about fourfold greater in fast- compared to slow-twitch muscle (*13*). If the steady myoplasmic Ca^{2+} is 3 to 7 μM during tetanus (*3*), then the fast/slow ratios of k_{tr} that we measured at similar concentrations of Ca^{2+} are comparable to those obtained in living fast- and slow-twitch muscles. Our results, however, do not exclude the possibility that the Ca^{2+} transient or subsequent thin filament activation steps are slower in slow- than in fast-twitch muscles.

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A Voltage-Dependent Chloride Current Linked to the Cell Cycle in Ascidian Embryos

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A voltage-dependent chloride current has been found in early ascidian embryos that is a minor conductance in the oocyte and in interphase blastomeres but that increases transiently in amplitude by more than tenfold during each cell division. Repeated cycles in the density of this chloride current could be recorded for up to 6 hours (four cycles) in cleavage-arrested embryos, whether they were activated by sperm or calcium ionophore. These data suggest that there is a direct link between the cell cycle clock and the properties of this channel, a link that results in pronounced cyclical changes in the electrical properties of early blastomeres.

THE RAPID CELL CYCLE OF EARLY embryos is driven by an endogenous clock that involves the synthesis and degradation of the protein cyclin, activation and inactivation of the kinase maturation-promoting factor, and release and reuptake of calcium ions from intracellular stores (*1, 2*). Changes in ion fluxes across the plasma membrane or in membrane electrical properties during embryonic cell divisions have been demonstrated in several preparations (*3–6*). In most cases, these are either permanent changes that are caused by the addition of new cleavage-furrow membrane to the cell (*4, 5*) or transient changes linked to cytokinesis that are caused, for example, by the mechanical strain of cleavage-furrow formation (*6*). It is not known whether the cell cycle oscillator can drive transient changes in the electrical or ionic properties of the plasma membrane that are unique to the time of cell division, but independent of the actual process of cytokinesis. We describe in this report a class of voltage-dependent Cl^- channels that is functional only at the time of embryonic cell divisions. Large oscillations in the functional density of these channels persist when cytokinesis is blocked, demonstrating that the cell cycle clock can exert a direct influence on the electrical properties of the plasma membrane.

Our experiments were done on cells from early embryos of the ascidian *Boltenia villosa*. Ascidians are primitive marine chordates (subphylum Urochordata) that have been used extensively in embryological and electrophysiological studies (*7–9*). Three major voltage-dependent currents are recorded from voltage-clamped *Boltenia* oocytes before fertilization (*8*): inward Na^+ and Ca^{2+} currents, activated by depolarizing voltage pulses from holding potentials more negative than −60 mV, and an inwardly rectifying K^+ current, activated at voltages more negative than −60 mV (Fig. 1). When the inwardly rectifying K^+ current was blocked by external Ba^{2+} (10 mM) (*10*), another inwardly rectifying current was revealed that had slower activation kinetics than the K^+ inward rectifier and was present at much lower density (Fig. 1). This Ba^{2+}-insensitive inward rectifier began to increase dramatically in amplitude about 100 min after fertilization (about 20 min before first cleavage at 12°C) (Fig. 2, A and B); by the time of cleavage the current density had increased by a factor of 12. In other cells, the current increased by factors of 10 to 30 as first cleavage approached.

Ion substitution experiments done near the time of cleavage, when the current was large enough to measure accurately, showed that the current was carried by Cl^- ions. Tail currents recorded in normal external Cl^- (530 mM) reversed at about −25 mV (Fig. 2C), a value significantly different from the equilibrium potentials of either K^+ (−70

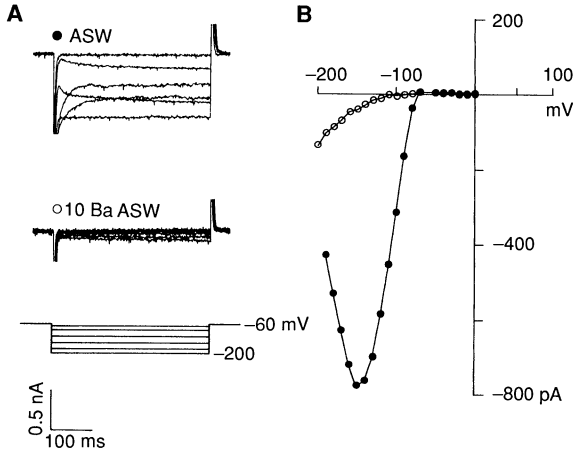

Fig. 1. Ba^{2+}-sensitive and insensitive components of the inward rectifier in the unfertilized *Boltenia* oocyte. (**A**) Current records during voltage-clamp pulses to −70, −90, −120, −150, −180, and −200 mV from a holding potential of −60 mV. Top records taken in ASW (*9*), bottom records in 10 Ba ASW. Top traces represent primarily the K^+ inward rectifier; the decline in current at the two most negative voltages is caused by a voltage-dependent block of the current by Na^+ ions (*20*). The bottom records represent the small inwardly rectifying Cl^- current. (**B**) Steady-state current-voltage (*I-V*) relations for the two currents in (A). Closed circles represent currents in ASW, predominantly the K^+ inward rectifier; open circles are currents in 10 Ba ASW, showing the Cl^- inward rectifier. In this cell the K^+ inward rectifier density was 5 pA/pF at −130 mV, and the Ba^{2+}-insensitive inward rectifier density was 0.2 pA/pF at −200 mV, values that were typical.

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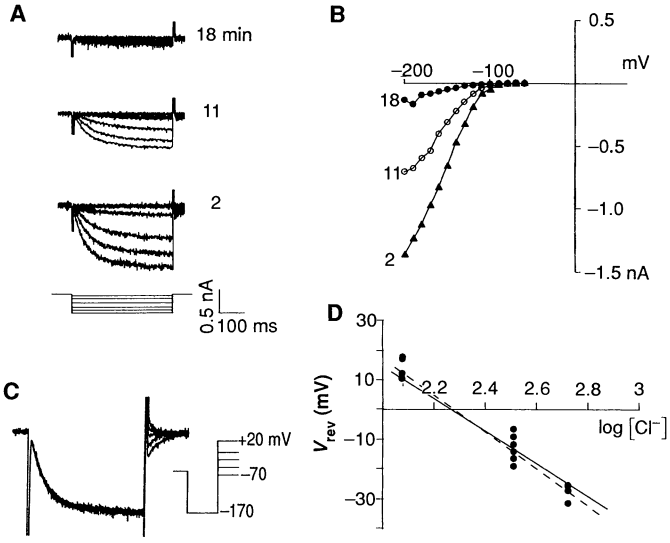

Fig. 2. Increase in the amplitude of the inwardly rectifying Cl^- current as the fertilized egg approaches first cleavage. (**A** and **B**) Voltage clamp records and steady-state *I-V* relations from a single fertilized egg at 18, 11, and 2 min before first cleavage (2 min before cleavage is approximately 120 min after fertilization). Same pulse paradigm as in Fig. 1. All records were taken in 10 Ba ASW to block the K^+ inward rectifier. (**C** and **D**) Identification of the Ba^{2+}-insensitive inward rectifier as a Cl^- current. (C) Tail currents taken at −70, −50, −30, −10, and +20 mV after a 400-ms prepulse to −170 mV in Na^+-free 10 Ba ASW. (D) Plot of tail current reversal potential as a function of external Cl^- concentration [Cl^-] taken from several cells, including the one in (C). Solid line is the least-squares best fit to the data; dashed line is a Nernst relation. Experiments in (C) and (D) were done near time of cleavage so currents were large enough to measure accurately.

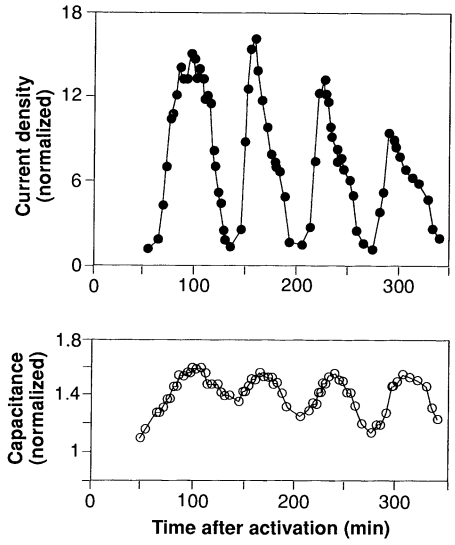

Fig. 4. Oscillations in Cl^- current and capacitance in an unfertilized egg activated with the Ca^{2+} ionophore A23187. Egg was dechorionated and then treated for 5 min at time 0 with A23187 as described (*9*). Data from a continuous recording in a single oocyte. Normalized currents and capacitance were measured as in Fig. 3.

mV; measured from K^+ inward rectifier tail currents) or Na^+ (+40 mV; measured from the Na^+ current reversal potential). When the external concentration of Cl^- was changed, the tail current reversal potential changed with an almost ideal Nernst slope (Fig. 2D). Variations in external Na^+, K^+, or Ca^{2+} concentrations had minimal effects.

Although the Cl^- current increased dramatically in the egg just before cleavage, it was seldom seen at significant levels in interphase blastomeres from the two-cell stage or later embryos. Large Cl^- currents did, however, reappear in two- or four-cell stage blastomeres as they approached cleavage. We conclude that the Cl^- current increases abruptly as each blastomere approaches cleavage and then decreases again in both daughter cells after cleavage. It was difficult to record this decrease in Cl^- current after cleavage because we could not be certain of adequate space clamp in cell pairs, given the voltage- and time-dependence of gap junctional conductance (*11*).

To circumvent this problem and to determine whether the oscillations in Cl^- current were a direct result of cytokinesis, we made continuous voltage-clamp recordings from fertilized eggs that had been cleavage arrested with cytochalasin B (*12*). Figure 3 shows the results of one such recording that spanned the time during which the first three cleavages occurred in control embryos. Near the time of each predicted cleavage, the Cl^- current increased abruptly and then immediately began to decrease, reaching almost its original low level before beginning the next cycle. Simultaneous measurements of cell capacitance as an indication of surface area showed periodic increases of about 20 to 25%, approximately in phase with the oscillations of Cl^- current density (Fig. 3A). Similar results were obtained in nine other cells.

Like the embryonic cell cycle oscillator, the oscillations in Cl^- current density could be set in motion by artificial activation of eggs, without sperm penetration (*1*). Figure 4 shows four complete oscillations in Cl^- current density, each representing a greater than tenfold change, recorded from an unfertilized oocyte that was activated by a 5-min exposure to a Ca^{2+} ionophore (at t = 0). Membrane capacitance showed oscillations of 15 to 20% at the times of expected cleavage (ionophore-activated eggs seldom cleave), approximately in phase with the changes in Cl^- current density. Similar results were obtained in six other cells (*13*). For both the cleavage-arrested fertilized eggs (Fig. 3) and the artificially activated eggs (Fig. 4), the times of minimum capacitance and Cl^- current density in each oscillation were identical (the difference was 2.2 ± 2.5 min; n = 8). The time of maximum Cl^- current density preceded the capacitance maximum somewhat (by 12.2 ± 4.9 min; n = 14).

The changes in Cl^- current density do not seem to be caused directly by a rise in intracellular Ca^{2+} activity with each cleavage (*2*). Application of the Ca^{2+} ionophore did not cause an immediate increase in Cl^- current in unfertilized oocytes, and exposure of fertilized eggs to ionophore well before first cleavage prevented, rather than accelerated, subsequent oscillations. The Cl^- chan-

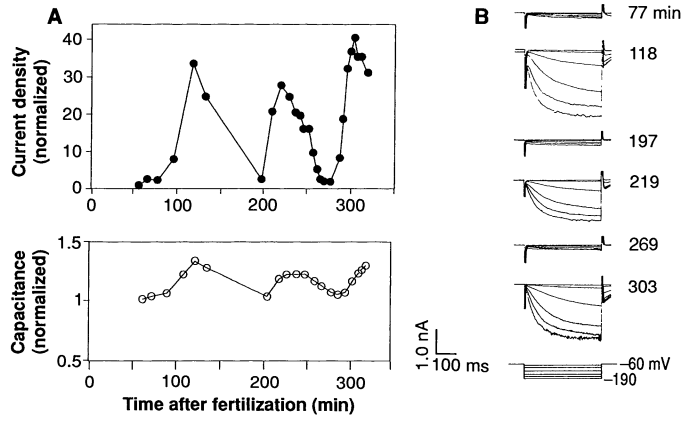

Fig. 3. Oscillations of Cl^- current and membrane capacitance in a fertilized, cleavage-arrested egg. Continuous whole-cell voltage-clamp recording from a single egg. After fertilization, the chorion was removed and the egg placed in cytochalasin B (2 μg/ml); 10 Ba ASW was used to block the K^+ inward rectifier. Recording was begun 55 min after fertilization. (**A**) Current density at −160 mV and capacitance both normalized to their values at 55 min after fertilization. The first three cleavages of control embryos began at 120, 200, and 270 min after fertilization. (**B**) Current record from the same experiment at various times after fertilization. Holding potential was −60 mV; pulses to −70, −90, −120, −150, −180, and −190 mV.

nels could be made available for activation by phosphorylation during the oscillations in kinase activity that are an integral part of the cell cycle (*1, 14*). Alternatively, the oscillations in Cl^- current density could result from insertion and removal of channels from the plasma membrane with each cell cycle. This mechanism is consistent with the small changes in surface area we recorded (Figs. 3 and 4) (*15, 16*), although it does not account for the time lag between the maximum Cl^- current and maximum capacitance.

Our experiments indicate a close association between the electrical properties of the plasma membrane and the endogenous cell cycle clock of the early embryo. It may also be true of other embryos that electrical properties recorded in a given blastomere depend critically on the state of the cell relative to its cleavage cycle. Thus in the interpretation of such experiments, permanent developmental changes in ion channel properties should be distinguished from transient ones. The significance of the oscillations in functional Cl^- channel density in the ascidian embryo may involve their role in promoting increased fluxes of HCO_3^- (*17*) and water across the plasma membrane, which could contribute to changes in intracellular *p*H and the maintenance of osmotic balance that occur at cleavage (*18, 19*). These changes may be important to maintain the intracellular environment in a state that permits the completion of mitosis.

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9. General methods of animal collection and maintenance, gamete preparation, and whole-cell recording were as described (*8*). In experiments in which extracellular Cl^- concentration was changed, bath potentials were recorded with a KCl microelectrode and subtracted from the patch pipette voltage. Artificial seawater (ASW) had the following composition: 400 m*M* NaCl, 10 m*M* KCl, 10 m*M* $CaCl_2$, 50 m*M* $MgCl_2$, and 10 m*M* Hepes, *p*H 8.1. To block the K^+ inward rectifier, Ba^{2+} was substituted for Ca^{2+} (10 Ba ASW). For Cl^- replacement, gluconate was the substitute. To measure tail currents of the Cl^- current, Na^+-free ASW (choline substitution) was used to eliminate the Na^+ current. Cytochalasin B (Sigma) was dissolved in ethanol at 1 mg/ml and diluted for use at a final concentration of 2 to 4 μg/ml. A23187 (Sigma) stock solution (4.5 m*M* in dimethyl sulfoxide) was diluted to a final concentration of 2.7×10^{-5} *M* in ASW for activation. Pipette (internal) solution contained 400 m*M* KCl, 10 m*M* NaCl, 10 m*M* EGTA, and 20 m*M* Hepes, *p*H 7.3. All experiments were performed at 10° to 12°C.
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13. Several control experiments were done to ensure that the cyclical changes in Cl^- current were not due to prolonged whole-cell recording or exposure to Ba^{2+} ions. Prolonged whole-cell recordings from unfertilized oocytes showed no significant change in the Cl^- current; brief whole-cell recordings initiated at various times after fertilization or prolonged two-microelectrode recordings showed the same increase in Cl^- current as cleavage approached. Similar results were obtained without Ba^{2+} in standard artificial seawater, in which the Cl^- current could be resolved as a slowly activating component superimposed on the K^+ inward rectifier. The oscillatory changes in current and capacitance did not result from changes in access resistance. Series resistance compensation was adjusted before each measurement, and usually only small adjustments were needed to maximize clamp speed. The small changes observed were steady increases in access resistance during the experiment, probably caused by clogging of the pipette tip by yolk droplets in the cytoplasm.
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15. Although many cell properties differentiate in apparently normal fashion in cytochalasin-arrested ascidian embryos (*12*), capacitance and ultrastructural measurements indicate that the addition of new membrane is substantially disrupted [T. Hirano and K. Takahashi, *J. Physiol. (London)* **386**, 113 (1987)]. The small oscillations in membrane capacitance are superimposed on a large ramplike increase as cleavage approaches. With cytochalasin treatment, the ramp increase disappears, leaving the small oscillation. The ramplike increase may be responsible for the continuous addition of functional Ca^{2+} and inwardly rectifying K^+ channels to the early embryo, so that the density of these currents remains constant through the eight-cell stage, despite a threefold increase in total embryo membrane area (*8*). Scanning electron micrographs of ascidian embryos show a cyclical extension and retraction of microvilli with each cleavage cycle, which may represent the ultrastructural correlate of our cytochalasin-resistant capacitance oscillations. The timing of these cannot be explained by a model based on the advanced insertion of membrane to be used later in furrow formation [N. Satoh and T. Deno, *Dev. Biol.* **102**, 488 1984)], and the function of this membrane, which seems more closely associated with the cell cycle oscillator, is not known. In *Xenopus* embryos, permanent changes in K^+ permeability occur during early cleavages. These changes are cytochalasin resistant, but unlike the changes in ascidian embryos, the cytochalasin treatment does not appear to block the addition of new membrane in *Xenopus* (*4*).
16. Several experiments indicate that newly added membrane in cleaving embryos can have different populations of functional ion channels or pumps than the old membrane [P. J. Woodward, *J. Gen. Physiol.* **52**, 509 (1968); (*5*); A. Ohara, K. Murayama, Y. Doida, Y. Marunaka, *Med. Sci. Res.* **15**, 1401 (1987)].
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Induction by Soluble Factors of Organized Axial Structures in Chick Epiblasts

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Inductive action of soluble factors was tested on isolated chick epiblasts. An assay was developed wherein conditioned medium derived from the *Xenopus* XTC cell line induced the formation of a full-length notochord and rows of bilaterally symmetric somites. Basic fibroblast growth factor, epidermal growth factor, retinoic acid, and transforming growth factor type β1 and β2 were not capable of inducing axial structures. Thus, soluble factors can elicit the development of polarity stored in the epiblast and behave as true morphogens since they can induce the formation of the organized complex structures that constitute the embryonic axis.

THE CHICK BLASTULA [STAGE XIII OF Eyal-Giladi and Kochav (*1*)] is formed of two physically distinct layers. The upper layer consists of the primary ectoderm or epiblast, from which all embryonic structures develop (*2*), plus the peripheral area opaca and marginal zone regions. The lower layer consists in its entirety of the primary endoderm or hypoblast. Interaction between the epiblast and the polarized hypoblast (*3*) results in the formation of axial mesodermal structures. In the absence of the hypoblast and the marginal zone, cells from the competent epiblast proceed to form nonaxial mesoderm, such as blood islands, but are not capable of generating any type of

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