Chapter 10

MOLECULAR CHANGES IN HARD TISSUE CELLS IN RESPONSE TO BIOELECTRIC PROLIFERATIVE SIGNALS*

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Introduction

ELECTRICAL STIMULATION is increasingly used clinically to promote hard tissue repair or regeneration. The therapeutic objective is to promote osteogenesis in areas where fibrous tissue seems to replace or preclude bone development. To initiate bone formation, the electric perturbation (EP) should promote either one or all of the following cellular processes: cell migration, proliferation, and ultimately differentiation. The mechanism by which this is accomplished is not well understood. One of these events, cellular proliferation, has been extensively studied in bone and other tissues. The rate and extent of proliferation, is controlled by growth factors, hormones, nutrients, pH, temperature, density dependent inhibition, and the influence of other regulatory cells acting through these or other mechanisms. Many of these regulatory effects are mediated by the cell membrane. It is our hypothesis that the cell membrane is the site at which EP regulates cell function in hard tissues promoting proliferation and other responses that ultimately lead to osteogenesis. The EP effects may involve alterations in membrane permeability and membrane potential, or modulation of membrane enzymes such as adenylate cyclase of Na^+, K^+, ATPase. These sensitive regulatory mechanisms, once perturbed, initiate a host of biochemical and morphological changes that can be assayed, timed, quantitated, and manipulated by pharmacological promoters or inhibitors. We have developed an *in vitro* model to study the effect of capacitative coupled EP on embryonic calcified tissues. This chapter is a review of previous data and tentative conclusions from preliminary data. From this overview, we hope to construct a crude model for part of the mechanism of how EP may affect hard tissue cells.

Methods and Materials

Instrumentation

The electric perturbation for our experimental system was generated by a capacitative coupled device consisting of two curved copper electrode

* Supported by PHS Grant #DE-04327.

plates (15 mm × 27 mm) facing each other, 1.5 cm apart in their inner diameter, to the inner surface of a Plexiglas® well (Norton, 1975, 1977, 1979). The electrodes were connected by 26-gauge Teflon®-insulated leads to a Hewlett Packard 6516A D.C. power supply. The square wave pulse was regulated by a Grass (54GR) physiologic stimulator. The peak electric field was 1750 V or 1166 V/cm delivered at 5 Hz, 0.1 seconds on and 0.1 seconds off. The measured rise time for the voltage was 1850 μsec (Fig. 10-1).

Tissue Preparation

In these experiments both freshly isolated and cultured cells were employed. Where cartilage cells were used, the epiphyseal plate of sixteen-day-old chick embryo tibiae were dissected free of muscle. They were then immersed in a Krebs-Ringer-Tris buffer solution (pH 7.4) containing 2

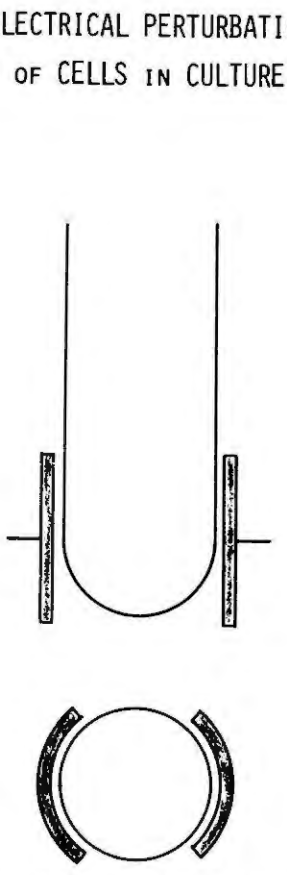

Figure 10-1. Schematic diagram of electrical perturbation apparatus. A well drilled in Plexiglas secures the curved copper capacitors to an inner diameter of 15 mm. The outer surface of the culture tubes comes in contact with the capacitor plates.

mg/ml of glucose at 4°C. The proliferative layers were separated, collected, and enzymatically digested in a collagenase-hyaluronidase solution consisting of calcium-magnesium-free Hank's solution, 2 mg/ml collagenase, and 1 mg/ml hyaluronidase (Rodan, 1974). Cell viability was tested with trypan blue for dye exclusion and cells were counted in a Neubauer chamber. The cell suspension was divided into experimental and control samples of at least 10^5 cells each. Cellular cyclic AMP determinations were made by radioimmunoassay (Rodan, 1975).

Bone cells were obtained from nineteen day rat embryo calvaria, scraped free of periosteum. The calvaria were minced in Krebs-Ringer solution and digested in the collagenase-hyaluronidase mixture at 37°C for twenty-minute sequential periods. The cells were collected by centrifugation, resuspended, and counted in a Coulter counter. A trypan blue exclusion test for viability was done on an aliquot of the suspension. The cells were attached on the inner surface of 17 × 100 mm glass or polypropylene culture tubes by placing 0.5 ml of the cell suspension in each tube and rotating them almost horizontally in a roller drum apparatus turning at 6 cycles/hour. The cells were then cultured for forty-eight hours at 37°C in ambient air using MCDB medium supplemented with 10% fetal calf serum (FCS) and 1% antibiotic-antimycotic solution to approximate confluency. The cell culture height on the sides of the tube corresponds to a standing level of 3 ml of media. In a typical experiment, 3 ml of new tissue culture containing medium (MCDB) with antibiotics but without FCS plus ^{3}H-thymidine, 3.3×10^6 CPM/ml (1.0 mc/0.0044 mg thymidine) was added to each of sixteen tubes. This dosage of ^{3}H should have little effect on the proliferative process (Pollack, 1979). The entire apparatus including controls and experimental samples were placed on a shaking platform (95 RPH) in a 37°C incubator at ambient air and vapour pressure. Seven tubes were exposed to the electrical perturbation, seven serve as controls placed outside the electrodes, and two tubes were placed on ice as a time zero for cell growth changes. During the experiments, the cells were gently agitated and incubated at 37°C with ambient air with 25 percent humidity. In another experiment, designed to determine whether an anode or cathode produced greater biological activity, the cells were grown on one side of the culture tube in a nonrotating drum producing a confluent cell "strip" for exposure to only one electrode.

DNA Bioassay

The primary bioassay for evaluation of the EP effect used in these experiments was the measurement of DNA synthesis as described by Burton (1968) or by Ash and Francis (1977). Cells were recovered from the culture tubes by trypsin (0.25%) at 37°C, resuspended in medium (or the starting point of a cell suspension experiment), and centrifuged to a pellet

at 2000 RPM for five minutes. The collected cells were lysed by sonication and the DNA precipitated with 2 ml of 5% trichloroacetic acid (TCA) plus 200 μg of bovine serum albumin. The solution was left overnight at 4°C, centrifuged, washed twice with tCA, and redissolved in 250 μl of 23M formic acid. This solution was counted in 10 ml of scintillation cocktail (ACS) in a liquid scintillation counter.

Results and Interpretations

As stated above we hypothesized that the transduction of the electrical perturbation (EP) is likely to involve changes in the activity of membrane bound enzymes, in the distribution of membrane-associated ions, and in ion fluxes across the membranes. In our earliest experiments with electric perturbations, using proliferative-layer isolated cartilage cells, we examined the dose and response dependency of cAMP production on applied voltage. Results are shown in figures 10-2 and 10-3 (Norton, 1976). Cellular cAMP content decreased with increasing voltage. These data gave a clear indication that a capacitative coupled signal altered the biologic activity of cartilage cells, which is consistent with the results of other workers using a similar system (Hekkelman, 1975; Brighton, 1976). More recently, Davidovitch (1980) using a D.C. *in vivo* model showed histochemical evidence of this phenomenon. Decreased cAMP has been associated

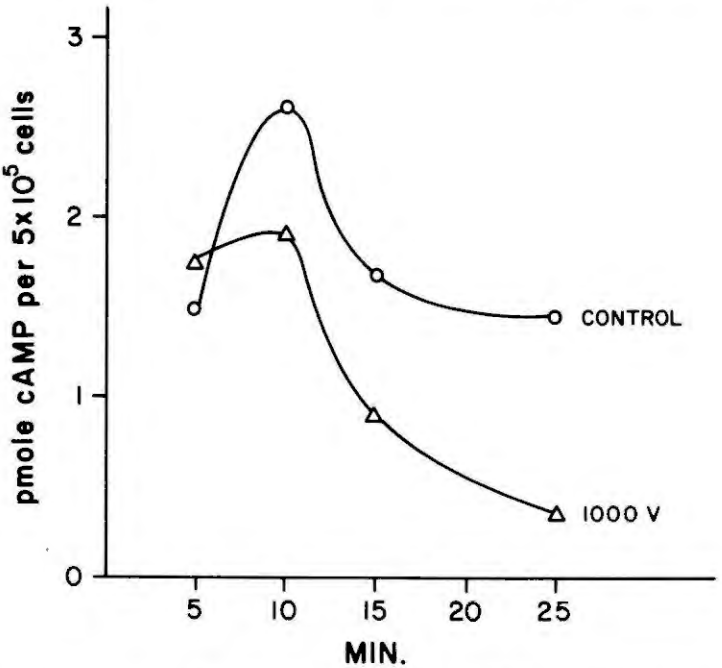

Figure 10-2. This graph indicated that the cellular cAMP concentration in cartilage decreased with time when exposed to an EP of 1000V access on 15 mm diameter capacitor. (From *Clin. Orthop., 124*:59-68, 1977.)

with cell proliferation. Therefore, using what we regarded as an optimal signal from our cAMP dose response data, 1166 volts/cm at 5 Hz, we measured ^{3}H-thymidine incorporation in cartilage cells after exposure to EP. Our data showed that there was an increase of 53 ± 13 percent after six hours of exposure. This was a reliable and repeatable observation (72 experiments, 216 samples) (Rodan, 1978). The difference in ^{3}H-thymidine was observed in material that was dioxyribonuclease digestible and was absent in TCA extractable material. Thus, the increased ^{3}H-thymidine was in the DNA. By exposing cells grown on only one side of a culture tube, we determined that the active electrode stimulating this effect was the cathode (Norton, 1979) (Fig. 10-4). In other experiments using a different system, Bassett (1968) and Herrmann showed similar results when they assayed for ^{3}H-thymidine uptake.

We assumed that the rapid rise and fall of potential differences across the electrodes transmitted through the culture glass to the cells briefly perturbed the cell membrane. This may be the result of (a) electric field effect acting on polar molecules in or around the cell membrane, (b) a transient current generated in the electrolyte culture media adjacent to the cells, or (c) changes in the surface charge of the glass directly influencing the adhering cell membrane. In all of these cases, ionic movement in and around the cell must be affected. Therefore, we hypothesized that ion

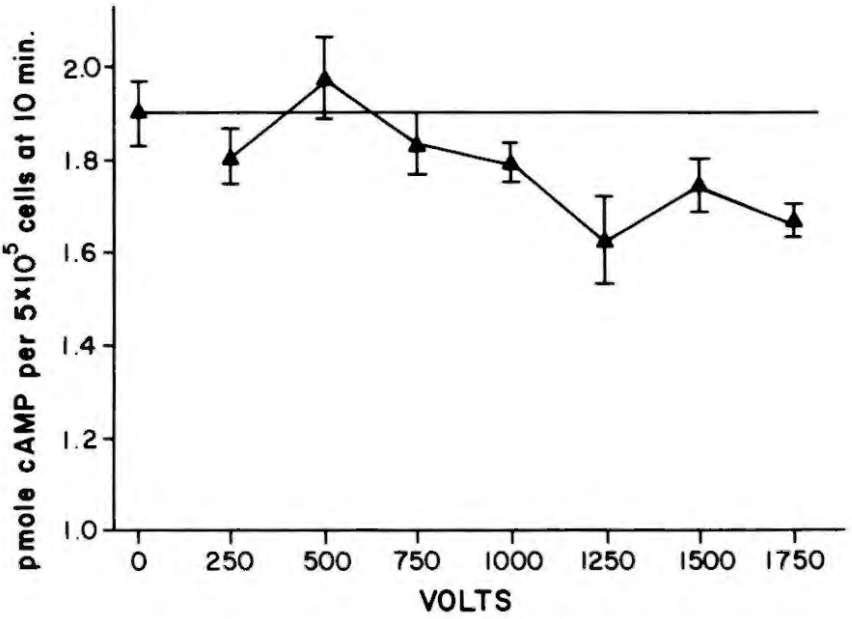

Figure 10-3. This graph illustrates the decrease of cAMP content in cartilage cells over incremental cartilage voltages. The vertical extension of the points are one standard deviation. (From *Clin. Orthop., 124*:59-68, 1977.)

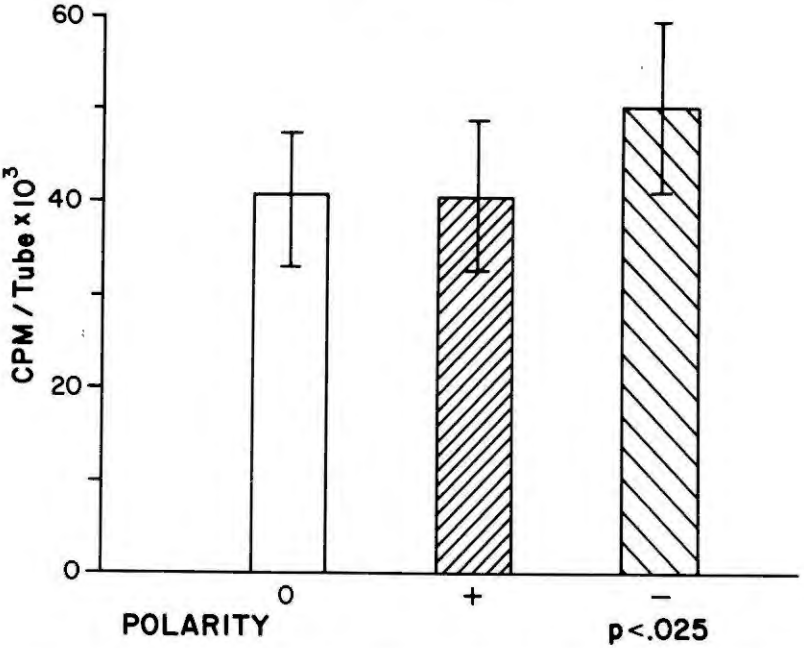

Figure 10-4. The bar graph shows that the biologically active electrode for increase in ^{3}H-thymidine uptake is the cathode. The quantitative date for this graph are seen in Norton (1979).

fluxes may be an early event associated with the EP effects. It has been postulated that the flux of calcium into the cell is a potential proliferative signal. Using ^{45}Ca prelabeled cells, we observed an increased Ca^{++} efflux into the media during exposure to EP (Fig. 10-5). To investigate the role of extracellular Ca^{++} in EP events, we investigated the effect of two concentrations of Ca^{++} in the media on ^{3}H-thymidine incorporation in control and EP cells. ^{3}H-Thymidine incorporation decreased with a decreased Ca^{++} concentration, but EP stimulation over its corresponding control retained approximately the same ratio at these concentrations (Table 10-I). The effect of a number of ion flux blockers or promoters on EP stimulation was examined. Verapamil, a Ca^{++} and to some extent Na^{+} flux inhibitor, blocked the EP effect at 10^{-6}M (Rodan, 1978). In similar

TABLE 10-I
EFFECT OF CALCIUM CONCENTRATION ON ELECTRIC FIELD STIMULATION OF ^{3}H-THYMIDINE INCORPORATION

CA Conc. (M)	10^{-6}	10^{-3}
Control	1557 ± 114	1849 ± 102
Electric Field (1166 V_{cm}^{-1}, 5 Hz)	2046 ± 154*	2408 ± 128*

* $p < .05$.

experiments, tetrodotoxin, a specific inhibitor of Na^+ channels in nerve tissue, was added to the media in varying concentrations. Tetrodotoxin produced a dose-dependent inhibition of ^{3}H-thymidine incorporation in control cells and a larger inhibition in EP cells. At 10^{-6}M, the EP effect was completely abolished. In other ion flux experiments, 5×10^{-8} valinomycin, a K^+ ionophore, negated the 58 percent increase in ^{3}H-thymidine uptake stimulated by EP over controls (Fig. 10-6).

The relation between Na^+ and K^+ fluxes and their dependence on Ca^{++} is well known. Changes in the distribution of these ions, in or on the cell membrane, appear to be necessary for the proliferative event to take place. These ion flux alterations occur very early in the series of cellular metabolic events leading to proliferation. They may be the primary mechanism of electrical perturbation transduction or, as seen in serum growth stimulatory systems, expressed in the modulation of the Na^+, K^+, ATPase (Elligsen, 1974; Tupper, 1977; Smith, 1978). Preliminary data, using ^{86}Rb to monitor K^+ flux, suggest that the latter enzyme axis is also affected by EP. The direct evidence accumulated so far does not single out a particular cation as the triggering agent for the EP effect but indicates that the various cations and the membrane are involved in the action of EP.

The capacitative coupled perturbation increased the cell diameter of the

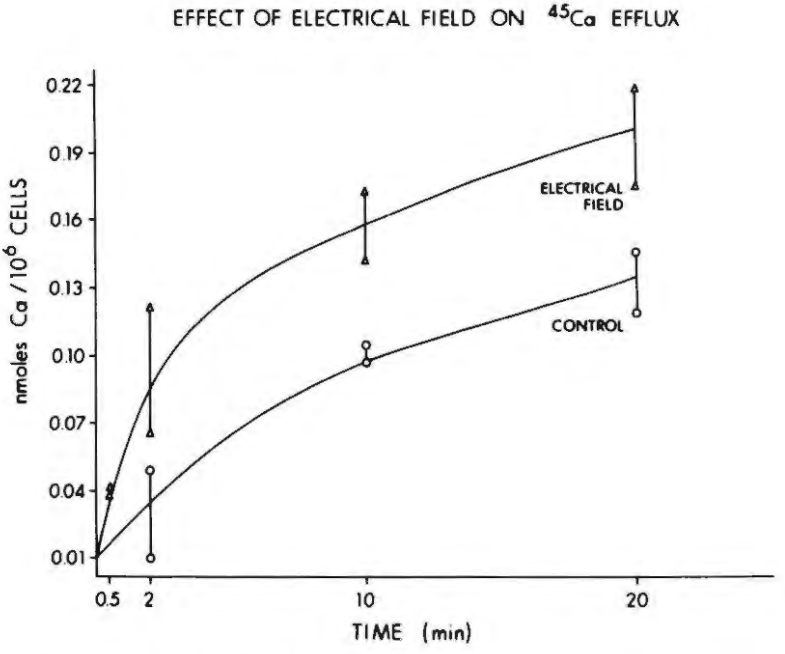

Figure 10-5. ^{45}Ca efflux in the presence of EP is shown to increase with time when compared to control concentrations.

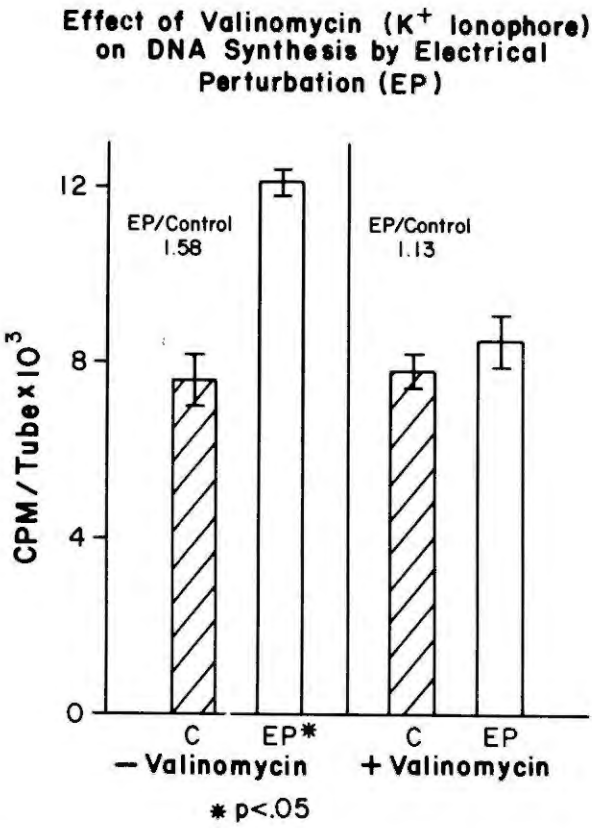

Figure 10-6. This graph indicates that 0.05 μM valinomycin abolishes the ability of the cell to increase ^{3}H-thymidine uptake in the presence of EP.

exposed cells by approximately 1.5 times (Fig. 10-7). This may be indicative of cells entering the M phase of the cell cycle. EP also increased the adhesivity of bone cells to the glass tubes. The rate of adherence rose from $t_{1/2}$ = 39 minutes to $t_{1/2}$ = 17 minutes (Fig. 10-8) and the release of cells by collagen digestion was substantially slower after twenty-four hour exposure to EP (Norton, 1979). The adhesivity findings strongly indicate cell membrane involvement in the EP effect.

We have also examined the effect of EP on cell cycle parameters. Preliminary data indicate that if synchronized cells are exposed to EP about 50 percent more cells are recruited from G_1 to S than in the respective control populations. There is no effect on the apparent length of the G_1 period, the peak DNA synthesis occurring in both groups at about twenty hours. Similarly, more cells already in the G_2 phase enter mitosis (M phase) in the presence of EP. Those highly selective conditions differ from the *in vivo* situation, where cells are heterogeneous, in all phases of the cell cycle and

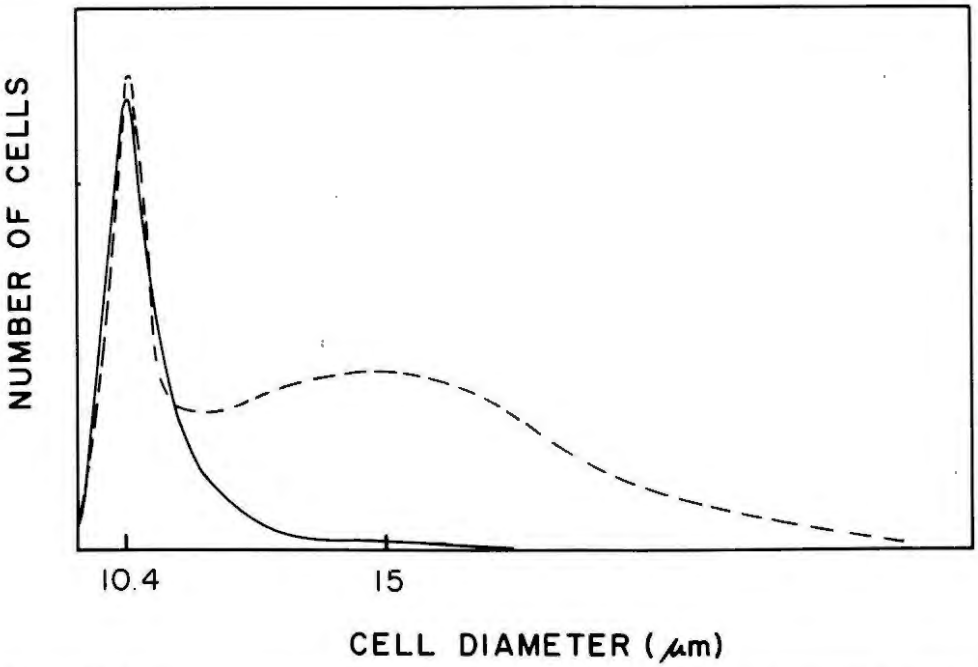

Figure 10-7. This is a Coulter counter channelyzer plot of the frequency distribution of cell size for a sample of control cells. The EP cells (— — — — —) have a bimodal distribution in cell diameter. (From Brighton, C. T., Black, J., and Pollack, S. R. (Eds.), *Electrical Properties of Bone and Cartilage*, 1979. Courtesy of Grune and Stratton, New York.)

subject to homeostatic influences. The presence of growth factors or hormones, cellular age or degree of differentiation, and position in the cell cycle all play a role in determining whether EP stimulation occurs and influences the magnitude of the EP effect.

TABLE 10-II

Time Scale	*Effect*
M Sec	Na^+, K^+ flux
Minutes	cAMP ↑ Ca^{++} (intracellular) Ca^{++} Efflux Cell adhesivity ↑ G_2 to M ↑
6-20 hours	^{3}H-thymidine uptake ↑ Mobilize cells in G_0 to G_1
20-24 hours	number of cells in S phase ↑ cell size ↑ Cell adhesion/probable cell matrix output ↑

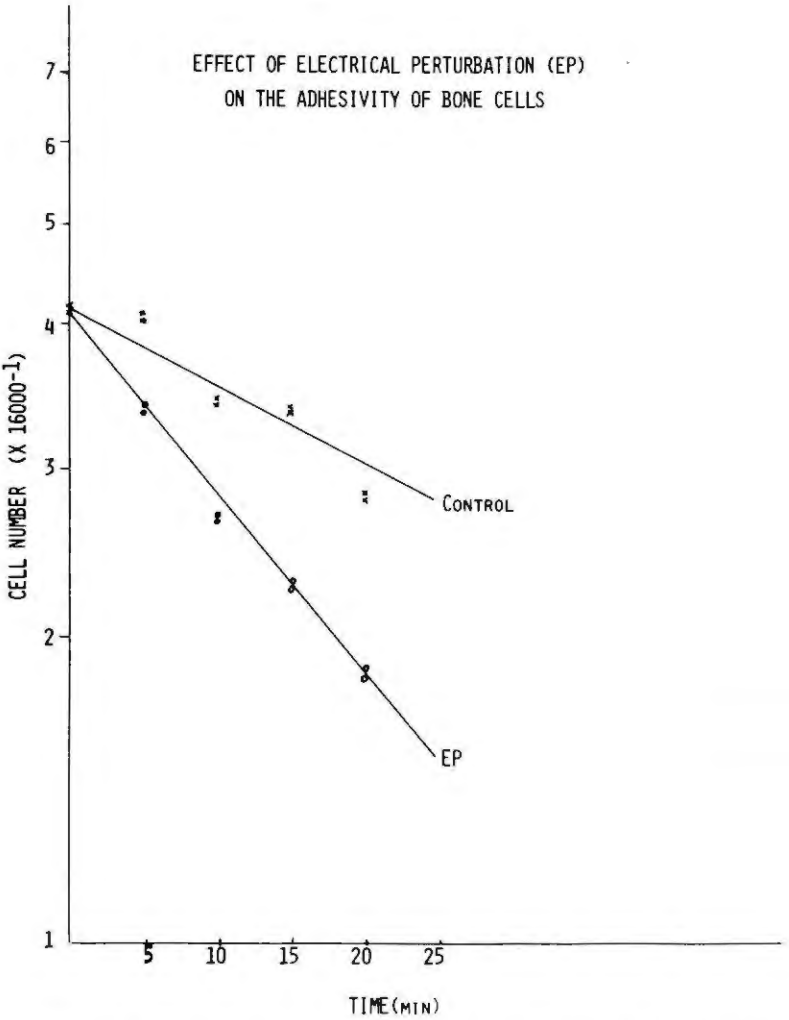

Figure 10-8. This plot shows that EP affects the rate of adhesivity of bone cells to culture tubes. The upper line shows the rate of attachment of the controls as compared to the EP cells seen in the lower line. (From Brighton, C. T., Black, J., and Pollack, S. R. (Eds.), *Electrical Properties of Bone and Cartilage*, 1979. Courtesy of Grune and Stratton, New York.)

Conclusions

The data collected so far allow us to speculate about the sequence of events that may take place when hard tissue cells are exposed to a capacitative coupled electric perturbation. A brief summary is seen in Table 10-II. Cells are affected by exposure to the cathode only. Probably within milliseconds, the Na^+, K^+, and ATPase produce changes detectable within minutes. Intracellular cAMP and Ca^{++} flux changes occur within the same time frame. Free cells adhere more readily when they strike a charged surface. Some evidence suggests that cells in G_2 may be recruited

into the M phase of the cell cycle. After six hours of perturbation, the uptake of ^{3}H-thymidine into DNA is statistically detectable, and unpublished data indicate that the peak is at twenty hours. During this time, cells are being mobilized from G_0 to G_1. Finally, after twenty hours, the number of cells in S phase is greatly increased. Some cells are enlarged and have altered adhesivity characteristics, possibly from increased production of matrix components or binding of fibronectin.

The physical basis for the EP effects observed in these studies is still subject to speculation. Two kinds of electrical changes occur in this system, depending on voltage, pulse frequency, capacitance, and the internal resistance of the power source: (a) charging of the test tube surface and (b) short-lived (μsecs) currents through the electrolyte solution. Surface charge is known to affect cell attachment and shape, which in turn were shown to modulate proliferation. Electric currents through the medium were shown to produce electrophoretic movement of molecules in the cell membrane, similar to patching, also known to modulate cell activity. Either could produce some or all of the changes observed.

One cannot assume that all events described in a highly controlled *in vitro* system represent a real portrayal of *in vivo* clinical events. However, the sequence and events outlined in this chapter are consistent with events observed in other cell populations when given proliferative stimuli. It is likely that electrical perturbations work in a similar manner. A great deal of study must be done to elucidate the mechanism of action of this unusual stimulatory signal on skeletal cells.

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