Calcif Tissue Int (1994) 55:376–380

Calcified
Tissue
International

Combined Magnetic Fields Increased Net Calcium Flux in Bone Cells

R. J. Fitzsimmons,[1] J. T. Ryaby,[2] F. P. Magee,[2] D. J. Baylink[1]

[1]Department of Medicine, Loma Linda University and Department of Research 151, Jerry Pettis VA Hospital, 11201 Benton Street, Loma Linda, California 92354
[2]Research and Development, OrthoLogic Corp, Phoenix, Arizona 85034

Received: 16 March 1994 / Accepted: 29 April 1994

Abstract. Low energy electromagnetic fields (EMF) exhibit a large number of biological effects. A major issue to be determined is "What is the lowest threshold of detection in which cells can respond to an EMF?" In these studies we demonstrate that a low-amplitude combined magnetic field (CMF) which induces a maximum potential gradient of 10^{-5} V/m is capable of increasing net calcium flux in human osteoblast-like cells. The increase in net calcium flux was frequency dependent, with a peak in the 15.3–16.3 Hz range with an apparent bandwidth of approximately 1 Hz. A model that characterizes the thermal noise limit indicates that nonspherical cell shape, resonant type dynamics, and signal averaging may all play a role in the transduction of low-amplitude EMF effects in biological systems.

Key words: Electromagnetic — Bone — Calcium — Osteoblast.

Low-amplitude electromagnetic fields (EMFs) are gaining more attention as an ever-increasing number of studies are revealing possible biological effects of these EMFs [1, 2]. An important question resulting from these studies is "What is the lower threshold for EMF detection by cellular or tissue systems?" Because bone cells may be particularly sensitive to EMFs—based on the hypothesis that bone cells detect naturally produced EMFs in the form of stress-generated potentials due to mechanical loading [3, 4]—we investigated the effects of EMF exposure on bone cells.

As an endpoint we assessed net ^{45}Ca uptake into bone cells. The rationale for using calcium uptake as an index for an affect is that low-amplitude EMFs have been reported to alter calcium flux in a number of cell types [5, 6]. In addition, these effects have been described for a wide range of electromagnetic exposure signals ranging from sinusoidal AC magnetic fields [7] to combined AC/DC magnetic fields (CMF) [8, 9]. Furthermore, calcium is an important regulator of cellular events [10], and changes in calcium flux have important regulatory consequences in bone cells [11] as well as other tissues [12]. In these studies we found that a CMF with an extremely low energy content was capable of increasing net ^{45}Ca into bone cells. These studies indicate that the lower threshold for cellular detection of an EMF is, at the most, in the low microtesla range inducing potential gradients in the range of 10^{-5} V/m.

Correspondence to: R. J. Fitzsimmons

Materials and Methods

Dulbecco's modified Eagles' medium (DMEM), trypsin, and calf serum were purchased from Gibco (Santa Clara, CA), and 24-well tissue culture plates were from Falcon Labware (Lincoln Park, NJ). $^{45}Calcium$ was obtained from New England Nuclear (Danvers, MA). All other chemicals were from Sigma (St. Louis, MO).

Cell Culture Conditions

Cells from either the human osteosarcoma cell line TE-85 or SaOS-2 were grown as previously described [13, 14] and maintained by weekly passage in DMEM containing 10% calf serum. For use in experiments, the cells were rinsed once with PBS, trypsinized for 3 minutes, DMEM (1:1 volume) was added, and cells were pelleted by centrifugation and plated in 24-well plates at 20,000 cells/well in 1.0 ml DMEM containing 1% calf serum. The following day the medium was changed to 0.5 ml unsupplemented serum-free DMEM, and the cultures were allowed to incubate for an additional 24 hours before being used in an experiment. This serum depletion partially growth arrested the cell cultures at the Go/G1 transition point [14]. At the time of the experimental procedure, the cells were subconfluent covering approximately 25% of the surface of the tissue culture wells. However, the center of the tissue culture wells contained approximately 50% of the cells in a dense population with each cell in direct cell contact with three or more cells. Two SaOS subpopulations with low and high alkaline phosphatase (ALP) specific activities [15] were also used to determine if the differentiation state of osteoblastic cells as measured by ALP influenced CMF responsiveness.

Magnetic Field Exposure System

The combined magnetic field exposure system was composed of an FG2 function generator and UC-10 wave counter from Circuitmate, a division of Beckman Co. (Palo Alto, CA), DM 2220 magnetometer from Schonstedt Instrument Co (Reston, VA), T921 oscilloscope from Tektronix (Beaverton, OR), and a custom-designed Helmholtz coil apparatus. This apparatus consisted of a round (30 cm diameter) Helmholtz coil pair made from 400 turns of #28 AWG magnet wire supported by a nonconducting nonmagnetic polymeric frame. The coils were oriented horizontally and produced a vertically oriented magnetic field, with the static and dynamic magnetic fields created by the same pair of Helmholtz coils (static and dynamic magnetic fields were parallel to each other). The amplitude and DC offset controls of the function generator were used to control the amplitude (40 μtesla peak-to-peak, 5.3 mA RMS through coil) of the AC (time varying) magnetic field and the amplitude (20 μtesla, 7.5 mA through coil) of the DC (static) magnetic field, respectively. The magnitude and directions of the ambient horizontal and vertical geomagnetic fields were measured and recorded during each experiment. Average value for the vertical DC component was 40 ± 2

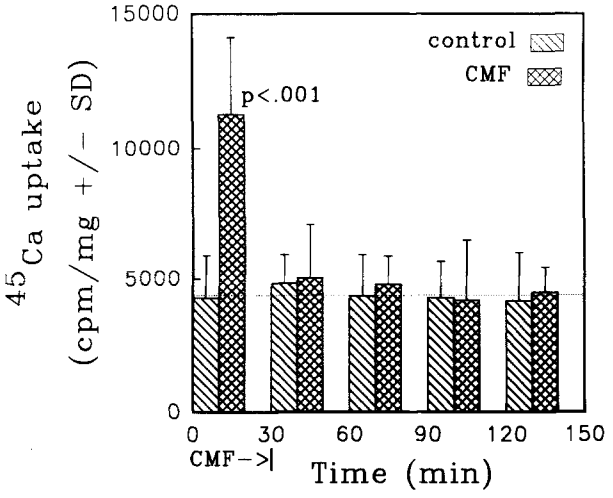

Fig. 1. CMF increased net ^{45}Ca flux during but not after CMF exposure. Cultures of TE-85 cells were assessed for net ^{45}Ca flux during a 30-minute incubation period with ^{45}Ca at 37°C. The cell cultures were exposed to a 15.3 Hz CMF for 30 minutes at time t = 0. At various times including t = 0, 30, 60, 90, and 120 minutes relative to the start of CMF exposure, ^{45}Ca was added and the cultures terminated 30 minutes later. Each group consisted of six replicates. Control values are represented by hatched bars and CMF values are represented by cross-hatched bars. The average of control values was calculated and are represented by the dashed line.

μtesla (which was reduced to 20 μtesla for experimental runs) and the horizontal component was 15.0 ± 1.6 μtesla.

Experimental Design

The cell cultures, after 24 hours of incubation in unsupplemented serum-free DMEM, were exposed to a CMF by placing the 24-well plates coplanar in the center of the Helmholtz coil apparatus in a CO_2 incubator at 37°C. The cultures were exposed for 10–40 minutes (as indicated in the figures); in post-exposure recovery experiments (Fig. 1), the cultures were transferred back to their original CO_2 incubator. As expected from such a low-amplitude field, there were no detectable changes in temperature (i.e., heating due to CMF) with a temperature probe with an accuracy of ±0.1°C. Control cultures were run in parallel to exposed cultures in a separate incubator (paired samples).

Net ^{45}Ca Uptake

^{45}Ca uptake was performed as previously described [11]. Briefly, cells were grown in 6-well culture plates in DMEM. The reaction was initiated by adding ^{45}Ca to a final concentration of 3 μCi/ml in a total volume of 600 μl. The reaction was subjected to CMF exposure, as indicated in figure legends. The reaction was terminated by rinsing four times with ice-cold Hanks solution containing 0.1 mM $LnCl_3$. The cell layer was extracted with 0.03% Triton X-100, and aliquots were analyzed for ^{45}Ca incorporation by LSC and protein content by dye-binding assay [16]. Data are expressed as cpm/mg protein. Statistical analysis was performed using student's *t*-test because of the paired nature of the experiments.

Energy Calculations

The maximum potential gradient, E_{emf}, induced in the culture media by the sinusoidal (AC) CMF, can be calculated from the following formula:

$$E_{emf} = \pi f B a \qquad \text{(eq 1)}$$

where f is the frequency of the magnetic field in cycles/second (Hz), B is the peak value of the AC magnetic field (T), and a = radius in meters (m) of a single tissue culture well. With f = 16.3 Hz, B = 4×10^{-5} T, and a = 1.8×10^{-2} m, then the induced field is $E_{emf} = 4 \times 10^{-5}$ V/m. E_{min} is the maximum field in the culture media which the surface of a cell may experience. The field induced inside a single cell with a radius of 10 μm is calculated to be 2×10^{-8} V/m. However, as the number of cells that are electrically coupled (e.g., by gap junctions) increases, the effective radius also increases with a maximum of 1.8×10^{-2} m, the radius of a tissue culture well.

This value can be compared to the value of the external field which would produce a signal-to-noise ratio (SNR) of unity when the noise is due to random thermal fluctuations. Assuming a spherical biological cell of 10 μm, this value is given by:

$$E_{min} = \tfrac{2}{3}[kTd/4\pi\epsilon_o Km]^{1/2}\, 1/r^2 \qquad \text{(eq 2)}$$

where $kT = 4.3 \times 10^{-21}$ J, d = thickness of cell membrane = 5×10^{-9} m, $\epsilon_o = 8.85 \times 10^{-12}$ C/V-m, Km = membrane dielectric constant = 2, and r = radius of cell = 1×10^{-5} m. Substituting appropriate values of the parameters gives Emin = 2 V/m. This is orders of magnitude greater than the induced field, E_{emf}. However, following Weaver and Astumian [17], the effects on E_{min} of non-spherical cell shape, reduced bandwidth (δf), and signal averaging can be incorporated. In this case E_{min} is given by:

$$E_{min} = \frac{2\,(2^{1/2})\,[p_m kT\delta f/\pi]^{1/2}}{r^{1/2}L^{3/2}(f\tau)^{1/2}} \qquad \text{(eq 3)}$$

p_m = membrane resistivity = 10^5–10^7 ohm-m, δf = the bandwidth of the transducing mechanism, r = radius of cylindrical cells, L = length of cylindrical cell, and fτ = number of cycles of field seen during the exposure. Substituting $p_m = 10^5$ ohm-m, δf = 1 Hz, r = 2.5×10^{-5} m, L = 1.5×10^{-4} m, and fτ = (16.3 Hz) (60 s/minute) (30 minutes) = 2.9×10^4, we obtain from equation 3, $E_{min} = 1.5 \times 10^{-6}$ V/m. Therefore, the value of the induced field, $E_{emf} = 4 \times 10^{-6}$ V/m, is larger than the thermal noise limit if cell shape, bandwidth, and signal averaging are incorporated into the analysis.

The argument presented above is focused on decreasing the electrical noise level which, as a result, increases the sensitivity of the cells to EMFs. Another means of increasing the signal-to-noise ratio for a given EMF is to amplify the EMF signal detected by the cells. It has been hypothesized that if numerous cells were electrically coupled, the EMF signal at the level of the cell may be amplified [18]. Such amplification could result from formation of a phased array or transmission line behavior.

Results

Previous data have shown that a 30-minute exposure to CMF increased cell proliferation in the osteosarcoma cell line TE-85, which were growth arrested by serum depletion [19]. Previous experiments in this laboratory have demonstrated that serum-depletion results in a larger percentage of growth-arrested (i.e., partially synchronized) cells than would exist in the presence of serum. However, we have not investigated this requirement for an observable effect of CMF exposure. Because the signaling mechanism for EMF stimulation remains unknown and calcium is an important intracellular second messenger [10], we initially investigated the possibility that a 30-minute CMF exposure affected calcium flux in TE-85 cells. As shown in Figure 1, net ^{45}Ca uptake into TE-85 cells was increased during exposure to the CMF but returned to control levels immediately post-CMF exposure.

Because the CMF has such low energy, it is reasonable to assume that it is coupling to a specific process(es) which may have specific frequency windows [20]. In order to determine

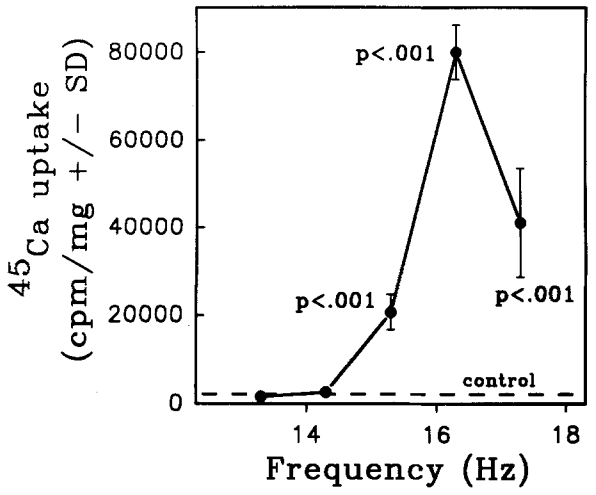

Fig. 2. CMF increased ^{45}Ca in a frequency-dependent manner. TE-85 cells were assessed for net ^{45}Ca uptake during a 30-minute incubation period with ^{45}Ca at 37°C. Cell cultures were exposed to a CMF at the indicated frequency (1 Hz frequency spacing between groups) during the incubation with ^{45}Ca. Each frequency was compared to its own control group for statistical analysis. Each group consisted of six replicates for both control and experimental groups. The peak frequency was at 16.3 Hz.

whether the increase in net ^{45}Ca uptake was frequency dependent, TE-85 cells were exposed to a CMF at the indicated frequencies for 30 minutes, and net ^{45}Ca uptake was assessed during the CMF exposure period. As shown in Figure 2, CMF exposure increased net ^{45}Ca flux in a frequency-dependent manner, with a peak near 16.3 Hz. Furthermore, we repeated these experiments with another osteosarcoma cell line, SaOS, which had been subcloned into populations with low and high ALP content. As shown in Table 1, both low and high ALP-containing SaOS cells demonstrated a frequency-dependent increase in net ^{45}Ca uptake peaking at 15.3 Hz, with increases of 4.73 ± 0.40- and 2.03 ± 0.63-fold increase of control values for low and high ALP SaOS cells, respectively.

In order to determine the time course for CMF-stimulated net ^{45}Ca uptake, TE-85 cell cultures were exposed to a CMF at 16.3 for various periods of time in the presence of ^{45}Ca. As shown in Figure 3, net ^{45}Ca uptake increased with time in control cultures, with a plateau occurring between 10–20 minutes. In contrast, CMF exposure significantly increased net ^{45}Ca uptake with exposures up to 40 minutes.

Discussion

The lower threshold for detection of an EMF by cells may be set at the resting membrane potential of a cell (10–100 mV). However, a lower threshold is evident for cellular detection of EMFs and this limit has been proposed to be the thermal noise level. The minimum detectable EMF with a signal-to-noise ratio of 1 has been calculated at 10^{-2} V/m [17]. However it is known that birds and fish can detect EMFs as low as 5×10^{-5} V/m [21]. This is in disagreement with the thermal noise level unless a number of additional factors are considered. Weaver and Astumian [17] included nonspherical dimensions, narrow bandwidth, and signal averaging which reduces the minimum detectable field into the range of

Table 1. CMF increased ^{45}Ca in SaOS cells in a frequency-dependent manner

Frequency (Hz)	Net ^{45}Ca uptake into cells (cpm/mg protein ± SD)	
	SaOS-L	SaOS-H
Sham	7360 ± 1186	2204 ± 843
14.3	23040 ± 2915 313 ± 40% $P < 0.001$	1973 ± 1296 90 ± 59% NS
15.3	34801 ± 2937 473 ± 40% $P < 0.001$	4485 ± 1386 203 ± 63% $P < 0.01$
16.3	22129 ± 2961 301 ± 40% $P < 0.001$	3283 ± 1292 149 ± 59% NS
17.3	15965 ± 1320 217 ± 18% $P < 0.001$	1239 ± 1040 56 ± 47% NS
18.3	13020 ± 2663 177 ± 36% $P < 0.001$	1141 ± 902 52 ± 41% NS

SaOS cells were assessed for net ^{45}Ca uptake during a 30-minute incubation with ^{45}Ca at 37°C. The cell cultures were exposed to a CMF at the indicated frequencies for 30 minutes in the presence of ^{45}Ca. SaOS cells had been previously subcloned into low and high ALP cells denoted as SaOS-L and SaOS-H, respectively. The ALP-specific activity for SaOS-L was <0.005 U/mg and 1.5 U/mg protein for the SaOS-H. Each group was composed of six replicates

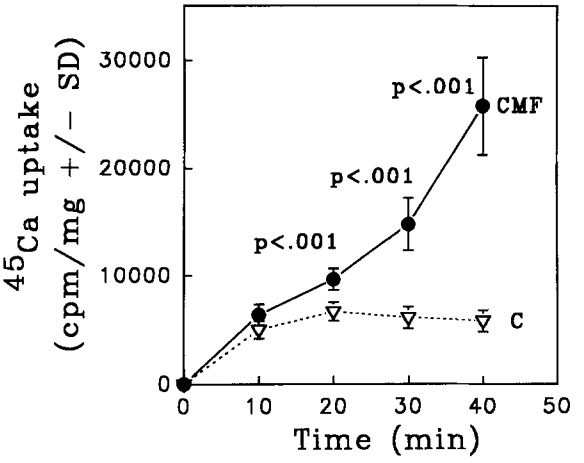

Fig. 3. Time course for CMF-stimulated ^{45}Ca uptake. TE-85 cells were assessed for net ^{45}Ca uptake during various time intervals as indicated in the presence of ^{45}Ca. A 16.3 Hz CMF exposure was applied to the experimental group for the duration of the ^{45}Ca incubation (i.e., ^{45}Ca and CMF were present for the same time periods). Each group consisted of six replicates.

10^{-6} V/m. The requirement for a narrow bandwidth and signal averaging may prove useful in formulating a mechanism for explaining the actions of low-energy fields. In addition, the greater a cell's dimension deviates from spherical the greater the sensitivity to an external EMF [i.e., from eq 2, the minimum detectable EMF decreases by a factor of (length/radius)$^{3/2}$].

The thermal noise level has gained acceptance as a lower barrier to EMF detection based on the assumption that the detection mechanism is linear. However, the known lower

limit for a biologically detectable EMF has been reduced considerably from 10^{-2} V/m as evidence is found that biological systems can detect energy levels that appear to be below the thermal noise level [17, 22]. One possibility is that a nonlinear mechanism is involved and that thermal noise is not the limiting issue. In order to determine the mechanism underlying this sensitivity, a number of characteristics describing the system need to be elucidated including the lower energy threshold, frequency dependence, and the temporal duration requirement for signal detection.

In these studies, net ^{45}Ca flux was used as a potential early marker of a response to low-amplitude EMF exposure in human bone cells. The EMF exposure system was composed of a DC magnetic field and an AC magnetic field which were initially calculated to couple to calcium, according to ion-resonance theory [23]. Even though ion-resonance theory has been refuted in subsequent theoretical studies [24, 25], it is interesting that the CMF used in these studies did indeed increase net calcium flux. This result suggests that further investigation is warranted into the possible role of the DC magnetic field in modulation of responses to low energy alternating (AC) magnetic fields.

The CMF applied in these studies is calculated to induce a maximum potential gradient of 10^{-5} V/m in a tissue culture well. With such a low potential gradient it is reasonable to assume that the site of interaction must be very specific in order to detect such a low-energy CMF, and it is not surprising that the response is highly specific. In these studies we observed frequency responses with peak affects between 15.3 and 16.3 Hz. The peak frequency was not dependent on the differentiation state of bone cells as measured by ALP. Frequency specificity has been observed in other model systems [20], and has been predicted by various theoretical approaches including ion resonance [26], Lorentz force/Zeeman Stark [27], quantum parametric resonance [28], and Larmor precession [29] models. However, the physical mechanism of this extreme frequency specificity remains unclear.

The ability of CMF to increase net ^{45}Ca uptake occurred only during exposure to the CMF and not post-CMF. This suggests that the effect is not secondary to a long-lived (i.e., greater than 30 minutes) primary event. Bassett et al. [30] also observed a similar time course for increased calcium flux in chondrocytes with a pulsed EMF. An increase in calcium flux could result if the CMF either increases the open time for a calcium channel or increases the probability for opening of a calcium channel [26]. In these studies, because the increase in net ^{45}Ca uptake was linear with respect to time, the data precludes a rapid stimulus of calcium mobilization, as observed with parathyroid hormone [31]. Furthermore, because the increase in calcium flux is absent in the 30-minute period immediately after termination of CMF exposure, the potential effect of CMF exposure to increase calcium channel kinetics is not due to stable channel modifications (i.e., a phosphorylation event with a lifetime >30 minutes).

In conclusion, these studies show that bone cells can detect a CMF as low as 40 microtesla, with a calculated potential gradient of 10^{-5} V/m. Furthermore, the detection mechanism is frequency dependent, with a peak response in the 15–16 Hz range. Further studies will be required to determine the role of calcium channels and the signal transduction mechanism (s) regulating the cellular response in low-amplitude and low-frequency exogenous EMFs.

Acknowledgments. We would like to thank Dr. Jonathan J. Kaufman for his comments concerning this manuscript. Funding was provided by Veterans Research Administration and OrthoLogic Corp, Phoenix, AZ.

References

1. Sagan LA (1992) Epidemiological and laboratory studies of power frequency electric and magnetic fields. JAMA 268:625–629
2. Luben RA (1991) Effects of low-energy electromagnetic fields (pulsed and dc) on membrane signal transduction processes in biological systems. Health Phys 61:15–28
3. Pollack SR (1984) Bioelectrical properties of bone. Orthop Clin NA 15:3–14
4. Bassett CAL, Becker RO (1962) Generation of electric potentials by bone in response to mechanical stress. Science 137: 1063–1064
5. Cadossi R, Bersani F, Cossarizza A, Zucchini P, Emilia G, Torelli G, Franceschi C (1992) Lymphocytes and electromagnetic fields. FASEB J 6:2667–2674
6. Walleczek J, Budinger TF (1992) Pulsed magnetic field effects on calcium signaling in lymphocytes: dependence on cell status and field intensity. FEBS Lett 314:351–355
7. Ozawa H, Abe E, Shibaski Y, Fukuhara T, Suda T (1989) Electric fields stimulate DNA synthesis of mouse osteoblast-like cells (MC3T3) by a mechanism involving calcium ions. J Cell Physiol 138:477–483
8. Yost MG, Liburdy RP (1992) Time-varying and static magnetic fields act in combination to alter calcium signal transduction in the lymphocyte. FEBS Lett 296:117–122
9. Rozek RJ, Sherman ML, Liboff AR, McLeod BR, Smith SD (1987) Nifedipine is an antagonist to cyclotron resonance enhancement of 45-calcium incorporation into human lymphocytes. Cell Calcium 8:413–427
10. Berridge MJ (1993) Inositol trisphosphate and calcium signaling. Nature 361:315–325
11. Farley JR, Hall SL, Herring S (1993) Calcitonin acutely increases net ^{45}Ca uptake and alters ALP specific activity in human osteosarcoma cells. Metabolism 42:97–104
12. Sato N, Wang X, Greer MA (1992) Protein kinase C modulates cell swelling-induced calcium flux and prolactin secretion in GH4C1 cells. Mol Cell Endocrinol 86:137–142
13. Fitzsimmons RJ, Strong D, Mohan S, Baylink DJ (1992) Low-amplitude, low-frequency electric field-stimulated bone cell proliferation may in part be mediated by increased IGF-II release. J Cell Physiol 150:84–89
14. Zhang K, Papageorge AG, Lowy DR (1992) Mechanistic aspects of signaling through Ras in NIH 3T3 cells. Science 257:671–674
15. Farley JR, Wergedal JE, Hall SL, Herring S, Tarbaux NM (1991) Calcitonin has direct effects on 3H-thymidine incorporation and ALP activity in human osteoblast-line cells. Calcif Tissue Int 48:297–301
16. Bradford MM (1976) A rapid and sensitive method for quantitation of microgram amounts of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–255
17. Weaver JC, Astumian RD (1990) The response of living cells to very weak electric fields: the thermal noise limit. Science 247: 459–462
18. Pilla AA (1993) State of the art in electromagnetic therapeutics. In: Blank M (ed) Electricity and magnetism in biology and medicine. San Francisco Press, San Francisco, pp 17–22
19. Fitzsimmons RJ, Baylink DJ, Ryaby JT, Magee FP (1993) EMF-stimulated bone-cell proliferation. In: Blank M (ed) Electricity and magnetism in biology and medicine. San Francisco Press, San Francisco, pp 899–901
20. McLeod KJ, Lee RC, Ehrlich HP (1987) Frequency dependence of electric field modulation of fibroblast protein synthesis. Science 236:1465
21. Kalmun AJ (1982) Electric and magnetic field detection in elasmobranch fishes. Science 218:916–918
22. Tenforde TS (1992) Microscopic dosimetry of extremely low-

frequency electric and magnetic fields. Bioelectromagnetics (suppl) 1:61–66
23. McLeod BR, Liboff AR (1987) Cyclotron resonance in cell membranes: the theory of the mechanism. In: Blank M, Findl E, (eds) Mechanistic approaches to interactions of electric and electromagnetic fields with living systems. Plenum Press, New York, 97
24. Halle B (1988) On the cyclotron resonance mechanism for magnetic field effects on transmembrane conductivity. Bioelectromagnetics 9:381–388
25. Adair RK (1991) Constraints on biological effects of weak extremely low-frequency electromagnetic fields. Phys Rev A 43: 1039–1048
26. McLeod BR, Liboff AR, Smith SD (1992) Electromagnetic gating in ion channels. J Theor Biol 158:15–31
27. Bianco B, Chiabrera A (1992) From the Langevin-Lorentz to the Zeeman model of electromagnetic effects on ligand-receptor binding. Bioelectrochem Bioenerg 28:355–363
28. Lednev VV (1991) Possible mechanism for the influence of weak magnetic fields on biosystems. Bioelectromagnetic 12:71–75
29. Edmonds DT (1993) Larmor precession as a mechanism for the detection of static and alternating magnetic fields. Bioelectrochem Bioenerg 30:3–12
30. Bassett CAL, Chohski HR, Hernandez E, Pawluk RJ, Strop M (1979) The effect of pulsing electro-magnetic fields on cellular calcium anc calcification of nonunions. In: Brighton CT, Black J, Pollack SR (eds) Electrical properties of bone and cartilage. Grune and Stratton, New York, pp 427–441
31. Fukayama S, Tashjian AH (1990) Stimulation by parathyroid hormone of ^{45}Ca uptake in osteoblast-like cells: possible involvement of alkaline phosphatase. Endocrinology 126:1941–1949