thoraces of 40 fleshflies (*Phormia terrae-novae*) or 30 honeybees (*Apis mellifica*) were used for the preparation of myofibrils[8]. All procedures were performed at 0 to 6 °C. Filament suspensions were obtained by either a 3 × 5-s homogenization (MSE homogenizer, 15,000 rpm) or a 15-s sonication (Bandelin Sonorex–Rapid GR 80, 40 kHz, 80/320 W) of myofibrils in 3 ml relaxing buffer (10 mM imidazole or 10 mM morpholinoethane sulfonic acid = MES, 10 mM KH_2PO_4, 10 mM $MgCl_2$, 10 mM ATP, 100 mM KCl, 2 mM EGTA, 1 mM DTE) per g muscle. Sonication or homogenization were followed by 10 min centrifugation at 20,000 × g. Another two sonication/homogenization and centrifugation cycles were run with the sedimented myofibrils. The supernatants were collected and assembled. In spite of extensive fragmentation of the filaments by the sonication procedure (fig. 1) this method was used because it gave a higher yield of free filaments.

250 µl of the filament suspension ($E_{310} \leq 1.0$) with 5% of the density medium was layered on top of 13 ml of a linear density gradient[9] and overlayered with 750 µl of the filament suspension. This system was centrifuged in a Sorvall OTD-65 swinging bucket rotor at 68,000 × g for 150 min. Eight equal-volume fractions were withdrawn from the bottom of the tube with the aid of a glass capillary and a peristaltic pump[9]. Separation of thick and thin filaments was checked by taking samples from individual fractions for negative staining (1% uranylacetate in H_2O) and electron microscopy (Zeiss EM 9) and by centrifuging the rest of the fractions for 2 h at 120,000 × g and using the sediment for SDS polyacrylamide gel electrophoresis (7.5% gels, Tris · HCl[9,10]).

To exclude an influence of endogenous proteases on the results of gel electrophoresis as far as possible, different protease inhibitors, such as leupeptin (Serva, 1 mg/l), Pepstatin A (Fluka, 10^{-6} M), phenylmethylsulfonyl fluoride (Serva, 0.1 mM), soybean trypsin inhibitor (Serva, 25 mg/l) and tosyl-L-phenylalanine chloromethyl ketone (Fluka, 20 mg/l) were added alone or in combination to the solutions during the preparation of myofibrils. No differences in the SDS gel pattern of myofibrillar proteins