Medaka, 3 (1985)17-23

PROPERTIES OF THE ISOLATED CORTEX OF THE MEDAKA EGG

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ABSTRACT — The release of cortical alveoli (CA) from the isolated egg cortex of the medaka, *Oryzias latipes*, was observed at the threshold level of 1 mM Ca^{2+} at pH 6.7, which on addition of phospholipase A_2 (PLase A_2) was lowered to 50 μM Ca^{2+} . The addition of ATP at 1 mM further lowered the threshold level to 1 μM Ca^{2+} . When the cortex was mechanically removed in a Ca-free medium, many protrusions were observed on the surface of the intact CA. The process of CA release in the isolated cortex was proposed.

INTRODUCTION

Fertilization in fish is marked by the cortical reaction, the most prominent of which is the breakdown of CA. After insemination of the egg, an invisible change, termed "fertilization wave" (Yamamoto, 1944), is propagated in the cortical cytoplasm. This triggers the wave-like breakdown of CA, starting from the animal pole, spreading over the egg surface and ending at the vegetal pole (Yamamoto, 1939). This wave is followed by the release of the alveolar contents into the perivitelline space and the elevation and hardening of the chorion (Nakano, 1956 ; Kudo, 1967 ; Iwamatsu, 1969).

Several studies have been devoted to the mechanism of CA release during fertilization. The success in the isolation of intact CA from the ovoplasm in the medaka egg (Nakano, 1956 ; Yamamoto, 1962 ; Iwamatsu and Ohta, 1976) has facilitated a study on the nature of the CA and factors which cause their dissolution. In fish eggs, it was observed that the presence of Ca^{2+} ions in the extracellular medium is necessary for sperm penetration into the egg and for activation of the egg (Yanagimachi and Kanoh, 1952 ; Yamamoto, 1954 ; Iwamatsu *et al.*, 1985). The eggs begin to take up ^{45}Ca about 30 sec after insemination with little uptake after completion of the cortical reaction (Iwamatsu, *et al.*, 1985). The release of Ca^{2+} ions from intracellular sources is believed to be a part of the trigger mechanism which initiates alveolar exocytosis (Steinhardt *et al.*, 1977 ; Gilkey *et al.*, 1978 ; Schuel, 1978). The injection of Ca^{2+} ionophore A23187, which carries Ca^{2+} ions through the membranes, induces alveolar or granular breakdown in the egg. In addition, the explosive rise in free Ca^{2+} ions during activation has been determined by a light-emitting protein, aequorin, in the eggs of the medaka (Ridgway *et al.*, 1977 ; Gilkey *et al.*, 1978) and of the sea urchin (Steinhardt *et al.*, 1977).

The present study was undertaken to determine the threshold level of extracellular Ca^{2+} which can trigger CA release in the medaka egg. Instead of intact eggs, the isolated cortex was used to facilitate exposure to extracellular Ca^{2+} . This procedure also allows the study of the mechanism of CA exocytosis by means of SEM observations on the morphological changes related to CA release.

MATERIALS AND METHODS

Obtaining Eggs and Isolation of the Cortex

Eggs of the orange-red variety of the medaka, *Oryzias latipes*, were used. Fish were reared under a controlled photoperiod of 14 hr light and 10 hr darkness at a water temperature of about 26℃. The spawning females were separated from the males the day before the experiments and the eggs were obtained by laparotomy from the ovarian lumen and placed in isotonic salt solution (Yamamoto, 1944).

Isolation of the cortex is designed to ensure

the exposure of the egg cortex to various media and to permit the direct observation of CA release of the egg cortex by SEM. For each batch, 5 to 6 eggs were transferred to a depressed glass slide and washed twice in ACA medium (refer to the next section) prior to the isolation process. The cortex of the egg was isolated in ACA medium by cutting the egg at the vegetal pole with fine iridectomy scissors and pushing it gently out of the chorion.

Preparation of Media and the Handling of the Isolated Cortex

The ACA medium was formulated by modifying the medium for sea urchin eggs (Sasaki and Epel, 1983). It consisted of 200 mM ε -aminocaproic acid (ACA), 5 mM NaCl, 2 mM $MgCl_2$, 10 mM EGTA or 1 mM $CaCl_2$ and 10 mM Good's buffer (for pH 5.7, MES ; pH 6.7, PIPES ; pH 9.0, CHES). For the study of effects of Ca^{2+} on CA release of the isolated cortex, either 10 mM EGTA or 1 mM $CaCl_2$ was used with the other components listed above.

To determine the threshold level of Ca^{2+} which can trigger CA release, Ca-buffer solutions were prepared. Since the cytoplasmic pH requirement at the threshold Ca^{2+} concentration to cause "calcium wave" during normal activation is between 7.0 to 7.2 (Gilkey, 1981), pH 6.7, which was assumed as the resting pH before CA release, was used for the exposure of the isolated cortices to various treatments. The desired levels of free Ca^{2+} and Mg^{2+} were calculated using the association constants for EGTA at pH 6.7 of 6.01×10^5 for Ca^{2+} and 18.4 for Mg^{2+} to produce the designated micromolar level of Ca^{2+} (Steinhardt *et al.*, 1977). In the succeeding series of experiments, 1 μg/ml phospholypase A_2 (PLase A_2) and 1 mM or 10 mM ATP were added, separately or together, to the ACA media with various Ca^{2+} concentrations at pH 6.7 to determine the factors which could trigger CA release in the isolated egg cortex. The isolated cortex was incubated in the above media for 10 min at room temperature (about 26 ℃). For comparison, direct observations were also carried out using a light microscope with Nomarski optics.

Preparation of Scanning Electron Microscopy (SEM)

After a 10 min incubation, the isolated cortices were fixed in 3.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), overnight at 4℃, washed in phosphate buffer and post-fixed in 1% osmium tetraoxide in 0.1 M phosphate buffer (pH 7.2) for 1-1.5 hr at 0℃ (Iwamatsu and Ohta, 1976). After washing in phosphate buffer, the samples were gradually dehydrated in the ethanol series and finally in isoamyl acetate. The specimens were critical point-dried in carbon dioxide and coated with gold (about 250 Å thickness) using an ion coater (IB-2 Eiko Eng. Co.). Observations were done with the SEM (Model Alpha 10, Akashi).

RESULTS

1. The Isolated Egg Cortex in Isotonic Salt Solution

The isolated CA appeared as smooth hyaline spheres as described by Nakano (1956, 1969). Under the Nomarski optics, the CA and oil droplets (OD) were distinctly observed (Fig.1). The CA have diameters ranging from 10-40 μm, whereas the OD have an average diameter of 80 μm, which may become much larger after fusion of contiguous droplets. Numerous hemispherical elevations and fine microvilli were observed on the surface of ripe unfertilized eggs in the isotonic salt solution (Fig.2a and b). On the other hand, in normal indentations, possibly those of the spherical bodies (SB) of CA in the process of release, or the crater-like pits of CA to be released were observed (Fig.2c and d).

2. Release of Cortical Alveoli (CA)

a. Effects of H^+ and Ca^{2+} on CA Release

The exposed surface of isolated cortex showed the presence of released and unreleased CA. The pockets with or without SB were counted as released CA, while the alveolar elevations were considered as unreleased CA (Fig.3). In the ACA medium, the presence of 1

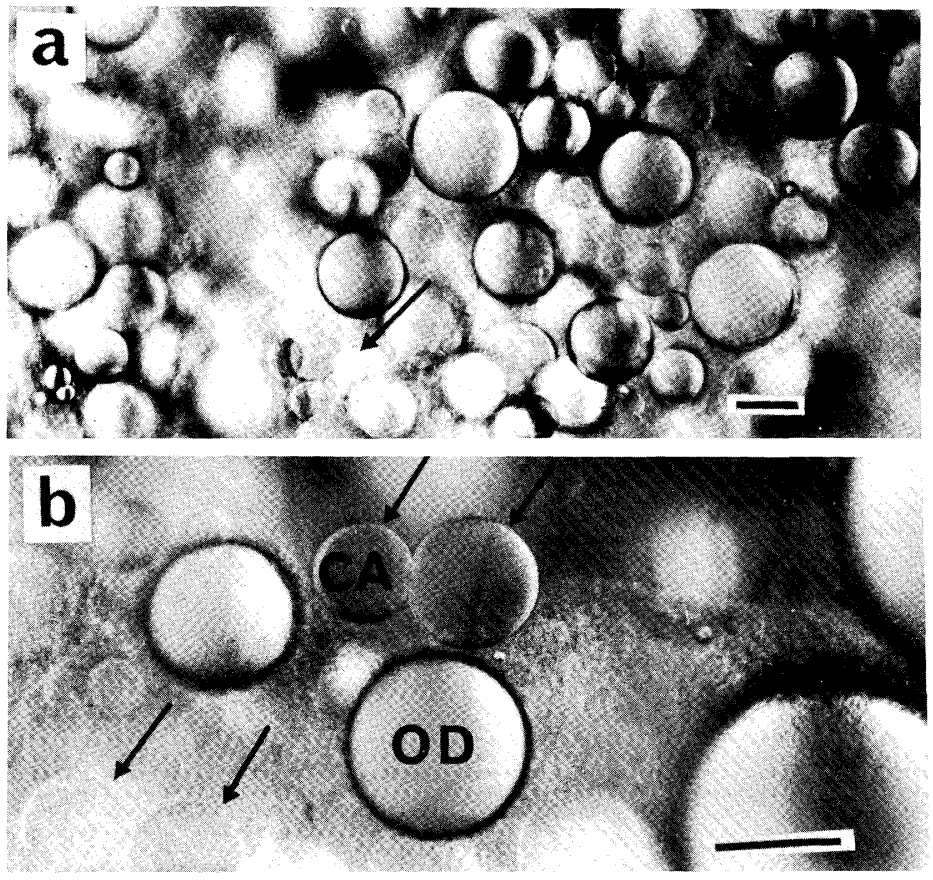

Fig.1 Surface of the isolated egg cortices viewed with Nomarski optics. (a) Low power magnification of the isolated cortices ; (b) Higher magnification of the same. Arrows point to the cortical alveoli (CA) ; OD, oil droplet. Scale bar : 50 μm.

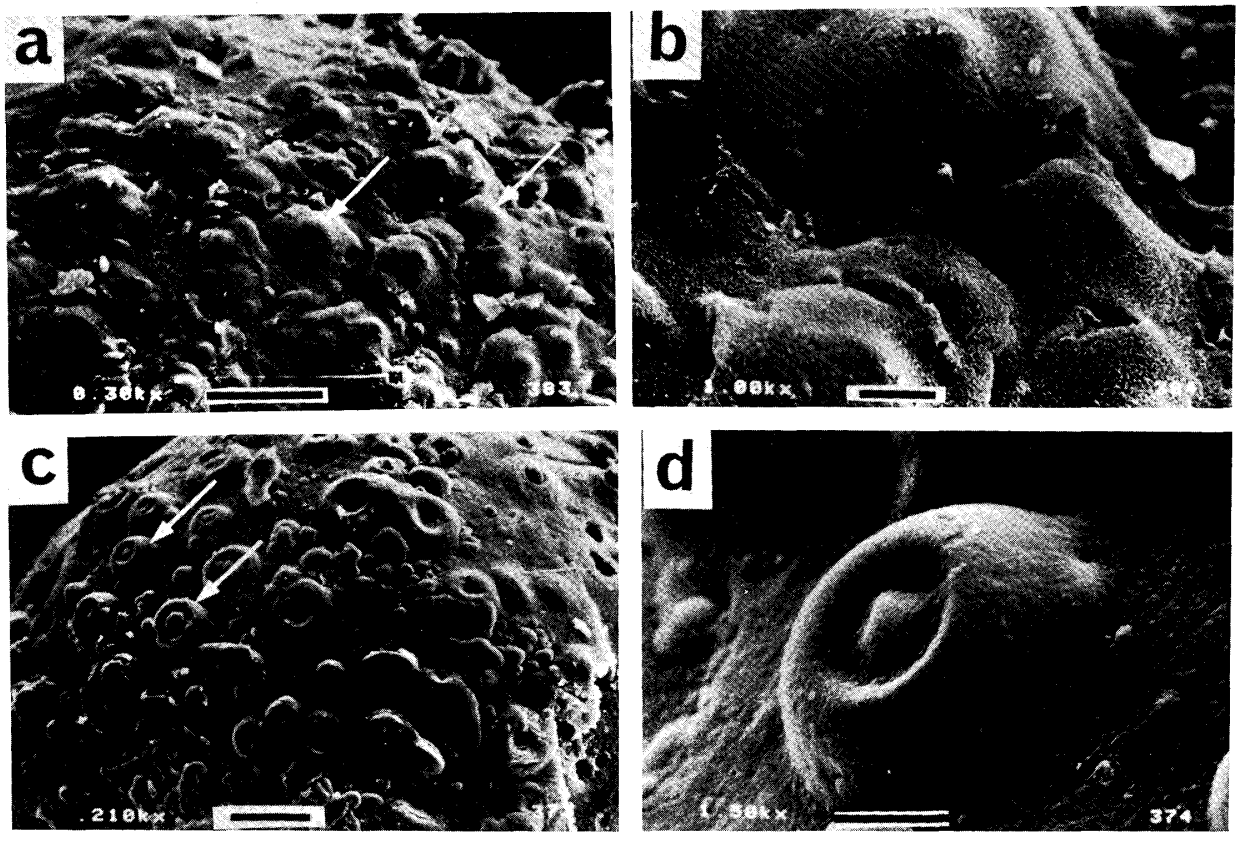

Fig.2 SEM micrographs of unfertilized and fertilized egg surfaces incubated in isotonic salt solution. (a,b) unfertilized egg cortices ; (c,d) fertilized egg cortices. Arrows point to the CA elevations in "a" and to the releasing CA with spherical bodies (SB) in "c". Note fewer microvilli and a smoother surface over the CA elevation in "b". Scale bar : (a,c) 50 μm ; (b, d) 10 μm.

mM Ca^{2+} at pH 6.7 resulted in the greatest number of released CA, followed by pH 9.0. The least number of CA release was observed at pH 5.7. No CA release occurred in the absence of Ca^{2+} in all the ranges of pH tested. Based on this result, pH 6.7 was used in all media in succeeding experiments. No breakdown of CA was observed in cortices isolated in the ACA medium with Ca^{2+}, ranging from 0.1 to 750 μM at pH 6.7, while CA release was recorded in 1 mM Ca^{2+}.

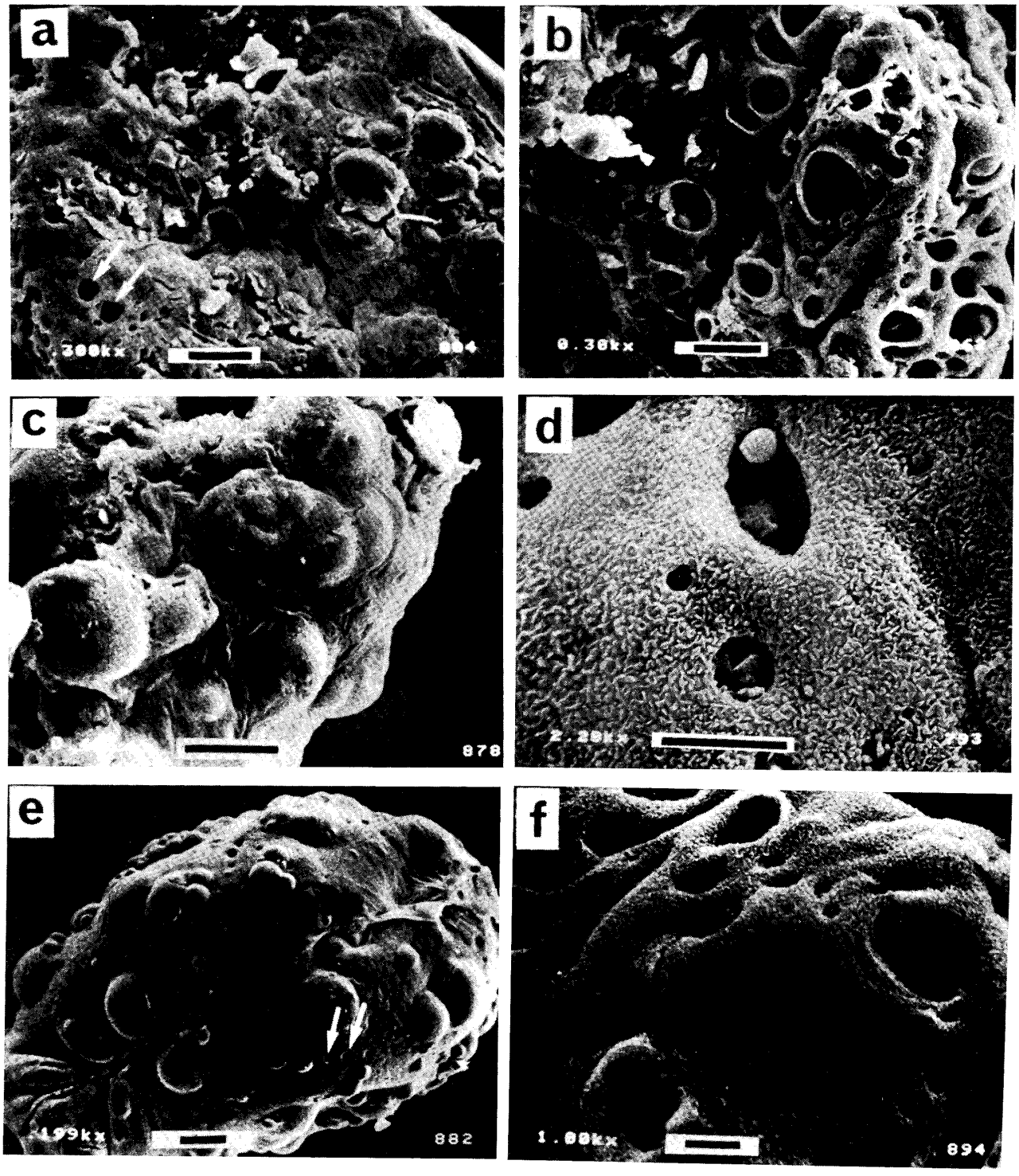

Fig.3 SEM micrographs of egg cortices isolated in 1 mM Ca^{2+} or Ca-free ACA medium at different pHs. (a,c,e) in Ca-free ACA medium ; (b,d,f) in 1 mM Ca^{2+} ACA medium. (a,b) pH6.7 ; (c,d) pH5.7 ; (e,f) pH9.0. (a,e) arrows point to a few released CA. Scale bar : (a,c,e) 50 μm ; (b,d,f) 10 μm.

b. Effects of ATP and PLase A_2 on CA Release

The effect of exogenous ATP on CA release is shown in Table 1. When 1 mM and 10 mM ATP were added separately to various ACA media at pH 6.7, the threshold level was considerably lowered from 1 mM (without ATP) to 1 μM Ca^{2+} . Even in the Ca-free medium with 1 mM and 10 mM ATP, some CA release was observed. The addition of PLase A_2 (1μg/ml) to the ACA medium resulted in the lowering of the threshold level of Ca^{2+} for CA release to 50 μM Ca^{2+} . Table 1 also shows the effect of the combination of Ca^{2+} PLase A_2 and ATP on CA release. The addition of 1 μg/ml PLase A and 10 mM ATP to the ACA medium resulted in a 1 μM Ca^{2+} threshold level for CA.

3. The Unreleased CA

The CA were observed in the process of release or in the unreleased condition (Figs.3b, f and 4b). The unreleased CA were covered with the cortex (Figs.2a and 3c), and the surface of the CA could not be observed. When the cortex isolated in the CA-free ACA medium and its surface layer was mechanically removed, protrusions (diameters ranging from 0.25-0. 60μm) were observed on the surface of the CA (Fig.4). These protrusions were not observed on the surface of CA in the isolated cortex in Ca^{2+} containing solution.

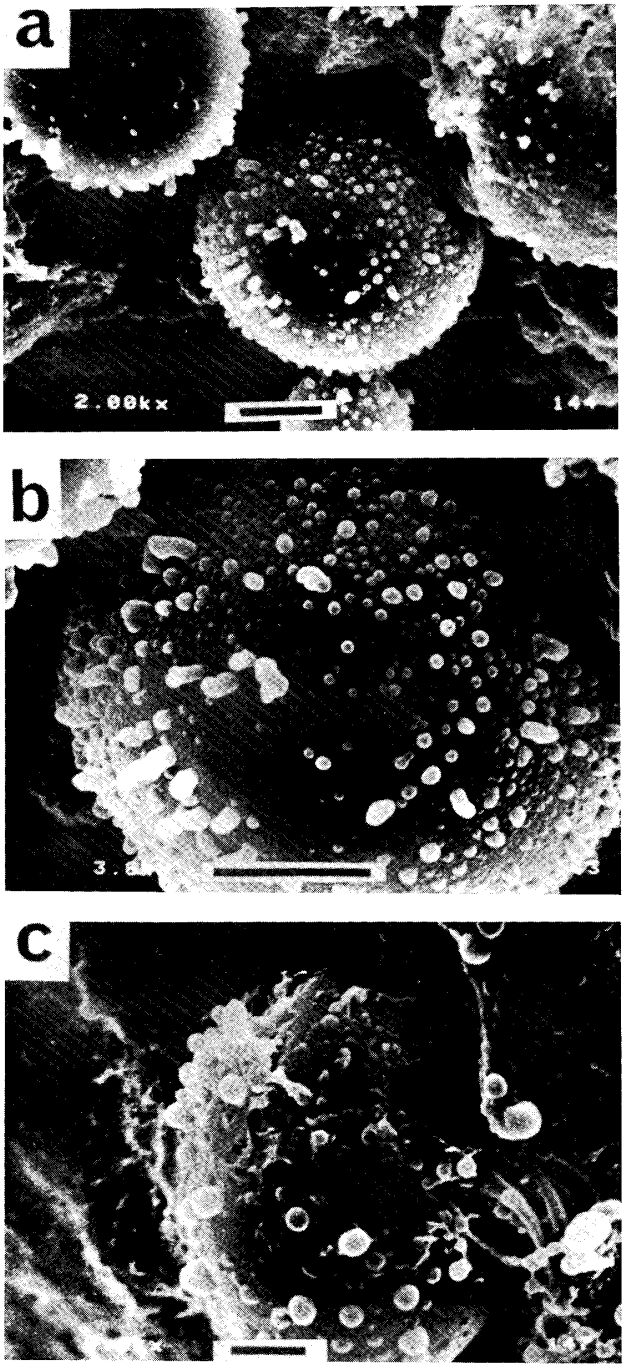

Fig.4. SEM micrographs of intact CA showing granules. The surface layer of egg cortex was removed mechanically in Ca-free ACA medium. Scale bar : 1 μm.

DISCUSSION

The results show that the isolated cortex of the medaka exhibits the same properties as the cortex of the intact egg. In the cortex isolated in the ACA medium (pH 6.7), the release of CA was observed at 1 mM Ca^{2+} . The presence of 1 mM Ca^{2+} facilitates CA exocytosis, without the addition of ATP and PLase A_2 . The addition of PLase A_2 (1 μg/ml) lowered the threshold level of Ca^{2+} to 50 μM and ATP (1 mM or 10 mM) further lowered it to 1 μM.

One of the factors which affect CA exocytosis was the pH of medium. Jaffe (1980) and Gilkey (1981) reported that the pH of the medium does not interfere with activation and normal development, although the cytoplasmic pH affects the calcium wave. Gilkey (1981) noted that the calcium wave was decelerated in the pH buffer below pH 7.1, while it was accelerated in the pH buffer above pH 7.1. The present results in which CA release is relatively greater in pH 9.0 than in pH 5.0-7.0 are in agreement with Gilkey's findings. In ayu *(Plecoglossus altivelis)* eggs, Ito (1960) found that CA release is accelerated in acidic Ringer's

Table 1 Effects of Ca^{2+}, ATP and PLase A_2 on CA release.

Ca μM	Relative Degree of CA Release			
	1 mM ATP	10 mM ATP	1 μg/ml PLase A_2	1 μg/ml PLase A_2 +10 mM ATP
0	±	±	−	−
1	+	+	−	+
50	+	++	+	+
500	++	NO	++	++
1000	+++	NO	NO	NO

±=with only a few released CA.
NO=not observed due to the tendency of the isolated cortices to become fragile.

solution (pH3.0-5.0), while inhibited in alkaline Ringer's solution (pH 7.8-9.4). However, it is suggested that there are different mechanisms of the breakdown of CA in this species.

Using fertilized or ionophore-activated eggs of the medaka injected with aequorin, Gilkey *et al.* (1978) and Gilkey (1981) found that the threshold free Ca^{2+} required to elicit the calcium wave is between 1-10 μM Ca^{2+} at pH 7.0 or near 10 μM at pH 6.5. These values are much below the 30 μM Ca^{2+} reached during the calcium wave. As the present data were based on CA release, higher values may be obtained. In the isolated egg cortex of sea urchin eggs, Steinhardt *et al.* (1977) observed that the Ca^{2+} level required for cortical granule release is higher than the Ca^{2+} level during the calcium wave in the intact eggs. The higher Ca^{2+} level required for CA release in the present study may be due to a considerable lowering of the ATP level during the isolation of the cortex. Hence, the addition of ATP provided a source of energy for the maintenance of the cytoplasm in the Ca^{2+} -loaded state (Baker and Whitaker, 1978) and enhanced calcium sensitivity of the isolated cortex. This is consistent with the results of Baker and Whitaker (1978) in the sea urchin egg cortex. They found that with 10 mM ATP, 1-3μM Ca^{2+} in the glycine medium was sufficient to cause exocytosis of the granules of cortex prepared from cyanide-poisoned eggs. Sasaki and Epel (1983) also observed that the ability to release granules could be maintained by 1 mM ATP at a threshold level of approximately 6 μM Ca^{2+} in a medium containing potassium gluconate and glycine.

The Ca^{2+} threshold level to induce CA release was also lowered to 50 μM by addition of PLase A_2 . This enzyme may cause the dissolution of the CA envelope, which is rich in lipid (Yamamoto, 1951 ; Aketa,1954). In human erythrocytes, PLase A_2 , which attacks the lecithin component of the cell membrane, is a lytic agent (Zwaal *et al.*, 1973). In the CA isolated from the rest of the ovoplasm, Yamamoto (1962) reported the dissolution of half of CA in 5% steapsin-Ringer's solution within 4 min at 28 ℃. He proposed that during fertilization in the medaka egg, there is an enzymatic dissolution of the CA envelope by an esterase system which seems to be lecithinase-like rather than lipase. The protrusions observed on the surface of CA seem to be related to this process, although there is a possibility that these protrusions were formed during fixation.

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