

Effects of electromagnetic stimuli on bone and bone cells *in vitro*: Inhibition of responses to parathyroid hormone by low-energy low-frequency fields

(electromagnetic field/cAMP/collagen synthesis/hormone response/nonequilibrium interaction)

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ABSTRACT Low-energy electromagnetic fields pulsed at frequencies of 10–90 Hz significantly increase healing of chronic fracture nonunions in man. These fields are effective at tissue current levels several orders of magnitude lower than those required for transmembrane depolarization of normal cells. We have examined the effects of two clinically used pulsed electromagnetic fields on cultures of the osteoblast-like mouse bone cell line MMB-1. Both fields significantly reduced cellular production of cAMP in response to parathyroid hormone and osteoclast activating factor. Neither basal nor fluoride-activated levels of adenylate cyclase were altered in membranes from cells cultured in the fields; however, the same membrane preparations exhibited markedly inhibited responses to parathyroid hormone. The fields blocked the inhibitory effects of the hormone on collagen synthesis by MMB-1 cells. However, there was no effect on the inhibition of collagen synthesis by 1,25-dihydroxyvitamin D₃, which is believed to act primarily by a nuclear, rather than by a membrane-dependent, mechanism. No significant differences were noted between effects of the two fields, one generating continuous pulse trains (72 Hz) and the other generating recurrent bursts (15 Hz) of shorter pulses. We hypothesize that these field effects are mediated primarily at the plasma membrane of osteoblasts, either by interference with hormone–receptor interactions or by blocking of receptor–cyclase coupling in the membrane. These responses occurred with induced extracellular fields of 1 mV/cm or less, even though transmembrane potential gradients are typically 10⁵ V/cm.

Clinical studies (1–5) have demonstrated the usefulness of electromagnetic fields in stimulating healing of chronically ununited fractures in humans. Devices generating such fields have been approved for clinical use. However, the mechanisms of action of these fields are not clear. Oscillating electromagnetic fields proven effective in clinical use generate electrochemical gradients in the tissue fluid surrounding cells (6), but these gradients are considerably weaker than the levels required to depolarize cell membranes. Typically, the devices in question impose ≈20-G pulsed magnetic fields, which induce current densities of ≈1 μA/cm² and associated electric gradients of 1–10 mV/cm in extracellular fluids (2). Because of the high resistance of cell membranes, any transmembrane electrical components of imposed fields would be lower than the extracellular gradients by two to three orders of magnitude (6) and thus as much as six orders of magnitude less than the typical excitatory

threshold currents of 1 mA/cm² observed for axonal depolarization (7).

It would therefore appear that the effectiveness of such weak stimuli in generating cellular processes must depend on a series of amplification mechanisms, either before or during the transmembrane coupling of the initial stimulus (8). Likely loci for amplification and extension of weak electrochemical triggering events at the cell membrane may involve glycoprotein molecules on the external surface of the plasma membrane (9), especially those molecules that constitute specific receptor sites for various extracellular molecules. In the present study, we have cultured bone and bone cells in the presence of clinically useful electromagnetic fields and have examined the responses of the cells to hormones that either do or do not appear to act primarily via plasma membrane receptors. The data are consistent with substantial changes in the activity of membrane processes as a result of exposure to the fields, suggesting that the clinical effectiveness of the fields may be mediated by changes in the sensitivity of cells *in vivo* to endogenous hormones. In addition, the data appear to be consistent with previously proposed nonequilibrium models for the responses of cells to weak electrochemical stimuli.

MATERIALS AND METHODS

For experiments in which isolated bones were cultured, the cranial bones were dissected from 3-day-old mice and cultured as described (10). For monolayer osteoblast cultures, the bone cells used were the MMB-1 osteoblast-like cell line developed in this laboratory (11) from primary cultures of mouse cranial bone cells. The cells were cultured in Eagle's minimal essential medium/10% fetal bovine serum (GIBCO) in 5% CO₂/95% air at 37°C. For experiments, cells were subcultured in multiwell plates (Falcon) at 20,000/25-mm well. The cells were allowed to attach for at least 18 hr before any further treatment. Dishes containing cells to be exposed to electromagnetic fields were placed in the central space of the 10 × 10 cm square coil of a Bi-Osteogen clinical field generator (furnished by Electro-Biology, Fairfield, NJ). The coil was placed in a tissue culture incubator, while the pulse generator unit was outside the incubator at normal room temperature and humidity. Control cultures were kept in a separate incubator under identical conditions or were placed in the same incubator as the treated cultures but isolated from the field by layers of Styrofoam and

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Abbreviations: PTH, parathyroid hormone; OAF, osteoclast-activating factor; SPP, single pulse, patient; PTP, pulse train, patient; ELF, extremely low frequency (<1.0 kHz).

stainless steel. The isolation conditions produced at least 40-dB attenuation of the field, as measured by inductive probes at various locations in the incubator. No differences in activities were noted between control cultures incubated in a separate incubator and cultures incubated under the isolation conditions described above.

After the specified times in the fields, control and treated cultures were removed from the field and the tissue culture medium was replaced with fresh medium containing 0.01 mM theophylline. The cells were then incubated for 30 min to allow temperature and gas equilibration before hormone treatment. Hormones were added and assays—cAMP accumulation, adenylate cyclase activity, and collagen synthesis—were carried out as described (10). Hormones used for the studies were bovine parathyroid hormone (1–34) (PTH) synthetic peptide (Bachem Fine Chemicals, Torrance, CA), osteoclast-activating factor (OAF) prepared in this laboratory (12), and 1,25-dihydroxyvitamin D₃ (Hoffmann–La Roche).

The general characteristics of the electromagnetic fields in these experiments have been described (13). The fields are generated by pulses of positive current applied to paired square Helmholtz coils 10 cm on a side, arranged coaxially and 6.3 cm apart. In one configuration, a continuous train of single pulses was produced at a frequency of 72 Hz (designated here SPP—“single pulse, patient”). Each magnetic pulse had an initial component 325 μ sec long, with a drop of 20% between the peak of the rising phase and the onset of the falling phase. The falling phase had an overshoot of opposite polarity with a typical peak amplitude 20% of the initial deflection. This overshoot decayed nonlinearly to zero over 5 msec. In the second field-exposure system (designated here PTP—“pulse train, patient”), bursts of pulses were produced at a 4-kHz rate, each burst lasting 5 msec and being repeated at a 15-Hz rate. The initial pulse was 200 μ sec long, and it was followed by a deflection of opposite polarity lasting 18.5 μ sec and limited in amplitude to 20% of the initial deflection. We have determined the characteristics of the magnetic field produced by this generator by a three-step process: (i) calibration of a search coil by using a sinusoidal waveform to energize the Helmholtz coils, determining the magnitude of the magnetic field by using a Hall effect probe and associated gauss meter; (ii) measurement of the emf induced in the search coil during excitation of the Helmholtz coil by the pulsed current generator; and (iii) modeling of the emf waveform by a series of linear segments and calculation of the magnetic field with constants determined in steps i and ii.

Calculated from the search coil output, the rising phase of the initial pulse changed at a rate of 0.92 G/ μ sec and, when measured from the emf across a resistance in series with the search coil, varied at a rate of 1.02 G/ μ sec. These rates of flux change indicated field components in the megahertz range of the frequency domain. We have therefore measured these high-frequency components for the 72-Hz generator by rf spectrometry and by digital spectral analysis. Energy distribution was essentially uniform (± 3 dB) over the range 0.8–9.5 MHz, with a sharp cutoff at ≈ 11 MHz and no evidence of significant output at higher frequencies. It should be emphasized that there is no evidence linking electromagnetic fields at frequencies above the extremely low frequency (ELF; <1.0 kHz) range with low-level bioeffects of the type described here. To the contrary, there is good evidence that, in the absence of low-frequency modulation, radio and microwave fields are without effect on binding and release of calcium in cerebral tissue (14) and in its synaptosomal fractions (15) and are similarly ineffective in modulating cell-mediated cytotoxicity of T lymphocytes (16), phenomena that are strongly responsive to radio and microwave fields amplitude modulated at frequencies <100 Hz. We have

confirmed that these fields produced an electrical gradient of ≈ 1.0 mV/cm around a 1-cm loop in the spatially homogeneous portion of the field between the coils and that the expected peak extracellular current density in homogeneous conducting electrolytes would be $\approx 1.0 \mu$ A/cm² (13). By reason of higher cell membrane impedance, transmembrane components of these fields would be substantially less. In all experiments described here, the cultures were exposed to the fields continuously for the indicated times prior to testing for hormone responses. All experiments were carried out on triplicate or quadruplicate cultures, and significance of the results was analyzed by Student's *t* test.

RESULTS

Isolated bone and bone cells treated in culture with PTH show markedly increased levels of cAMP (17). When the MMB-1 osteoblast-like cell line was cultured in the electromagnetic fields, the PTH-stimulated accumulation of cAMP was markedly reduced (Table 1). At doses normally found to produce maximal stimulation of cAMP accumulation, inhibition was $\approx 90\%$. Inspection of the dose-response curves for cAMP response suggested that the PTH dose required for half-maximal response was increased 50- to 100-fold. The fields also inhibited accumulation of cAMP stimulated by OAF (Table 1).

The inhibited accumulation of cAMP observed in the cells was not due to increased export of cAMP into the medium or to activation of phosphodiesterase (data not shown) but apparently was caused by decreased activation by PTH of adenylate cyclase in the cell membranes (Fig. 1). These data show that inhibition of PTH and OAF responsiveness persisted after the cells were removed from the fields; the experiments were carried out 30–60 min after removal of the cultures from exposure to the fields. Interestingly, however, the total adenylate cyclase catalytic activity in the membranes, as measured by activation with fluoride (Table 2), was not altered by culture of cells in the fields. These data suggest that the inhibition of responsiveness may be due to effects of the fields on cell surface receptors for PTH and OAF rather than on the cyclase itself.

To further test the hypothesis that the effects of the fields might be localized to cell surface receptors, we examined other intracellular responses to PTH that are believed to be mediated by the increased cAMP concentrations produced by PTH treat-

Table 1. Effects of fields on cAMP accumulation in bone cell monolayers

Agent	Dose, ng/ml	cAMP, pmol per 10 ⁶ cells		
		No field	SPP field	PTP field
None		2.1 \pm 0.3	3.1 \pm 0.6	2.3 \pm 0.5
PTH	10	5.7 \pm 0.8*	3.3 \pm 0.4	2.6 \pm 0.4
PTH	30	8.3 \pm 1.0*	4.1 \pm 0.8	3.5 \pm 0.6
PTH	100	11.6 \pm 1.8*	5.3 \pm 0.5*	4.9 \pm 0.8*
OAF	1	9.3 \pm 0.9*	4.2 \pm 0.6	3.3 \pm 0.5
OAF	10	13.8 \pm 2.1*	5.6 \pm 0.7	4.3 \pm 0.8*

Dose-response relationship for effects of PTH and OAF on accumulation of cAMP in MMB-1 cell monolayers with and without culture in electromagnetic fields. MMB-1 cells were plated in multiwell culture dishes (Falcon 2.5-cm 12-well dishes) at 20,000/well and then cultured in either SPP or PTP fields for 72 hr. At the end of culture, cells were removed from the fields and the medium was replaced with medium containing 0.01 mM theophylline. After 30 min of incubation, PTH or OAF was added at various doses for 5 min and then the cells were disrupted and cAMP was measured by radioimmunoassay. Results are mean \pm SEM of quadruplicate cultures.

* Significantly different from control (no field), $P < 0.05$. Paired *t* tests indicated no significant differences between effects of SPP and PTP fields at any dose.

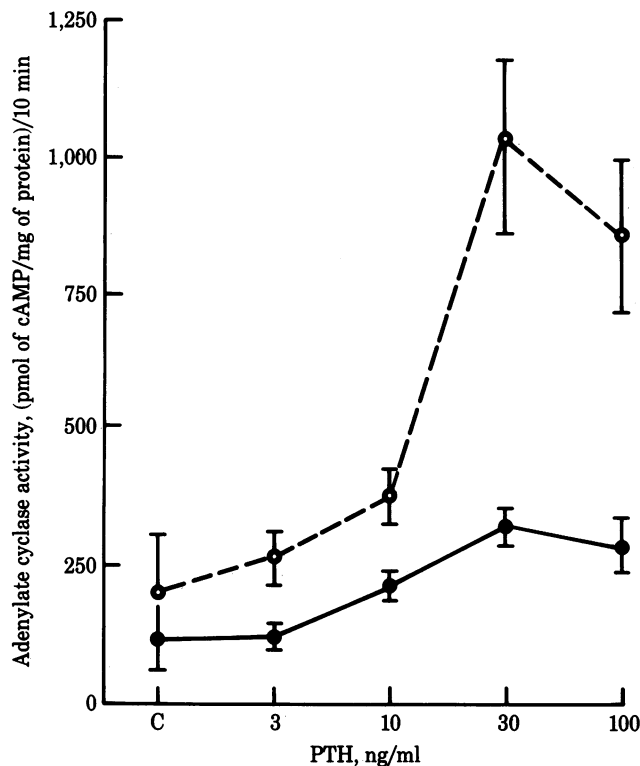


FIG. 1. Dose-response relationships for effects of PTH on adenylate cyclase activity of bone cells in culture. MMB-1 cells were grown in the presence (●) or absence (○) of the SPP field for 90 hr without hormone treatment. The cells were then disrupted and a membrane preparation was isolated for adenylate cyclase assay as described (6). The membranes were treated for 10 min with the indicated concentrations of PTH without exposure to the field, and then the production of cAMP was determined. Results are mean \pm SEM.

ment. The specific inhibition of collagen synthesis by PTH (18) was blocked by treatment of cells with the fields (Fig. 2). However, the fields did not block the effects on collagen synthesis of 1,25-dihydroxyvitamin D_3 , a hormone that apparently acts via a cytoplasmic rather than a membrane receptor (19).

As with cell cultures, isolated mouse cranial bones exhibited significantly decreased accumulation of cAMP when treated with PTH or OAF after culture in the fields (Table 3).

DISCUSSION

It is reasonable to postulate that the osteoblast, the bone forming cell, is specifically sensitive to electromagnetic fluxes of

Table 2. Effects of fluoride on adenylate cyclase activity with and without fields

Treatment	Cyclase activity, pmol of cAMP/mg protein
None (no field)	98.5 \pm 18.8
None (SPP field)	112.4 \pm 22.6
PTH (30 ng/ml)	1,024 \pm 246
PTH (30 ng/ml), SPP field	213.5 \pm 43.5
NaF (10 mM)	1,144 \pm 231
NaF (10 mM), SPP field	1,379 \pm 306

MMB-1 cells were grown in the presence or absence of the SPP field for 72 hr and then removed, and the cell layers were disrupted to prepare membranes for adenylate cyclase assay (6). The membranes were treated in the absence of fields for 10 min with either PTH or 1 mM NaF, and then the amount of cAMP formed was determined by radioimmunoassay. Results are mean \pm SEM.

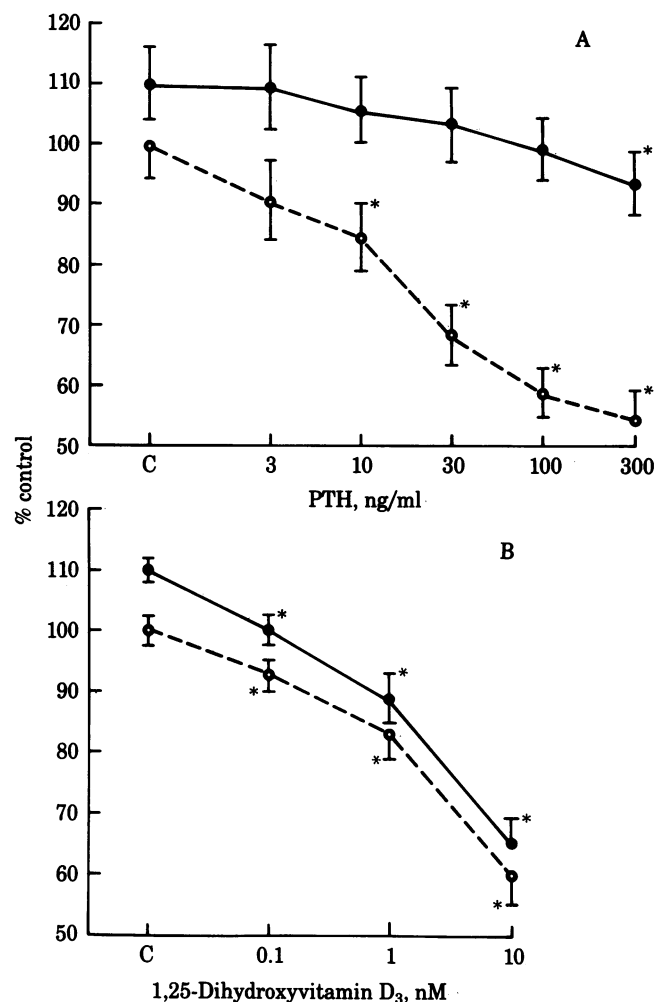


FIG. 2. Effects of fields on synthesis of collagen by bone cells. MMB-1 cells were grown in the presence (●) or absence (○) of the SPP field for 12 hr, and then various concentrations of PTH (A) or 1,25-dihydroxyvitamin D_3 (B) were added in fresh culture medium. Culture was continued in or out of the field for 48 hr, with the final 4 hr being in the presence of [3H]proline at 1 μ Ci/ml (1 Ci = 3.7×10^{10} becquerels). Cells were disrupted and collagen synthesis was measured by a collagenase digestion technique (11). C, control. *Significantly different from control (no field), $P < 0.05$.

both exogenous and endogenous origin. Electromagnetic fields are effective in stimulating healing of bones *in vivo* (1-3). Treatment schedules (field time on vs. time off) have varied considerably. The present studies were carried out with continuous field exposure; future studies should evaluate variations in treatment schedule. Low-energy electric currents produced by piezoelectric responses to stress on bone appear to play a role both in normal bone remodeling and in healing of fractures under normal treatment regimens (5). The present data suggest that the effects of fields on bone involve subtle changes in the response of bone cells to their surroundings. The ability of the bone cells to respond to added PTH or OAF with increased cAMP formation was inhibited dramatically by the fields; the same result was observed for isolated mouse cranial bones cultured under similar conditions. Since the effects of short-term treatment with PTH are osteolytic, blocking of the effects of PTH *in vivo* should lead to net increases in the rate of bone formation. In a case in which only small localized areas of bone are rendered resistant to PTH action, there would likely be no significant changes in the overall circulating concentrations of calcium or PTH, leading to increased bone density in the affected

Table 3. Effects of fields on cAMP accumulation in cultured mouse calvariae

Agent	Dose, ng/ml	cAMP, pmol per half calvaria		
		No field	SSP field	PTP field
None		4.2 ± 0.7	3.7 ± 0.1	3.3 ± 0.2
PTH	10	4.3 ± 0.3	4.6 ± 0.3	3.0 ± 0.2
PTH	30	7.9 ± 0.8*	3.0 ± 0.4	6.6 ± 0.3*
PTH	100	22.2 ± 3.2*	8.0 ± 2.3	6.5 ± 1.8
OAF	1	13.1 ± 2.2*	3.6 ± 0.4	3.8 ± 0.3
OAF	10	24.4 ± 3.3	7.6 ± 1.3*	8.3 ± 1.1*

cAMP accumulation was assayed in neonatal mouse calvariae cultured for 72 hr in the presence or absence of fields and then treated for 5 min with various doses of PTH or OAF. Culture and assay conditions were as described in Table 1. Results are mean ± SEM of triplicate cultures.

*Significantly different from control (no field), $P < 0.05$.

areas without systemic effects on mineral balance. This in fact has been observed clinically (1, 2).

The current results do not directly indicate the electrochemical mechanism by which osteoblasts respond to electromagnetic fields. However, the evidence points to the cell membrane as a primary site of interaction. Although the fields inhibited the activities of the peptide agents PTH and OAF, we did not observe changes in the activity of 1,25-dihydroxyvitamin D₃, a hormone that does not appear to act via plasma membrane receptors (19). In addition, the earliest enzyme involved in PTH action, PTH-activated plasma membrane adenylate cyclase, was substantially inhibited by the fields. In contrast, the total amount of adenylate cyclase catalytic units in the membrane (as assessed by activation with fluoride) was not decreased, nor was the basal cyclase activity altered by exposure of cells to the field. This finding argues against long-term direct effects of the fields on the cyclase itself and suggests that the fields may be interfering with the binding of hormone to receptor, the ability of the hormone-receptor complex to activate cyclase, or both.

Another possible locus for the effects of electromagnetic fields is the coupling of the hormone-receptor complex to adenylate cyclase in the membrane. Such an effect could be mediated either directly, by effects on the intrinsic membrane coupling protein(s) (20), or indirectly, by modification of other membrane functions. It is of particular interest in this regard that many investigators have postulated a role for membrane transport of Ca²⁺ in the mechanism of PTH action of bone cells. Activation of adenylate cyclase by PTH is accompanied by influx of calcium ions (21) and, conversely, some of the hormonal actions of PTH may be mimicked by calcium ionophores (22). Recent findings (23) indicate that calcium influx in bone cells may be linked with a Na⁺-Ca²⁺ exchange mechanism and thus with the transmembrane gradients of Na⁺ and K⁺ maintained by the Na⁺/K⁺ ATPase pump. These findings are supported by previous studies of the effects of electromagnetic fields on the binding and release of Ca²⁺ in isolated cerebral tissue (13, 24, 25), in cerebral synaptosome fractions (14), in pancreatic islets (26), and in modification of cytotoxicity of T lymphocytes exposed to low-frequency modulated microwave fields (15). There is evidence that initial events in coupling of weak oscillating electromagnetic fields to cell membranes occur on membrane surface polyanionic glycoproteins, producing transient coherent states of nearest-neighbor fixed-charge sites (27). These coherent states may exist for considerable distances along the membrane surface (28), and thus would appear likely sites for the first steps in transductive coupling. Binding and release of Ca²⁺ at these membrane surface sites correlate closely with

exposure to certain weak electromagnetic fields. Sensitivity to these fields in narrow frequency and amplitude "windows" (14, 29–31) supports a series of models based on cooperative or dissipative interactions at cell membrane surface glycoproteins. Evidence from Ca²⁺-H⁺ interactions in the presence of these fields raised the possibility of proton tunneling, perhaps at the margins between coherent and incoherent fixed-charge zones (25). Dispersive (reaction-diffusion) interactions may also occur at cell membrane surfaces between linear macromolecules and electromagnetic fields (32–34) in the form of soliton waves and act as a substrate for coupling extracellular events to the intracellular domain (35). It seems reasonable that future studies of the effects of electromagnetic fields on bone cells should focus on the fluxes of cations across the cell membrane as well as on the effects on intracellular metabolic activities. It will be important to demonstrate not only that the effects on hormone responsiveness that we have observed *in vitro* can also be observed *in vivo* but also that there is consistency between responses of isolated cells and living tissues.

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