EFFECTS OF LOW-INTENSITY PULSED ELECTROMAGNETIC FIELDS ON THE EARLY DEVELOPMENT OF SEA URCHINS

C. FALUGI,* M. GRATTAROLA,‡ AND G. PRESTIPINO§
*Institute of Comparative Anatomy, University of Genova, 16126 Genova, Italy; ‡Department of Biophysical and Electronic Engineering, University of Genova, 16145 Genova, Italy; and §Institute of Cybernetics and Biophysics, c/o Department of Physics, University of Genova, 16146 Genova, Italy

ABSTRACT The effects of weak electromagnetic signals on the early development of the sea urchin *Paracentrotus lividus* have been studied. The duration and repetition of the pulses were similar to those used for bone healing in clinical practice. A sequence of pulses, applied for a time ranging from 2 to 4 h, accelerates the cleavages of sea urchin embryo cells. This effect can be quantitatively assessed by determining the time shifts induced by the applied electromagnetic field on the completion of the first and second cleavages in a population of fertilized eggs. The exposed embryos were allowed to develop up to the pluteus stage, showing no abnormalities.

INTRODUCTION

Recent studies have shown that a variety of low-frequency electromagnetic (em) signals can affect cell behavior in vitro (1–4), and a number of clinical applications have been proposed (5). Presently, despite many experimental findings, a sound explanation of these phenomena is not available. Among the proposed mechanisms is a possible modification of Ca^{++} binding to the cell surface, induced by exposure to the em signals (1, 2, 4).

We chose early sea urchin (*Paracentrotus lividus*) embryos in the fertilized egg stage as an appropriate biosystem for elucidating this type of phenomena. We studied the effects of weak em signals continuously applied to developing embryos and larvae, starting from the stage of the unfertilized egg. The reasons for choosing are as follows: (*a*) the ease of finding a large number of cells undergoing the same biological events (egg activation and first cleavages) synchronously, and the fact that these events have been studied in echinoderm eggs and embryos from both the biological (6) and biophysical points of view (7–9); (*b*) sensitivity of the early developmental stages to exogenous physical perturbations (10, 11). It is also worth noting that the influence of weak em fields on the development of the early chick embryo has been reported (12); (*c*) the role played, in the control of embryonic development, by ionic currents in the cells, as suggested in reference 15. In fact, waves of increased cytoplasmic free Ca^{++} start egg activation (8, 13) and are supposed to control development (14). Moreover, electric fields are generated by developing embryos (16). Finally, ionic current patterns were computer-reproduced by simulating the effect of a weak electric field on a nonlinear membrane model (17).

MATERIALS AND METHODS

Eggs and sperm were obtained from mature specimens of *Paracentrotus lividus* (routinely collected from the Gulf of Genoa). The eggs were suspended in filtered seawater inside petri dishes. During each experiment, the control and treated dishes were left at 19°C in a thermostated room. Before fertilization, the treated eggs were exposed for 2 h to an em signal, to which they were continuously exposed after fertilization, which was obtained by adding 0.2 ml of a sperm suspension to the dishes containing the eggs in 5 ml of seawater. The sperm suspension was obtained by adding 0.1 ml of concentrated sperm to 20 ml of seawater. The exposure system consisted of two coils (21-cm inner diam, 30 loops each, 1.5-mm wire diam, 6 cm apart) between which the petri dishes containing the eggs were inserted. The temperature between the two coils, as measured with a thermocouple, showed no detectable difference above random fluctuations (up to 0.2°C). The em signal used was a sequence of 20-μs pulses, repeated every 200 μs. During repetition, the em signal changed sign and remained at a constant value, which was ~0.1 times the pulse value, the time average of the electric field being zero. The magnetic field intensity varied from 0 to ~20 gauss, with a mean value of ~10 gauss. The maximum intensity of the induced electric field, at 2.5 cm from the dish center, was ~20 mV/cm, as measured by using the open-circuit voltage of a loop probe (~0.5 cm in diameter) in a dummy petri dish. The duration of the pulses and the number of times they were repeated were the same as used for bone healing in clinical practice (5). A dummy couple of coils, disconnected from the generator, was used for the control dishes. The electrical field experienced by the control dishes, as measured with the loop probe, was less than 0.001 times the value of the signal experienced by the exposed dishes. The power spectrum of the signal, as obtained by a spectrum analyzer, is shown in Fig. 1. It displays a

Send offprint requests to Dr. Grattarola.

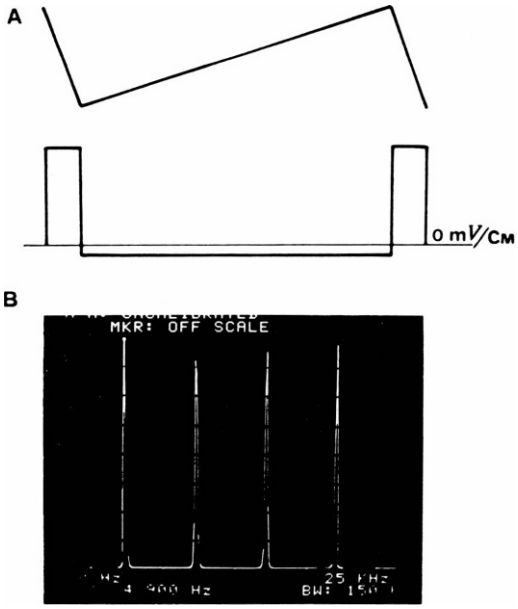

FIGURE 1 (*A*) Waveforms of the signal: magnetic induction field (*top*); time-varying electric field (*bottom*). The peak magnetic field is ~20 gauss. The mean electric field value is zero. The pulse duration is ~20 μs and the repetition time is ~200 μs. (*B*) Power spectrum of the electric signal.

maximum value of ~5 KHz, which is repeated for multiple frequency values.

In all the experiments, two petri dishes containing 5 ml of filtered seawater and ~2,000 eggs/ml were placed under the exposure system, while two identical dishes contained control eggs. After fertilization, one dish for each group was periodically examined by phase-contrast microscopy. As soon as eggs in either of the examined dishes started the first cleavage, a standard quantity of seawater containing ~1,000 eggs was sampled from the other dishes and poured into blind score test tubes. Concurrently, the eggs in the test tubes were fixed by adding 2% glutaraldehyde to the seawater (final concentration). Such sampling was repeated six times, every 4 min. The same sequence was repeated when microscopic inspection showed the beginning of the second cleavage. To show the nuclear and cytoplasmic dynamics, the collected samples were then stained by the cholinesterase method suggested by Karnovsky and Roots (18) using acetylthiocholine iodide (Sigma Chemical Co., St. Louis, MO) as substrate. This method allows the staining of nuclear material, nuclear membrane, and caryomere membrane by dark precipitation (19). The embryos were stained to facilitate scoring of the percentages of embryos belonging to different developmental stages.

The completion of cytokinesis was considered the necessary condition for an embryo to be assigned to a specific development stage (i.e., two, four, eight, . . . blastomeres). This means that all intermediate stages between two cleavages were assigned to the preceding stage. A detailed description of stages is given in the next section and in Fig. 3. Some of the exposed and control embryos were allowed to develop until the larval stage (48 h), in the same petri dishes, which have been manufactured to allow for respiratory exchanges (Heraeus Petriperm, Milano, Italy).

TABLE I
DIFFERENCES BETWEEN THE PERCENTAGES OF EXPOSED AND CONTROL CLEAVED EGGS

Cleavage	Exposed	Control
Third	86.8 ± 11.5 (4)	4.8 ± 1.3 (4)

Percentages of cloven embryos (third cleavage) in exposed and control samples; the moment of sampling was chosen after microscopic inspection of controls. The differences between the data are highly significant, as shown by the test performed (Student's t) ($P < 0.001$). Inside the parentheses the number of experiments is given.

RESULTS

Fig. 2 shows the time sequences of the first and second cleavages in control eggs and in eggs exposed according to the protocol described in the Materials and Methods section. The four curves represent the percentages of eggs that, at each time, have already completed the first cleavage (curves *A* and *B*) and the second cleavage (curves *C* and *D*). About 300 eggs were scored for each data point.

These data show that most of the exposed eggs and embryos (curves *A* and *C*) cleave earlier than the control ones (curves *B* and *D*). This effect is more evident for the second cleavage; in fact, the time delay between curves *C* and *D*, at the moment when 50% of the embryos have already completed cytokinesis, is 5–6 min. The curves referring to the second cleavage were reproduced three times, and for each data point, the mean values are indicated in Fig. 2 together with the SD. The Student's t test shows that, at the points of maximum difference, the results are highly significant ($P < 0.01$).

Four experiments were extended to the third cleavage. In this case, only one sampling was carried out, contemporaneously with the beginning of the third cleavage in the

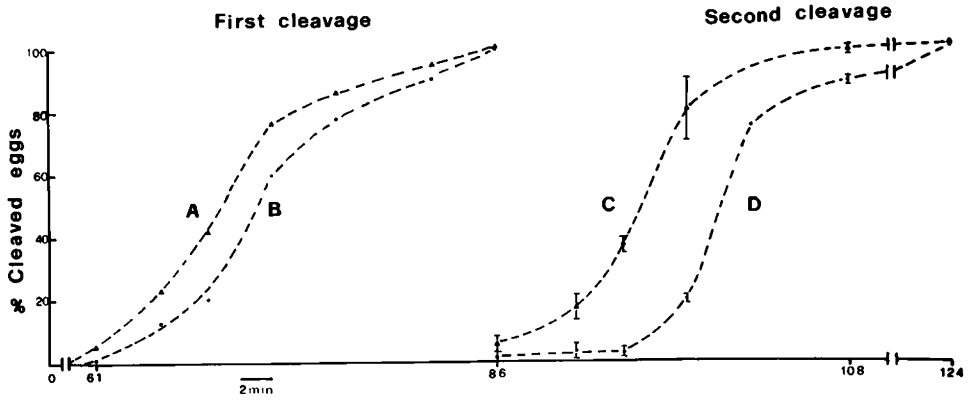

FIGURE 2 Time sequences of the first and second cleavages in sea urchin embryos. Immature oocytes, easily recognizable by nuclear and nucleolar features, were not considered in the calculation of percentages. Control embryos (curves *B* and *D*); exposed embryos (curves *A* and *C*). The time scale indicates minutes after fertilization.

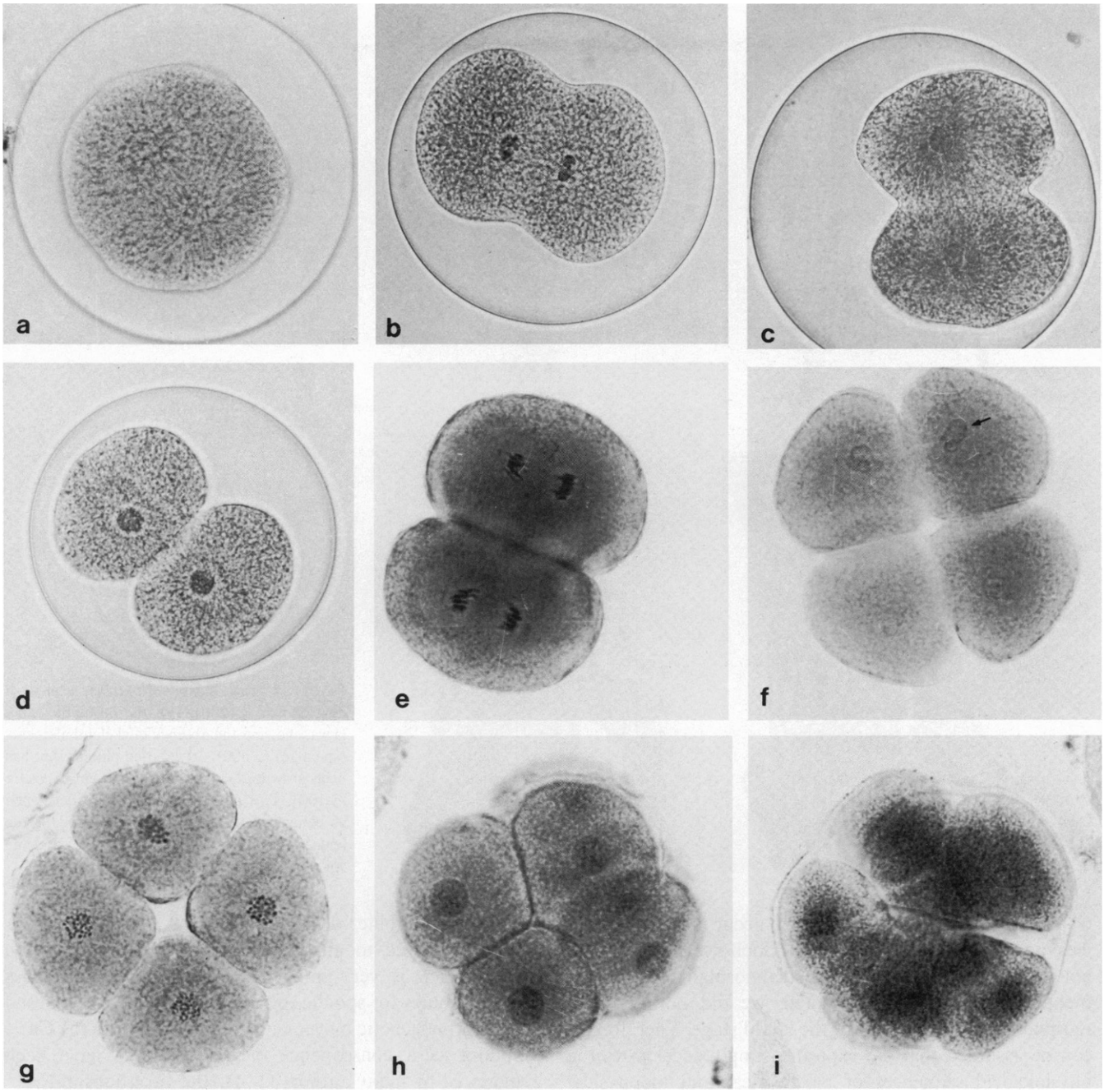

FIGURE 3 Developmental stages of early *P. lividus* embryos. (*a–c*) All of these embryos belong to the first stage (one blastomere), because the cytokinesis is not complete. (*d–f*) Two-blastomere stage (first cleavage); the arrow in *f* shows karyomeres, the presence of which indicates that cytokinesis is not complete. (*g*) Four-blastomere stage (second cleavage); the metaphasic nucleus indicates that the stage is advanced. (*h* and *i*) Eight-blastomere stage (third cleavage). The dark staining was performed by the method of Karnovsky and Roots (18) for cholinesterases.

blastomeres of control embryos, as observed by microscopic inspection. The resulting values are summarized in Table I. Fig. 3 exemplifies the first three stages of development (one, two, four blastomeres) of sea urchin embryos studied in this paper. In two experiments (Fig. 4), the development was followed until the larval stage (pluteus). No evidence of any morphological abnormality was found in both the control and exposed plutei, while the exposed plutei exhibit some morphological details that suggest a more advanced stage as compared with controls.

DISCUSSION

The experiments described in this paper show that the exposure of sea urchin eggs before and during fertilization to low-frequency em fields, similar to those used for bone healing in clinical practice (5), causes the first three

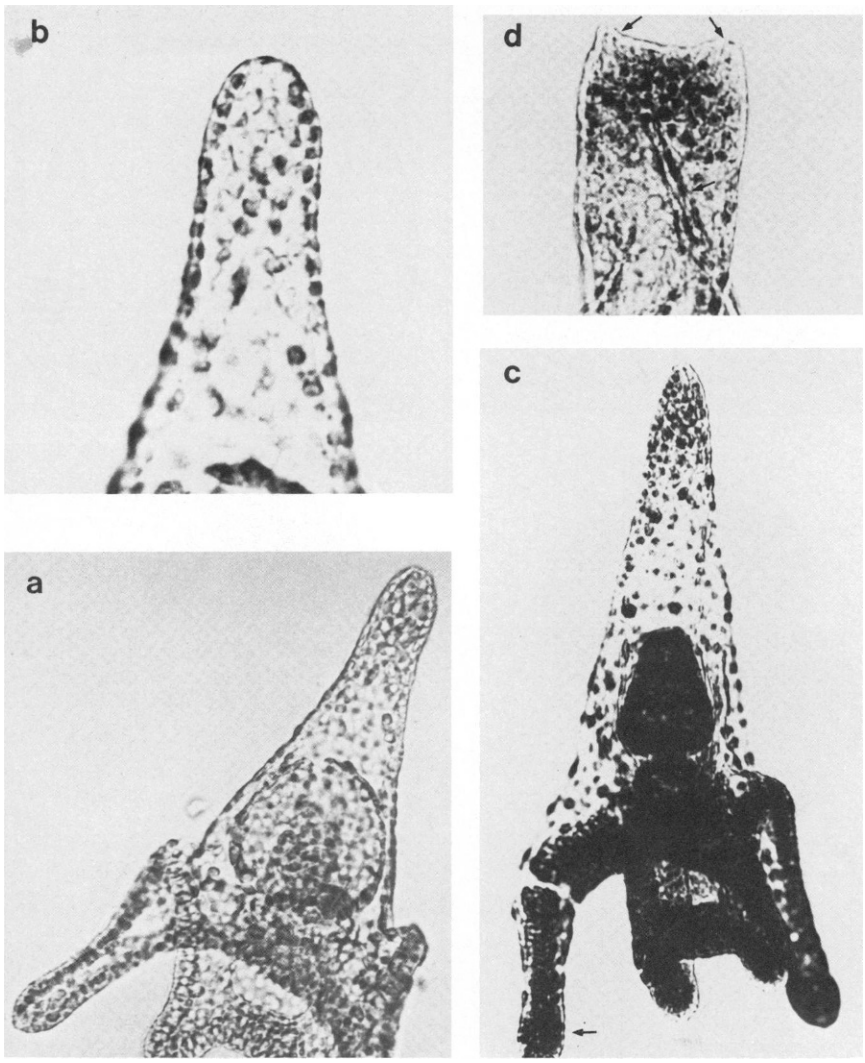

FIGURE 4 (*a*) Unexposed pluteus, after 48-h development (×250); (*b*) the top of the same larva, showing the absence of skeletal elements (spiculae) (×400); (*c*) exposed pluteus that has been growing for 48 h under the em exposure (×100). This pluteus appears more developed as compared with the unexposed one, for the length of the appendages (*arrow* in *c*) and the spiculae, shown in *d* (*arrows*, ×400).

cleavages of the treated embryos to occur earlier than the cleavages of the control embryos. Such acceleration does not seem to depend on any induced abnormality in development. In fact, as already noted, we did not find any morphological anomaly until the larval stage. Therefore, it can be assumed that em exposure simply accelerates the physiological sequences of biochemical and biophysical developmental events. Because there was no measurable thermal drift in the exposed samples, we deem it extremely improbable that a "macroscopic" thermal effect should be induced by em exposure.

A few preliminary experiments (not shown), performed by exposing the eggs before and not during fertilization, show a similar type of acceleration, thus ruling out a major effect of the em fields on the sperm. Among the egg structures, possible areas for the action of em fields are the Ca^{++} binding sites on the cell membrane and on the smooth endoplasmic reticulum vesicles membrane, where, under the effect of the em fields, the release of sequestered Ca^{++} may increase. Calcium release is one of the earliest and most important signals to which eggs can respond during embryonic development (8, 9, 13). Ca^{++} was recently proved to affect also cytoskeletal organization (20), which is very important for both cell cleavages (microtubules for spindle formation, actin for cytokinesis) and morphogenetic movements. Modifications to the Ca^{++} binding as a consequence of em exposure have been suggested by many authors (1–4), and variations in Ca^{++} flux rates have been measured in synaptosomes exposed to low-frequency amplitude-modulated microwave fields (21) and in brain tissue exposed to extremely low frequency fields (4).

Presently it would be premature to speculate further on the possible mechanisms of interaction between em fields and specific cellular structures inside the sea urchin egg and embryo.

The main goal of this paper is to draw the attention of the biophysical community to a biological system that we consider particularly suitable for testing the biological effects of weak em fields. We hope that our results may stimulate other researchers to duplicate and improve them, thereby contributing to the development of the research

and clinical applications based on the interactions between weak low-frequency em fields and biosystems.

The authors thank Prof. A. Chiabrera for many helpful discussions. The assistance of Dr. A. Raveane in setting up the exposure system and the help of Mr. G. Ferri are also acknowledged. Special thanks are due to Prof. A. Monroy for useful advice and to the unknown referees for their constructive criticism.

This work was supported by the National Research Council (CNR) of Italy and by the Ministero della Pubblica Istruzione.

Received for publication 25 February 1986 and in final form 26 September 1986.

REFERENCES

1. Dixey, R., and G. Rein. 1982. H-Noradrenaline release potentiated in a clonal nerve cell line by low-intensity pulsed magnetic fields. *Nature* (*Lond.*). 296:253–255.
2. Luben, R. A., C. D. Cain, M. Chen, D. M. Rosen, and W. R. Adey. 1982. Effects of electromagnetic stimuli on bone and bone cells in vitro: inhibition of responses to parathyroid hormone by low-energy low-frequency fields. *Proc. Natl. Acad. Sci. USA.* 79:4180–4185.
3. Blackman, C. F., S. G. Benane, J. R. Rabinowitz, and D. E. House. 1985. A role of the earth's magnetic field in biological effects caused by elf electromagnetic signals. *Biophys. J.* 47:245a. (Abstr.)
4. Blackman, C. F., S. G. Benane, L. S. Kinney, W. T. Joines, and D. E. House. 1982. Effects of ELF fields on calcium-ion efflux from brain tissue in vitro. *Radiat. Res.* 92:510–520.
5. Bassett, C. A. L., R. J. Pawluk, and A. A. Pilla. 1974. Augmentation of bone repair of inductively coupled electromagnetic fields. *Science* (*Wash. DC*). 184:575–577.
6. Giudice, G. 1985. The Sea Urchin Embryo. A Developmental Biological System. Springer-Verlag, Berlin, Heidelberg, New York, Tokyo.
7. Tyler, A., A. Monroy, C. Y. Kao, and H. Grundfest. 1956. Membrane potential and resistance in the starfish egg before and after fertilization. *Biol. Bull.* 1:153–177.
8. Shapiro, B. M., and E. M. Eddy. 1980. When sperm meets egg: biochemical mechanisms of gamete interactions. *Int. Rev. Cytol.* 66:257–302.
9. Whitaker, M. J., and R. A. Steinhardt. 1982. Ionic regulation of egg activation. *Q. Rev. Biophys.* 19:593–666.
10. Rustad, R. C. 1964. U.V.-induced mitotic delay in the sea urchin egg. *Photochem. Photobiol.* 3:529–538.
11. Rustad, R. C. 1970. Variation in the sensitivity to x-ray-induced mitotic delay during the cell division cycle of the sea urchin egg. *Radiat. Res.* 42:498–512.
12. Delgado, J. M. R., J. Leal, J. L. Monteagudo, and M. G. J. Gracia. 1982. Embryological changes induced by weak, extremely low-frequency electromagnetic fields. *J. Anat.* 134:533–551.
13. Jaffe. L. F. 1983. Sources of calcium in egg activation: a review and a hypothesis. *Dev. Biol.* 99:265–276.
14. Jaffe, L. F. 1979. Control of development by ionic currents. *In* Membrane Transduction Mechanisms. R. A. Cone and J. E. Dowling, editors. Raven Press, New York. 199–231.
15. Stern, C. D., and D. O. Mackenzie. 1983. Sodium transport and the control of epiblast polarity in the early chick embryo. *J. Embryol. Exp. Morphol.* 77:73–98.
16. Jaffe, L. F. 1979. Strong electrical currents leave the primitive streak of chick embryos. *Science* (*Wash. DC*). 206:569–571.
17. Casaleggio, A., L. Marconi, G. Morgavi, S. Ridella, and C. Rolando. 1984. Evaluation of ionic fluxes in a cell, with non-linear membrane, stimulated by an electric field. *J. Bioelect.* 3:305–328.
18. Karnovsky, M. J., and L. Roots. 1964. A "direct colouring" thiocholine method for cholinesterase. *J. Histochem. Cytochem.* 12:219–221.
19. Falugi, C., G. Costelli, and P. Catellani. 1985. Attivitá di colinesterasi nello aviluppo precoce di riccio di mare. *Atti del XXI Congr. Soc. It. Istochim.* 61. (Abstr.)
20. Sha'afi, R. I., J. Shefcyk, R. Yassin, T. F. P. Molski, M. Volpi, P. H. Naccache, J. R. White, M. B. Feinstein, and E. L. Becker. 1986. Is a rise in intracellular concentration of free calcium necessary or sufficient for stimulated cytoskeletal-associated actin? *J. Cell Biol.* 102:1459–1466.
21. Lin-Liu, S., and W. R. Adey. 1982. Low-frequency amplitude-modulated microwaves fields change calcium efflux rates from synaptosomes. *Bioelectromagnetics.* 3:305–322.