Cytosolic Free Calcium-Ion Concentration in Cleaving Embryonic Cells of *Oryzias latipes* Measured with Calcium-selective Microelectrodes

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ABSTRACT Calcium-selective microelectrodes were used to measure the free calcium-ion concentration ($[Ca^{2+}]_i$) in early-cleaving embryonic cells of the golden medaka, *Oryzias latipes*, a fresh water teleost fish. Embryos could be dechorionated as early as the four-cell stage using a three-step technique consisting of removal of some yolk to enlarge the perivitelline space, partial digestion of the chorion with pancreatin, and removal of the weakened chorion with forceps. Dechorionated embryos underwent cleavage at a normal rate.

Intracellular cytosolic $[Ca^{2+}]_i$ was monitored by impaling blastomeres first with a microelectrode filled with 5 M potassium acetate to measure membrane potential, and a few minutes later with a calcium-selective microelectrode. During nine rounds of cytokinesis from a total of six different embryos, cytosolic $[Ca^{2+}]_i$ remained constant (with apparently random fluctuations of $<\pm 0.1$ μM). During two successive cleavages in one embryo, however, $[Ca^{2+}]_i$ rose transiently fourfold above the original resting level to 1.32 and 1.20 μM in synchrony with each period of cytokinesis and returned after each rise to submicromolar levels. Because a calcium-selective microelectrode can detect $[Ca^{2+}]_i$ changes only in the immediate vicinity of its 2-μm tip, we interpreted these data to suggest that, although $[Ca^{2+}]_i$ in most areas of the cytosol remains between 0.01 and 0.40 μM (mean of 0.14 μM), there may be small regions of the cell in which $[Ca^{2+}]_i$ undergoes a substantial increase at the time of cleavage. Evidence also is presented to suggest that the membrane potential in these blastomeres undergoes a slow net hyperpolarization during early cleavage stages.

The free calcium-ion concentration in the intracellular cytosol ($[Ca^{2+}]_i$)[1] has been studied intensely in marine invertebrate eggs and found to play many roles in embryonic development (1-3). The presence of contractile proteins in cleavage furrows (4, 5) suggests a possible role for calcium in cytokinesis. Application of calcium ionophores (6, 7) may induce formation of cleavage furrowlike structures, perhaps by increasing intracellular $[Ca^{2+}]_i$, whereas injection of calcium chelator solutions into cells (8) may interfere with cytokinesis by reducing $[Ca^{2+}]_i$ (although in none of these studies was $[Ca^{2+}]_i$ actually quantitated). Recently, several techniques for measuring $[Ca^{2+}]_i$ have been described (9, 10). Use of one such technique, calcium-selective microelectrodes (CSMs), has indicated that there is no change in $[Ca^{2+}]_i$ during cytokinesis in *Xenopus laevis* (11). However, injection of the calcium-sensitive fluorescent protein, aequorin, into eggs of the golden medaka, *Oryzias latipes*, detected very small changes in fluorescence that might be associated with cell division (12). Unfortunately, the aequorin technique used was not sensitive enough to demonstrate convincingly that a correlation existed between changes in $[Ca^{2+}]_i$ and cytokinesis. The sensitivity of CSMs to very low levels of $[Ca^{2+}]_i$ in vivo surpasses that of aequorin (9, 10, 13-16), and provides the additional advantage of allowing direct and continuous observation of cytokinesis.

The embryos of teleosts, including those of *O. latipes* are surrounded by a tough, protective chorion. A procedure for its removal, without damage to the embryo, is required before CSMs can be used to measure cytosolic $[Ca^{2+}]_i$ during cytokinesis. Although a slow and difficult microdissection of the

[1] *Abbreviations used in this paper:* $[Ca^{2+}]_i$, intracellular cytosolic free calcium-ion concentration; CSM, calcium-selective microelectrode; E_m, membrane potential.

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chorion with ultrasharp forceps or irridectomy scissors is possible after fertilization and enlargement of the perivitelline space, the technique was actually devised for the larger embryos of *Fundulus heteroclitus*, and even granting experience and manual dexterity, is never applied to *O. latipes* with much success (17, 18). Few chemical methods for removing teleost chorions are successful when applied to the medaka. A mixture of pancreatin, plus the hatching enzyme found in the buccal tissues near the time of hatching (19), has been reported to dechorionate embryos with no adverse effects (20). However, collection of adequate quantities of hatching enzyme is time consuming and requires a large, steady supply of embryos. Pronase has been applied successfully to embryos at later stages of development (21), but results in lysis of the early-cleaving embryo (22). Thus, heretofore no routinely successful and convenient method has been available for dechorionating teleost embryos, especially at very early stages of cleavage and in a type of teleost available in the laboratory year-round.

The dechorionation method described herein combines mechanical and enzymatic techniques modified from a procedure used by Sakai (20). This procedure is more rapid than most solely enzymatic methods and requires less practice and dexterity than purely mechanical techniques. It provides embryos that are still in the early stages of development (often at the four-cell stage) and which develop at a normal rate. Application of CSMs to dechorionated embryos during early cleavage has allowed direct measurement of cytosolic $[Ca^{2+}]_i$ and shown that the resting levels of $[Ca^{2+}]_i$ are very low and rise significantly during cytokinesis, although the rise appears to be a localized one.

MATERIALS AND METHODS

Microelectrodes: Single-barrel pyrex capillary tubing (1.5-mm outer diameter [o.d.], 1.1-mm inner diameter [i.d.]) (Glass Co. of America, Millville, NJ) containing an internal fiber was used in the construction of microelectrodes for measuring the resting membrane potential (E_m). Tubing without an internal fiber was used for CSMs. All capillary tubing was cleaned with 75% ethanol followed by a boiling water bath (23) before pulling into submicron-tipped micropipettes. Particulate matter was removed from backfill solutions by filtering them through rinsed Millipore filters of 0.45-μm pore size (Millipore Corp., Bedford, MA). The tapered end of all microelectrodes was painted with silver conducting paint diluted with ethanol (Electrodag 416, Acheson Colloids Co., Port Huron, MI) leaving only the terminal 0.5 mm of the tip uncoated. At the shoulder of the microelectrode, a thin ring of vacuum grease was applied so as to overlap the silver conducting paint, thus preventing the development of short circuits through moisture that might accumulate on the outer surface of the glass. Several centimeters of one end of a thin silver wire (0.005 in diam) (Medwire Corp., Mt. Vernon, NY) was electrolytically chlorided in 3 M KCl. Droplets of KCl solution were rinsed from the Ag/AgCl wires with triple-glass distilled water. To reduce clogging of microelectrode tips with AgCl precipitate (24), the chlorided wires were not inserted into microelectrodes until immediately prior to use.

Micropipettes for measuring membrane potential were backfilled with 5 M potassium acetate (Fluka Chemical Corp., Hauppauge, NY) using an attenuated, integral plastic needle (25). Tip resistance was 12–60 MΩ in Yamamoto's medium (12). Results were discarded if tip resistance was altered by cell membrane penetration.

Tips of micropipettes for CSMs were beveled at a 25° angle from the horizontal in a milky suspension of distilled water and 0.05 μm Alumina powder (Union Carbide Corp., Indianapolis, IN) (model BV-10 micropipette beveler, Sutter Instrument Co., San Francisco, CA). Tips were sharp and had a 2–3-μm diam opening. These micropipettes were placed tips up in a Coplin jar filled with 6 × 50-mm test tubes. The open Coplin jar containing ~20 micropipettes, along with a ground glass lid, was slowly heated to 200 °C. *N,N*-dimethyl-trimethylsilylamin (5 μl) (Fluka Chemical Corp.) was added to the jar, which was immediately sealed with the hot glass lid and placed back into the oven. After 15 min the Coplin jar was opened and the silanized micropipettes were slowly returned to room temperature while remaining in the oven with its door slightly ajar. Next, we injected a backfill solution of 0.1 M $CaCl_2$ into a silanized micropipette by using an attenuated integral plastic needle. The large end of the micropipette was cemented into the tip of a 1-ml disposable plastic syringe (No. 5623, Becton Dickinson & Co., Rutherford, NJ) with molten sealing wax. The syringe was filled with backfill solution and pressure was applied in order to fill the micropipette tip. The filled micropipette was cut free from the syringe and observed to assure the absence of air bubbles. CSMs contained 12% ETH 1001 (calcium ligand, Fluka Chemical Corp.), 6% tetraphenylarsonium tetrakis (*p*-biphenylyl) borate (extremely hydrophobic salt, W-P Instruments, Inc., New Haven, CT), 72% *o*-nitrophenyl octylether, and 10% high molecular weight polyvinylchloride (a gift from Dr. W. Simon from the Max-Planck-Institute, FRG). This solution was mixed 1:2 (wt:wt) with tetrahydrofuran (15). A droplet of the neutral-carrier ligand solution was drawn into a large (~300 μm i.d.) micropipette by capillary action, and the enlarged micropipette was attached to a micromanipulator. The tip of a microelectrode previously backfilled with 0.1 M $CaCl_2$ was briefly inserted into the ligand-containing pipette. The operation was observed with a Nikon MS inverted microscope (Nikon Inc., Garden City, NY). The dipping process was repeated until, by capillary action, the microelectrode tip became filled with a 200–325-μm-long column of the final ligand solution. Bubbles in the calcium-selective ligand solutions in the tip of the electrode could sometimes be removed by repeated and rapid dipping of tips into the ligand mixture. Because CSM tips were so large, it was necessary that all ligand solution was removed from the external surfaces of the tips (using a rinse of tetrahydrofuran, or by manual scraping) to achieve stable intracellular records.

In order to avoid problems of drift (23), pCa 6 buffer solution (15) was used to continuously bathe CSM tips for 1–2 d before an experiment. CSMs were discarded 5 d after construction because sensitivity to $[Ca^{2+}]$ decreased, and hysteresis increased dramatically after this time, presumably due to increased tip resistance (26).

CSMs were calibrated in a series of buffered solutions (See Table I of reference 15 for recipes) before and after cellular impalements. Data were rejected when the microelectrode response to $[Ca^{2+}]$ changed by >5 mV/decade $[Ca^{2+}]$.

Embryo Dechorionation: Embryos were separated from female medakas (Carolina Biological Supply Co., Burlington, NC) with watchmaker's forceps immediately after spawning. Six fertilized eggs were placed into a 60 × 15-mm bacteriological plastic petri dish (Falcon No., 1007, Oxnard, CA) containing 10 ml of Yamamoto's medium at pH 7.3 (12). With observations being made through a dissecting microscope, two sets of No. 5 watchmaker's forceps were ground under oil to long fine points on a soft Arkansas stone, followed by a final sharpening under oil on a hard Arkansas stone, to produce paired tips no more than 2 μm in diameter.

All observations of embryos were made with the use of a Wild M-5 dissecting microscope (Wild Heerbrugg Instruments Inc., Farmingdale, NY) and transmitted light. Embryos always remained in a dish of Yamamoto's medium at room temperature (24 ± 1 °C) unless otherwise specified. As soon as the embryos completed first cleavage, the dechorionating manipulations were begun (manipulation of the yolk cell before first cleavage prevented further development). First, under a dissecting microscope at × 17.5, one pair of ultrasharp watchmaker's forceps was brought to each side of the embryo. The embryo was positioned with the vegetal and animal poles to the left and right, respectively. One set of forcep tips was held closed and pressed against the vegetal pole, while those on the right side were held open so as to contact the chorion almost tangentially above the equator between animal and vegetal poles. Slight pressure was applied tangentially across the chorion so that one of the forcep tips over the animal hemisphere side of the equator penetrated into the perivitelline space. Next, the penetrating forcep tip was gently pushed ~20 μm through the yolk cell membrane near the equator. This produced a small wound. The forceps were gently removed and the yolk cell was slowly and gently squeezed to release a small volume of yolk before the membrane resealed itself. The embryos, now with shrunken yolk cells, were immediately placed onto a 50-μl droplette of frozen 3% pancreatin (Sigma Chemical Co., St. Louis, MO) in a phosphate buffer composed of 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, and 4.2 mM $NaHCO_3$, pH 6.0, in a small plastic test tube. The droplet immediately thawed (submerging the embryo) and was incubated at 24 °C for 95 min. This brief exposure to cold seemed to cause a pause in development, so that embryos were still in very early stages of development when dechorionated. The enzyme apparently entered the slit in the chorion and began digesting the zona radiata interna (27), for when the four-celled embryo was returned to Yamamoto's medium at the end of the digestion period, the remaining chorion, retaining its external decorations, had been changed into a semiflaccid sac. In such embryos, the yolk cell membrane remained taut, although reduced in diameter to ~0.89 mm from an original 1.09 mm. Therefore, only ~54% of the original yolk cell volume remained. Embryos were rinsed well with at least two 10-ml rinses of Yamamoto's medium to halt further enzymatic digestion. Then the chorion was removed carefully

using ultrasharp forceps and either the manipulative steps described by Trinkaus (18), or by simply tearing away very small patches of the chorion, gradually forming a hole through which the embryo could easily roll out.

Embryos dechorionated in the manner described above (heareafter referred to as dechorionated embryos) were kept in Yamamoto's medium, together with various control groups. Each group was observed at 30-min intervals until the commencement of epiboly, and daily thereafter. Control and test groups were obtained from the same clutch in experiments in which the rates of development were compared. It is possible to dechorionate immediately after wounding (eliminating enzymatic digestion), thus obtaining freed two-celled embryos, however, the tough bits of chorion cling tenaciously to the forceps making the process quite difficult.

Measurement of $[Ca^{2+}]_i$ and E_m: Embryos at the four- to eight-cell stage were placed into a small plastic dish, dechorionated, and held in position in a notch cut into the edge of a microscope slide. (Because these embryos cleave rapidly [every 20–30 min at 24 °C] all reached the eight-cell stage before measurement of $[Ca^{2+}]_i$). All manipulations were done in the same dish, because dechorionated embryos were very fragile. A dividing cell was penetrated first with a microelectrode filled with 5 M potassium acetate to allow recording of E_m. After several minutes, a CSM was inserted into the same cell, or an adjacent cell of the same embryo, to allow recording of CSM potential (adjacent blastomeres are electrically coupled in *F. heteroclitus* [28]). Preliminary current injection experiments (not shown) indicated that all blastomeres of the two- to eight-celled medaka embryo, like those of *Fundulus*, are >85% electrically coupled. Changes in E_m are very small relative to voltage changes needed to indicate a change in $[Ca^{2+}]_i$ during an experiment, and so even if coupling were not as complete as in *Fundulus*, only a very small error would be introduced.

The circuit used for measuring potentials consisted of a grounded calomel reference electrode (13-639-79, Fisher Scientific Co., Pittsburgh, PA) in 3 M KCl connected via a salt bridge (3 M KCl in 2% agarose gel) to either the calibration solution or the dish of Yamamoto's medium used for cell impalements. Microelectrodes, with their tips in the test solutions, were connected by a Ag/AgCl wire in the backfill solution to a dual-channel high-impedance differential electrometer with driven probes (model F-223, W-P Instruments, Inc.). Outputs were displayed as traces on a two-pen strip chart recorder (No. 7132A, Hewlett-Packard Co., Palo Alto., CA): E_m (from the potassium acetate microelectrode) and corrected CSM potential (the CSM potential minus the E_m; subtraction performed automatically by the electrometer). Both voltages were recorded simultaneously until the completion of sixth cleavage, or until a substantial drop in E_m indicated damage to the cell membrane. The two electrodes were often partitioned into different cells as cytokinesis proceeded, but the cells probably remained electrically coupled (28). However, changes in E_m were not directly related to cytokinesis in any manner that should have affected the results presented here, even if cells had been uncoupled. Also, it has been previously shown that membrane potential changes are not involved in early cleavage in the marine mudsnail, *Ilyanassa obsoleta* (29). All microelectrode manipulations were performed inside a grounded Faraday cage.

In one set of experiments, dimethylsulfoxide (DMSO) in Yamamoto's medium was added (without stirring) during a stable impalement to give a final DMSO concentration of not <0.5% surrounding the embryo. In another set of experiments, a calcium ionophore solution (A23187 [Sigma Chemical Co.] dissolved in DMSO and Yamamoto's medium) was added, without stirring, during a stable impalement so as to give a final concentration of at least 9.5 μM A23187 and 0.5% DMSO in the medium surrounding the embryo. Each experiment was done repeatedly, with the impaling electrode being either a CSM, or a potassium acetate-filled microelectrode. Such single microelectrode impalements were resorted to after numerous techniques for adding ionophore solution all failed to be gentle enough for maintaining most doubly impaled embryos in a healthy state. No other experiments were designed to measure pCa_i^{2+} without dual-impaling microelectrodes.

To measure E_m in embryos that had neither been dechorionated nor treated with enzyme, a micropipette with a tip diameter <30 μm was inserted through the chorion into the perivitelline space above the embryo. The micropipette then was broken just above the outer surface of the chorion. Finally, a micropipette for measuring E_m was guided through this chorionic shunt and inserted through the plasma membrane of the embryo (Fig. 1). E_m was successfully recorded from 19 embryos that had received chorionic shunts.

RESULTS

Effects of Dechorionation

The rate of development of dechorionated embryos was compared with that of control embryos that were simply transferred from the spawning female to a dish of Yamamoto's medium at 24 °C ± 1 °C. Embryos in these two groups were always found to develop at the same rate ± one stage (as defined by Kirchen and West [30]) until the experiment was terminated (Fig. 2). Epibolic movements occurred at the same time in both groups, and were soon followed by rhythmic contractions over the yolk cell. Daily observations of a group of 12 dechorionated embryos revealed that 75% began actively swimming and feeding by the 17th day after fertilization (25% depleted their yolk cells and died during the 2nd or 3rd wk of development). Dechorionated embryos produced smaller than normal fry. Successful microelectrode penetrations did not alter these results. A control group of untreated embryos held at 4 °C in 3% pancreatin solution for 95 min stopped cleaving until being returned to room temperature. Cytokinesis then resumed at a normal rate within 1 h. Because these chilled embryos went on to become normal hatchlings, it was assumed that placing embryos to be dechorionated on a 50-μl droplette of frozen pancreatin buffer solution, which reached room temperature within minutes, did nothing more than briefly retard development so that, at the time of dechorionation, embryos were still at the four- to eight-cell stages.

Because in another teleost fish, *F. heteroclitus*, it has been noted that the embryo proper is less damaged by wounding than is the yolk cell (28), one group of six embryos was wounded by puncturing an early cleaving embryonic cell, rather than the yolk cell. After enzyme treatment and chorion removal, daughter cells of the undamaged parent cell underwent rounds of cytokinesis at a normal rate. However, daughter cells of the cell that had received the wound were slow to develop, and often cleaved at an angle to the normally expected cleavage plane. These embryos ceased development before eight rounds of cytokinesis were completed. In contrast, of those embryos subjected to the standard dechorionation procedure, only 1 of 20 examined showed any skew from the normal cleavage pattern, and all developed at the same rate as control (intact) embryos. The only visible developmental difference between dechorionated and intact embryos was a change in the path of contractile waves over the yolk cell just after completion of epiboly (stage 18). In control embryos, contractile waves initiated at the lateral surface of the embryo proper, and propagated to the vegetal polar region where they often slowed down, halted, and relaxed. In dechorionated embryos, contractile waves initiated at the lateral surface of the embryo proper, propagated in a slightly anterior or posterior direction to the vegetal polar region, and then continued around the shrunken yolk cell to the opposite side of the

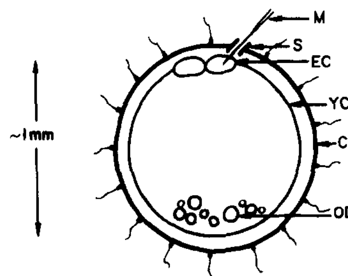

FIGURE 1 This illustration depicts a cross-sectional view of a two-celled medaka embryo surrounded by a chorion (*Ch*) that has been shunted by a glass tube (*S*) to allow for recording of the E_m without subjecting the embryo to the dechorionation process. A microelectrode (*M*) is shown penetrating a cell of the embryo proper (*EC*). The yolk cell (*YC*) and oil droplettes of the yolk (*OD*) are also shown. Shunt is shown disproportionately large for clarity.

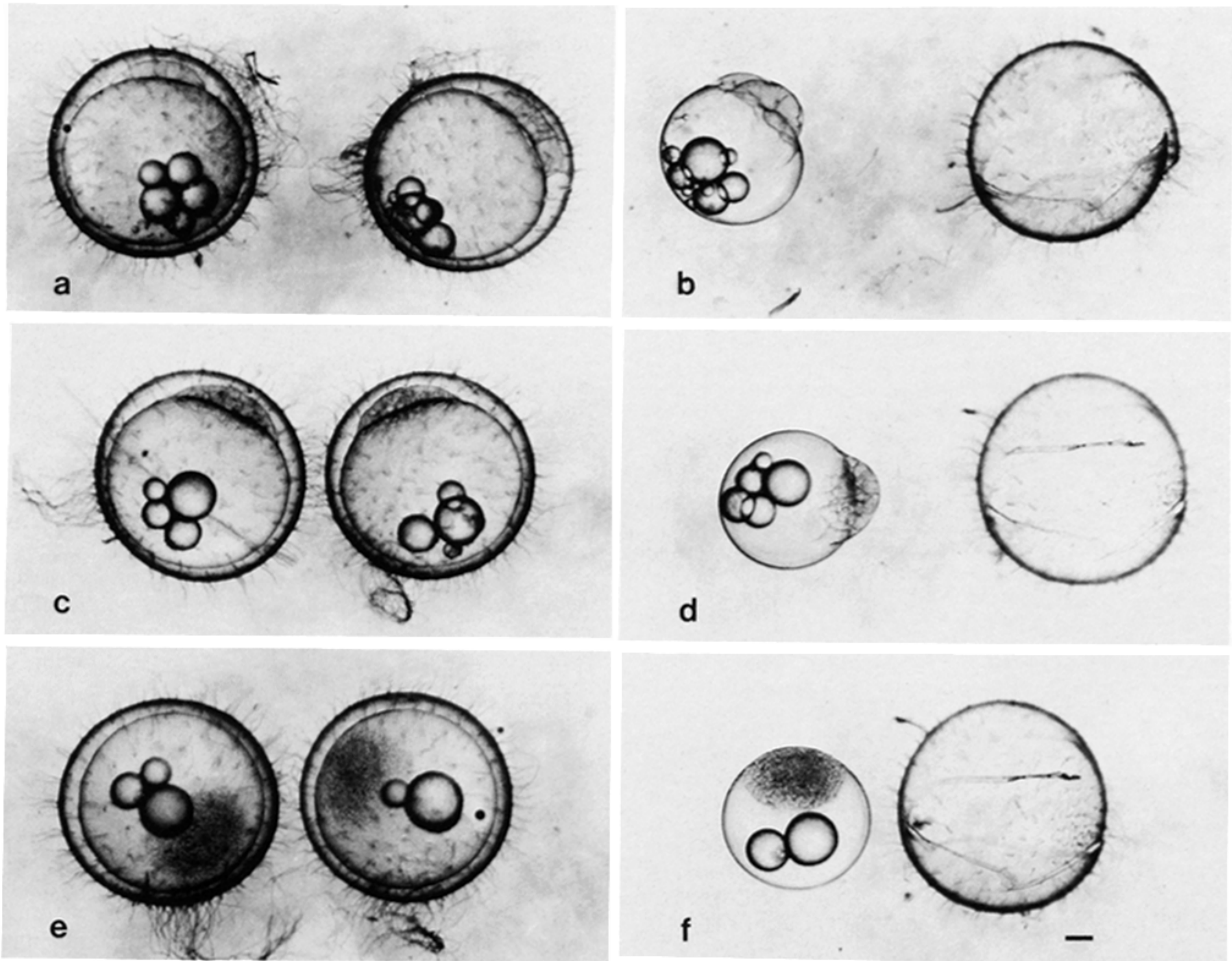

FIGURE 2 Untreated medaka embryos (*a*, *c*, and *e*) and dechorionated embryo, beside its empty chorion (*b*, *d*, and *f*) taken from the same clutch develop in Yamamoto's medium at 24 °C. The eight-cell stage (*a* and *b*) and the 64-cell stage (*c* and *d*) were reached at 3 and 4.5 h postfertilization, respectively. By 12 h postfertilization (*e* and *f*), embryos had reached the flat blastula stage. Bar, 175 μm. × 23.4.

embryo at the animal polar region. In summary, these experiments indicated that development of dechorionated embryos was essentially normal, especially during the first day of development, when the microelectrodes were used to measure $[Ca^{2+}]_i$ and E_m during cleavage.

Calcium-selective Microelectrodes

The CSMs in these experiments displayed almost no hysteresis and had nearly Nernstian slopes when the $[Ca^{2+}]$ was ≥10 μM (Fig. 3). Below 10 μm $[Ca^{2+}]$, there was only slight hysteresis; however, responses became substantially more sub-Nernstian with each 10-fold decrease in $[Ca^{2+}]$ (Fig. 3). As a result, this technique could not reliably quantitate $[Ca^{2+}]$ <0.10 μM. Thus, values <0.10 μM are necessarily estimates. CSM sensitivity to $[Ca^{2+}]$ in the presence of interfering ions found in vivo was good, as has been shown by others using these types of CSMs (11, 15, 23, 31, 32). An accurate pCa^{2+} of 2.74 was indicated for pure Yamamoto's medium containing Na^+ and K^+ concentrations >2 mM. When distilled water was introduced on dissecting instruments into the 1–2 ml of solution bathing an embryo during some dechorionations, the CSMs again responded well, indicating a pCa^{2+} >2.74 (Fig. 4).

Cytosolic $[Ca^{2+}]_i$ during Cytokinesis—Generally No Change

Embryos were penetrated with 5 M potassium acetate microelectrodes, and resting E_m was recorded for several minutes before a CSM was also placed into the same or adjacent cell of the embryo. After impalement of an embryonic cell at the 8- to 16-cell stage with a CSM, a rapid drop in the CSM potential usually occurred equivalent to ~pCa_i^{2+} 6, followed by a slow decrease in intracellular $[Ca^{2+}]_i$ over many minutes (Fig. 4). For six embryos these changes were followed by a stable, nonfluctuating recording of the resting level of cytosolic $[Ca^{2+}]_i$, whereas the embryonic cells continued to undergo one round of cytokinesis every 20–40 min throughout the experiment and for several hours thereafter. In six separate experiments cytosolic $[Ca^{2+}]_i$ was found to be 0.01–0.40 μM (Table I) throughout a total of nine rounds of cleavage. Within the cells of a single embryo $[Ca^{2+}]_i$ remained constant, but it varied significantly among embryos. Small fluctuations of $[Ca^{2+}]_i$ (≤ ±0.10 μM) did not correlate with the occurrence of cytokinesis in number or in time.

Yamamoto's medium containing 0.5% DMSO caused no change in E_m (two trials) or pCa_i^{2+} (two trials). However, addition of ionophore solution (see Materials and Methods)

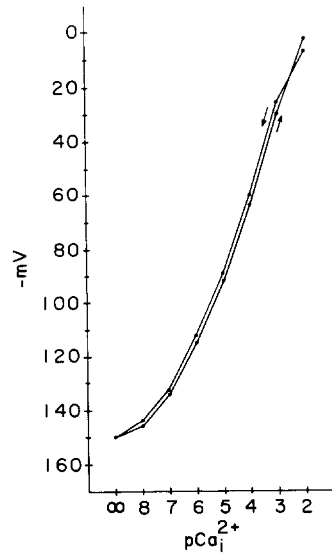

FIGURE 3 Calibration curve for CSM with 1.5-μm o.d. tip. This CSM was used for the experiment shown in Fig. 4. Arrows indicate the order in which solutions were changed during calibration. Changes in $[Ca^{2+}]$ can be detected below the micromolar range despite sub-Nerstian responses.

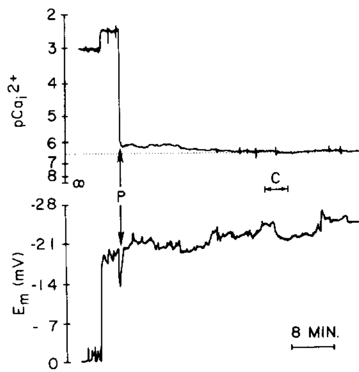

FIGURE 4 Pen recorder traces of pCa_i^{2+} and E_m in an embryonic cell of *O. latipes* during the fifth cleavage. Vertical blips on pCa_i^{2+} trace are a result of changing channels in order to observe digital readouts on the differential potentiometer used. Penetration (*P*) of the cell with the large CMS produced a temporary drop in the E_m. The cell quickly recovered and E_m continued to undergo a net hyperpolarization. After penetration, a rapid drop in $[Ca^{2+}]_i$ is shown, followed by a slow decline over many minutes. Subsequent measurements indicated $[Ca^{2+}]_i$ remained approximately constant at 0.40 μM, indicated by the dotted line. Cytokinesis (C) was seen to occur over a period of ~4 min as indicated.

TABLE I

$[Ca^{2+}]_i$ and E_m Levels during Early Cleavages

Rounds of cytokinesis observed	E_m at cleavage	$[Ca^{2+}]_i$
	−mV	*nM*
1	15	10
4	12–20	14
1	20	51
1	23	63
1	16	126
1	22	400

Summary of data from six experiments in which $[Ca^{2+}]_i$ remained constant, even during cytokinesis. Membrane potential (E_m) underwent a net hyperpolarization in each experiment. The value of E_m given is the mean E_m during the period of time when cleavage was occurring. Only the third through sixth cleavages were examined.

to embryos with single microelectrode impalements (two trials), and in a doubly impaled blastomere in one case, indicated that, had large changes in cytosolic $[Ca^{2+}]_i$ occurred, they would have been readily detected by the CSMs used. Addition of ionophore solution to embryos between the 16- and 32-cell stages indicated that in six trials E_m was affected by not >± 2 mV by the ionophore solution, and a slow net hyperpolarization was still indicated regardless of the drug's presence. However, when an embryo at the 64-cell stage was presented with the ionophore solution, microelectrode readings rapidly became more positive (Fig 6). Because the drugs had almost no effect on E_m, and because addition of the same concentration of ionophore solution to calibration solutions had no effect on CSM response, the positive deflection must have been due to a real rise in $[Ca^{2+}]_i$.

The E_m in the dechorionated embryos always followed a course of gradual net hyperpolarization with small transient depolarizations of several millivolts interspersed in a seemingly random manner. An experiment was performed to determine whether this was an artifact of dechorionation. Chorions surrounding intact, nontreated embryos, received chorionic shunts (a glass tube through the chorion as described above) through which the membrane potential electrode was inserted (Fig. 5). Embryos which appeared least damaged by dechorionation (i.e., the puncture in the yolk cell membrane sealed cleanly rather than forming a plug of xanthic material) usually displayed E_m with ±5 mV of the values from nondechorionated, i.e., shunted, ones. Therefore, the data suggest that the dechorionation procedure per se does not cause the steady hyperpolarizations observed. Instead, steady hyperpolarization of the embryonic cells appears to represent a normal developmental phenomenon. Not shown in Fig. 5 are values of E_m obtained from two embryos, with shunted chorions, at approximately the seventh cleavage (~128 cells). In these instances, E_m was −40 mV and −50 mV, indicating that net hyperpolarization of medaka blastomeres probably continues into the range of the so-called "normal" resting potentials (~−70 mV) found in most cells of most organisms.

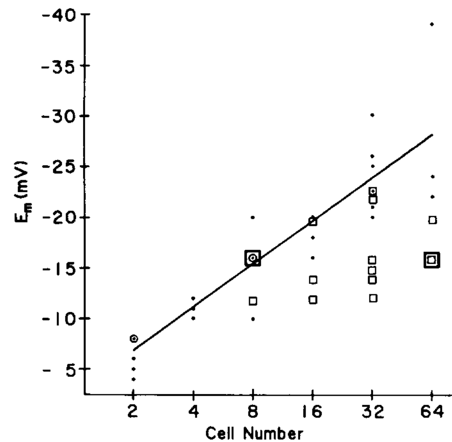

FIGURE 5 A chorionic shunt was used to determine E_m (in millivolts; circles), which is shown for 17 different embryos (overlapping data at open symbols). A net hyperpolarization (indicated by linear regression line; $r = 0.90$) was observed in embryos retaining their chorions, indicating that the net hyperpolarization found to occur in cells of all dechorionated embryos is not an artifact of the dechorionation process. Squares indicate E_m of the six CSM-impaled embryos from Table I. The difference between the mean membrane potentials for these two groups is ≤ 6.7 mV during the 8- through 32-cell stages, and 11.2 mV at the 64-cell stage.

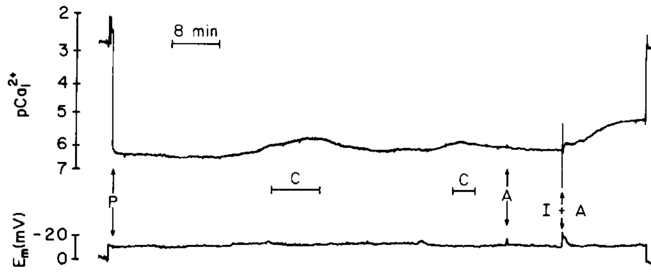

FIGURE 6 Simultaneous recordings of pCa^{2+} and E_m for an embryo during early cleavage stages. Penetration (*P*) of the blastomer by the CSM caused a small, transient fall in E_m indicating that (*a*) the CSM had penetrated the membrane of the same blastomere that already contained the 5 M K acetate electrode, and (*b*) that the membrane did seal around the CSM. Resting $[Ca^{2+}]_i$ was ~0.32 μM until just before the fifth cleavage. Then it rose to 1.32 μM. Afterwards, $[Ca^{2+}]_i$ fell to 0.63 μM until the sixth cleavage, when it again rose to 1.20 μM. Arrows at *A* and (*I* + *A*) indicate artifactual spikes in the traces caused when *I* touched the Faraday cage. *I* indicates the time of addition of the calcium ionophore A23187. Ionophore caused no change in E_m, but caused $[Ca^{2+}]_i$ to rise to 7.59 μM. E_m increased from −10 to −16 mV during this experiment. Cytokinesis was visible during the time bracketed by the bar under each *C*.

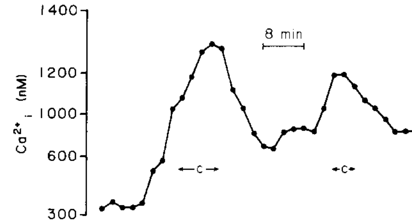

FIGURE 7 $[Ca^{2+}]_i$ at the time of cleavages in Fig. 6 is plotted at 2-min intervals with a 10-fold expansion of the vertical axis. $[Ca^{2+}]_i$ is indicated in nanomolar concentrations. The periods during which rounds of cytokinesis (C) were visible are indicated by arrows. Cleavages were clearly associated with transient increases in $[Ca^{2+}]_i$ in this embryonic cell.

Cytosolic $[Ca^{2+}]_i$ during Cytokinesis— Occasionally A Sharp Change

In a seventh embryo, cleavage was examined two more times. In this embryo an initial resting level of $[Ca^{2+}]_i$ was found to be 0.32 μM. During the time periods when this impaled blastomere could be seen to undergo fifth and sixth cleavages, the $[Ca^{2+}]_i$ rose to 1.32 and 1.20 μM, respectively (Fig. 6). Between successive rounds of cytokinesis, $[Ca^{2+}]_i$ returned to submicromolar levels (albeit increasing). When the data from Fig. 6 were replotted on an expanded scale of $[Ca^{2+}]_i$, the rises in $[Ca^{2+}]_i$ that occur in synchrony with cytokinesis could be seen more clearly (Fig. 7).

DISCUSSION

The results suggest that a practical technique for dechorionation now allows access to the very early cleaving medaka embryo for microelectrode penetrations and other studies. Opportunities, therefore, are available for studying cytokinesis in a vertebrate embryo available in the laboratory year-round. Using CSMs it was found that overall cytosolic $[Ca^{2+}]_i$ in this teleost is held constant (±~0.10 μM) during successive rounds of cytokinesis between the 8- and 64-cell stages, inclusive, although there appears to be a localized rise in $[Ca^{2+}]_i$ during cleavage. Resting $[Ca^{2+}]_i$ in such embryos was held at a value probably very near 0.10 μM. In contrast, the E_m undergoes a gradual net hyperpolarization during these stages. It would seem that these embryos may regulate cytokinesis through large but localized changes in $[Ca^{2+}]_i$.

Effects of Dechorionation

Cleavage rate, epiboly, gastrulation, and organogenesis appeared in dechorionated embryos to be no different from those in untreated control embryos, suggesting that basic cellular processes such as cytokinesis also were occurring in a normal manner. However, three specific properties of the embryos were altered. First, dechorionation reduced the mean E_m by ~8 mV (Fig. 5). This indicates that the membranes were sometimes made somewhat leaky by the treatment. However, because E_m was near control levels in those embryos in which $[Ca^{2+}]_i$ was measured, membrane damage should have been minimal in those cases. Second, dechorionation was associated with altered cleavage planes in 5% of dechorionated embryos. This has not been reported previously when chemical methods (21, 33, 34), mechanical methods (17, 18, 35), or a combination of both methods (20) have been used for removing chorions from teleost embryos. It is possible that the resulting skewed cell arrangements may occur even in 5% of normal, nondechorionated embryos (as is well known to occur in frog embryos), but have simply never been reported. Alternatively, the skewing may represent a phenomenon unique to *O. latipes*. However, because most enzymatic dechorionation treatments require long incubation times, embryos may contain hundreds of cells before observations are made, and so it is likely that any skewing of early cleavage planes may have been missed in other systems. Third, the contractile waves normally seen in the yolk cell and enveloping layer of cells appeared at the normal time and location, and seemed to travel at control rates, but traveled around the entire embryo in a path that was not quite perpendicular to the neural tube of the embryo proper. This may have arisen because of the reduced size of the yolk cell in dechorionated embryos. Except for the three features noted above, dechorionated embryos develop in a fashion which still allows for their experimental analysis.

Response of Embryos to Impalement with Microelectrodes

The CSMs used in these experiments responded well to submicromolar levels of Ca^{2+} for 3–7 d after their fabrication. Successful impalements with these electrodes showed an initial rapid response to micromolar $[Ca^{2+}]_i$, followed by a slow drop to submicromolar resting levels within several minutes. The slow decline may represent cellular buffering of small quantities of exogenous Ca^{2+} introduced by microelectrode impalement. The normal E_m net hyperpolarization is reattained quickly after CSM impalement, indicating an adequate sealing of the membrane around the CSM. Therefore, the intracellular measurements of cytosolic $[Ca^{2+}]_i$ presented here should be representative of normal intracellular levels. Rapid response to addition of calcium ionophore A23187 indicated that the CSMs were capable of measuring changes in intracellular $[Ca^{2+}]_i$. Differences of $[Ca^{2+}]_i$ between 0.01 and 0.40 μM for different embryos probably indicates that the cells suffered various degrees of membrane damage even under the

best conditions. Therefore, values in the lower end of this range are most likely to represent the genuine values for $[Ca^{2+}]_i$ in vivo.

Does $[Ca^{2+}]_i$ Change during Cytokinesis?

There were no changes in $[Ca^{2+}]_i$ >0.10 μM during nine rounds of cytokinesis in the medaka embryos examined here. However, a localized or short-lived calcium transient involved in cytokinesis would have been very difficult to detect in the present experiments because the CSMs only monitor a small region of cytoplasm, and required 1–10 s to respond to a 10-fold change in $[Ca^{2+}]_i$. In fact, a study of $[Ca^{2+}]_i$ in cleaving embryonic cells of *X. laevis* between the two-cell and 64-cell stages concluded that CSMs did not detect a change in $[Ca^{2+}]_i$ at the time of cytokinesis (11). In that case, cleaving embryonic frog cells impaled with CSMs showed a relatively constant value of 0.079 μM $[Ca^{2+}]_i$, similar to what is reported in the present paper. The investigators in that study also felt that damage to cell membranes caused by poor CSM impalements was responsible for very high resting values of $[Ca^{2+}]_i$ observed in some embryos (a mean $[Ca^{2+}]_i$ of 0.30 μM was given). Using calcium-stimulated aequorin fluorescence in order to determine $[Ca^{2+}]_i$ in cleaving starfish embryos, it has been determined that there are no resolvable calcium transients associated with cleavage (36), however, aequorin has more limited sensitivity to $[Ca^{2+}]_i$ than CSMs. For the first time, we are now able to compare aequorin (12) and CSM data for cleaving embryonic cells in the same organism (*O. latipes*). In the present study, CSMs indicated that there are probably no $[Ca^{2+}]_i$ transients associated with most of the cytosol during cytokinesis in embryonic cells of *O. latipes*, whereas, in an earlier study (12), aequorin fluorescence suggested that a small transient rise in $[Ca^{2+}]_i$ might occur with each round of cytokinesis in these embryos. Why would experiments using aequorin (12) and CSMs suggest apparently different correlations between $[Ca^{2+}]_i$ and cleavage in early cleaving medaka embryos? There are several possibilities. First, the apparent resting and transient concentrations of Ca_i^{2+} determined during cleavage were estimates made at the very limit of detection for the aequorin technique (9, 10, 14). The values obtained therefore were only approximations (12). One might still expect calcium transients to play a role in cytokinesis however, because increases in intracellular $[Ca^{2+}]_i$ do induce the formation of cleavage furrowlike structures (6, 7, 37). A second possibility is that although the $[Ca^{2+}]_i$ increases seen with aequorin occurred during the first and second cleavage, they do not occur after the second cleavage (an important difference between the two experiments is that the aequorin study ceased at the second cleavage, whereas the CSM penetrations could not begin until the third cleavage). Thirdly, it has been shown that anesthetics such as urethane and tetracaine enhance aequorin activity at constant $[Ca^{2+}]_i$ (38). Likewise, if a cellular mechanism were present for increasing the activity of calcium-binding proteins during cytokinesis and recognized the calcium-binding aequorin as a substrate, then transient increases in fluorescence might be seen with the aequorin technique, but not be matched by any change in CSM response. Finally, the aequorin experiments may have detected a real rise in $[Ca^{2+}]_i$, which was localized to a small region of the cell and thus unlikely to have been detected by the small, but sensitive microelectrode tip. This final explanation seems to be the appropriate one, because in one embryo out of seven studied in the present experiment, the CSM tip did detect a very large transient rise in $[Ca^{2+}]_i$, which was directly correlated in time with two rounds of cytokinesis. Resting cytosolic $[Ca^{2+}]_i$ in this embryo was unusually high (0.32 to 0.63 μm), indicating that a small quantity of extracellular ions was probably leaking into the egg. Even so, the cleavage-related Ca^{2+} transients were large enough to be clearly visible. The simplest explanation of this positive result is that a localized $[Ca^{2+}]_i$ transient does accompany cytokinesis.

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