

CYTOSOLIC FREE CALCIUM ION CONCENTRATION
IN CLEAVING EMBRYONIC CELLS OF Oryzias latipes
MEASURED WITH CALCIUM-SELECTIVE MICROELECTRODES

by

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TABLE OF CONTENTS

	<u>page</u>
I. Acknowledgements-----	1
II. Cytosolic Free Calcium Ion Concentration In Cleaving Embryonic Cells of <u>Oryzias latipes</u> Measured with Calcium-Selective Microelectrodes-----	2
A. Introduction-----	3
B. Materials and Methods-----	5
1. Microelectrodes-----	5
2. Embryo Dechoriation-----	8
3. Measurement of $[Ca^{2+}]_i$ and E_m -----	10
C. Results-----	14
1. Effects of Dechoriation-----	14
2. Calcium-Selective Microelectrodes-----	17
3. Cytosolic $[Ca^{2+}]_i$ During Cytokinesis-----	22
D. Discussion-----	29
1. Effects of Dechoriation-----	29
2. Response of Embryos To Impalement With Microelectrodes-----	30
3. Does $[Ca^{2+}]_i$ Change During Cytokinesis?---	31
E. References-----	34
III. Appendices-----	38
A. Preparation of Solutions-----	39
B. Filling the Silanized Micropipette-----	44
C. Pulling Micropipettes-----	45
D. Ideal Breeding Conditions for <u>O. latipes</u> -----	46

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LIST OF FIGURES

	<u>page</u>
Figure 1. Illustration of a chorion shunted with a glass tube-----	12
Figure 2. Early development of untreated and dechorionated embryos-----	15
Figure 3. Calibration record of a calcium-selective microelectrode-----	18
Figure 4. Calibration curve of a calcium-selective microelectrode-----	20
Figure 5. Pen recorder trace of pCa_i^{2+} and membrane potential-----	23
Figure 6. Membrane potential <u>vs</u> cell stage-----	27

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INTRODUCTION

Free calcium ion concentration ($[Ca^{2+}]_i$) 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 furrow-like 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 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 which 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 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 affects (20). However, collection of adequate quantities of hatching enzyme is time-consuming and requires a large, steady supply of embryos. Pronase has been successfully applied 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 other than those of Fundulus, especially at very early stages of cleavage and in a type of teleost available in the laboratory year-round.

The dechoronation 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 which are still in the early stages of development (often at the four cell stage) and which develop at a normal rate until depletion of the yolk cell contents. Eight of twelve dechorionated embryos permitted to develop to full term became actively swimming, feeding 'hatchlings' in 17 days. 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 remain essentially constant during cytokinesis.

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, New Jersey) 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) prior to 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 Filter Corp., Bedford, Mass.). 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 which might accumulate on the outer surface of the glass. Several centimeters of one end of a thin silver wire (0.005 inch diameter) (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. In order 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 Chem. Corp., Hauppauge, NY) using an attenuated, integral plastic needle (25). Tip resistance was 12 to 20 megohms in

Yamamoto's medium (12). Results were discarded if tip resistance was altered by cell membrane penetration.

With the aid of a Leitz micromanipulator, tips of micropipettes for CSMs were touched to a piece of tissue culture plastic (Falcon, Oxnard, CA). Tips were smoothly broken to 2.5 μm or 1.5 μm O.D. These micropipettes were placed tips up in a Coplin jar filled with 6X50 mm test tubes. The open Coplin jar containing approximately 20 micropipettes, along with a ground glass lid, was slowly heated to 200°C. Tri-n-butyl chlorosilane (15 μl) (Pfaltz and Bauer Inc., Stanford, Conn.) was added to the jar, which was immediately sealed with the hot glass lid and placed back into the oven. After 15 minutes 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, a backfill solution of 0.1 M CaCl_2 was injected into a silanized micropipette using an attenuated integral plastic needle. The large end of the micropipette was cemented into the tip of a 1 ml disposable plastic syringe (#5623, Becton Dickinson and 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.

Two different types of CSMs were tried. Neutral-carrier calcium ligand mixture for use in type a CSMs with 2.5 μm tips was made from a stock mixture containing 10% ETH 1001 (calcium ligand, Fluka Chem. Corp., Hauppauge, NY), 89% o-nitrophenyl octylether, (Fluka Chem. Corp., Hauppauge, NY), and 1% sodium tetraphenyl borate (Aldrich, Milwaukee, WI). It was made by the method of Lanter et al. (16) with two slight alternations: The stock mixture contained only a 10% (w:w) addition of high molecular weight polyvinyl chloride (PVC) (a gift from Dr. W. Simon), and the stock mixture was then

dissolved in no more than twice its weight of tetrahydrofuran (Aldrich, Milwaukee, WI) to make the final solution. Then a droplet of the final neutral-carrier ligand solution was drawn into a large (approximately 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. The dipping process was repeated until, by capillary action, the microelectrode tip became filled with a 350 μm long column of the final ligand solution. Type b CSMs had tips of 1.5 μm O.D. and were dipped as above until they contained a 200 to 325 μm long column of final ligand solution at the time of filling. The latter contained 3% tetraphenylarsonium tetrakis (p-biphenyl) borate (extremely hydrophobic salt, W.P. Instruments Inc., New Haven, Conn.), 73% o-nitrophenyl octylether, and 12% high molecular weight PVC. This solution was mixed 1:2 (w:w) with tetrahydrofuran (15). 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) in order to achieve stable intracellular records. Because both types of CSMs appeared to respond equally well to $[\text{Ca}^{2+}]_i$ in standard solutions, those with 1.5 μm tips (type b) were used for all later intracellular records in order to minimize damage to the cell membrane.

In order to avoid problems of drift (23), pCa 6 buffer solution (15) was used to continuously bathe CSM tips for two to five days prior to an experiment. Type a CSMs were discarded 7 days after construction and type b CSMs were discarded 5 days after construction because sensitivity to $[\text{Ca}^{2+}]_i$

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 #1 of ref. 15 for recipes) before and after cellular impalements. Data were rejected when the microelectrode response to $[Ca^{2+}]_i$ changed by more than five mV/decade $[Ca^{2+}]_i$.

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 X 15 mm bacteriological plastic petri dish (Falcon #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 number 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 of no more than 2 μ m in diameter.

All observations of embryos were made with the use of a Wild M-5 dissecting microscope and transmitted light. Embryos always remained in a dish of Yamamoto's medium at room temperature ($24^{\circ}C \pm 1^{\circ}C$) 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 X, 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 about 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 in order 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 droplet of frozen 3% pancreatin (Sigma, 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 minutes. 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 semi-flacid sac. In such embryos, the yolk cell membrane remained taut, although reduced in diameter to approximately 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 in order 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 (hereafter referred to as dechorionated embryos) were kept in Yamamoto's medium, together with various control groups. Each group was observed at 30 minute 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.

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 minutes at 24°C) all reached the eight-cell stage prior to 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 5M potassium acetate to allow recording of membrane potential (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)).

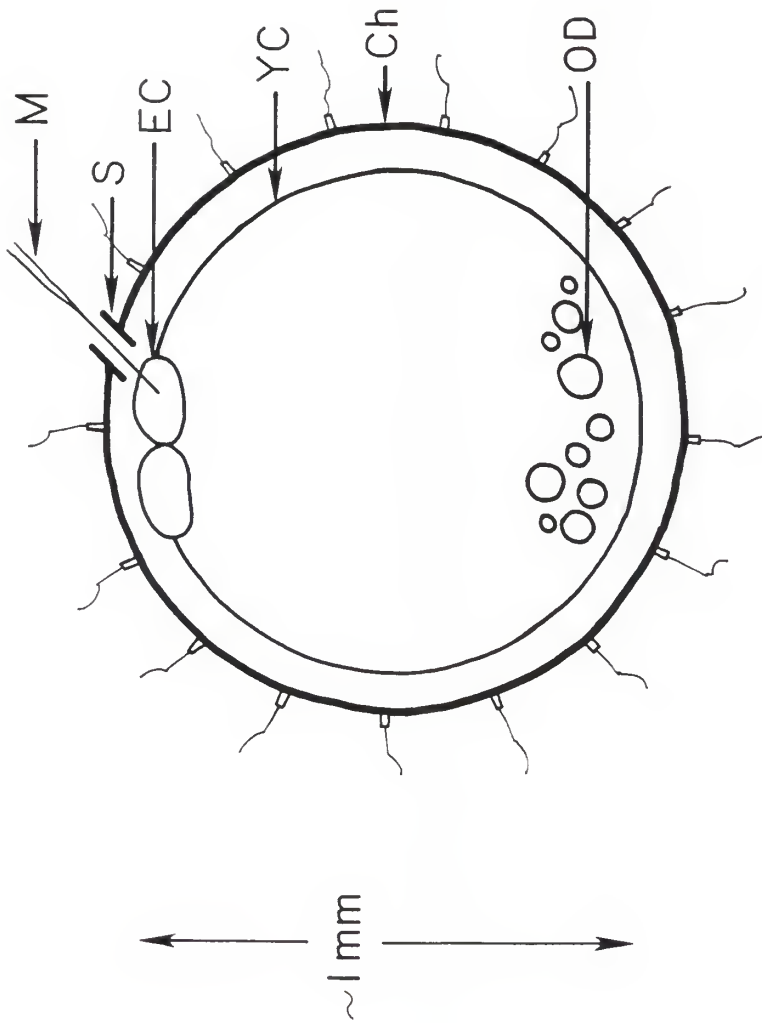
The circuit used for measuring potentials consisted of a grounded calomel reference electrode (13-639-79, Fisher Scientific Co., Pittsburgh, PA) in 3 M KCl connected via a salt bridge (3 M KCl in 2% agarose gel) to either the calibration solution or the dish of Yamamoto's medium used for cell impalements. 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, New Haven, CT). Outputs were displayed as traces on a two-pen strip chart recorder (#7132A, Hewlett-Packard, San Deigo, 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 early high blastula stage, or until a substantial drop in E_m indicated damage to the cell membrane. The two

electrodes were eventually 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 which should have affected the results presented here, even if cells had been uncoupled. All microelectrode manipulations were performed inside a grounded Faraday cage which was isolated from most mechanical vibrations by several inflated rubber inner tubes.

In one experiment, dimethylsulfoxide (DMSO) in Yamamoto's medium was added (without stirring) during a stable impalement to give a final DMSO concentration of not less than 1% surrounding the embryo. In another set of experiments, a calcium ionophore solution (A23187 (Sigma, St. Louis, MO) 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. This was done twice, with the impaling electrode being a CSM the first time, and a potassium acetate-filled microelectrode the second time. Such single microelectrode impalements were resorted to after numerous techniques for adding ionophore solution all failed to be gentle enough for maintaining doubly-impaled embryos in a healthy state. No other experiments were intentionally designed to measure $p\text{Ca}_i^{2+}$ without dual impaling microelectrodes.

In order to measure E_m in embryos which had neither been dechorionated nor treated with enzyme, a micropipette with a tip diameter less than 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 three embryos having received chorionic shunts.

Figure 1: This illustration depicts a cross-sectional view of a two-celled Medaka embryo surrounded by a chorion (Ch) which has been shunted by a glass tube (S) in order to allow for recording of the E_m without subjecting the embryo to the dechoriation 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.



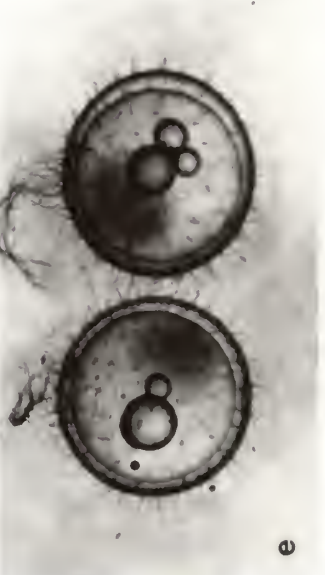
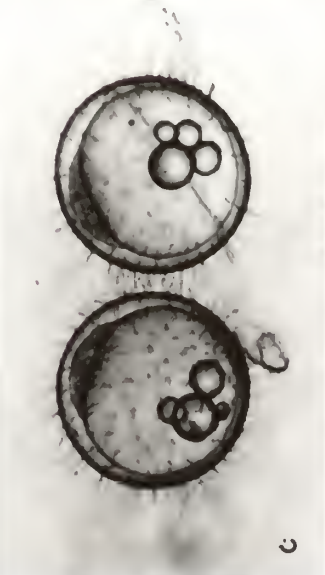
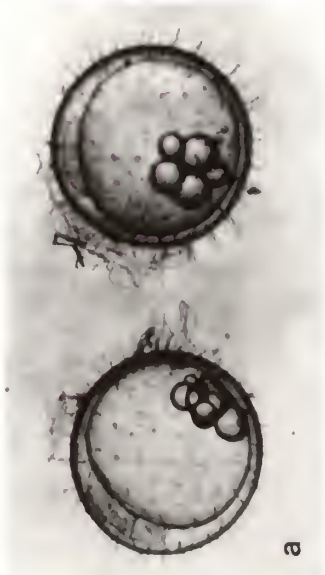
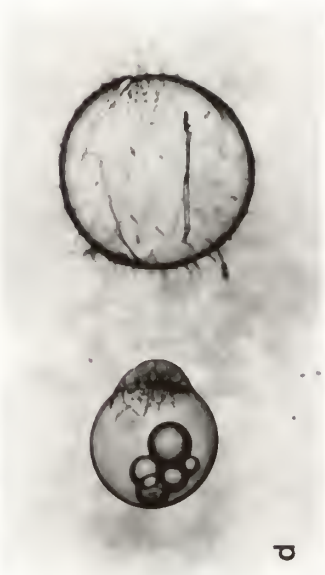
RESULTS

EFFECTS OF DECHORIONATION

The rate of development of dechorionated embryos was compared with that of control embryos which were simply transferred from the spawning female to a dish of Yamamoto's medium at $24^{\circ}\text{C} \pm 1^{\circ}\text{C}$. Embryos in these two groups were always found to develop at the same rate \pm one stage (as defined by Kirchen and West (29)) 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 twelve dechorionated embryos revealed that 75% began actively swimming and feeding by the seventeenth day after fertilization (25% depleted their yolk cells and died during the second or third weeks of development). Successful microelectrode penetrations did not alter these results. A control group of untreated embryos held at 4°C in 3% pancreatin solution for 95 minutes stopped cleaving until being returned to room temperature. Cytokinesis then resumed at a normal rate within one hour. Because these chilled embryos went on to become normal hatchlings, it is assumed that placing embryos to be dechorionated on a 50 μl droplet 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, Fundulus 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

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 8-cell stage, a, and b, and the 64-cell stage, c, and d, were reached at 3 and 4.5 hours post-fertilization respectively. By 12 hours post-fertilization, e, and f, embryos had reached the flat blastual stage. Enlargement X 23.4, bar represents 175 μm .



b

p

f

a

c

e

cytokinesis at a normal rate. However, daughter cells of the cell which 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 one out 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 slowed down, halted, and relaxed. In dechorionated embryos, contractile waves initiated at the lateral surface of the embryo proper, and 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 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 (Fig. 3) and had nearly Nernstian slopes when the $[Ca^{2+}]_i$ was 10 μM or greater (Fig. 4). Below 10 μM $[Ca^{2+}]_i$, there was only slight hysteresis; however, responses became substantially more sub-Nernstian with each ten-fold decrease in $[Ca^{2+}]_i$ (Fig. 2 and 3). As a result, this technique could not reliably quantitate $[Ca^{2+}]_i$ below pCa_i^{2+} 7. Thus, values below 0.10 μM are necessarily estimates. CSM sensitivity to $[Ca^{2+}]_i$ in the presence of interfering ions found in vivo

Figure 3: Calibration record shows typical response of pre-impalement CSM with 1.5 μm O.D. tip. Vertical blips on the plateaus are artifacts caused by the antennae properties of persons near the Faraday cage. Changes in $[\text{Ca}^{2+}]_i$ can be detected well below the μM range despite sub-Nerstian responses.

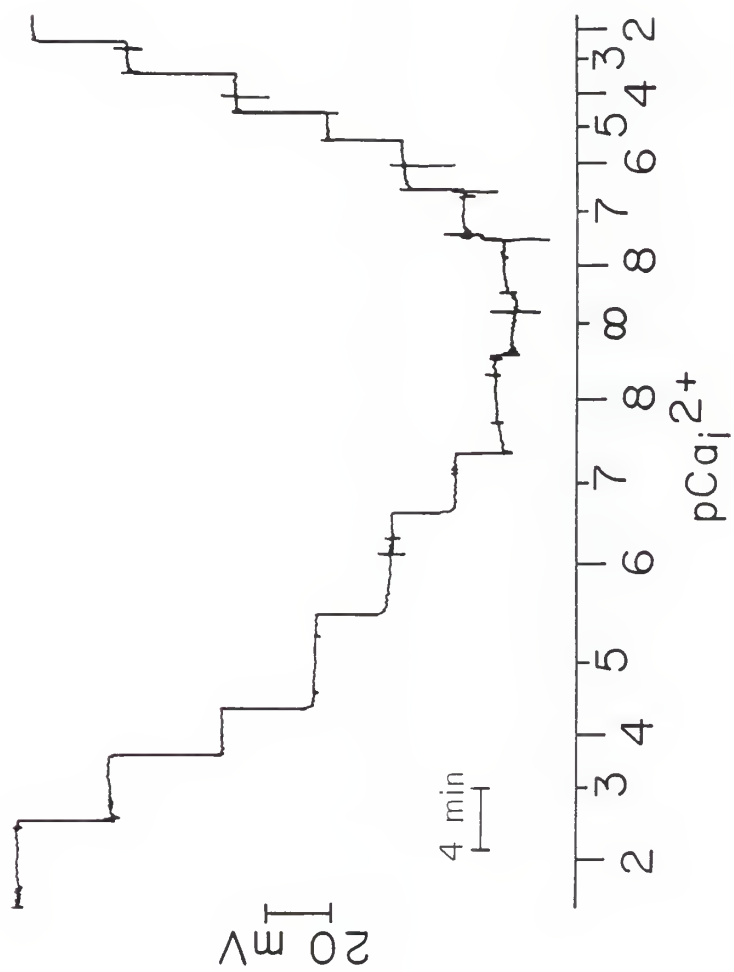
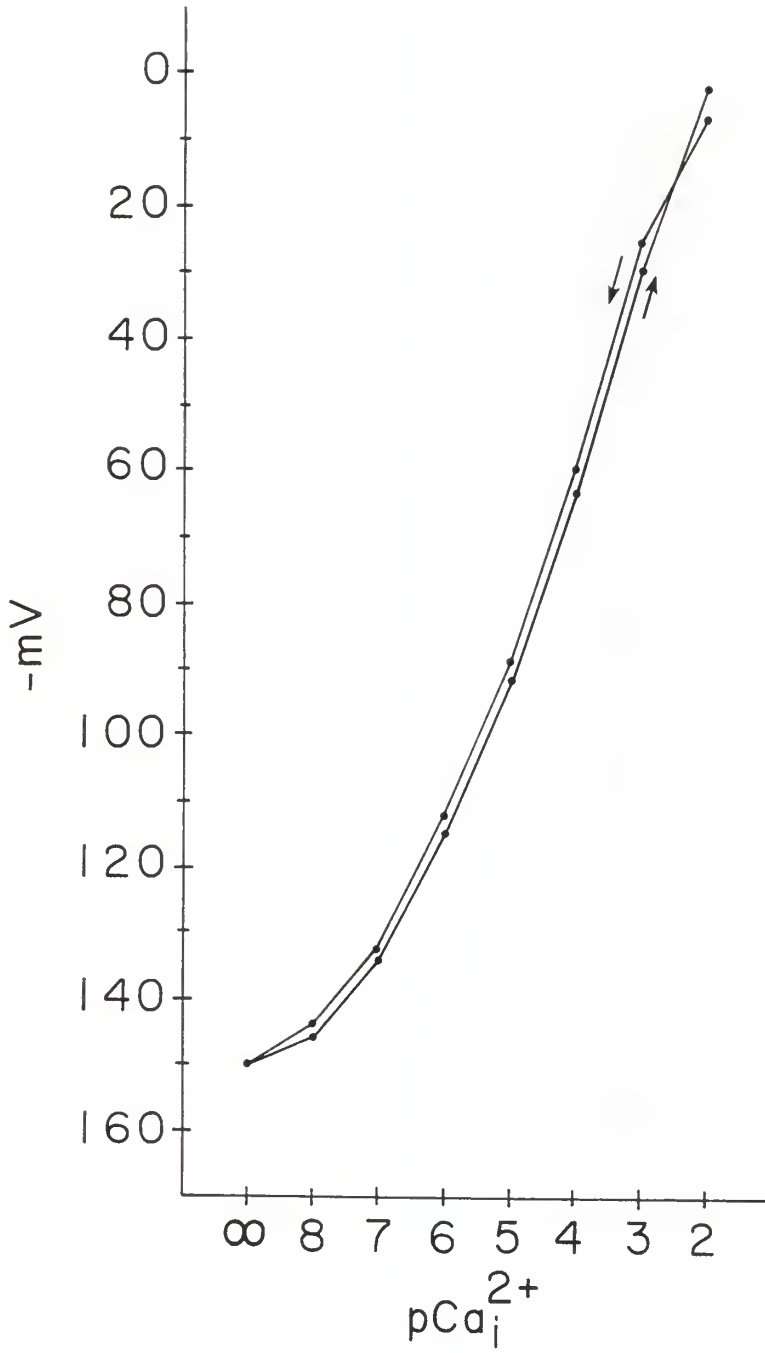


Figure 4: CSM preimpalement curve constructed from data shown in fig. 3. Minor hysteresis is indicated. This CSM was used for the experiment shown in fig. 5. Arrows indicate the order in which solutions were changed during the two traces.

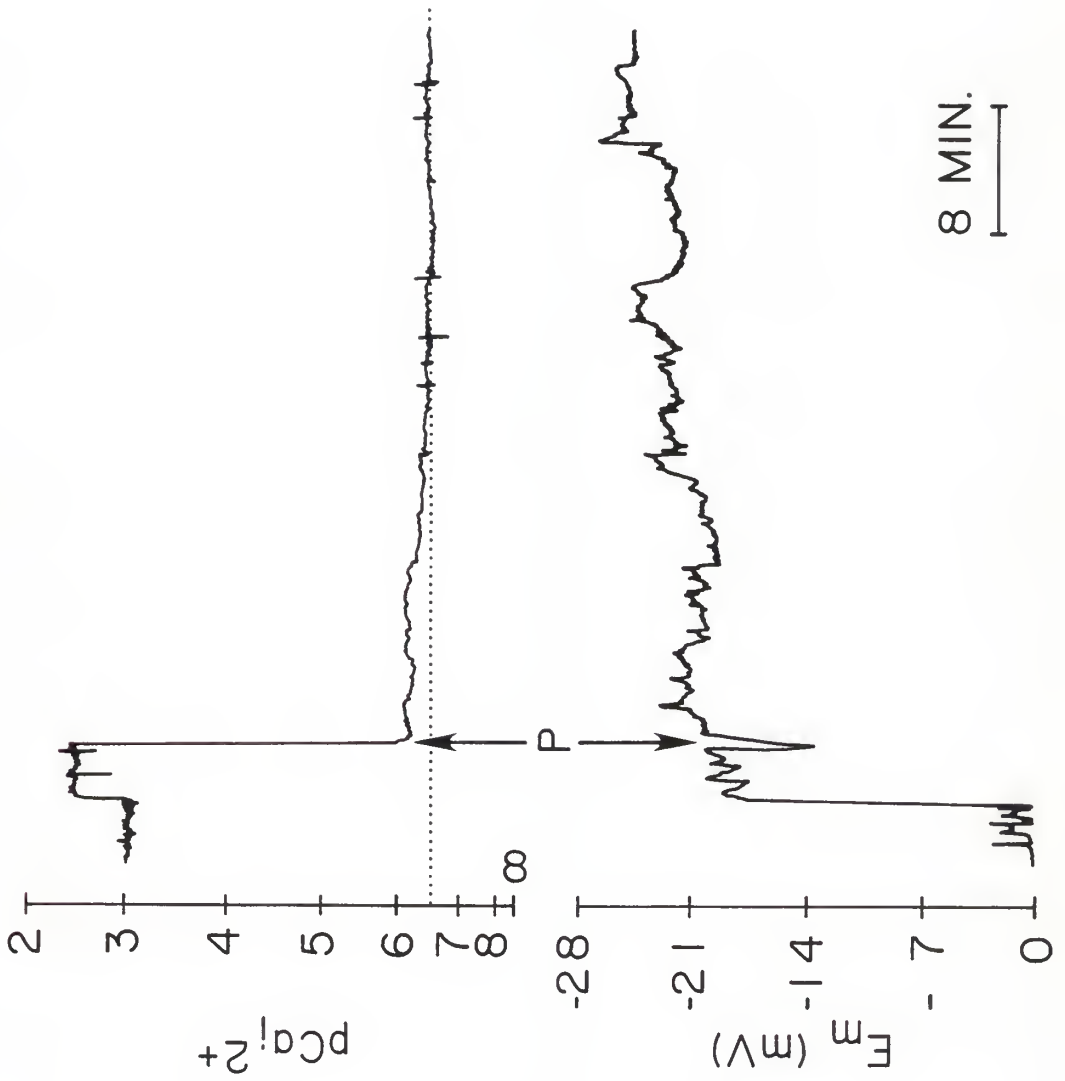


was good, as has been shown by others using these types of CSMs (11,15,23,30, 31). An accurate pCa_i^{2+} of 2.74 was indicated for pure Yamamoto's medium containing Na^+ and K^+ concentrations in excess of 2 mM. When distilled water was introduced on dissecting instruments into the 1 to 2 ml of solution bathing an embryo during some dechorionations, the CSMs again responded well, indicating a $pCa_i^{2+} < 2.74$ (Fig. 5).

CYTOSOLIC $[Ca^{2+}]_i$ DURING CYTOKINESIS

Embryos were penetrated with 5M 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. Following impalement of an embryonic cell at the 8- to 16-cell stage with a CSM, a rapid drop in the CSM potential occurred equivalent to approximately $pCa_i^{2+} 6$, followed by a slow decrease in intracellular $[Ca^{2+}]_i$ over many minutes. These changes were followed by a stable, non-fluctuating recording of the resting level of cytosolic $[Ca^{2+}]_i$, while the embryonic cells continued to undergo one round of cytokinesis every 20 to 30 minutes throughout the experiment. In two separate experiments cytosolic $[Ca^{2+}]_i$ was found to be 0.40, and 0.014 μM , respectively. Within the cells of a single embryo $[Ca^{2+}]_i$ remained constant, but it varied significantly between embryos. Small fluctuations of $[Ca^{2+}]_i$ ($\pm 0.10 \mu M$ or less) did not correlate with the occurrence of cytokinesis in number or in time. In two other experiments good CSM penetration was achieved, however, problems with membrane potential microelectrodes prevented automatic subtraction of E_m from pCa_i^{2+} . In these two cases, however, E_m was successfully determined at the end of the experiments, and manually subtracted from the final CSM values. In these instances, $[Ca^{2+}]_i$ was found to be 1.00 μM . These single electrode recordings indicated that either a slow net hyperpolarization, or a slow

Figure 5: Pen recorder traces of pCa_i^{2+} and E_m in an embryonic cell of O. latipes during fifth and sixth cleavages. 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 CSM 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 $0.40 \mu M$, indicated by the dotted line.



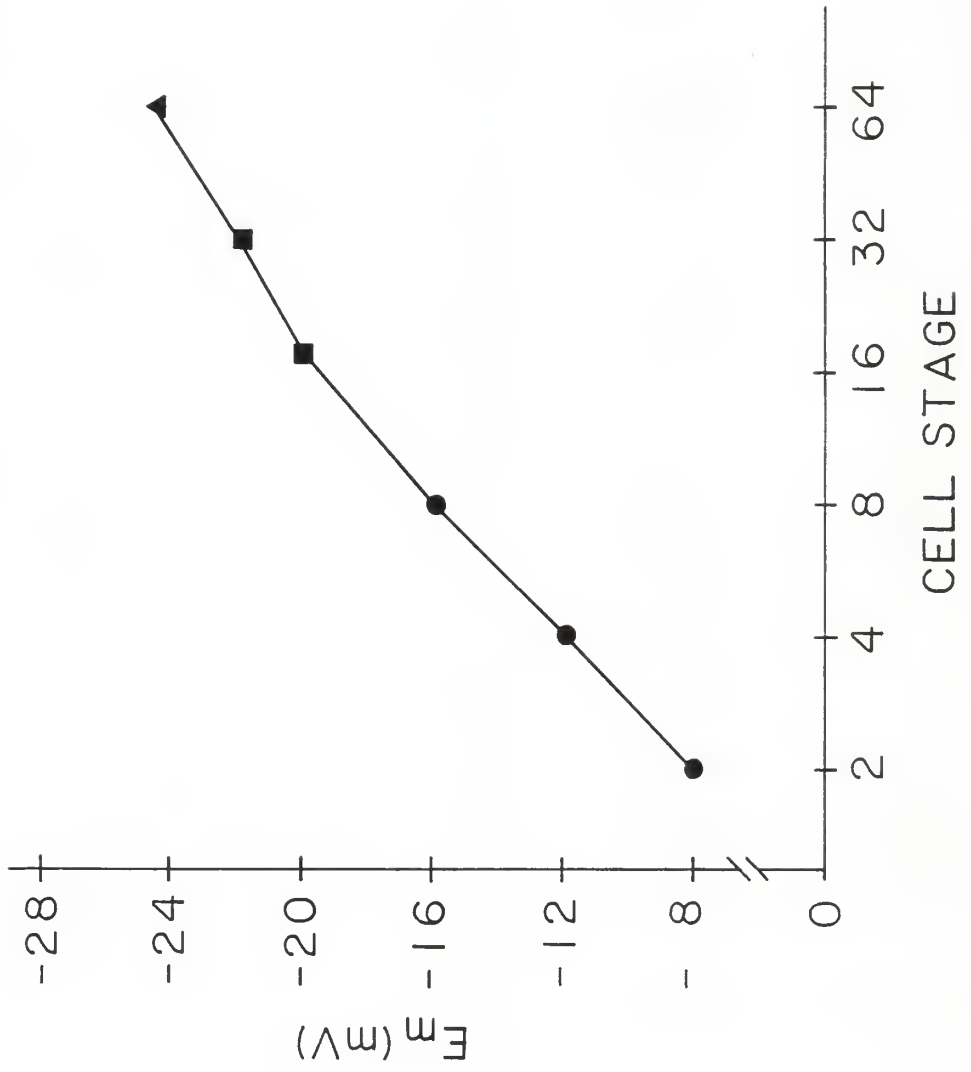
minute rise in $[Ca^{2+}]_i$, or a combination of both had occurred during development. Cytokinesis was observed 9 times in these 4 experiments, and was never marked by a $[Ca^{2+}]_i$ transient.

Yamamoto's medium containing 1% DMSO caused no change in E_m or pCa_i^{2+} . However, addition of ionophore solution (see Methods) to embryos with single microelectrode impalements 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 E_m was unaffected by the ionophore solution, i.e., a slow net hyperpolarization was indicated, regardless of the drug's presence. However, when an embryo between the 16- and 32-cell stages impaled with a CSM was presented with the ionophore solution, microelectrode readings rapidly became more positive. Because the drugs had 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 rise in $[Ca^{2+}]_i$. If one assumes a typical E_m of -19 mV for the CSM-impaled embryo at this stage of development, then the ionophore solution caused a clearly resolved increase in intracellular cytosolic $[Ca^{2+}]_i$ from approximately 0.01 μM to 3.2 μM within one minute from the time of the addition of the drug.

The membrane potential in the dechorionated embryos always followed a course of gradual net hyperpolarization with small transient depolarizations of several mV interspersed in a seemingly random manner. An experiment was performed in order to determine whether this was an artifact of dechorionation. Chorions surrounding intact, non-treated embryos, received chorionic shunts (a glass tube through the chorion as described above) through which the membrane potential electrode was inserted. Dechorionated embryos and those with chorionic shunts (Fig. 1) showed net hyperpolarizations of -8.4

and -8.6 mV respectively, between the 8- and 64-cell stages (stages during which measurements were obtainable from both groups), with the mean E_m being 8 mV less hyperpolarized at each stage for a group of eight dechorionated embryos than for intact embryos. However, embryos which appeared least damaged by dechorionation displayed E_m within 2 mV of the values from non-dechorionated ones (compare Figs. 5 and 6). 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.

Figure 6: Using a chorionic shunt, E_m (in mV) was measured for three different embryos (●, ■, ▲) retaining their chorions. A net hyperpolarization was observed, indicating that the net hyperpolarization found to occur in cells of all dechorionated embryos is not an artifact of the dechorionation process.



DISCUSSION

The results suggest that a practical technique for dechoriation 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 cytosolic $[Ca^{2+}]_i$ in this teleost is held constant (\pm approximately 0.10 μ M) during successive rounds of cytokinesis between the eight cell stage and that of the early high blastula. Resting $[Ca^{2+}]_i$ in such embryos was held at a value probably very near 0.014 μ M. In contrast, the E_m undergoes a gradual net hyperpolarization during these stages. It would seem that these embryos do not regulate cytokinesis through large changes in overall cytosolic $[Ca^{2+}]_i$. The results obtained with CSMs do not eliminate the possibility that rapid or highly localized changes in $[Ca^{2+}]_i$ occur during cytokinesis.

EFFECTS OF DECHORINATION

Cleavage rate, epiboly, gastrulation, and organogenesis appeared to be no different in dechorionated than 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, dechoriation reduced the mean E_m by approximately 8 mV. This indicates that the membranes were sometimes made somewhat leaky by the treatment. However, E_m was very near control levels in those embryos in which pCa_i^{2+} was measured, and so membrane damage should have been minimal in those cases. Second, dechoriation was associated with altered cleavage planes in 5% of dechorionated embryos. This has not been reported previously when chemical methods (21,32,33), mechanical methods (17,18,34),

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, non-dechorionated embryos, but have simply never been observed previously. Alternatively, the skewing may represent a phenomenon unique to O. latipes. However, since most enzymatic dechorionation treatments require long incubation times, embryos may contain hundreds of cells before observations are made, and so 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 travelled around the entire embryo in a path which 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_i^{2+} for three to seven days 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_i^{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 0.014 and 1.00 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 $[Ca^{2+}]_i$ greater than 0.10 μM during as much as nine rounds of cytokinesis in the Medaka embryos examined here. It is conceivable that such small fluctuations could play a role in cytokinesis if it were not for the fact that these small fluctuations had no consistent temporal relationship with cytokinesis. Therefore, there is no evidence provided by these experiments to indicate that changes in cytosolic $[Ca^{2+}]_i$ are involved in regulation of cytokinesis in vivo in early cleaving Medaka embryos. Although injection of chelators has shown that Ca^{2+}_i is necessary for cell divisions in some systems (8), the present experiments suggest that this requirement may be provided for adequately by resting levels of Ca^{2+}_i (11). However, if a short-lived calcium transient is involved in cytokinesis, it would not have been detected in the present experiments because the CSMs used required 1 to 10 seconds to respond to a ten-fold change in $[Ca^{2+}]_i$.

A study of $[Ca^{2+}]_i$ in cleaving embryonic cells of Xenopus laevis between the 2-cell and 64-cell stages has produced results similar to those presented here (11). It was reported that cleaving embryonic frog cells impaled with CSMs showed a relatively constant value of 0.079 μM $[Ca^{2+}]_i$. The investigators in that study also felt that damage to cell membranes caused by poor CSM impalements was responsible for higher values of $[Ca^{2+}]_i$ observed in most 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 (35). 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 no $[Ca^{2+}]_i$ transients associated with cytokinesis in embryonic cells of O. latipes, whereas in an earlier study (12), aequorin fluorescence suggested that a small transient rise in $[Ca^{2+}]_i$ occurs 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^{2+}_i determined during cleavage were estimates made at the very limit of detection for this 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 furrow-like structures (6,7,36). A second possibility is that the aequorin experiments detected a significant, but localized increase in $[Ca^{2+}]_i$ unlikely to be detected by the small tip of a micro-electrode. A third possible explanation is that although the $[Ca^{2+}]_i$ increases seen with aequorin occurred during first and second cleavage, they do not occur after second cleavage (an important difference between the experiments is that the aequorin study ceased at second cleavage, whereas the CSM penetrations could not begin until third cleavage). Finally, it has been shown that anesthetics such as urethane and tetracaine enhance aequorin activity at constant $[Ca^{2+}]_i$ (37). 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. In conclusion, if transient increases in cytosolic $[Ca^{2+}]_i$ really do play a role in cytokinesis in animal cells, however small, brief, and localized, they remain to be demonstrated.

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III. Appendices

Appendix A - Preparation of SolutionsYamamoto's Medium (see ref. 12)

7.488 g NaCl

0.194 g KCl

0.411 g CaCl₂

500 ml H₂O

Adjust to pH 7.3 with 0.5 M sodium bicarbonate.

Bring to 1000 ml. Sterilize through a rinsed 0.45 Millipore filter. Can be stored 6 months at room temperature.

3% Pancreatin Solution

Hank's Phosphate Buffer:

0.0225 g Na₂HPO₄·7H₂O

0.0150 g KH₂PO₄ (Monobasic)

0.0875 g NaHCO₃

Bring to 250 ml with H₂O

Filter through washed 0.45 Millipore filter.

97 parts Hank's phosphate buffer was mixed with 3 parts (w/w) pancreatin. The pH of the solution was not adjusted. Can be stored at least 6 months at -20°C.

pCa Buffers (see Table 1 of ref. 15)

Store solutions at 4°C.

KOH solutions used to adjust pH were 1.00 M, 0.50 M, 0.10 M, and 0.01 M.

Abbreviations: EGTA = Ethyleneglycol-bis-(β -aminoethylether)-N,N'-tetraacetic acid.
HEEDTA = N-(2-hydroxyethyl) ethylenediamine triacetic acid.
MOPS = 4-morpholinepropanesulfonic acid.
HEPES = N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
NTA = Nitritotriacetic acid
TAPS = 3-[tris(hydroxymethyl)methylamine]-l-propanesulfonic acid.

pCa2

50 ml of a 1000 mM KCl stock solution
50 ml of a 100 mM HEPES stock solution
0.7351 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$
Bring to pH 7.80 with KOH solutions
Bring to 500 ml with deionized double distilled water.

pCa3

5 ml of a 100 mM CaCl_2 stock solution
49 ml of a 1000 mM KCl stock solution
50 ml of a 100 mM MOPS stock solution
Bring to pH 7.30 with KOH solutions
Bring to 500 ml with deionized double distilled water.

pCa4

25 ml of a 100 mM CaCl_2 stock solution
45 ml of a 1000 mM KCl stock solution
50 ml of a 100 mM HEPES stock solution
0.9557 g NTA
Bring to pH 7.39 with KOH solutions
Bring to 500 ml with deionized double distilled water.

pCa5

25 ml of a 100 mM CaCl₂ stock solution
45 ml of a 1000 mM KCl stock solution
50 ml of a 100 mM TAPS stock solution
Bring to pH 8.42 with KOH solutions
Bring to 500 ml with deionized double distilled water.

pCa6

25 ml of a 100 mM CaCl₂ stock solution
50 ml of a 100 mM HEEDTA stock solution
45 ml of a 1000 mM KCl stock solution
50 ml of a 100 mM MOPS stock solution
Bring to pH 7.70 with KOH solutions
Bring to 500 ml with deionized double distilled water.

pCa7

25 ml of a 100 mM CaCl₂ stock solution
45 ml of a 1000 mM KCl stock solution
50 ml of a 100 mM MOPS stock solution
1.902 g EGTA
Bring to pH 7.29 with KOH solutions
Bring to 500 ml with deionized double distilled water.

pCa8

25 ml of a 100 mM CaCl₂ stock solution
45 ml of a 1000 mM KCl stock solution
50 ml of a 100 mM HEPES stock solution
1.902 g EGTA
Bring to pH 7.80 with KOH solutions
Bring to 500 ml with deionized double distilled water.

pCa9

50 ml of a 1000 mM KCl stock solution

50 ml of a 100 mM HEPES stock solution

1.902 g EGTA

Bring to pH 7.80 with KOH

Bring to 500 ml with deionized double distilled water.

Simon's Neutral-carrier Calcium Ligand Solution (for tip OD \geq 2 μ m)

Solution A:

50 mg ETH 1001

445 mg o-Nitrophenyl octyl ether

5 mg Na-tetraphenylborate

60 mg high molecular weight polyvinylchloride

Add 1.5 parts (w/w) tetrahydrofuran to 1 part solution A.

Store in small, evacuated, air-tight vessel. Lasts 1 week at room temperature.

Tsien's Neutral-carrier Calcium Ligand Solution (for tip OD \geq 1.5 μ m)

Solution A:

50 mg ETH 1001

304 mg o-Nitrophenyl octyl ether

12.5 mg tetraphenylarsonium tetrakis (p-biphenyl) borate

50 mg high molecular weight polyvinyl chloride.

Add 2 parts (w/w) tetrahydrofuran to one part solution A.

Store in small, evacuated, air-tight vessel. Lasts 1 month at room temperature.

1% DMSO

Add 40 μ l dimethyl sulfoxide to 2000 μ l Yamamoto's medium.

Add all of above solution to \sim 2 ml Yamamoto's medium in experimental dish to give 1% DMSO.

Ionophore Solution

Solution A =

1 mg A23187 calcium Ionophore

1 ml dimethylsulfoxide

Add 20 μ l solution A to \sim 4 ml Yamamoto's medium in experimental dish to give \sim 9.5 μ M A23187 and 0.5% DMSO.

Appendix B - Filling the Silanized Micropipette

When silanized micropipettes lacked internal fibers, the backfill solution did not fill the micropipette tip of its own accord. Once 0.1M CaCl_2 had been injected into the silanized micropipette with an attenuated integral plastic needle, the back 0.5 cm of the barrel was inserted into a 1 cc disposable tuberculin syringe (plunger previously removed and metal tip discarded) (#5623, Becton-Dickinson and Co., Rutherford, NJ). A few drops of hot sealing wax was dripped onto the micropipette-syringe joint, and the hot wax was worked into the gap by twisting the micropipette. The sealing wax hardened within a few minutes, after which time, the syringe was filled with 0.1 M CaCl_2 and the plunger was reinserted. Pressure was applied to the plunger until several droplets of backfill solution emerged from the micropipette tip. (Caution: occasionally the sealing wax gives way, and a micropipette projectile is released!) While still applying pressure, the syringe barrel was cut with a razor blade. This permitted air to enter the syringe as the micropipette was removed, so that a vacuum did not draw the backfill solution away from the micropipette tip. Having been filled and freed from the syringe, the micropipette tip normally remained filled for 5 to 10 minutes. Neutral-calcium carrier had to be added before the backfill solution receded from the hydrophobic tip.

Appendix C - Pulling Micropipettes

Although a large number of settings will give suitable micropipettes, the settings given below will pull omega-dot pyrex capillary tubing (1.5 mm OD and 1.1 mm ID) to submicron tips of 12 to 18 megohms and also pull regular pyrex tubing (1.5 mm OD and 1.1 mm ID) to tips which can be easily broken to 1 to 5 μm . A vertical pipette puller (#700C, David Kopf Instruments, Tujunga, CA) was used. Solenoid was 65. Heat was 21 Amps. The left arm allowed for a 3.8 mm drop before solenoid was turned on, and the right arm permitted a total drop of 24 mm before turning the solenoid off again.

Appendix D - Ideal Breeding Conditions for *O. latipes*

Conditions were initially those described by Kirchen and West (29). The temperature cycle which they describe becomes very important in August when the natural breeding season ends. However, other conditions such as the extended light cycle seemed to stress the fish too much. Stresses due to the 16 hour light, 8 hour dark cycle or overfeeding both resulted in outbreaks of fungal diseases. Therefore, conditions were modified so as to minimize stress while maintaining optimal spawning.

O. latipes spawned freely during June and July under a 15 hours light: 9 hours dark cycle, accompanied by light tri-daily feedings upon dry tropical fish flakes (~ 30 flakes per 10 gallon tank containing ~ 20 fish per feeding). In order to maintain breeding after July, conditions must be carefully manipulated. A 100 watt aquarium thermostat (controlled by the same time dial which regulated the light cycle) was used to cause the temperature to rise to 25-28°C during the light cycle, and to fall to 23-25°C during the dark cycle. In addition to dry tropical fish flakes, minced frozen shrimp (~ 20 bite-sized pieces) was given once or twice each day. While food had to be both abundant and of high quality during Fall and Winter, overfeeding quickly resulted in fouled aquarium water and outbreaks of disease. Use of an external aquarium filter produced strong currents and reduced spawning. On the other hand, mild currents produced by an under-gravel filter caused spawning to increase. Finally, Elodea plants seemed to improve spawning activity, while overcrowding (as many as 24 fish per 10 gallons) produced no observed detrimental affects.

CYTOSOLIC FREE CALCIUM ION CONCENTRATION
IN CLEAVING EMBRYONIC CELLS OF Oryzias
latipes MEASURED WITH CALCIUM-SELECTIVE
MICROELECTRODES

by

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ABSTRACT

Calcium-selective microelectrodes were used to measure 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: first, removal of a portion of the yolk in order to enlarge the perivitelline space; second, partial digestion of the chorion with 3% pancreatin in a phosphate buffer; and third, removal of the chorion using two sets of ultra-sharp watchmaker's forceps. Dechorionated embryos underwent cleavage at a normal rate until epiboly, approximately 14 hours post-fertilization (at 25°C). Visual observations indicated normal development. At 17 days post-fertilization, as yolk cell contents became depleted, actively swimming embryos began to feed on powdered commercial tropical fish food.

Intracellular cytosolic $[Ca^{2+}]_i$ was monitored by impaling blastomeres first with a microelectrode filled with 5M potassium acetate to measure membrane potential, and a few minutes later with a calcium-selective microelectrode. Simultaneous recordings from such pairs of electrodes within single blastomeres indicated that there was no apparent correlation between a change in cytosolic $[Ca^{2+}]_i$ and the occurrence of cytokinesis between the eight cell stage and the beginning of the early high blastula stage. During impalements of the blastomeres within a single embryo, cytosolic $[Ca^{2+}]_i$ remained constant during cytokinesis, with occasional apparently random fluctuations of 0.1 μ M or less. The technique does not eliminate the possibility that rapid or highly localized changes in $[Ca^{2+}]_i$ occur in these cells. Steady state levels of $[Ca^{2+}]_i$ in different embryos ranged from 1.00 μ M to 0.014 μ M. During the same period of development, resting membrane potential underwent a gradual net hyperpolarization in both chorionated and dechorionated embryos.