Structural basis of the activation wave in the egg of *Xenopus laevis*

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SUMMARY

A series of changes in the surface of activated *Xenopus* eggs was observed. Within a few seconds of prick activation a light area appears near the pricking point and expands as a circular light zone (light wave). Some 60 s later this is followed by a dark area expanding as a circular dark zone (dark wave). Both waves travel at a rate of about $9 \mu m/s$ at 21 °C. In the light zone, cortical granules are breaking down, microvilli are elongating, and the egg surface is expanded. On the other hand, the elongated microvilli are reshortening to become globular and the egg surface is contracted in the dark zone.

INTRODUCTION

Using time-lapse cinematographic technique, Hara & Tydeman (1979) demonstrated wave-like propagation of dark–light–dark circular zones of brightness from the site of sperm entrance in artificially inseminated *Xenopus* eggs. This change in brightness of egg surface, called the 'activation wave (AW)' by them, started some 5–8 min after artificial insemination and travelled over the animal hemisphere towards the vegetal region at about 0·6 mm/min at 21 °C. Hara & Tydeman suggested that the AW reflects the propagation of the front of cortical granule breakdown and related phenomena.

A detailed observation of the AW was made in the present study. Surface changes were analysed by use of time-lapse cinematography or time-lapse video tape recording (VTR). The dark–light–dark zones described by Hara & Tydeman were preceded by an additional light zone. The AW was accompanied by contraction and relaxation of egg surface. Observations by electron microscopy revealed a correlation of the AW with the ultrastructure of the egg surface.

MATERIALS AND METHODS

Preparation of unfertilized eggs

Unfertilized eggs of the frog, *Xenopus laevis*, were squeezed from females following injection of 300 i.u. human gonadotrophic hormone ('Gonatropin' Teikoku Zoki Ltd., Tokyo). Eggs were dejellied by being gently swirled in 1 %

sodium thioglycolate at pH 9–10 for 1 min. Preliminary observation by SEM revealed that treatment with 0·3 % pronase for 3 min is enough to remove the vitelline membranes completely. In routine experiments eggs were treated with 0·3 % pronase at pH 6·8 for 5 min. The denuded eggs were settled on a flat surface of agar in modified Steinberg's solution buffered to pH 6·8 by 3 mM-HEPES-NaOH in place of Tris-HCl.

Time-lapse recording

Eggs were allowed to rest in agar-coated vessels for observation with the animal hemisphere facing upward in the modified Steinberg's saline. The materials were illuminated aslant from two directions and imaged from above or occasionally from the side either with a time-lapse cine camera (Bolex H-16M equipped with Nikon CFMA) or with a time-lapse video system (National NV-8030) through a microscope. As described in the results section, we activated the eggs under recording mainly by pricking with a glass needle rather than by insemination, since this method enabled us to determine the exact site and time of prick activation.

In order to relate our observations to those by Hara & Tydeman, time-lapse recording was also made on artificially fertilized eggs. Unfertilized eggs with intact jelly and vitelline membranes, settled in observation vessels in full-strength modified Steinberg's solution, were artificially inseminated and the subsequent changes in the egg surface were recorded.

Carbon marking

To detect any local stretching and contraction of the surface, denuded eggs were loaded with minute carbon particles (Norit, 'extra') on their animal hemispheres. Movement of the particles after pricking of the eggs was analysed by measuring the distances between adjacent particles or between each particle and the point of pricking either on still pictures taken at intervals of 15 s or on frames of VTR.

Electron microscopy

A few minutes after pricking, the eggs under recording with VTR were removed from the observation vessels and transferred into a solution of 2·0 % or 2·5 % glutaraldehyde (GA) and 0·8 % paraformaldehyde in 0·05 M-phosphate buffer (pH 7·2) at room temperature. 2·0 % GA was used for scanning electron microscopy (SEM) and 2·5 % GA for transmission electron microscopy (TEM). The time required for the transfer was less than 10 s. The last frame of VTR was used to determine the position of the AW travelling on the egg surface at the time of fixation. After fixation for about 12 h eggs were postfixed in 1 % OsO_4 in 0·05 M-phosphate buffer (pH 7·2) for 1 h at 4 °C.

For SEM eggs were slowly dehydrated through a graded series of acetones and

dried by the critical-point method. The dried eggs were attached to stubs with silver conducting paste, coated with gold and observed under SEM (Jeol JSM-T20).

For TEM the postfixed eggs were dehydrated in a graded ethanol series and embedded in Spurr resin (Spurr, 1969). Thin sections were made with an LKB Ultrotome III 8800 and stained with 1 % aqueous uranyl acetate and with lead citrate. Observations were carried out with a Hitachi HU-11D-1 transmission electron microscope.

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RESULTS

The AW in artificially inseminated eggs

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By time-lapse cinematography or video recording we observed a dark spot appearing some 5–10 min after insemination on the animal surface of the egg, which propagated as a circular dark zone along the egg surface at a rate of about 9 μm/s at 21 °C. Comparison of the time of appearance and the speed of propagation of
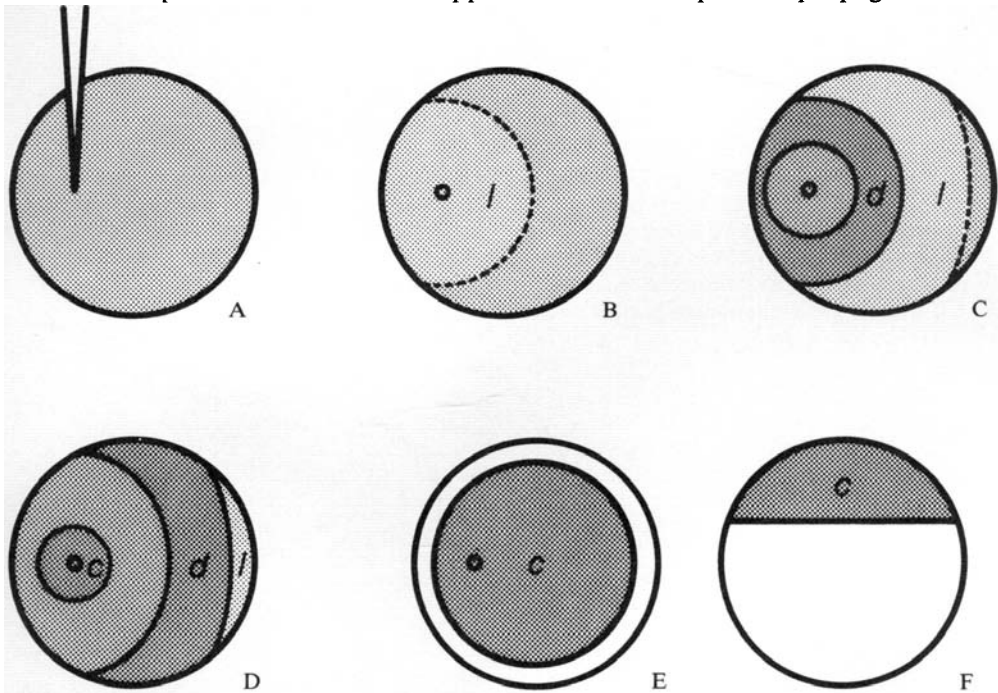

Fig. 1. Diagrammatic representation of the AW, which consists of light and dark waves, propagating on the animal surface of the egg of *Xenopus*. (A–C) 0, 1 and 2 min after pricking respectively. The light wave (*l*) and the dark wave (*d*) are represented as circular light and dark zones respectively. The front of the light wave is shown by a dotted line and the dark wave is located between two solid lines. (D) 3 min after pricking. The dark wave is propagating. The cortical contraction (*c*) begins from the pricking point. (E) and (F) 4 min after pricking, as viewed from above (E) and from the side (F). The dark wave has passed away and now the animal hemisphere is contracting.
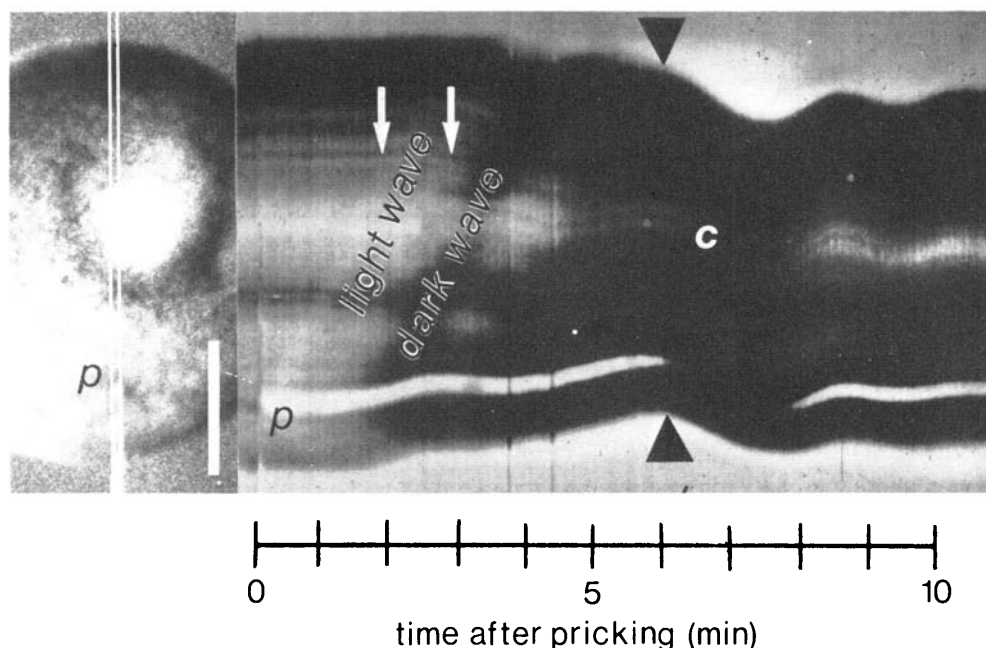
Fig. 2. Photokymogram of the AW in an activated egg at 21 °C. A frame of the cinematographic film recording the AW is shown in the left photograph. This film was projected through a narrow slit onto photographic paper on a rotating drum, by which a change in the brightness of the narrow rectangular portion of the egg surface (marked between two parallel lines in the left photograph) was visualized on a still picture (right 'photokymogram'). A pricking point (*p*) is seen as a white horizontal band in the photokymogram. Arrows show the front of the light and dark waves. The former appears immediately after pricking, and the latter about 60 s after the former. Both waves propagate on the egg surface from the pricking point. After the passage of the dark wave the egg surface regains its normal brightness and then the whole pigmented surface contracts to make the animal surface darker again (cortical contraction: *c*). During this contraction, the egg sways several times (cf. Fig. 4). In this photokymogram, this swaying begins about 6 min after pricking and is visualized as undulating margins of animal pigmented zone (arrow heads).

this wave with the respective values reported by Hara & Tydeman (1979), makes it certain that this dark zone is the first part of what Hara & Tydeman called the 'activation wave'. After passage of the dark zone the egg surface regained its normal brightness. Then the whole pigmented surface contracted to make the animal surface darker again. This contraction has been called 'cortical contraction'. These serial changes in brightness are apparently identical with what Hara & Tydeman described as the wave-like propagation of 'dark–light–dark' zones. The second dark zone is actually a result of the contraction of the whole pigmented surface of the animal hemisphere. It was noticed, moreover, that some 60 s before the appearance of the first dark zone another circular light zone appeared and propagated at the same rate.

By artificial fertilization, however, it is usually hard to determine the exact

time of activation, so that a consistent timing of the appearance of the AW cannot be found. To overcome this difficulty we activated the eggs by pricking instead of insemination. As will be shown, the AW is still observed in these eggs, and its pattern and speed of the propagation are identical to those seen in artificially inseminated eggs. Routine observations to follow were made on the eggs activated by glass needle pricking.

The AW in artificially activated eggs

In artificially activated eggs the circular light zone appeared first and the dark zone followed it, as in inseminated eggs. These two waves will be termed the 'light wave' and the 'dark wave' hereafter. A schematic representation of these phenomena is given in Fig. 1, and a photokymogram which visualizes propagation of the AW is shown in Fig. 2. The AW (including both light and dark waves) was found to originate at the point of pricking, in the same way as the AW in inseminated eggs starts from the site of sperm entrance (Hara & Tydeman, 1979).

These characters of the AW are consistent with those observed in inseminated eggs. A new finding is that the light wave appears immediately after activation, as seen in Fig. 2. The only difference in appearance of the AW's between inseminated and pricked eggs is that the front of the light wave in pricked eggs was clearer than that in inseminated eggs. This, however, will be due to the absence of jelly and vitelline membrane in pricked eggs.

The light wave started to expand as a circular light area and travelled on the animal hemisphere for about 150 s at an average speed of 9 µm/s (ranging from 8·1 to 9·8 µm/s in 11 eggs) at 21 °C. The dark wave appeared about 60 s after pricking and propagated as a circular dark zone at a rate nearly the same as the light wave (7·9–9·7 µm/s in 11 eggs). After the passage of these waves the animal pigmented surface contracted from the pricking point. The AW resembles 'surface contraction waves (SCWs)' (Hara, 1971; Sawai, 1982; Yoneda, Kobayakawa, Kubota & Sakai, 1982) in that both AW and SCWs are circular expansions of light and dark zones along the surface of amphibian eggs, though the AW propagates about 10 times faster than the SCWs. Even so its propagation is still too slow to be identified in real time, but it is observable from films or records speeded up about 24 times.

Movement of the egg surface accompanying the AW

Movement of carbon particles on the surface of the animal hemisphere is shown in Fig. 3. The distances were measured on still pictures taken at intervals of 15 s. Fig. 3A shows the distances between the pricking point and each particle, these distances first shorten rapidly, then lengthen a little and reshorten slowly. Fig. 3B shows the changes of local contraction rates. The egg surface first relaxes, and then contracts, after which it tends to relax again. These serial changes propagate on the egg surface from the pricking point. To find the correlation
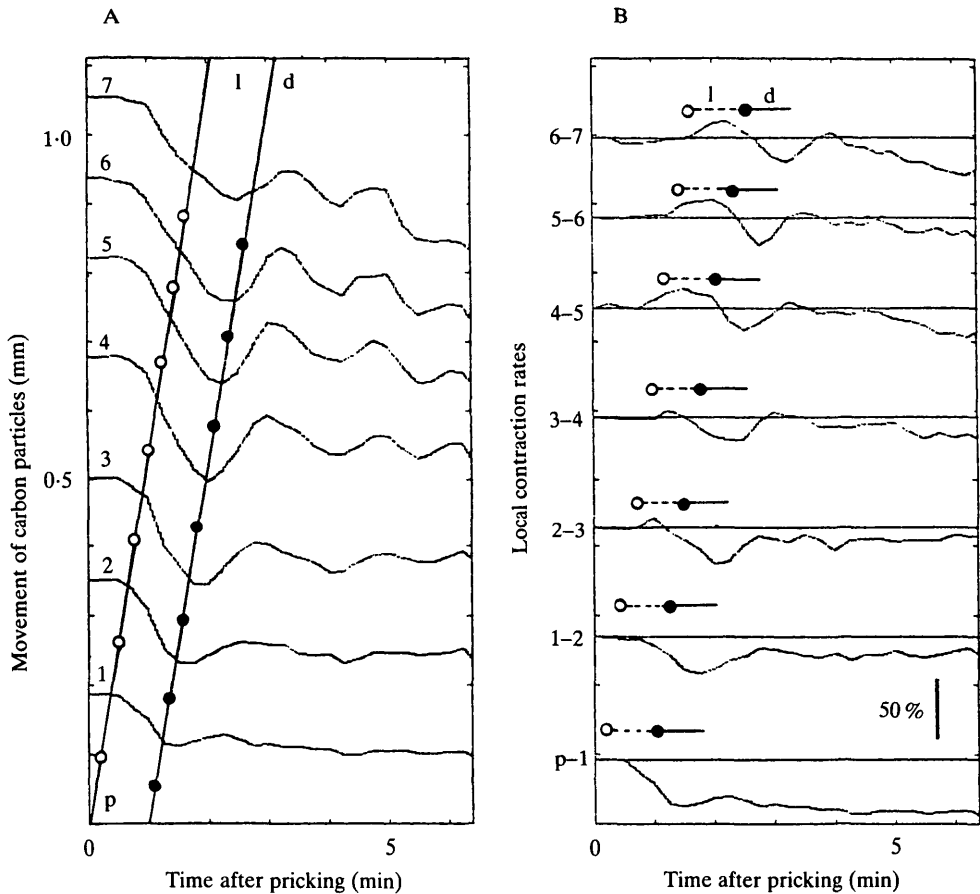
Fig. 3. (A) The movement of carbon particles, which are aligned in a single diameter and numbered from 1 to 7, is shown as distances from the pricking point (p). Oblique lines show the propagation of light and dark waves (l, d). Open and solid circles show the times when the fronts of the light and dark waves pass the middle point of each interval. (B) Distances between adjacent points (p, 1–7) showing local stretching and contraction of the egg surface. Distances are expressed as relative values with reference to the initial distances (horizontal lines). Bar equals 50 %. Open and solid circles, corresponding to those in Fig. 3A, indicate the times of arrival of the light and dark waves at the middle point of each segment. Thus broken and solid lines refer to the passage of light and dark waves respectively.

between these changes of each segment and the passage of the AW, in Fig. 3A the normalized course of the AW (the speed is taken as 9 μm/s, and the interval between the light and dark waves as 60 s) is drawn as slant lines. In Fig. 3B the times of arrival of the light (open circles) and dark (solid circles) waves at each segment, estimated from Fig. 3A, are also shown. It is seen that the period of relaxation of each segment coincides fairly well with the passage of the light wave, and the period of contraction with the dark wave. The same correspondence of light–dark with relaxation–contraction was also found by analysis on

frames of VTR. These results suggest that changes in the brightness of the egg surface are ascribed to those in the density of pigment granules in the subcortical region. After the passage of the dark wave, temporary relaxation and cortical contraction follow.

Time-lapse observation of cortical contraction

Time-lapse observation revealed that maximum contraction occurred about 6 min after pricking and then the cortex relaxed slowly during another 10 min. During this contraction, eggs swayed first to the pricked side then to the opposite side, as seen in Figs 2 and 4. This swing was repeated several times. This oscillation was also observed in artificially inseminated eggs within their vitelline membranes. Although its significance is not clear, it is possible that it might have some role in the rotation of intact eggs in the perivitelline space.

Ultrastructural changes

Figs 5, 6, 7 and 8 show the ultrastructure of the animal hemisphere surface of the eggs. These eggs were fixed 1, 2, 3 and 4 min after pricking respectively and observed by SEM. Observations by TEM were also carried out on the animal cortex of eggs fixed 2 min after pricking (Fig. 9). The changes in these eggs had been recorded from above by time-lapse video until just before fixation so that the exact position of light and dark waves in the fixed eggs could be located.
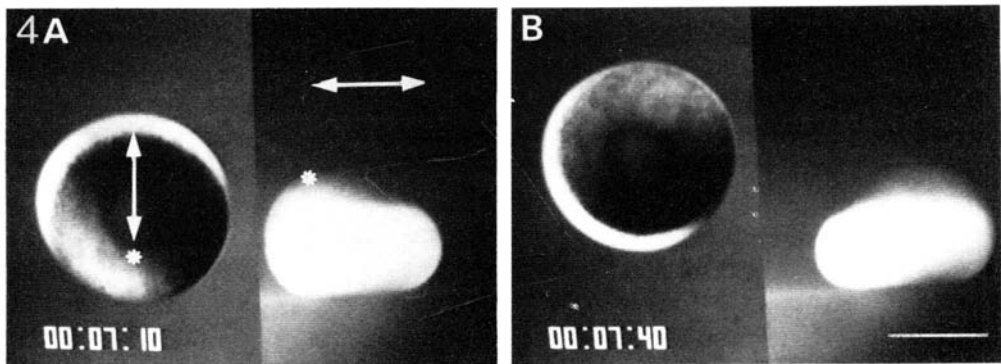

Fig. 4. Swing movement of an activated egg. Eggs are viewed both from above and from the side and recorded by a video system onto a single video tape by a special image processor (National WJ-545, cf. Yoneda *et al.* 1982). The top view is shown in the left half of each frame. The side view from the right side of the top view is shown in the right half. Asterisks show the point of pricking. (A) 7 min 10 s after pricking. The pigmented animal hemisphere leans towards the pricked side. (B) 7 min 40 s after pricking. Now the animal hemisphere leans towards the opposite side. The amplitude of this swing is variable among eggs and this sample is one of the very obvious cases. The direction of this oscillation (arrows) always lies along the line connecting the point of pricking and the opposite side of the egg. Bar equals 1 mm.
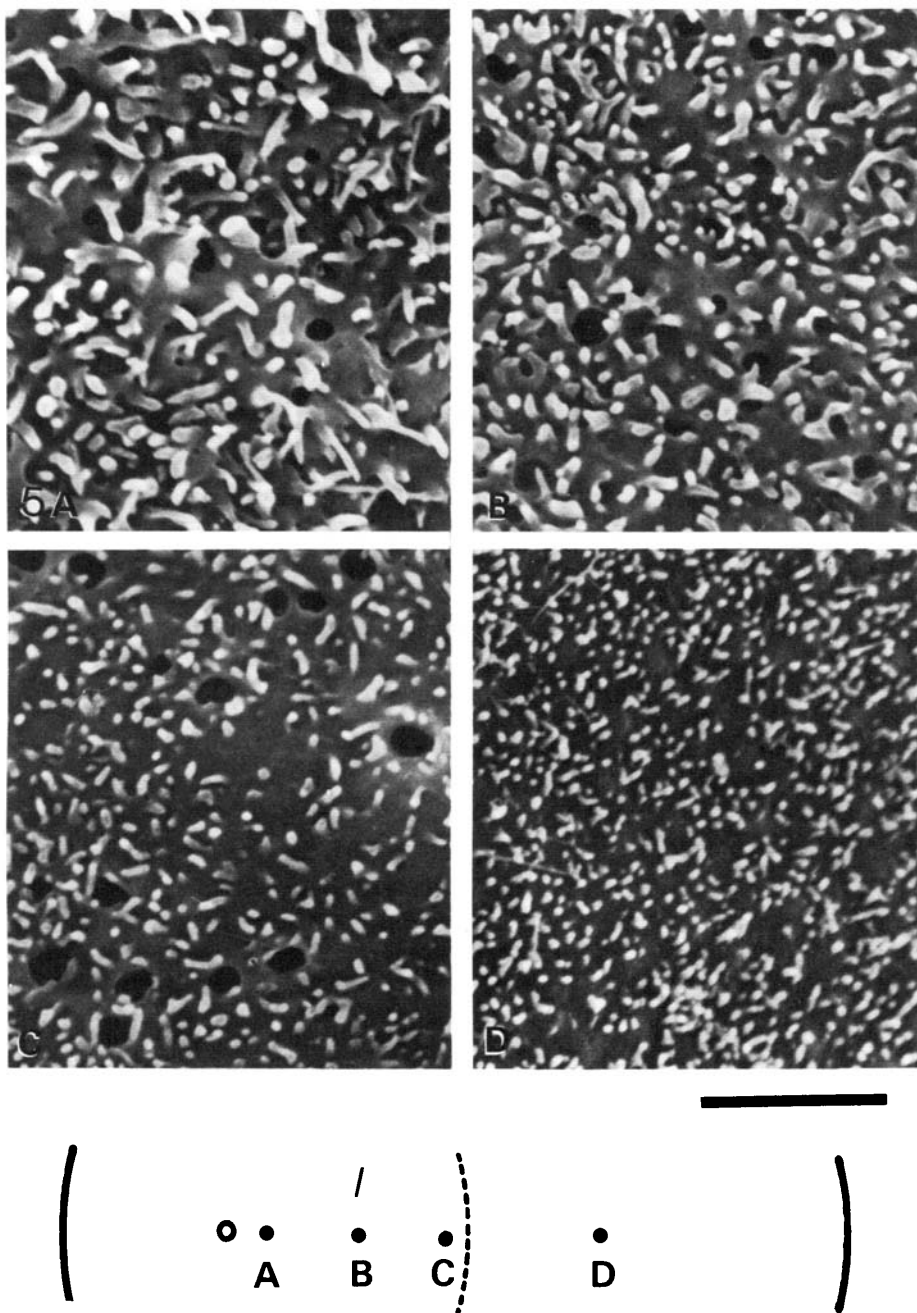
Fig. 5. Views of the surface of the animal hemisphere of an egg fixed 1 min after pricking. The position of each micrograph on the egg surface is shown in a line drawing (below). An open circle: the pricking point. A dotted line: the front of the light wave (*l*). Elongation of microvilli and exocytosis of cortical granules are observed in the light wave area (A, B, C). Outside this area, microvilli are short and stubby (D). This is the same feature as observed in unactivated eggs (cf. Fig. 9A). Bar equals 5 μm.
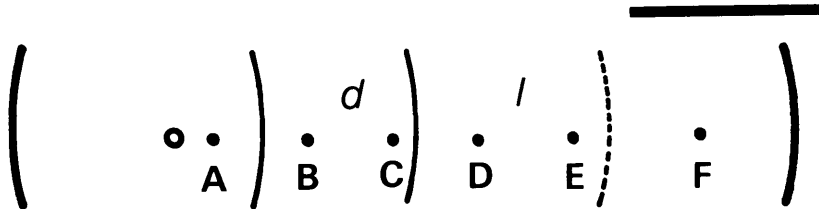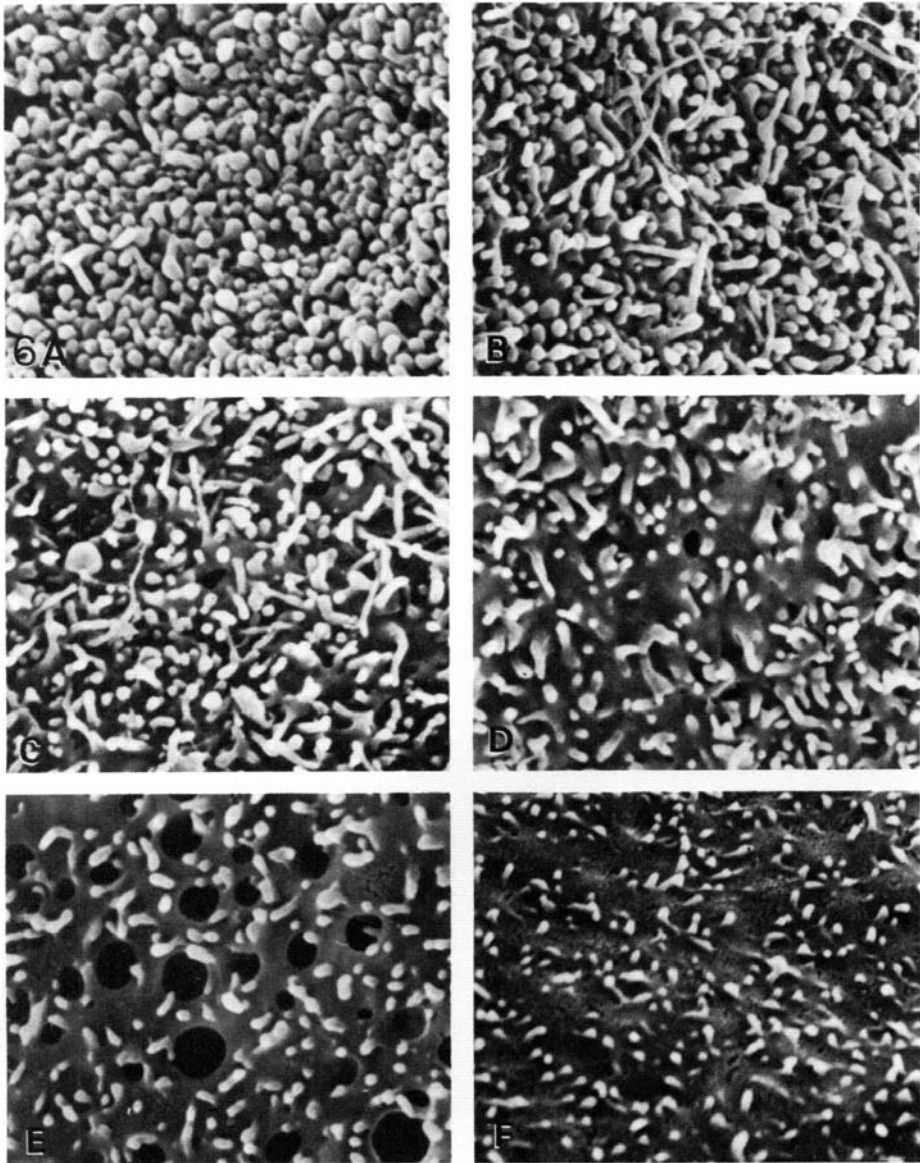
Fig. 6. 2 min after pricking. After the passage of the dark wave microvilli become globular (A). In the dark wave area (*d*), which is located between two circular solid lines in the diagram below the photographs, elongated microvilli are reshortening (B, C). Exocytosis of cortical granules is observed in the light wave area (D, E) and stubby microvilli outside it (F). Bar equals 5 μm.
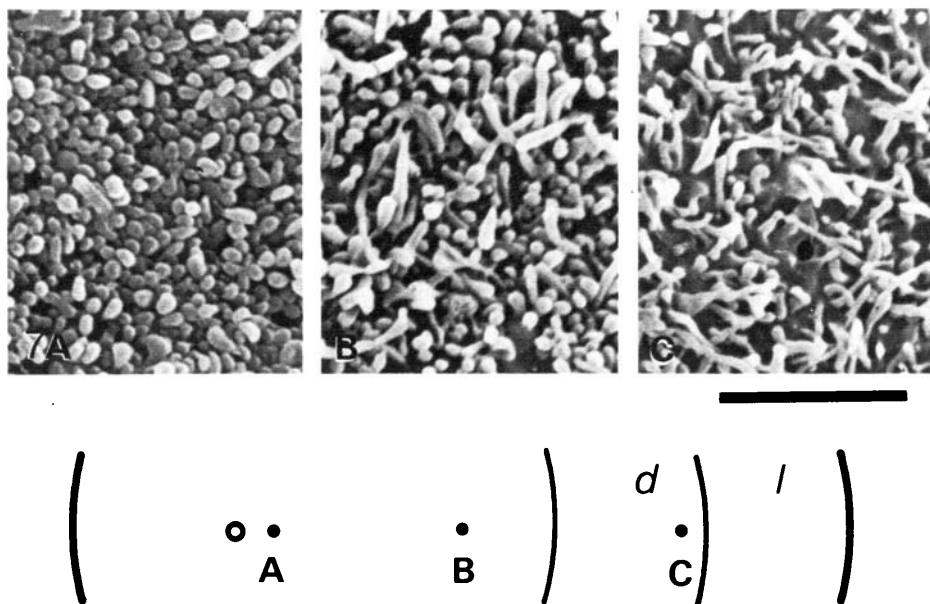
Fig. 7. 3 min after pricking. Microvilli are most elongated at the front of the dark wave (C) and become short and globular (A, B). Bar equals 5 μm.
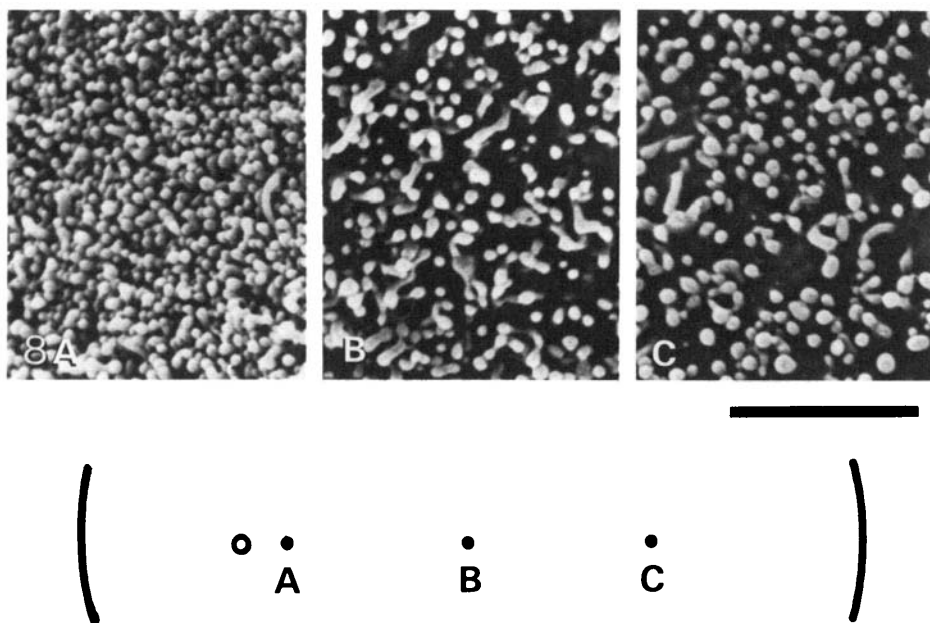

Fig. 8. 4 min after pricking. The dark wave has passed away. No elongated microvilli are observed. Bar equals 5 μm.
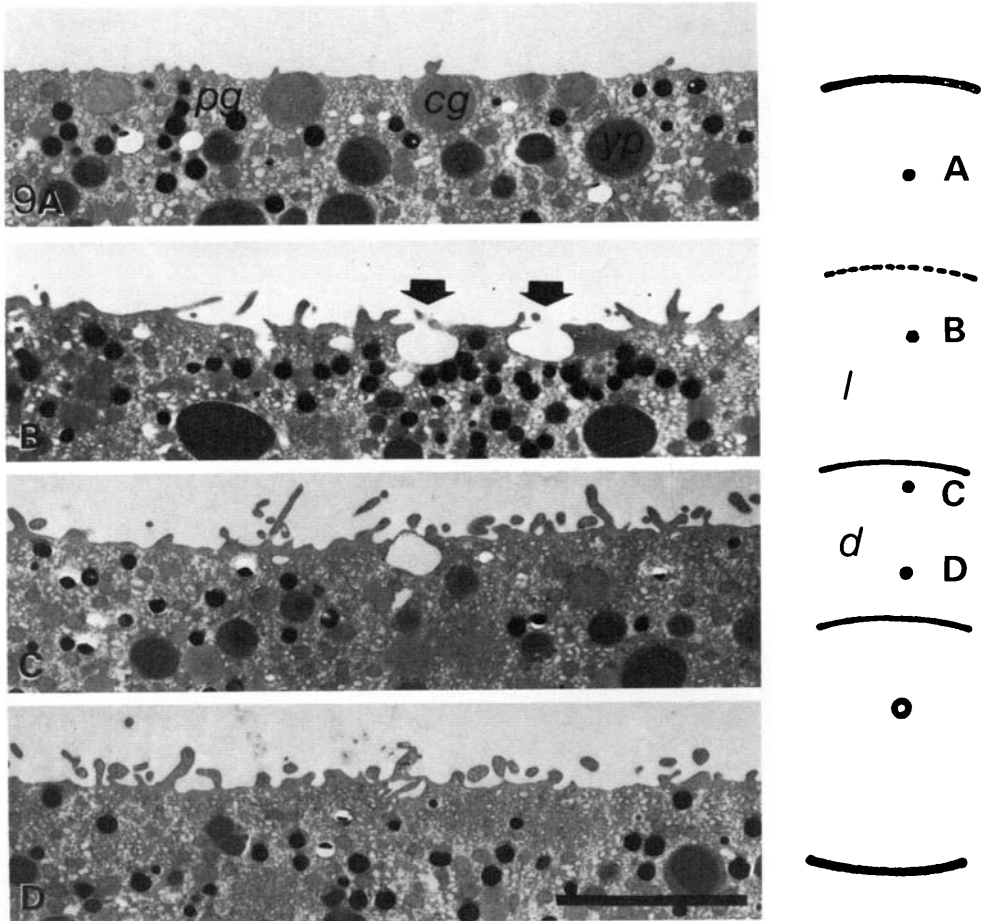
Fig. 9. Exocytosis of cortical granules in an egg fixed 2 min after pricking. There are intact cortical granules (*cg*) before the arrival of the light wave (A). They are breaking down (arrows) in the light zone (B). Elongation and reshortening of microvilli are observed in the dark area (C, D). Bar equals 5 μm. *pg*: pigment granule, *yp*: yolk platelet.

Before the arrival of the light wave, the egg surface has short and stubby microvilli distributed uniformly in the animal hemisphere (Figs 5D, 6F, 9A). In the light wave area we observed the appearance of dark circular holes, indicating the breakdown of cortical granules (Figs 5C, 6D, E). Observation by TEM of the eggs 2 min after pricking confirms the exocytosis of cortical granules (Fig. 9). Before the arrival of the light wave, the cortical granules exist in the cortex (Fig. 9A). They are breaking down in the light zone (Fig. 9B) and few of them are left in the dark zone (Fig. 9C, D).

In the light wave area microvilli begin to elongate (Figs 5A, B, C, 6D, E). At the front of the dark wave microvilli are most elongated (Figs 6C, 7C). Then they reshorten and become globular in the dark zone (Figs 6B, 7B). After the passage
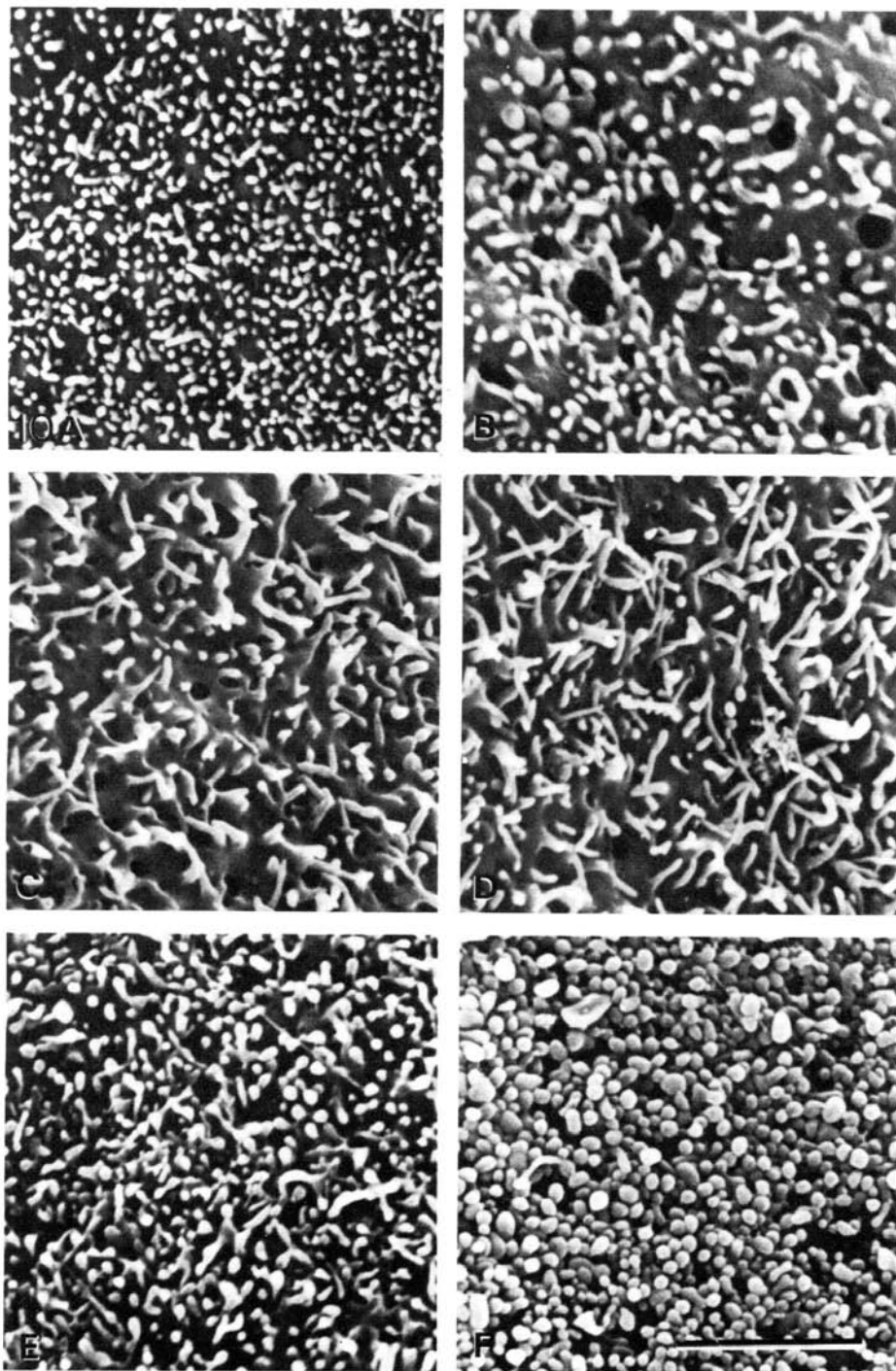
Fig. 10. Serial changes in ultrastructure. (A) Animal hemisphere of an unactivated egg. (B–F) Eggs fixed 30 (B), 60 (C), 90 (D), 120 (E) and 180 (F) s after pricking respectively. A corresponding position close to the pricking point of each egg is shown. Bar equals 5 μm.

of the dark wave they completely shorten to form a globular shape (Figs 7A, 8A, B, C). A few eggs fixed 5–10 min after pricking were also observed. These eggs showed a similar appearance to those fixed 4 min after pricking. No remarkable change seems to have occurred after 4 min.

It is also noticed from these figures that the density of microvilli changes as the AW propagates on the egg surface. The density of microvilli in the light zone (Figs 5C, 6C) is lower than that of the unactivated surface (Figs 5D, 6F), while in the dark zone (Figs 6B, 7C) microvilli are more crowded than in the unactivated area. The apparent high density of microvilli in the dark zone and the lowering of this density in the light zone will be due to relaxation in the light zone and contraction in the dark zone of egg surface as has been shown by the movement of attached carbon particles.

Fig. 10A shows the animal surface of an unactivated egg and Fig. 10B–F represent the ultrastructure of the positions close to the pricking points of eggs fixed 30, 60, 90, 120 and 180s after pricking. Fig. 10 reveals a series of changes which a part of the egg surface seems to undergo; exocytosis of cortical granules, elongation and reshortening of microvilli.

DISCUSSION

Propagation of the AW in *Xenopus* eggs activated either by pricking or by insemination was observed in the present study. The AW has been first reported as a wave-like propagation of dark–light–dark zones (Hara & Tydeman, 1979), but it has now been shown that a 'light wave' appears immediately after pricking, so that a series of changes of 'light–dark–light–dark' (light wave, dark wave, relaxation, cortical contraction) occurs in the brightness of the egg surface. The first light wave has been unnoticed in the work of Hara & Tydeman (1979) perhaps because it is vaguer in intact eggs with jelly and vitelline membranes than in denuded eggs.

Stewart-Savage & Grey (1982) reported that contraction begins as early as about 1 min after pricking. However, they mentioned that the cortical contraction is the first observed change in the pigment pattern. The 'contraction' at 1 min after pricking might be actually the first appearance of the dark wave. The result obtained by Hara & Tydeman (1979) and the present result (Fig. 2) clearly show that the dark wave is separated from the following cortical contraction by the presence of the relaxation zone. Since this zone of relaxation is noticed only by time-lapse recording, it is possible that Stewart-Savage & Grey (1982) regarded the dark wave and the cortical contraction as continuous phenomena. We think that the dark wave and the cortical contraction are two different kinds of phenomenon although both begin from the pricking point; the former propagates as a circular dark zone whereas the latter is the contraction of the whole pigmented surface towards the animal pole.

It has been reported from SEM study that club-like microvilli and the large

hemispherical protrusions carrying them exist in the unfertilized eggs of *Xenopus laevis* (Monroy & Baccetti, 1975). In the present study similar microvilli were observed on the unactivated surface (Figs 5D, 6F, 9A, 10A), but such protrusions were not found.

A series of changes in the ultrastructure of the activating egg surface, as revealed in this study, has been also found in *Rana* (Elinson, 1980; Goldenberg & Elinson, 1980) and *Bufo* (Iwao, 1982). In these studies cortical granule breakdown and subsequent elongation of microvilli were also observed by SEM. Essentially the same changes have been reported in sea urchins (Eddy & Shapiro, 1976; Schroeder, 1978, 1979; Chandler & Heuser, 1979, 1981; Kidd & Mazia, 1980). In *Xenopus*, cortical granules and their breakdown have been observed by TEM (Grey, Wolf & Hedrick, 1974; Wolf, 1974; Campanella & Andreuccetti, 1977). The present study related these morphological changes of the egg surface to the AW and movement of the egg surface; in the light wave area the surface is expanded and both exocytosis of cortical granules and elongation of microvilli are observed. In the dark wave area the surface is contracted and the elongated microvilli are shortening to form a globular shape.

In the present study the eggs were activated mainly by pricking with a glass needle rather than by artificial insemination. This choice enabled us to demonstrate more closely the temporal correlation of the AW with ultrastructural changes in the egg surface, with enhanced visibility of the AW.

A question may be raised whether or not the temporal correlation found in prick-activated eggs applies also to normally fertilized eggs, since prick-activated *Xenopus* eggs do not cleave (Hara, Tydeman & Kirschner, 1980). The similarity of the AW in pricked and fertilized eggs with respect to its speed and light–dark pattern as well as its site of initiation was already mentioned. The corresponding comparative investigation of ultrastructural changes was not done in the present study: we found it very difficult to remove the vitelline membrane at such early stages from fixed fertilized eggs of *Xenopus laevis*, for observation by SEM. The recent success by Picheral & Charboneau (1982) and Charboneau & Picheral (1983) in observing serial ultrastructural changes in fertilized eggs of *Rana* revealed that the process of cortical reaction is identical to those reported so far in artificially activated anuran eggs.

Although we have not directly demonstrated this, we expect that the temporal correlation of the AW with ultrastructural changes, i.e., cortical granule breakdown and the elongation of microvilli during the passage of the light wave, and the reshortening of microvilli as the dark wave propagates, still persists in normally fertilized *Xenopus* eggs.

It has been known that free calcium increases explosively after activation of eggs in many species (reviewed by Epel, 1980). In sea urchin eggs this increase causes both exocytosis of cortical granules (Vacquier, 1975) and elongation of microvilli (Begg, Rebhun & Hyatt, 1982). Further, Gilkey, Jaffe, Ridgway & Reynolds (1978) showed that a free Ca^{2+} wave preceding the exocytotic wave

traverses the egg of medaka from the site of sperm entry to the opposite side as a narrow band at a speed of about 12·5 μm/s. In *Xenopus*, it has been known that contractions of egg cortex are triggered by, or need, calcium (Gingell, 1970; Schroeder & Strickland, 1974; Merriam & Sauterer, 1983). The present study suggests that relaxation and contraction of the egg surface, exocytosis of cortical granules and morphological changes of microvilli, all of which proceed on the egg surface as a wave-like propagation, are co-regulated by a common mechanism related to the free Ca^{2+} change, although details must be investigated further.

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