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Net calcium and acid release at fertilization in eggs of sea urchins and ascidians

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Abstract — Sea urchin eggs lose about 10–30% of their total calcium content upon fertilization. We have investigated the mechanism of this calcium-loss with an ion-selective vibrating probe system. Upon fertilization of *Arbacia punctulata* and *Lytechinus pictus* eggs we could measure a calcium efflux signal with an average duration of 204 ± 26 s and 146 ± 46 s, respectively. Measurements of hydrogen ion signals in normal and in low sodium media showed that the release of cortical vesicle material from these eggs lasts for about 30 and 50 s, respectively. The data indicate that most of the calcium that is lost from sea urchin eggs originates from the cytosol in which it is released during fertilization and then pumped out through the plasma membrane. Calcium loss due to cortical granule release accounts for less than 14% of the total loss measured.

We also measured a substantial post-fertilization calcium efflux in eggs of *Phallusia mammilata*, with an average duration of 265 ± 18 s followed by smaller periodic effluxes that corresponded to oscillations in the $[Ca^{2+}]_i$ during contractile waves in these eggs. These data, together with the lack of cortical granules in ascidian eggs, indicate that *Phallusia* eggs also pump out a substantial amount of calcium through the plasma membrane after fertilization.

Fertilization of a mature egg results in a large transient increase in the concentration of cytoplasmic free calcium. It has been established in eggs of a wide variety of different species that this calcium pulse originates at the sperm entry point and travels as a wave across the egg toward the opposite pole of the egg (*see* e.g. [1–6]). Such a calcium wave is now believed to cross all deuterostome eggs during fertilization [7]. The mechanism of this calcium wave is thought to be a chain reaction in which locally released free calcium causes the release of yet more calcium from a nearby cytoplasmic compartment, probably the endoplasmic reticulum (*see* [8–12] for reviews).

Although it seems clear that the wave calcium originates from the endoplasmic reticulum (ER) [13], there are uncertainties about how the $[Ca^{2+}]_i$ is

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brought back to the resting level. Possibilities are that the calcium is pumped back into the ER or into mitochondria [3, 14, 15]. However, based on the measurement of total calcium in recently fertilized sea urchin eggs, Azarnia and Chambers [16] reported that – depending on the species – up to about 30% of the total egg-calcium is lost after fertilization. Due to technical restrictions they were not able to make measurements during the first 3 min after fertilization. X-ray microprobe analysis and total calcium measurements by Gillot et al. [9, 17] show that cortical granules contain high concentrations of calcium. They therefore suggested that at least part of this calcium is lost from the egg at fertilization [9].

In sea urchin eggs, the wave of calcium is immediately followed by exocytosis of the cortical granules. Cortical granules are specialized vesicles that lie in the cortex of the unfertilized egg. Exocytosis sheds their contents into the perivitelline space. This process is clearly dependent on the rise of cytoplasmic calcium [18, 19]. Since there is thus both a massive exocytosis of calcium rich cortical granules and a rise of intracellular free calcium immediately after fertilization, it is difficult to assess the contribution(s) of both processes in the calcium that is lost from the egg.

In contrast to sea urchin eggs, the eggs of the ascidian *Phallusia* do not contain cortical granules and no massive exocytosis occurs (*see* [5, 6, 20–22]). In these eggs fertilization triggers a contractile or peristaltic wave that starts at the animal pole and sweeps over the egg, reaching the vegetal contraction pole in about 2 min. This contractile wave is somehow initiated by a typical fertilization or activation wave of calcium that starts at the point of sperm entry [5, 6, 21].

For this paper, we have used ion-specific vibrating probe measurements [23] to show that calcium is actively pumped out of a fertilized egg whenever the $[Ca^{2+}]_i$ goes up. By comparing the time course of calcium efflux obtained from fertilized sea urchin eggs with time courses obtained from eggs of the ascidian *Phallusia*, we were able to determine the source of the calcium that is lost during fertilization. Furthermore, by utilizing a H^+-ion specific electrode we measured the duration of the cortical granule release in sea urchin eggs. The results indicate that the calcium that leaves the egg after fertilization must be actively pumped out and that only a small fraction of the measurable calcium efflux can be due to cortical granule release. An abstract of part of this work has been published before (Gillot et al. (1990) Biol. Bull., 179, 224).

Materials and Methods

Animals and gametes

Adult specimens of *Arbacia punctulata* were collected near Woods Hole (MA, USA). Specimens of *Lytechinus pictus* were purchased from Marinus, Inc. (Long Beach, CA, USA). Both sea urchin species were kept in aquaria with running sea water. To prevent spawning, the sea water was cooled to about 14°C. Gametes were obtained by intracoelomic injection of 0.5 M KCl. The jelly of the eggs was removed by washing them for a few minutes with acidified sea water (pH 4). The eggs were washed twice with artificial sea water (ASW) and then stored in ASW at room temperature. Dry sperm was stored in an ice bath until use.

Phallusia mammilata was obtained from Sete and Roscoff (France) and kept in aquaria at 15°C. The ascidian *Phallusia* is self-fertile; therefore care was taken that the oviduct and spermiduct of one animal were tapped separately. The sperm was stored dry on ice and the eggs were dechorionated in a 0.1% solution of trypsin (porcine pancreas; Sigma, St Louis, USA) in sea water for 1–2 h [24]. The dechorionated eggs were rinsed in filtered sea water and kept in gelatin coated dishes to prevent sticking and lysis [23].

Solutions

Artificial sea water (ASW) was prepared according to the Marine Biological Laboratory (MBL) formula: 425 mM NaCl, 9 mM KCl, 9.3 mM $CaCl_2$, 19.9 mM $MgCl_2$, 25.5 mM $MgSO_4$ and 2.1 mM $NaHCO_3$ in deionized water. Calcium free ASW (CaFASW) was prepared according to the MBL formula for osmotically balanced CaFASW: 436.8

mM NaCl, 9 mM KCl, 22.9 mM $MgCl_2$, 25.5 mM $MgSO_4$ and 2.1 mM $NaHCO_3$ in deionized water. Low calcium ASW was prepared by adding the appropriate amount of a stock-solution of 1 M $CaCl_2$ to CaFASW. Low sodium ASW (LSASW) was based on choline substitution (modified from [25]): 480 mM choline chloride, 10 mM KCl, 5.5 mM $CaCl_2$, 20 mM $MgSO_4$, 27 mM $MgCl_2$ and 2.1 mM $NaHCO_3$ in deionized water, pH 7.8.

Vibrating ion-specific probes

Details of the vibrating ion-specific probe technique have been published elsewhere [23]. Briefly, glass micropipettes with a tip diameter of about 4 μm are pulled from TW150–4 glass capillaries (World Precision Instruments, New Haven, CT, USA) on a Mecanex BB-CH electrode puller (Mecanex, Geneva, Switzerland). The micropipettes are silanized with dimethylaminotrimethylsilan vapor (Fluka Chemical Corp., Ronkonkoma, NY, USA) for 20 min. at 175°C. For calcium-specific electrodes, the micropipettes are front filled with electrolyte (100 mM $CaCl_2$) by applying suction with a syringe. The tip is then front filled with a 20 μm long column of calcium sensor (Calcium Ionophore I, Cocktail A; Fluka Chemical Corp.). For H^+-ion specific electrodes, the electrolyte is 50 mM PIPES-buffer in 50 mM KCl (pH 7.1 with NaOH); whereas the H^+-ion sensor is Fluka's Hydrogen ion ionophore I, Cocktail B. The electrodes have a linear response with slopes that are close to Nernstian (about 28 mV per decade for Ca^{2+}-electrodes and about 58 mV per decade for H^+-electrodes). Electrical contact is established by inserting a Ag/AgCl wire into the back of the ion-selective electrode. The return electrode is a WPI Ag/AgCl half cell, connected to the solution by a fine-tipped pipette filled with 3M KCl and 0.5% agar. The tip signal is fed into a high impedance voltage follower, which in turn is connected to a x1000 amplifier.

The ion-selective electrode is vibrated at 0.5 Hz in a plane by an assembly of piezo electrical micro stages (PZS–100, Burleigh Instruments, USA), which is in turn held by a micromanipulator for positioning purposes. The whole setup is built on top of a Zeiss IM35 inverted microscope equipped with a video camera. The electrode potential at the two extremes of vibration is digitized and the potential difference is calculated by a computer. In the figures, this difference is referred to as the Phase Sensitive Detector (PSD) output and is depicted on the Y-axis. The result is superimposed on the video image and recorded on a JVC BR-9000U timelapse videorecorder as well as on disk.

Flux measurements

Measurement of the ionic fluxes during and after fertilization was performed as follows. An egg was placed in a 35 mm plastic petri dish, containing about 3 ml of artificial sea water (normal, low Ca^{2+} or low Na^+, depending on the experiment). The vibrating ion-selective electrode was positioned close to the egg, such that the distance between the probe tip and the egg surface at closest proximity was 5–10 μm. A small amount of dry sperm (2–4 μl) was diluted into 0.5 ml of the same artificial sea water solution. Small differences in the Ca^{2+} or H^+ concentration between the sperm solution and the bathing solution would result in small artificial gradients that would be picked up by the probe. Therefore, one drop of the sperm solution was added at some distance (about 0.5 cm) away from the egg. This allowed for enough time for small artificial gradients to dissipate before the spermatozoa reached the egg. Control experiments showed that in this way disturbances in the PSD-output were limited to a few microvolts. In order to add the sperm, the Faraday cage that contains the instrument had to be opened. This caused a large temporary artefact in the PSD-output. In the graphs, the time axis starts as soon as this artefact is gone. Time 'zero' is thus actually about 5 s after insemination. The spermatozoa needed a variable time to swim to the egg and fertilize it. Since the acrosome reaction of the sperm is calcium dependent, the lag-period between insemination and fertilization is particularly long in low calcium ASW.

In sea urchin eggs, fertilization is followed by an elevation of the fertilization envelope. During this process, the distance between the probe-tip and this envelope was kept constant at 10 μm by adjusting the position of the probe. Repositioning of the probe was also needed during the contraction waves after fertilization of *Phallusia* eggs.

Calculation of fluxes from PSD-output data

Flux-data for calcium-measurements can be calculated from the PSD-output by applying the principles of diffusion theory. A full account of the theory of ion-specific vibrating probes can be found in Kühtreiber and Jaffe [23]. Briefly, the concentration difference between the two extremes of vibration can be calculated from:

$$\Delta V = \frac{S}{2.3} \cdot \frac{\Delta C}{C_{Average}} \cdot \frac{1}{Efficiency}$$

where ΔV is the voltage from the PSD-output; S is the slope of the ion-specific electrode; and $C_{Average}$ is the average concentration between the two extremes of vibration. Note that for small signals this is the same as $C_{Background}$. The efficiency depends on various electronic and physical parameters of the vibrating probe system and is currently about 0.5.

From ΔC, the flux can then be calculated using Fick's law:

$$J = -D\left(\frac{\Delta C}{\Delta X}\right)$$

where J is the flux; ΔX is the diffusion distance (i.e. the vibration amplitude); ΔC the concentration difference over distance X and D is the diffusion constant (D_{Ca} for calcium ions in aqueous solutions is 8×10^{-6} cm^2/s; D_{H^+} for H^+ ions is 9.4×10^{-5} cm^2/s; see [26]).

For H^+ ions, the situation is complicated by the presence of bicarbonate ions, which act as a substantial pH buffer. Specifically, the bicarbonate ions will reduce the H^+-ion gradient by picking up H^+-ions at a high $[H^+]$ and letting it go at a low $[H^+]$ according to:

$$H^+ + HCO_3^- \Leftrightarrow H_2CO_3$$

Modification of the equation for the facilitation of calcium diffusion by weak calcium buffers as derived in Speksnijder et al. [20] for bicarbonate, leads to:

$$r = \left(\frac{D_{HCO_3^-}}{D_{H^+}}\right) \cdot \left(\frac{[HCO_3^-]}{K_D}\right)$$

where r is the ratio of facilitated to direct H^+-ion flux down a gradient; $D_{HCO_3^-} / D_{H^+}$ is the ratio of the mobility of HCO_3^- to that of H^+ (about 0.12); $[HCO_3^-]$ is the local bicarbonate concentration and K_D is the true dissociation constant of carbonic acid (about 0.2 mM). The bicarbonate concentration in the ASW-solutions used was 2.1 mM. This yields a value of about 1.3 for r. The effective hydrogen ion flux in the media used is thus 2.3 times the flux calculated via Fick's law.

Results

Calcium is lost from fertilizing sea urchin eggs

Arbacia punctulata eggs were fertilized in ASW with 0.1 mM or 1 mM Ca^{2+}, whereas all *Lytechinus pictus* experiments were performed in the presence of 1 mM Ca^{2+}. The time course of the calcium efflux after the fertilization of *Arbacia* and *Lytechinus* was determined using a calcium specific vibrating probe. In both egg species, once fertilization occurred, the calcium efflux reached a maximum within a few seconds (Figs 1 and 2). The efflux then slowly decreased again over a period of a few minutes. The total signal-duration for *Arbacia* eggs was 204 ± 26 s (SD; n=6) and for *Lytechinus* eggs 146 ± 46 s (SD; n=7). The different background calcium (0.1 or 1 mM) did not appear to have an influence on the total duration of the calcium efflux in *Arbacia* eggs. However, numerical integration of the respective curves in *Arbacia* showed that the total efflux measured at 1 mM Ca^{2+} background (558 ± 128 pmol/cm^2; n=3) was roughly twice the total efflux measured at 0.1 mM Ca^{2+} (209 ± 84 pmol/cm^2; n=3). Numerical integration of the area under the *Lytechinus* curves (at 1 mM Ca^{2+}) yielded an average total signal of 4300 ± 1300 µV.s (SD; n=6), which corresponded to an average total efflux of 2800 ± 820 pmol/cm^2.

The eggs of *Lytechinus* are relatively transparent. Therefore, in these eggs it is possible to easily see the wave of cortical granule release. Also, the elevation of the fertilization envelope is particularly clear in these eggs, since it can elevate 50 µm or more. In *Arbacia* both phenomena are much less easily visualized because of the relative opaqueness

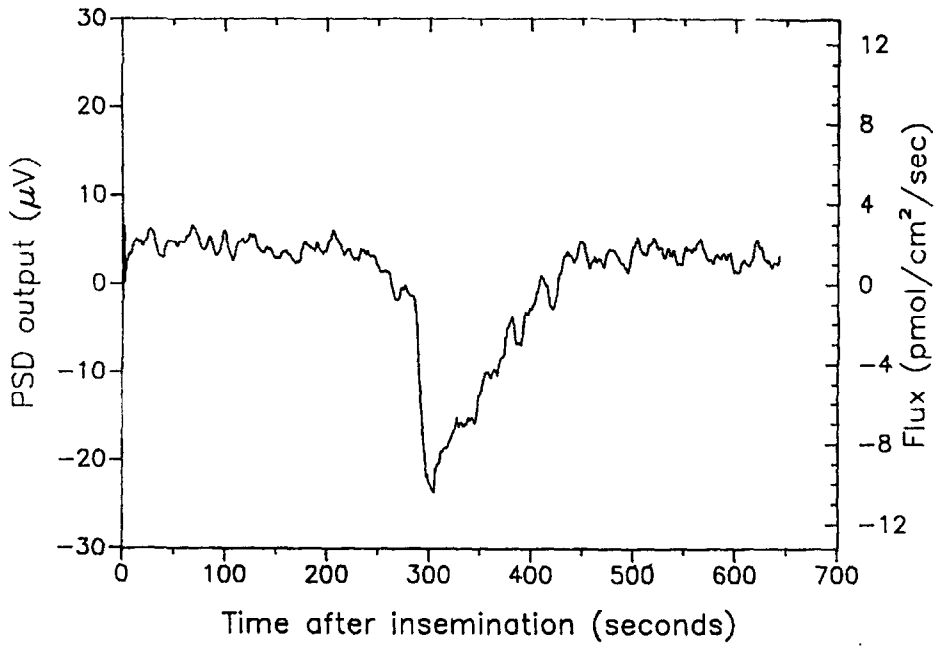

Fig. 1 Calcium efflux after fertilization of an *Arbacia* egg in artificial sea water with 1 mM Ca^{2+} background. The vibration amplitude of the calcium electrode was 30 μm. The total signal duration was 231 s.

of the egg and because the fertilization envelope only elevates a few microns. Since the PSD-output is superimposed on the video image, it is possible to exactly correlate the timing of the cortical granule release in *Lytechinus* and the calcium signal (*see* inset of Fig. 2). From such correlations it is clear that the calcium efflux signal has a much longer duration than the cortical granule release. In the experiment depicted in Figure 2, the envelope has clearly been formed long before the calcium signal is gone. Note that, although in this particular experiment the probe happens to be vibrating near an area where there is only a small elevation of the FE, the calcium signal persists for the same amount of time than in eggs where the FE elevates much more.

In other experiments, in which the first elevation of the fertilization envelope happened at the side of the egg opposite to the site of measurement, there was a time lag of about 14 s between the first moment of visible envelope elevation and the moment at which the calcium signal was first measurable (data not shown). This time lag was in good correspondence with the expected time of about 12 s for the calcium wave to cross the egg [27, 28]. There was no clear relation between the position of the vibrating probe with respect to the direction of the calcium wave and the magnitude of the calcium efflux signal measured.

Ascidian eggs lose calcium with the same temporal pattern as the periodic rise in internal calcium

Dechorionated *Phallusia* eggs were suspended in ASW with 0.1 mM $CaCl_2$ and fertilized with sperm that was pre-activated for about 20 min with normal (chorionated) eggs according to Sardet et al. [21]. Fertilization of *Phallusia* eggs is easily visible because the eggs undergo a dramatic shape change. The first visible event is a bulging at the animal pole of the egg, which is immediately followed by a wave of contraction which travels towards the vegetal pole at a speed of about 1.4 μm/s and reaches it in about 120 s (*see* Fig. 1 of Sardet et al.

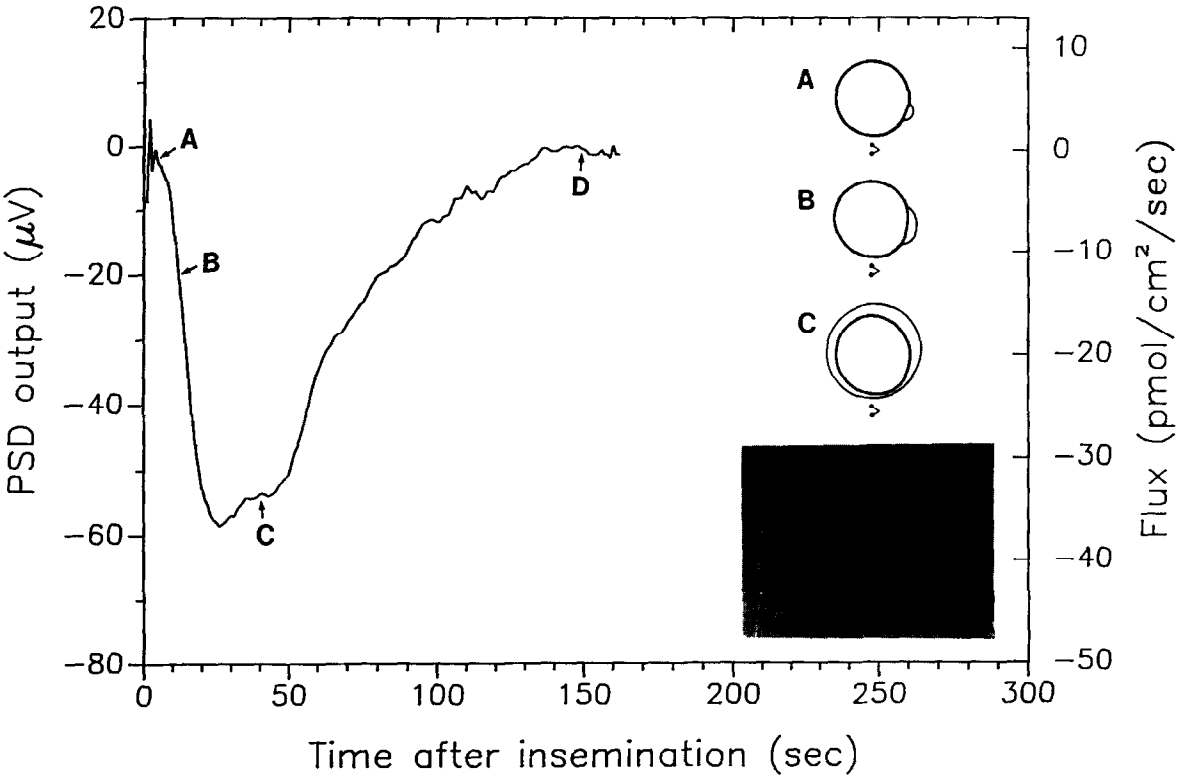

Fig. 2 Calcium efflux after fertilization of a *Lytechinus* egg in artificial sea water with 1 mM Ca^{2+} background. The vibration amplitude of the probe was 20 μm. The calcium efflux was measurable during about 130 s. The inset shows the progression of the elevation of the fertilization envelope where the 4 stages correspond to the labels A–D of the graph. The arrow in inset A indicates the fertilization point. The contrast of the fertilization envelope as seen by normal transmitted light microscopy in stages A–C, the fertilization envelope is still very low. This would be very difficult to reproduce in a journal. Therefore these stages are shown as drawings made from actual sceen shots.

[21]). The vibrating electrode was repositioned during the contraction in order to keep the distance between the probe and the egg surface approximately constant. A clear calcium efflux was measured upon fertilization, beginning during the contraction of the egg (*see* Fig. 3). The duration of the total calcium efflux signal that was associated with fertilization was 265 ± 18 s (SD; n=4). Numerical integration of this fertilization calcium-peak yielded an average efflux of 125 ± 60 pmol/cm^2 (SD; n=6).

The first contraction wave that is associated with fertilization is followed by a series of transient animal protrusions that form at regular time intervals. Each animal protrusion involves a flow of the centrally located cytoplasm in the animal direction [21]. Speksnijder et al. [20] have shown that the initial large calcium fertilization transient is immediately followed by a series of 12–25 post-fertilization calcium pulses that occur at regular intervals of 1–3 min during the completion of meiosis and that stop as soon as the second polar body is formed. The first few of these 'secondary pulses' are also detected by the vibrating calcium specific probe and are correlated with the formation of the secondary protrusions. Figure 3 shows 4 of such pulses at the vegetal pole that coincide with protrusion formation. Numerical integration of these pulses results in an average efflux per secondary pulse of 35 ± 20 pmol/cm^2 (SD; n=11).

Hydrogen ion efflux after fertilization of sea urchin eggs

Arbacia or *Lytechinus* eggs were suspended in ASW with a 2.1 mM $NaHCO_3$ buffer (although the buffer will interfere with the vibrating H^+-ion specific probe measurements, some pH buffering is needed to prevent uncontrolled pH changes). A drop of sperm, diluted with the same solution, was added at a distance of about 0.5 cm. Since the background H^+-ion concentration is very low at the pH of the sea water used (pH 7.8), the sensitivity of the H^+-ion vibrating probe is much higher than for calcium flux measurements. Therefore, the signals measured by the H^+-ion electrode are much larger (in excess of 1 mV; *see* Figs 4 and 5) than for calcium measurements. When *Arbacia* eggs are measured in this way, in most experiments 2 peaks of hydrogen ion efflux become apparent (*see* Fig. 4). The first peak has an average duration of 24 ± 3 s (SD; n=4); whereas the whole signal's duration is 181 ± 24 s (SD; n=6). Performing the same experiment with *Lytechinus* eggs did not reveal 2 peaks (*see* Fig. 5; one peak of 177 ± 41 s; SD; n=5). Presumably in these cases the time courses of the 2 peaks are such that they are not resolved. No hydrogen ion fluxes were detectable upon fertilization of *Phallusia.*

There are 2 possible sources for hydrogen ions early after fertilization of sea urchin eggs. One possibility, as mentioned above, is the acid contents of the cortical vesicles. The other is the Na^+/H^+-exchanger that becomes active after fertilization so as to raise the cytoplasmic pH of the egg [29, 30]. To determine whether the 2 hydrogen ion peaks after *Arbacia* fertilization represent respectively the cortical vesicle exocytosis and the Na^+/H^+-exchanger, fertilizations were performed in low Na^+ ASW (Figs 6 and 7). The rationale behind these experiments was that lowering the $[Na^+]$ to a point where the Na^+ present becomes rate-limiting for the Na^+/H^+-exchanger should result in a decreased hydrogen ion signal, since less hydrogen ions are now pumped out of the egg (pseudo Km for Na^+/H^+

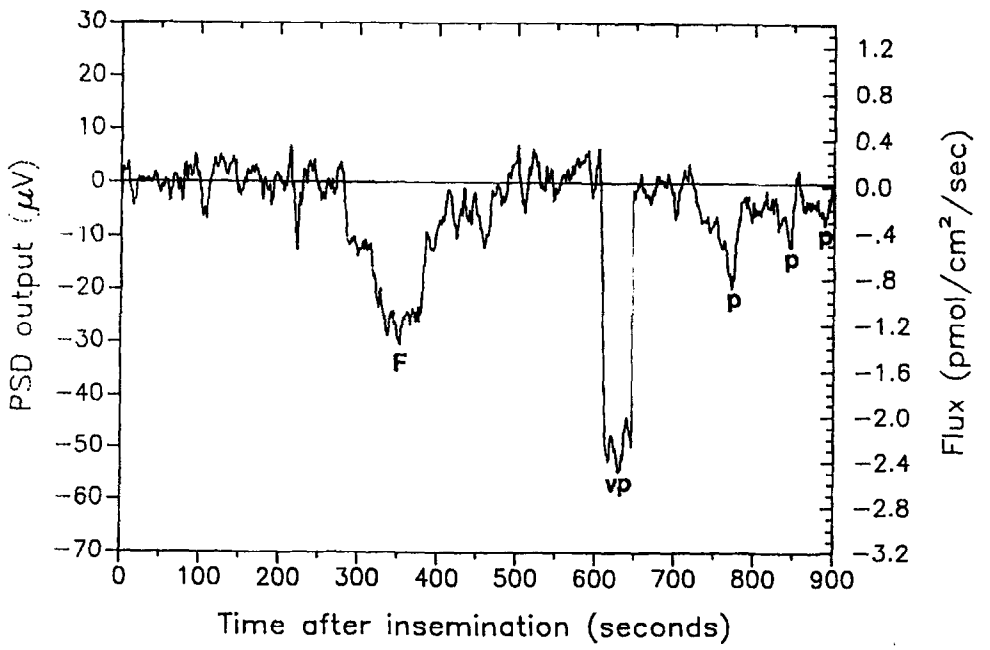

Fig. 3 Calcium efflux after fertilization of a *Phallusia* egg in artificial sea water with 0.1 mM Ca^{2+} background. The vibration amplitude of the calcium probe was 30 μm. The first peak measured corresponded to the fertilization peak (F). After fertilization, the probe was repositioned at the vegetal pole (vp). The peaks following fertilization (vp and p) were correlated with the post-fertilization contraction waves of the egg.

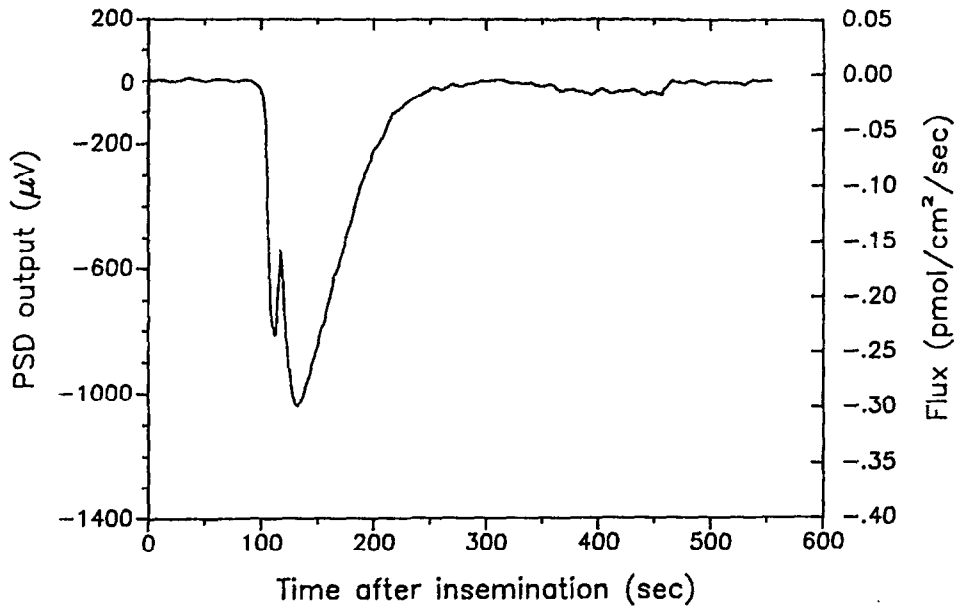

Fig. 4 Hydrogen ion efflux after fertilization of an *Arbacia* egg in artifical sea water (pH 7.8). Two separate peaks can be distinguished. The first peak corresponds to the cortical granule release, whereas the second peak is due to the activity of a Na^+/H^+ exchanger. The vibration amplitude of the H^+-ion selective probe was 10 μm. The cortical granule peak lasted for 23 s, whereas the whole signal duration was 176 s.

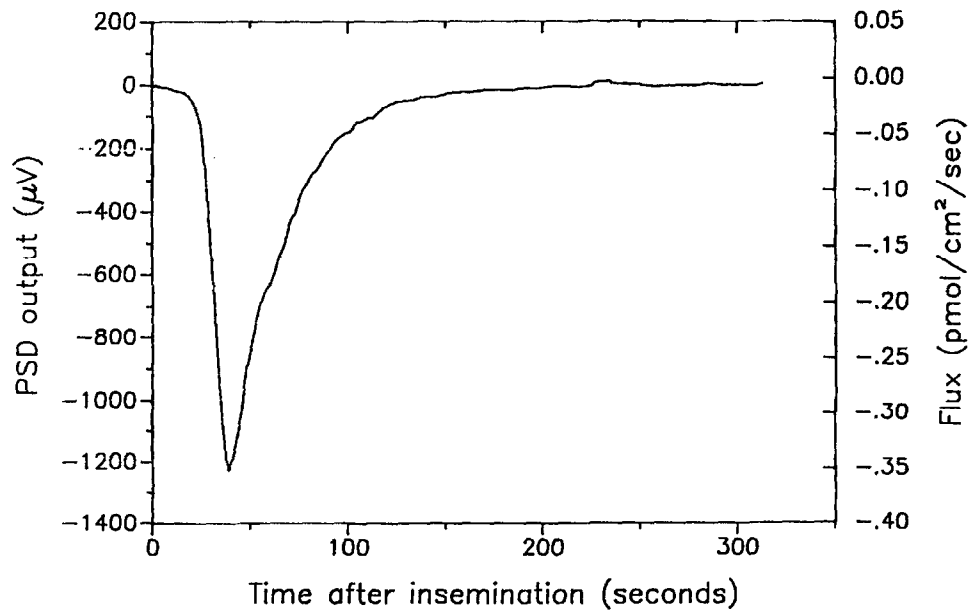

Fig. 5 Hydrogen ion efflux after fertilization of a *Lytechinus* egg in artificial sea water (pH 7.8). No separate cortical granule peak can be distinguished. Vibration amplitude was 10 μm. The total signal duration was 143 s.

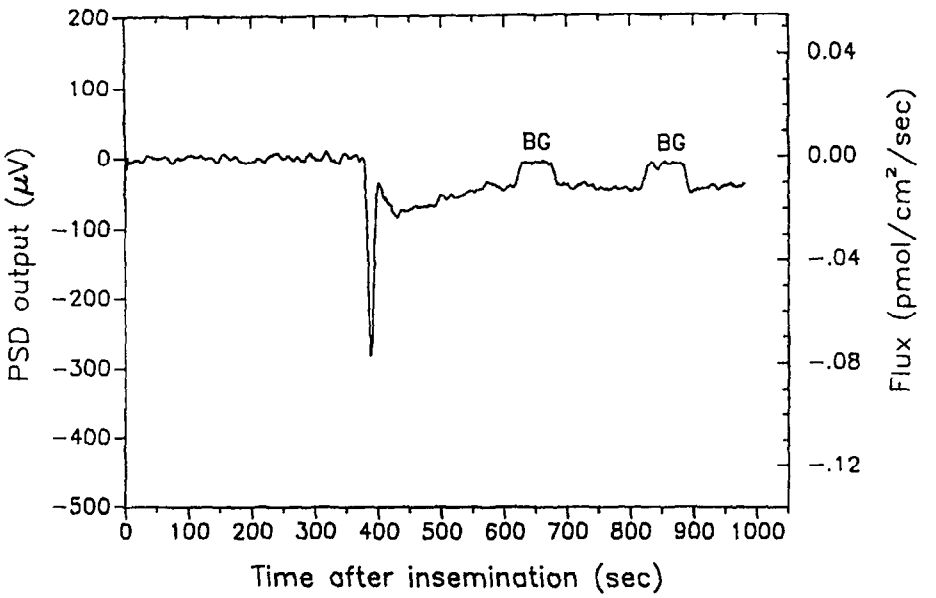

Fig. 6 Hydrogen ion efflux after fertilization of an *Arbacia* egg in low sodium artificial sea water (pH 7.8). While the cortical granule peak remained essentially unchanged, the Na^+/H^+ peak was very much reduced and of a much longer duration than is the case in normal sodium media. BG = measurement in a background position about 300 μm away from the egg. Vibration amplitude 10 μm.

exchanger is about 5 mM [30]).

Figure 6 shows an example of such an experiment with *Arbacia* eggs. The first hydrogen ion peak is unaltered and is thus due to cortical granule release. The average duration of the cortical granule peak in low Na^+ is 29 ± 7 s (SD; n=3). The magnitude of the second hydrogen ion peak is now dramatically reduced in magnitude, but much extended in duration. In the experiment depicted in Figure 6, the measurement was discontinued after 10 min, but the hydrogen ion efflux was still clearly measureable. Fertilizing *Lytechinus* eggs under low sodium conditions likewise results in a reduction of the hydrogen ion signal (Fig. 7). The amplitude of the peak becomes smaller and the hydrogen ion signal now lingers on for extended periods of time (up to about 25 min).

Discussion

It is well established that sea urchin eggs lose a large percentage of their total calcium contents upon fertilization. In the present study we have addressed the question of the *source* of the intracellular calcium that is lost from the fertilized egg. Gillot et al. [31] have shown that the cortical vesicles contain high levels of calcium (up to about 95 mM for *Arbacia lixula*), at least part of which is exchangeable. An estimate of the amount of calcium present in the cortical vesicles corresponds rather well with the total amount of calcium lost from the sea urchin egg [32]. Such data have therefore led to the hypothesis that this is at least in part the calcium that is lost from the egg [9]. Alternatively, since internal calcium transiently rises at fertilization, it must be expected that this calcium also could be extruded by a calcium pump in the plasma membrane of the egg. We have investigated to what extent both mechanisms contribute to the observed calcium loss after fertilization.

The duration of the calcium efflux in sea urchin eggs

Our present data of fertilizing sea urchin eggs show that the total calcium efflux peaks last on average 204 s for *Arbacia punctulata* and 146 s for

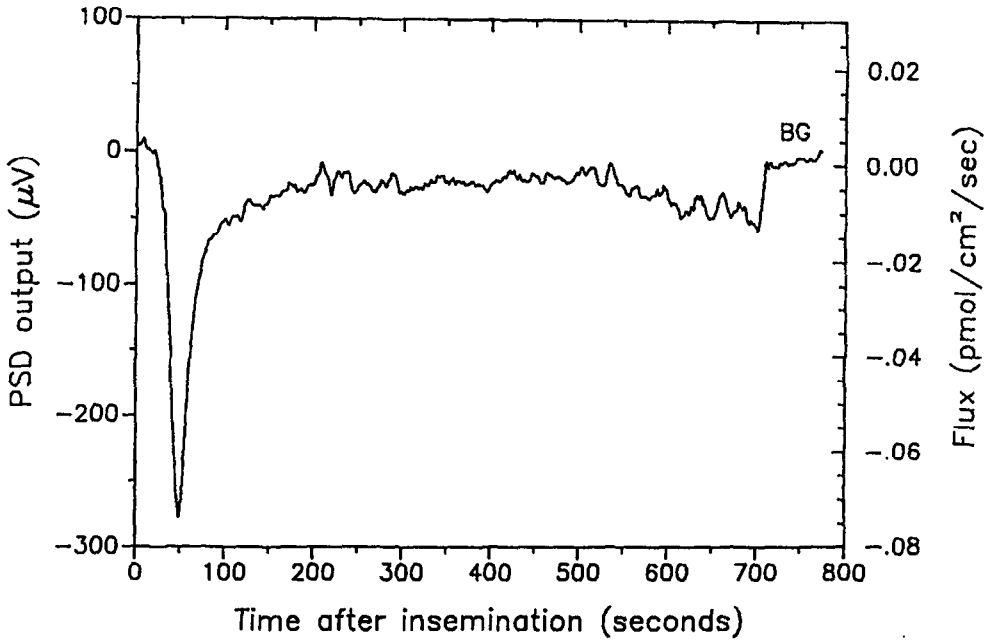

Fig. 7 Hydrogen ion efflux after fertilization of a *Lytechinus* egg in low sodium artificial sea water (pH 7.8). Under these conditions, the cortical granule peak is revealed, whereas the Na^+/H^+ exchanger peak is now very much reduced and extended in duration. BG = Background measurement. Vibration amplitude 10 μm.

Lytechinus pictus (Figs 1 and 2). From the literature it is known that the calcium wave that precedes the cortical granule wave last for about 9 s in *Arbacia punctulata* [33] and about 12 s in *Lytechinus pictus* [27]. Particularly careful measurements on *Arbacia punctulata* eggs have been carried out by Moser [34], who found that in these eggs the wave of cortical granule release is completed in on average 10 s at 25.6°C. Since the measured efflux thus is much longer then expected if cortical vesicle exocytosis would be the only source of calcium, this is the first indication that there must be another source, or at least an additional one, for the calcium that is lost from the egg.

The calcium efflux does not depend on the presence of cortical granules

We have attempted to block cortical granule release in sea urchin eggs by means of ultraviolet light treatment [35] or procaine treatment [36]. In all of these experiments a normal calcium signal was measured. However, in our hands a hint of a fertilization envelope was often visible after such treatments, indicating that cortical granule release was not completely blocked (data not shown). These results were thus not completely conclusive, although they appeared to be consistent with calcium loss via a plasma membrane pump rather than exocytosis. We therefore decided to investigate calcium signals from a species that does not have cortical granules. Measurements on fertilizing *Phallusia mammilata* eggs indeed showed that calcium efflux could take place in the absence of cortical granules (*see* Fig. 3). The most obvious source for this calcium is the fertilization calcium wave ([5, 6] *see also* [37]). Since this would imply the presence of an effective calcium pump in *Phallusia* plasma membrane, it seems plausible that the sea urchin egg also has such a pump. In addition, it is probable that the calcium pumps of the cortical vesicles [9, 31] might become incorporated in the egg plasma membrane during the

membrane fusion step of exocytosis and therefore also participate in generating the calcium efflux.

Speksnijder et al. [6] show that the (primary) calcium wave that is associated with fertilization is followed by a series of secondary calcium waves that are associated with contractions of the egg surface. Figure 3 shows that the calcium probe also measures several post-fertilization signals after fertilization of *Phallusia* eggs. The timing of these signals is in good agreement with the observations of the post-fertilization calcium waves described above. Apparently, plasma membrane associated calcium pumps are also involved in reducing the free calcium concentration back to its resting level at the end of such a wave. Only the first 3–5 of the total of 12–25 post-fertilization calcium pulses [6] are detected by the calcium-ion specific vibrating probe. It is possible that the egg relies more heavily on intracellular calcium-sequestration during later stages to prevent the loss of too much calcium.

A *Phallusia* egg loses 0.13 ± 0.06 pmoles of Ca^{2+} during the primary fertilization pulse and 0.04 ± 0.02 pmoles during the secondary post-fertilization pulses (*see* 'Quantification of the calcium loss' section later on in the Discussion). This 3-fold ratio between primary and secondary pulses is in good aggreement with the data on the intracellular free calcium concentrations as measured by aequorin luminescence by Speksnijder et al. [20], who report a maximum $[Ca^{2+}]_i$ of 6.7 μM in the fertilization peak and of 2.0–4.1 μM in the post-fertilization peaks.

Timing the cortical granule release by means of hydrogen ion efflux measurements

The above observations on ascidian eggs suggest that the observed calcium efflux from sea urchin eggs cannot be explained by cortical granule release alone. However, *Phallusia* belongs to a different phylum and hence the mechanisms involved might differ from those of sea urchins. To get quantitative data on the duration of the ionic signals that are associated with the cortical granule release in sea urchins, we decided to measure the hydrogen ion signals that are associated with fertilization. After fertilization, there are two processes that cause the loss of hydrogen ions from the egg. First, the contents of the cortical granules are acidic because of the presence of acid mucopolysaccharides [38, 40]. Second, under normal conditions, the egg increases its intracellular pH by means of a Na^+/H^+-exchanger. Our H^+-ion selective vibrating probe data show that in *Arbacia* eggs these 2 different hydrogen ion sources can actually be distinguished from each other (Fig. 4). We interpret the first peak as the cortical granule peak and the second one as being due to the activity of the Na^+/H^+-exchanger. The hydrogen ion signal that is associated with the cortical granule release thus slightly precedes the signal that is associated with the Na^+/H^+-exchanger. In *Lytechinus* eggs a separation into two peaks does not occur (Fig. 5). Interestingly, no hydrogen ion signals at all were detectable after fertilization of *Phallusia* eggs.

To verify this interpretation, hydrogen ion measurements were made during fertilization in low sodium ASW. Figures 6 and 7 show that the efflux of H^+ ions is much reduced; presumably because under such low sodium conditions the Na^+/H^+ exchanger is rate-limited. We can therefore be confident that the peak that is unaffected by the low sodium conditions must be due to cortical granule release, keeping in mind that the duration will be slightly overestimated due to the time constant (3 s) of the vibrating probe.

The resulting cortical granule hydrogen ion peak in *Arbacia* eggs fertilized in low sodium media is on average 29 s, which is considerably longer then expected from Moser's data [34]. Therefore, some residual exocytotic activity seems to be left for a short period of time after the cortical granule wave has passed. Since the hydrogen ion efflux rate in low sodium media is lower, it takes much longer for the egg to increase its internal pH, as is evidenced by the long trailing hydrogen ion signal in Figure 6. The same holds true for *Lytechinus* (Fig. 7). Since the hydrogen ion peak thus last for about 29 s, the calcium release from *Arbacia* eggs due to cortical granule release likewise should last for about this period of time. If calcium was free to diffuse away, it would only take less than a second to diffuse the distance of about 10–20 μm to the probe ($D_{Ca^{2+}}$ is 8 x 10^{-6} cm^2/s). A diffusional lag-period therefore cannot explain why the calcium peak is so much longer.

The duration of the calcium signal in eggs in which the FE only elevates slightly is comparable to the duration in eggs that show much more FE elevation (*see* Fig. 2). This argues against the possibility that the persisting calcium signal is due to chemical or enzymatic processes that occur during the modification of the FE [39]. This does not completely exclude the possibility, however, that some calcium might be released from calcium binding sites while the FE and the egg periphery are being modified [39]. There exist some indications in the literature that the calcium that is present in the cortical vesicles is bound. First, Gillot et al. [31] have shown that only part of the cortical vesicle calcium is exchangeable. Second, it has been shown that some of the cortical granule components actually bind calcium [38]. Third, Azamia and Chambers [16] show that the total calcium content of *Arbacia* eggs that were **not** washed in calcium free sea water was 15% higher than of eggs that were washed in this way. The authors explain this by assuming: 'The initial increase in Ca content represents the absorption of extracellular Ca to cortical granule materials sud- denly liberated after fertilization'. It seems there- fore plausible that at least part of the calcium that is present in the cortical granules will remain bound to the components of the cortical granules in the perivitelline space. This again argues against large scale calcium loss during FE modification.

Quantification of the amount of calcium lost from sea urchin eggs

If one assumes that the calcium efflux is more or less equal over the whole egg surface, it becomes possible to calculate the total amount of calcium that is lost from an egg — for *Arbacia* eggs, the total calcium efflux at a calcium background of 1 mM was 558 pmol/cm^2. The average diameter of these eggs is 74 μm. Taking the vibration amplitude of the electrode and its proximity to the egg surface into account, the probe was measuring the calcium efflux from a sphere with a surface area of approximately 40 800 μm^2. The amount of calcium lost from the egg is thus 0.23 pmoles. The amount lost at 0.1 mM was smaller (about 0.1 pmoles). However, the amount lost at 1 mM should be more reliable, since this is closer to the natural calcium concentration of about 10 mM of normal sea water.

In *Lytechinus* eggs the elevation of the fertilization envelope is much more pronounced. This makes it necessary to adjust the probe position as the fertilization envelope elevates. This complicates the calculations considerably. However, quite often the envelope elevates asymmetrically, such as in the example of Figure 2, where the egg happens to remain close to the fertilization envelope near the probe position. In such a case, the probe position does not have to be adjusted. Calculating the average amount of calcium lost from such a *Lytechinus* egg (diameter 111 μm) yields a value of about 2 pmoles. Similar calculations for *Phallusia* eggs yield values of 0.13 ± 0.06 pmoles of calcium lost during the primary fertilization peak and 0.04 ± 0.02 pmoles during the post-fertilization pulses. Note that the calcium signals that are picked up by the vibrating probe for *Phallusia* eggs are much smaller than for sea urchin eggs because the diameter of the *Phallusia* egg (140 μm), and thus the surface area, is much larger than for sea urchin eggs.

If one combines the sea urchin data from the H^+-ion efflux measurements with the data Ca^{2+}-ion efflux measurements, it becomes possible to address the question of how much of the calcium that is lost from the egg is due to cortical vesicle exocytosis. The average time for the first *Arbacia* H^+ peak is 24 s (*see* Figs 4 and 6). Numerical integration of the first 24 s of the *Arbacia* calcium curves yields an average of 14 ± 4% of the total calcium efflux (*see* Fig. 1). However, since during this first phase presumably both cortical granule release and active calcium-pumping is occuring, only part can be attributed to cortical vesicle exocytosis. How much calcium is freed into the sea water from the cortical vesicles will depend upon the equilibrium between the calcium that is bound to the granule matrix and the free calcium in the seawater. Although we had to do our measurements at a calcium concentration of 0.1 or 1 mM to get a reasonable signal-to-noise ratio, the calcium concentration in normal sea water is about 10 mM. Therefore, in normal seawater even less calcium should originate from the cortical granules than under the experimental conditions. For *Lytechinus* eggs the same estimation is less acurate because the H^+ peak that is due to cortical

granule release is not seperated from the H^+ peak that is due to the activity of the Na^+/H^+ exchanger (Fig. 5).

As calculated above, according to our calcium specific vibrating probe data, the *Arbacia* egg loses on average 0.23 pmoles calcium in 3.4 min. The total calcium content of an *Arbacia* egg is 0.64 pmoles/egg [16]. Therefore, the egg loses on average 36% of this calcium in the first 3.4 min. According to the theoretical analysis of Azarnia and Chambers, this should have been only about 9%. This is a surprisingly large discrepancy. Azarnia and Chambers [16] could not make measurements during the first 3 min after fertilization because they had to wash the eggs with calcium-free sea water to get reliable total calcium data. The calcium-loss during the first 3 min was extrapolated from the data of samples taken at later times. However, the mechanisms or their rate constants during the initial phases of calcium loss may be different from later phases. On the other hand, we did our experiments in low calcium ASW, whereas Azarnia and Chambers kept their stock of fertilized eggs in normal sea water. It is therefore quite possible that our method results in a larger calcium efflux and thus an overestimate of the amount of calcium normally lost from the egg. First, the calcium pumps would find it easier to pump against a lower external calcium concentration. Second, there should be a smaller amount of calcium leaking back into the eggs at lower external calcium concentrations. Unfortunately the resulting loss in sensitivity prohibits the use of the calcium selective vibrating probe in normal sea water.

To our knowledge, the total calcium content of *Lytechinus pictus* eggs has not been published (the value for *Lytechinus variegatus* is 3.1 μmol/ml; *see* [16]). It therefore remains unclear which percentage of calcium is lost from these eggs. The total amount of calcium lost per unit of surface area for *Lytechinus pictus* is 5 times higher than for *Arbacia punctulata* eggs (2800 ± 820 versus 558 ± 128 $pmol/cm^2$). On the other hand, the volume of a *Lytechinus pictus* egg (diameter 111 μm) is about 3.5 times the volume of an *Arbacia punctulata* egg (diameter 74 μm). On a per volume basis, the amount of calcium lost from both types of eggs are therefore about equal.

In summary we can conclude that a cell membrane calcium pump is responsible for most of the calcium that is lost from fertilizing eggs, while cortical granule release can account for less than about 14% of the calcium lost after fertilization of sea urchin eggs.

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