LOCALIZATION OF FLUORESCENTLY LABELED CALMODULIN IN LIVING SEA URCHIN EGGS DURING EARLY DEVELOPMENT

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ABSTRACT

Changes in the localization of calmodulin in the living eggs of the sand-dollar, Clypeaster japonicus, during fertilization and mitosis were investigated by observing the fluorescence of porcine brain calmodulin labeled with N-(7-dimethylamino-4-methylcoumarinyl)-maleimide (DACM) which was microinjected into the eggs. Calmodulin fluorescence was localized at the sperm aster in fertilized eggs. During mitosis, the fluorescence was associated with the mitotic apparatus, in which the spindle poles at metaphase were most intensely fluorescent. This fact was confirmed with mitotic apparatuses isolated from the eggs preloaded with DACM-calmodulin. During anaphase, the fluorescence around the poles spread to the astral rays and toward the central region of the spindle. The interzone became distinctly fluorescent at telophase. Calmodulin was distributed over the entire cell cortex, but no difference was found in fluorescence at the region of the cleavage furrow from the rest of the cell cortex. These results suggest that calmodulin plays significant roles in the motility of the cell during fertilization and mitosis.

KEY WORDS calmodulin / fertilization / microinjection / mitosis / sea urchin eggs

Calmodulin plays a central role in regulating many cellular activities in various eukaryotic cells as an intracellular intermediary for calcium ions (8, 12). Although calmodulin has been found in sea urchin eggs and purified (6, 13), its roles have not been investigated in detail. Because various calcium-regulated processes during fertilization and mitosis have been reported in sea urchin eggs (4, 14, 17, 18, 19), it is possible that calmodulin plays significant roles in them.

Localization of calmodulin investigated by immunofluorescence (1, 3, 5, 23, 24) and immunoelectron microscopy (10, 25) has been reported in mammalian cells. Immunocytochemistry, however, yields limited information on dynamic features of the distribution in living cells and the possibility of artifacts due to fixation should always be considered. In order to overcome this difficulty, we have investigated changes in the localization of calmodulin during fertilization and mitosis in living sea urchin eggs by the intracellular microinjection of a fluorescent conjugate of calmodulin.

MATERIALS AND METHODS

Gametes of the sand-dollar, Clypeaster japonicus, were obtained by the injection of 0.5 M KCl or sea water containing 1 mM acetylcholine into the coelomic cavity. The fertilization membrane and hyaline layer were removed by treating the eggs with 1 M urea for 1 min shortly after insemination.

Calmodulin was extracted and purified to homogeneity from porcine brain by the method of Teo et al. (21) with a slight modification; i.e., DEAE-cellulose was replaced by DEAE-Seph-
arose CL-6B. Calmodulin (3.5 mg/ml) in a solution of 0.5 M KCl/1.5% (v/v) dimethyl-sulfoxide/53 mM NaHCO₃, pH 8.65, was mixed with 1/20 volume of 21 mM N-(7-dimethylamino-4-methylcoumarinyl)-maleimide (DACM) (5 moles dye/mole protein) dissolved in acetone (cf. 26), and the mixture was shaken continuously at 20°C for 2 hr, and then dialyzed thoroughly against 50 mM KCl/0.1 mM dithiothreitol /20 mM N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid (HEPES)-NaOH, pH 7.0, to remove excess DACM. The solution containing 2.5 mg/ml DACM-calmodulin in the dialyzing solution was used for microinjection. As controls, 2.3 mg/ml DACM-α-lactalbumin (DACM-LA) and 6.0 mg/ml DACM-bovine serum albumin (DACM-BSA) were used.

Ca²⁺-dependent cyclic nucleotide phosphodiesterase was prepared from porcine brain by the method of Teo et al. (21), and purified using a calmodulin-Sepharose 4B column by the method of Watterson and Vanaman (22). Phosphodiesterase activity was measured by the method of Teo et al. (21). Protein was assayed by the methods of Lowry et al. (11) and Bradford (2) by using bovine serum albumin or bovine brain calmodulin as standards. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed according to Laemmli (9).

Microinjection was carried out by the method of Hiramoto (7) using a braking micropipette. The volume of microinjected liquid per egg was usually 20–40 pl, corresponding to 3–5% of the egg volume. Therefore, the concentration of DACM-calmodulin was 0.07–0.13 mg/ml in the egg cytoplasm.

In case of the isolation of mitotic apparatus, the egg microinjected with DACM-calmodulin was replaced from normal sea water to a solution consisting of 0.3 M glucose/0.3 M glutamic acid /2 mM MgSO₄/1 mM glycothreidamine-tetraacetic acid (EGTA)/5 mM piperazine-N',N'-bis(2-ethanesulfonic acid) (PIPES)-KOH, pH 7.0, and then the mitotic apparatus was isolated by squirting isolation medium consisting of 1 M glycerol /2 mM MgSO₄/1 mM EGTA/0.1% Triton X-100/5 mM 2-(N-morpholino)ethanesulfonic acid (MES)-KOH, pH 6.2, through a glass capillary to the egg (cf. 16).

Eggs and mitotic apparatuses were observed with an epifluorescence microscope (Nikon XF-EF) with UVF 40x (N.A. 1.30) or UVF 100x (N.A. 1.30) objective, a differential interference microscope (Nikon XF-NT) with Plan DIC 20x (N.A. 0.40) objective and a polarization micro-

RESULTS

The molar ratio of DACM to calmodulin was

Fig. 1 SDS-polyacrylamide gel electrophoresis of DACM-calmodulin. Ten micrograms of DACM-calmodulin were electrophoresed. Left: the Coomassie brilliant blue staining pattern. Right: the fluorescence pattern obtained by ultraviolet trans-illumination.

Fig. 2 Activation of brain Ca²⁺-dependent phosphodiesterase by DACM-calmodulin. 0.36 μg of phosphodiesterase was mixed with 0.02 units of S'-nucleotidase and 1 mM cyclic AMP. The reaction mixture was incubated at 30°C for 30 min. ○: DACM-calmodulin. •: unlabeled calmodulin.
determined to be 1.07 using the molar extinction coefficient ($\varepsilon = 19800$) for DACM at 380 nm according to Yamamoto et al. (26). It is considered that the label was incorporated in the lysine residues under the present conditions because DACM is known to react covalently with lysine residues at alkaline pH and because calmodulin does not contain any SH groups. The fluorescent band of DACM-calmodulin coincided with its Coomassie brilliant blue staining band by SDS-polyacrylamide gel electrophoresis (Fig. 1). No difference was observed in phosphodiesterase stimulating activity between DACM labeled and unlabeled calmodulin (Fig. 2). It is concluded that the properties of calmodulin are not changed by the labeling procedures.

Fig. 3 Calmodulin localization in an egg shortly after fertilization. a, c, e, and g: ordinary light micrographs. b, d, f, and h: fluorescence micrographs. Time after insemination; 4 min (a and b), 6 min (c and d), 11 min (e and f) and 22 min (g and h). A: sperm aster; N: egg pronucleus; and O: oil drop introduced at the time of microinjection. 1 division = 10 μm. Since the egg was considerably compressed, the fertilization membrane was not completely elevated.

Fig. 4 α-lactalbumin localization in an egg shortly after fertilization. An ordinary light micrograph (a) and a fluorescence micrograph (b) of an egg 10 min after insemination. A: sperm aster; N: egg pronucleus; and O: oil drop introduced at the time of microinjection. 1 division = 10 μm
DACM-calmodulin fluorescence was uniformly diffused in the egg cytoplasm within a few minutes after microinjection. Because the surfaces of eggs and blastomeres were clearly recognized in the fluorescent image, it is considered that calmodulin is slightly concentrated in the cell cortex. Meanwhile, the fluorescence of the egg pronucleus (Fig. 3) and the nucleus (Fig. 5) in the egg injected with DACM-calmodulin became more intense than that of the surrounding cytoplasm, suggesting that calmodulin readily enters into the nucleus. DACM-LA was also concentrated in the nucleus (Fig. 4), while DACM-BSA was not.

After fertilization, intense fluorescence caused by DACM-calmodulin appeared at the center of the sperm aster (Fig. 3). As the aster became larger, fluorescence was distinctly observed at the astral rays (Fig. 3f). When the aster was dividing into two, the fluorescence localized at the aster was also dividing with intensely fluorescent projections tangent to the nuclear membrane (Fig. 3h). In cases of DACM-LA (Fig. 4) and DACM-BSA, the intense fluorescence was not observed in the aster(s). Therefore, it may be concluded that calmodulin is localized specifically at the aster(s). After the division, the asters were located on the edges of the nucleus, and the intense fluorescence was also found in association with the astral centers and along the astral rays (Fig. 5).

After the nuclear membrane disappeared, distinct fluorescence was observed in the region of the mitotic apparatus in the eggs injected with DACM-calmodulin (Fig. 6). At metaphase (Fig. 6b), the fluorescence of the spindle poles and the area around the poles became most intense. The fluorescence around the poles became diffuse except in the intensely fluorescent central parts, and fluorescence was observed along the elongating astral rays during anaphase (Fig. 6d). During telophase (Fig. 6, f and h), the fluorescence of the central parts of the asters was most intense in the eggs, and the astral rays and the interzone distinctly fluoresced. Such a change in fluorescence localization was repeated during subsequent mitoses until 4 hr or more after injection, when embryos developed to blastulae. Therefore, it is inferred that injected calmodulin retains its activity at least for 4 hr in the egg cytoplasm.

The chromosomes did not exhibit fluorescence and the fluorescence at the region of the cleavage furrow was not different from that at the rest of the cell cortex (Fig. 6, f and h). Fluorescence was observed in the region free from yolk granules of the eggs injected with DACM-BSA (Fig. 7).

Fig. 8 shows a mitotic apparatus isolated from
Fig. 6 Calmodulin localization in a blastomere during 8-cell stage. a, c, e, g, and i: differential interference micrographs. b, d, f, h, and j: fluorescence micrographs. Differential interference micrographs and fluorescence micrographs were taken in pairs. a was taken 2 hr 48 min after insemination and a~j were taken in succession at one- to two-minute intervals in the alphabetical order. 1 division = 10 μm
LOCALIZATION OF CALMODULIN IN SEA URCHIN EGGS

Fig. 7 BSA localization in a blastomere of 4 cell stage at metaphase. A fluorescence micrograph (a) and an ordinary light micrograph (b). 1 division = 10 μm.

Fig. 8 Calmodulin localization in an isolated mitotic apparatus at metaphase. A fluorescence micrograph (a), a polarization micrograph (b), and a differential interference micrograph (c). 1 division = 10 μm.

an egg at metaphase which had been loaded with calmodulin. Fluorescent fibrous structures projecting toward, but not reaching the chromosomes can be observed near the spindle poles (Fig. 8a). This fluorescence distribution in the spindle is obviously different from the distribution of birefringence (Fig. 8b); the birefringence was found in the spindle region near the equator, but not at the spindle poles.

DISCUSSION

It has been found in the present study that DACM-calmodulin is a good molecular probe for the study of the intracellular localization of calmodulin for the following reasons. Calmodulin labeled with DACM retains the same biochemical activity as unlabeled calmodulin, and it is not rapidly degraded in cells. Calmodulin in sea urchin eggs closely resembles that in bovine brain as shown by amino acid analysis and peptide mapping of tryptic peptides (6), and that in porcine brain in respect to Ca²⁺-dependent inhibitory effect on microtubule assembly (13). Calmodulin is one of the proteins structurally and functionally conserved throughout the animal and plant kingdoms (8, 12). Therefore, injected DACM-calmodulin would be expected to function similarly to intrinsic calmodulin in the egg. The concentration of intrinsic calmodulin is calculated to be 0.16 mg/ml in the egg cytoplasm, using the calmodulin concentration of 0.1% of total protein (6) and the total protein concentration of 157 mg/ml (15) in sea urchin eggs. Although the total content of calmodulin in the eggs usually increased by a factor of 1.4–1.8 after injection in the present study because the amount of injected DACM-calmodulin was 0.07–0.13 mg/ml in the egg cytoplasm (cf. Materials and Methods), this excess calmodulin probably does not perturb the physiological activities of the eggs because normal development was observed even in the eggs injected with an amount of calmodulin that was 2.5 times the intrinsic calmodulin content in the egg cytoplasm.

Using DACM-calmodulin, we have investigated the changes in the distribution of calmodulin by in vivo molecular cytochemistry introduced by Taylor and Wang (cf. 20). We have found that in sea urchin eggs, calmodulin is mainly localized at aster(s) and mitotic apparatus during fertilization and mitosis. The specific fluorescence due to DACM-calmodulin is always localized at the aster(s) through early development. Calmodulin distribution during mitosis in this study is similar to the distribution obtained by the immunofluorescence study (1, 23, 24). Especially, the distribution of calmodulin in the isolated mitotic apparatus is quite similar to that in the mitotic apparatus in mammalian fixed cells (24). Calmodulin is distributed in association with the asters and the spindle poles, but not with the chromosomes or their vicinities. This calmodulin distribution in the mitotic apparatus is different from the distribution of birefringence shown in the present study, and that of tubulin described by Andersen et al. (1) and Welsh et al. (24). Calmodulin did not show a Ca²⁺-dependent inhibitory effect on sea urchin microtubule assembly (13). These facts suggest that calmodulin does not exert any direct effect on assembly and disassembly of microtubules. However, the specific localization mentioned above suggests that calmodulin plays...
significant roles in cell motility during fertilization and mitosis.

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