

# SIGNAL TRANSDUCTION IN ELECTRICALLY STIMULATED BONE CELLS

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**Background:** Electrical stimulation is used to treat nonunions and to augment spinal fusions. We studied the biochemical pathways that are activated in signal transduction when various types of electrical stimulation are applied to bone cells.

**Methods:** Cultured MC3T3-E1 bone cells were exposed to capacitive coupling, inductive coupling, or combined electromagnetic fields at appropriate field strengths for thirty minutes and for two, six, and twenty-four hours. The DNA content of each dish was determined. Other cultures of MC3T3-E1 bone cells were exposed to capacitive coupling, inductive coupling, or combined electromagnetic fields for two hours in the presence of various inhibitors of signal transduction, with or without electrical stimulation, and the DNA content of each dish was determined.

**Results:** All three signals produced a significant increase in DNA content per dish compared with that in the controls at all time-points ( $p < 0.05$ ), but only exposure to capacitive coupling resulted in a significant, ever-increasing DNA production at each time-period beyond thirty minutes. The use of specific metabolic inhibitors indicated that, with capacitive coupling, signal transduction was by means of influx of  $\text{Ca}^{2+}$  through voltage-gated calcium channels leading to an increase in cytosolic  $\text{Ca}^{2+}$  (blocked by verapamil), cytoskeletal calmodulin (blocked by W-7), and prostaglandin  $\text{E}_2$  (blocked by indomethacin). With inductive coupling and combined electromagnetic fields, signal transduction was by means of intracellular release of  $\text{Ca}^{2+}$  leading to an increase in cytosolic  $\text{Ca}^{2+}$  (blocked by TMB-8) and an increase in activated cytoskeletal calmodulin (blocked by W-7).

**Conclusions:** The initial events in signal transduction were found to be different when capacitive coupling was compared with inductive coupling and with combined electromagnetic fields; the initial event with capacitive coupling is  $\text{Ca}^{2+}$  ion translocation through cell-membrane voltage-gated calcium channels, whereas the initial event with inductive coupling and with combined electromagnetic fields is the release of  $\text{Ca}^{2+}$  from intracellular stores. The final pathway, however, is the same for all three signals—that is, there is an increase in cytosolic  $\text{Ca}^{2+}$  and an increase in activated cytoskeletal calmodulin.

**Clinical Relevance:** Electrical stimulation in various forms is currently being used to treat fracture nonunions and to augment spinal fusions. Understanding the mechanisms of how bone cells respond to electrical signals—that is, understanding signal transduction and the metabolic pathways utilized in electrically induced osteogenesis—will allow optimization of the effects of the various bone-growth-stimulation signals.

The concept of electrical stimulation to elicit fracture-healing has a long history, dating from 1812, when direct current was used to elicit the healing of a nonunion of a fracture<sup>1</sup>. Authors of other early reports have also described encouraging results with galvanopuncture (galvanic stimulation delivered through insulated needles) for the treatment of pseudarthrosis<sup>2,3</sup>. Despite many successes, however, the technology disappeared from mainstream medical research by the end of the nineteenth century because claims

regarding its efficacy had remained unsubstantiated.

Fukuda and Yasuda<sup>4</sup> rekindled interest in electrically induced bone growth in 1957 with a description of electrical fields generated by mechanical stress on bone. They suggested that stress on the crystalline components of bone produced a current flow that triggers healing processes. Yasuda<sup>5</sup> demonstrated that electrical signals similar to those generated by mechanical stress could enhance fracture-healing. These reports encouraged both laboratory and clinical research on electrically induced bone formation and healing with use of various forms of electrical stimulation<sup>6</sup>. The clinical effectiveness of bone-growth stimulation proved to be



TABLE I Cellular Proliferation in Response to Electrical Stimulation

Type and Duration of Stimulation	No. of Dishes	DNA/Dish* ( $\mu\text{g}$ )		Increase (%)	P Value†
		Control Group	Experimental Group		
Combined electromagnetic fields					
0.5 hr	10	13.3 $\pm$ 0.6	16.1 $\pm$ 1.1	21	0.05
2 hr	10	13.8 $\pm$ 0.6	16.8 $\pm$ 0.9	22	0.01
6 hr	10	13.6 $\pm$ 0.9	17.0 $\pm$ 1.1	25	0.01
24 hr	10	13.4 $\pm$ 1.0	17.4 $\pm$ 1.2	30	0.001
Inductive coupling					
0.5 hr	6	15.5 $\pm$ 1.0	17.8 $\pm$ 0.7	15	0.05
2 hr	6	16.8 $\pm$ 1.2	19.6 $\pm$ 1.6	17	0.01
6 hr	6	15.9 $\pm$ 1.0	19.2 $\pm$ 0.6	21	0.01
24 hr	6	15.8 $\pm$ 0.7	19.3 $\pm$ 1.0	22	0.001
Capacitive coupling					
0.5 hr	6	33.0 $\pm$ 3.2	38.6 $\pm$ 2.0	17	0.006
2 hr	6	32.7 $\pm$ 2.6	40.2 $\pm$ 2.1	23	0.001
6 hr	6	34.3 $\pm$ 3.1	43.0 $\pm$ 1.7	25	0.001
24 hr	6	33.0 $\pm$ 3.2	49.1 $\pm$ 3.6	49†	0.0001

\*The values are given as the mean and the standard error of the mean. The starting DNA was  $3.0 \pm 0.2 \mu\text{g}/\text{cm}^2$  for all dishes in all groups.

†The level of significance of the difference between the experimental group and the control group. ‡Significantly higher than all of the other groups ( $p = 0.05$ ).

easier to demonstrate than did the mechanism or mechanisms of action of electrically induced osteogenesis. Studies on the treatment of nonunion with direct current<sup>7-9</sup>, inductive coupling<sup>10-13</sup>, and capacitive coupling<sup>14,15</sup> as well as the treatment of spinal fusions with inductive coupling<sup>16</sup> and capacitive coupling<sup>17</sup> largely predated information on the mechanism or mechanisms of action.

The current study was undertaken to determine the biochemical pathways that are activated in signal transduc-

tion when various types of electricity are applied to bone cells. The types of electrical stimulation used were capacitive coupling, inductive coupling, and combined electromagnetic fields. The hypothesis tested was that if the initial transduction site during capacitive-coupling electrical stimulation is at or within the cell membrane and the initial transduction site during inductive-coupling or combined-electromagnetic-fields electrical stimulation is intracellular, then the dose-response of capacitive coupling as well as the

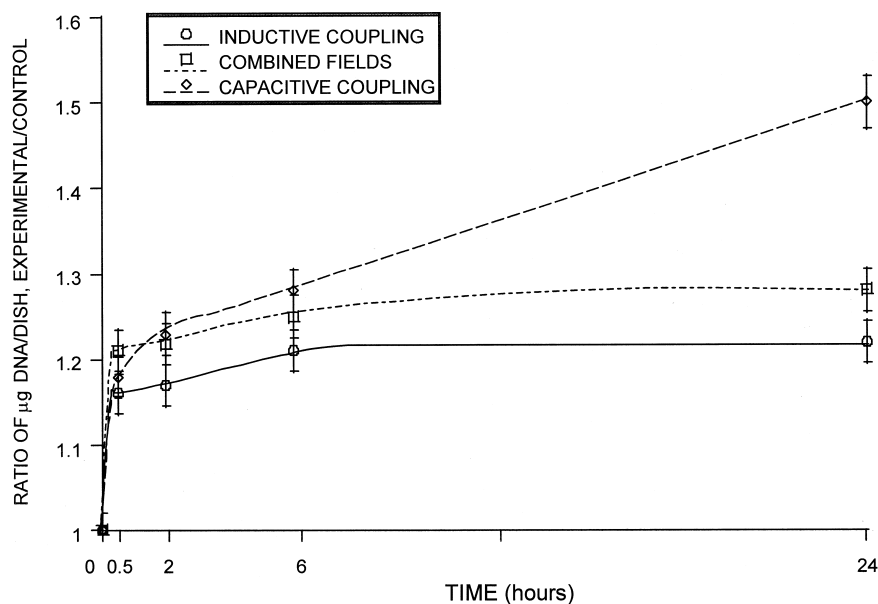


Fig. 1

Graph showing the mean increases in micrograms of DNA per culture dish of MC3T3-E1 bone cells after thirty minutes and after two, six, and twenty-four hours of electrical stimulation with capacitive coupling, inductive coupling, or combined electromagnetic fields. P values are given in Table I. The horizontal bars represent the standard error of the mean.

**TABLE II Cellular Proliferation in Response to Twenty-four Hours of Electrical Stimulation with All Cells Stimulated in Cooper Dishes**

Type of Stimulation	No. of Dishes	DNA/Dish* ( $\mu\text{g}$ )		Increase (%)	P Value	
		Control Group	Experimental Group		Difference Between Control and Experimental Groups	Difference Between Stimulation Groups
Capacitive coupling	18	21.3 $\pm$ 0.4	28.5 $\pm$ 0.5	33.8	<0.0001	<0.0001†
Combined electromagnetic fields	12	20.4 $\pm$ 0.6	24.4 $\pm$ 0.8	20.0	<0.0001	0.006†
Inductive coupling	12	20.1 $\pm$ 0.9	25.6 $\pm$ 1.1	27.4	0.0003	0.48§

\*The values are given as the mean and the standard error of the mean. The starting DNA was 3.0  $\pm$  0.2  $\mu\text{g}/\text{cm}^2$  for all dishes in all groups.  
†Capacitive coupling compared with combined electromagnetic fields. ‡Capacitive coupling compared with inductive coupling. §Combined electromagnetic fields compared with inductive coupling.

signal transduction and biochemical pathways activated by capacitive coupling will be different from that of the other two signals.

## Materials and Methods

### Part A: Dose-Response of Three Signals (Capacitive Coupling, Inductive Coupling, and Combined Electromagnetic Fields)

#### Cell Culture

MC3T3-E1 osteoblastic cells<sup>18</sup> from mice were cultured in 150-mm culture flasks in Dulbecco modified Eagle medium (D-MEM; Life Technologies, Grand Island, New York) supplemented with 10% newborn calf serum in 5% humidified carbon dioxide at 37°C. Media were changed every three days. Prior to confluence, the cells were subcultured and plated at a density of 50,000 cells/cm<sup>2</sup> either onto 35-mm tissue-culture dishes (Corning Glass Works, Corning, New York) for use in both the combined-electromagnetic-fields and inductive-coupling experiments or onto specially modified

Cooper dishes (Falcon, Oxnard, California) for use in the capacitive-coupling experiments. The cells were grown until two days postconfluence, with the media changed just prior to the beginning of the experimental treatments.

#### Capacitive Coupling

Capacitive-coupling electrical stimulation was performed as previously described<sup>19-21</sup>. Bone cells were plated in monolayer on the bottom of modified Cooper dishes, each fitted with glass coverslips on the top and bottom to which stainless-steel electrodes were attached, as previously described<sup>21</sup>. The electrodes were connected to a custom-built function generator with a blocking capacitor in the circuit and then to a power amplifier (model XL-500; Hafler, Tempe, Arizona). The experimental cell cultures throughout these studies were subjected to a 60-Hz sine-wave signal with an output of 44.81 V peak to peak. This produced a calculated electrical field strength in the culture medium of 2.0 V/m with a current density of 300  $\mu\text{A}/\text{cm}^2$ . Control cell-culture dishes were identical to the stimu-

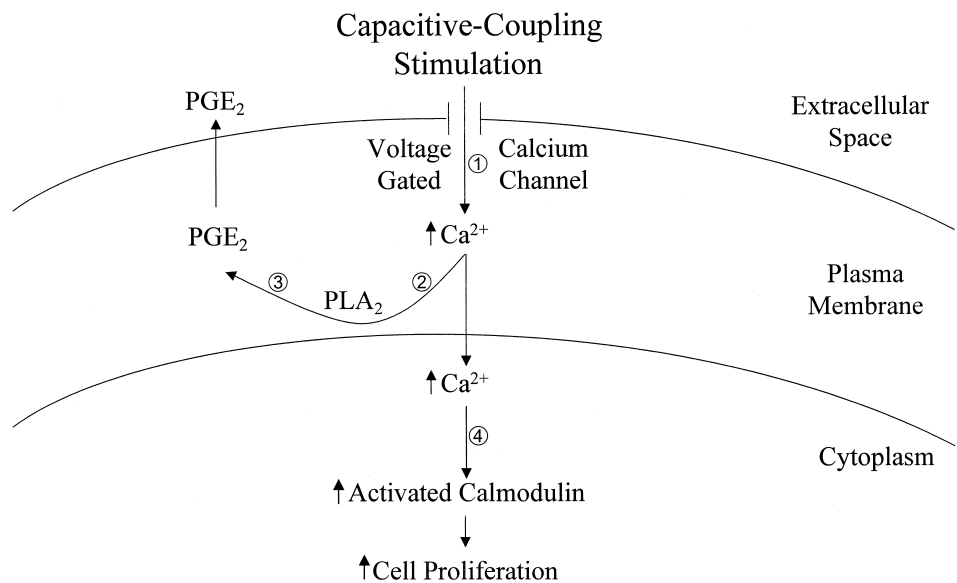


Fig. 2

Schematic drawing depicting the signal transduction pathway followed by capacitive-coupling electrical stimulation. The circled numbers indicate the inhibitor that blocks the pathway at that site: 1 = verapamil, 2 = bromophenacyl bromide, 3 = indomethacin, and 4 = W-7. PGE<sub>2</sub> = prostaglandin E<sub>2</sub>, and PLA<sub>2</sub> = phospholipase A<sub>2</sub>.

lated dishes except that the electrodes were not connected to the generator in the case of capacitive coupling and the electromagnetic fields were not turned on in the case of inductive coupling or combined electromagnetic fields.

#### Inductive Coupling

Inductive-coupling stimulation was generated by a commercial unit (EBI, Parsippany, New Jersey) that is used clinically. There was an arched copper coil over the top of each unit. A 17.8 × 20.2-cm Plexiglas stage held six 35-mm tissue-culture dishes within the unit. The stage was placed in the bottom of the unit at a distance that is typical during clinical use. The pulsed electromagnetic or inductively coupled field consisted of a 15-Hz burst of pulses with twenty pulses per burst and a pulse frequency of 4.3 kHz. The maximum value of the magnetic field amplitude generated at the culture dish was 22.5 ± 2.5 G, and the electrical field amplitude induced in the culture dish at 1.0 cm from the center of the dish was 0.16 ± 0.02 V/m.

#### Combined Electromagnetic Fields

Combined electromagnetic fields were generated by a com-

mercial bone-growth stimulator (OL 1000; OrthoLogic, Phoenix, Arizona) that is used clinically. The unit uses a pair of copper-coil transducers to generate colinear static and time-varying magnetic fields. A 15 × 17.5-cm Plexiglas stage held ten 35-mm tissue-culture dishes within the unit. The stage was placed in the middle of the unit, 9 cm from the top and bottom, a distance from the coils that is typical during clinical use. The combined-electromagnetic-fields condition is made up of a static or direct-current magnetic field combined with a colinear alternating-current sine-wave electromagnetic field. The measured magnetic field strengths used in these experiments were 340 ± 140 mG for the static field and 370 ± 47 mG for the alternating-current sine-wave time-varying magnetic field. The measured frequency of the sine wave was 76.6 Hz. The value of the electric field amplitude induced in each culture dish at a distance of 1.0 cm from the center of the dish was calculated to be 0.89 ± 0.16 × 10<sup>4</sup> V/m.

#### Experimental Design

For all three forms of stimulation, the experimental unit was designed to allow for thirty minutes, two hours, six hours, or

**TABLE III DNA Content per Dish of Bone Cells Stimulated with Various Electrical Fields with or without Inhibitor**

Type of Stimulation and Inhibitor	No. of Dishes	DNA/Dish* (μg)		Increase (%)	P Value†
		Control Group	Experimental Group		
<b>Capacitive coupling</b>					
None	12	19.9 ± 0.8	25.7 ± 0.1	29.1	<0.0001
Verapamil	12	20.1 ± 0.7	21.8 ± 1.2	8.5	NS
Indomethacin	12	19.0 ± 0.8	20.0 ± 0.8	5.3	NS
Bromophenacyl bromide	12	19.8 ± 1.1	20.8 ± 0.8	5.1	NS
W-7	12	21.6 ± 1.1	20.6 ± 1.1	0.0	NS
TMB-8	12	20.8 ± 0.7	26.5 ± 0.8	27.2	<0.05
Neomycin	12	19.8 ± 1.1	25.8 ± 0.5	30.3	<0.001
<b>Inductive coupling</b>					
None	10	16.8 ± 1.2	19.6 ± 1.6	16.7	<0.001
Verapamil	10	12.8 ± 0.5	15.0 ± 0.5	17.2	<0.05
Indomethacin	10	13.1 ± 0.4	16.0 ± 0.8	22.1	<0.01
Bromophenacyl bromide	10	12.7 ± 0.3	16.0 ± 1.0	26.0	<0.01
W-7	10	16.1 ± 0.3	16.0 ± 0.5	0.0	NS
TMB-8	10	13.4 ± 0.6	15.3 ± 1.4	14.2	NS
Neomycin	10	12.7 ± 0.3	15.1 ± 0.4	18.9	<0.05
<b>Combined electromagnetic fields</b>					
None	6	13.8 ± 0.6	16.8 ± 0.9	21.7	<0.02
Verapamil	6	10.5 ± 0.4	13.8 ± 0.5	31.4	<0.01
Indomethacin	6	11.0 ± 0.2	12.6 ± 0.1	14.5	<0.01
Bromophenacyl bromide	6	13.2 ± 0.7	18.6 ± 1.0	40.9	<0.001
W-7	6	10.2 ± 0.5	10.3 ± 0.3	1.0	NS
TMB-8	6	12.7 ± 0.6	13.1 ± 0.2	3.1	NS
Neomycin	6	14.2 ± 0.2	18.0 ± 0.6	26.8	<0.01

\*The values are given as the mean and the standard error of the mean. The starting DNA was 3.0 ± 0.2 μg/cm<sup>2</sup> for all dishes in all groups.

†The level of significance of the difference between the experimental group and the control group. NS = not significant.

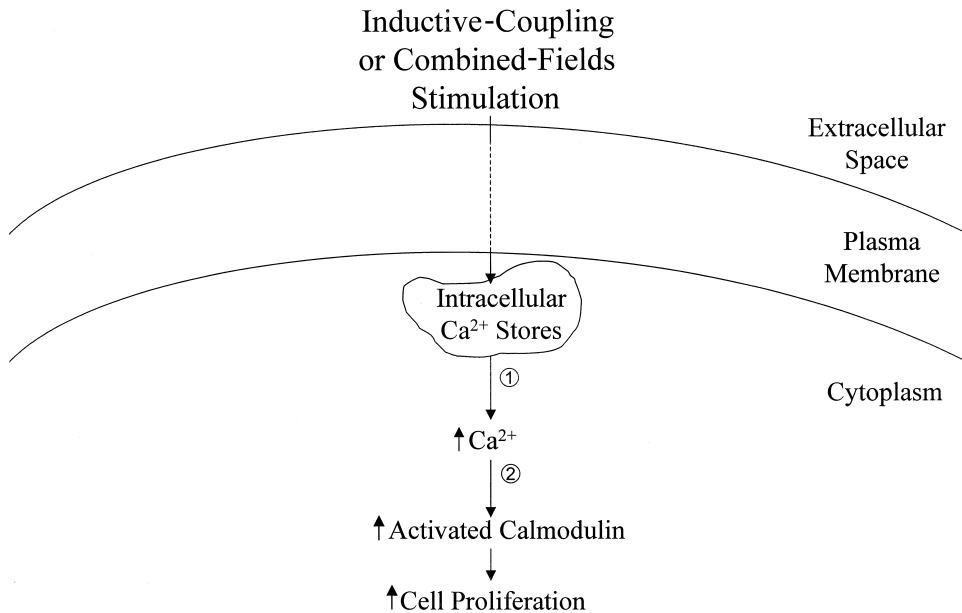


Fig. 3  
Schematic drawing depicting the signal transduction pathway followed by inductive-coupling and combined-electromagnetic-fields electrical stimulation. The circled numbers indicate the inhibitor that blocks the pathway at that site: 1 = TMB-8 and 2 = W-7.

twenty-four hours of stimulation. The units of each experiment were separated in matched incubators and placed in the same position and orientation in each of the incubators. A dual-channel thermometer (Fisher Scientific, Pittsburgh, Pennsylvania) was used to measure temperature within the sample dishes for each unit of each experiment. Preliminary experiments revealed that the temperature in the culture dishes during stimulation with combined electromagnetic fields and with inductive coupling, but not with capacitive coupling, was approximately 0.2°C warmer than that in the control dishes at thirty minutes. Accordingly, during the experiment proper, the temperatures of the incubators containing cells stimulated with combined electromagnetic fields or inductive coupling were adjusted to 36.8°C during the entire

treatment period to compensate for the 0.2°C heating of the media by the coils.

The cells were stimulated for thirty minutes and for two, six, and twenty-four hours and were harvested twenty-four hours after the beginning of stimulation. For example, cells stimulated for thirty minutes remained unstimulated in culture for another 23.5 hours. Control dishes were incubated in the control unit for the same time-period as the experimental dishes. Twenty-four hours after the beginning of stimulation, the cells from the experimental and control groups were harvested, by scraping, into phosphate-buffered saline solution and were used for measuring total DNA content as an index of proliferation<sup>22</sup>. Ten dishes were used at each time-period for each run with combined electromag-

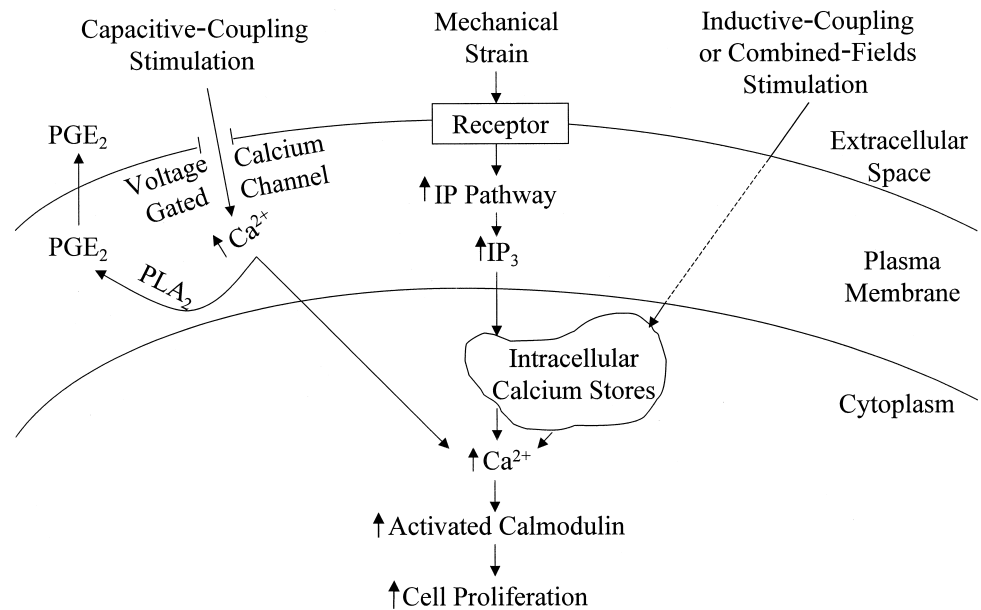


Fig. 4  
Schematic drawing showing the signal transduction pathways followed by the three forms of electrical stimulation compared with that followed by mechanical strain (cyclic, biaxial, 0.17% strain at 1 Hz). PGE<sub>2</sub> = prostaglandin E<sub>2</sub>, PLA<sub>2</sub> = phospholipase A<sub>2</sub>, IP = inositol phosphate, and IP<sub>3</sub> = inositol triphosphate.

TABLE IV Transduction of Electrical Signals in Bone Cells

	Type of Electrical Stimulation		
	Capacitive Coupling	Combined Electromagnetic Fields	Inductive Coupling
Signal transduction inhibitor*			
Verapamil	+	-	-
Indomethacin	+	-	-
Bromophenacyl bromide	+	-	-
W-7	+	+	+
TMB-8	-	+	+
Neomycin	-	-	-
Signal transduction pathway†			
Ca <sup>2+</sup> channel	+	-	-
Prostaglandin E <sub>2</sub>	+	-	-
Phospholipase-prostaglandin E <sub>2</sub>	+	-	-
Activated calmodulin	+	+	+
Intracellular Ca <sup>2+</sup> release	-	+	+
Inositol phosphate pathway	-	-	-

\*+ = inhibitor prevented increased cellular proliferation caused by electrical stimulation, and - = inhibitor did not prevent increased cellular proliferation caused by electrical stimulation. †+ = signal transduction pathway activated by electrical stimulation, and - = signal transduction pathway not activated by electrical stimulation.

netic fields, and six dishes were used at each time-period for each run with inductive coupling and for each run with capacitive coupling. Each run was repeated three or four times with each method of stimulation for the thirty-minute and twenty-four-hour time-periods, and each run was repeated two, three, or four times with each method of stimulation for the two and six-hour time-periods.

Because the Cooper dishes were of a different size, shape, and material (plastic and glass rather than plastic alone) than the 35-mm tissue-culture dishes, an additional experiment was done to make sure that any differences noted between capacitive coupling and inductive coupling or combined electromagnetic fields were real. In this experiment, all cultures, experimental and control, for assessment of all three signals were performed in Cooper dishes. The cells were grown until two days postconfluence as described above, and then all cultures were stimulated for twenty-four hours with capacitive coupling, inductive coupling, or combined electromagnetic fields as described above. At the end of the twenty-four-hour stimulation period, the cells were harvested and the DNA content per dish was determined as described above. The experiment was run three times with a total of eighteen dishes for capacitive coupling and twelve each for inductive coupling and combined electromagnetic fields.

### Part B: Transduction of the Three Signals

#### Signal Transduction Inhibitors

Six signal transduction inhibitors were used: verapamil (Sigma, St. Louis, Missouri), which blocks voltage-gated calcium chan-

nels in the cell membrane<sup>23</sup>; neomycin (Pharma-Tek, Huntington, New York), which blocks the inositol phosphate pathway in the cell membrane by inhibiting phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>)<sup>24</sup>; bromophenacyl bromide (Sigma), which inhibits phospholipase A in the cell membrane<sup>25</sup>; TMB-8 (Sigma), which inhibits Ca<sup>2+</sup> release from intracellular stores<sup>26</sup>; indomethacin (Sigma), which inhibits prostaglandin synthesis in the cell membrane<sup>27</sup>; or N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7; Sigma), a calmodulin antagonist<sup>28</sup>. The concentrations of the inhibitors used in this study, which were the same as those used in previous studies, were 20 μM of verapamil, 10 μM of neomycin, 15 μM of bromophenacyl bromide, 125 μM of TMB-8, 4 μg/mL of indomethacin, and 1 μM of W-7<sup>29,30</sup>. At these concentrations, the inhibitors had no effect on cell proliferation in control, nonstimulated cells. The total DNA content was determined in the absence and presence of the specific intracellular inhibitors in bone cells stimulated by the three signals.

#### Experimental Design

The bone cells were grown in the media and under the environmental conditions described above until two days postconfluence. At that time, the media were changed and each capacitive-coupling experimental run was divided into four groups: control, stimulated, control and inhibitor, and stimulated and inhibitor. Each combined-electromagnetic-fields and inductive-coupling experimental run was divided into

three groups: control, control and inhibitor, and stimulated and inhibitor. Inhibitor was added to the appropriate cultures at the concentrations presented above, and 50 µg/mL of sodium ascorbate was added to all cultures. The cells were stimulated for two hours. Twenty-four hours after the beginning of stimulation, the cells were harvested and were used for total DNA analysis. For each run with six inhibitors, six to twelve dishes were used for each time-period, and each run was repeated two, three, or four times.

#### Statistical Analysis

All data were analyzed with use of one-way analysis of variance and the Tukey-Kramer multiple-comparisons test for significant differences between groups.

### Results

#### Part A: Effects of the Three Signals on Cell Proliferation

All three signals produced significant increases in bone-cell DNA per dish compared with that in the controls at all time-points ( $p < 0.05$ ) (Table I and Fig. 1). However, use of capacitive coupling resulted in a significant, ever-increasing DNA production (17%, 23%, 25%, and 49%) compared with that in the controls at each time-period (thirty minutes, two hours, six hours, and twenty-four hours, respectively), whereas use of the other two signals resulted in only a minimal increase in DNA production after the first thirty minutes of stimulation (15%, 17%, 21%, and 22% for inductive coupling and 21%, 22%, 25%, and 30% for combined electromagnetic fields, respectively). The proliferative response of bone cells to capacitive coupling after twenty-four hours of stimulation increased the production of DNA by 123% compared with that in the cells exposed to inductive coupling fields ( $p < 0.05$ ) and by 63% compared with that in the cells exposed to combined electromagnetic fields ( $p < 0.05$ ).

In the experiment in which bone cells exposed to the three different signals for twenty-four hours were all grown in Cooper dishes in order to rule out any influence that the size, structure, or material of the culture dish might have on the results, the cells exposed to the capacitively coupled signal showed a significant increase in DNA content per dish compared with those stimulated with either inductive coupling ( $p = 0.006$ ) or combined electromagnetic fields ( $p < 0.0001$ ) (Table II).

#### Part B: The Effects of Signal Transduction Inhibitors on Cell Proliferation

The increase in cellular proliferation caused by capacitive-coupling stimulation was inhibited by blocking voltage-gated calcium channels with verapamil, by blocking either phospholipase A<sub>2</sub> with bromophenacyl bromide or prostaglandin synthesis with indomethacin, or by blocking Ca<sup>2+</sup> activation of cytoskeletal calmodulin with W-7. Neither blocking the release of Ca<sup>2+</sup> from intracellular stores with TMB-8 nor blocking the inositol phosphate pathway in the cell membrane with neomycin had any effect on cell proliferation (Table III).

None of the metabolic blockers (verapamil, bromophenacyl bromide, indomethacin, or neomycin) that act within the cell membrane had any effect on the increase in cellular proliferation produced by inductive coupling or combined electromagnetic fields. Inhibiting the release of Ca<sup>2+</sup> with TMB-8 or inhibiting the activation of cytoskeletal calmodulin with W-7, however, did block the increase in cellular proliferation otherwise produced by either of these two signals (Table III).

The data above indicate that the signal transduction pathway activated by the various forms of electrical stimulation is the pathway that is inhibited by a specific metabolic inhibitor or inhibitors (Table III). Thus, for capacitive coupling, signal transduction is by means of Ca<sup>2+</sup> ion translocation through cell membrane voltage-gated calcium channels leading to increases in prostaglandin E<sub>2</sub>, cytosolic Ca<sup>2+</sup>, and activated cytoskeletal calmodulin (Table IV and Fig. 2). For both inductive coupling and combined electromagnetic fields, signal transduction is by means of the intracellular release of Ca<sup>2+</sup> leading to increases in cytosolic Ca<sup>2+</sup> and activated cytoskeletal calmodulin (Table IV and Fig. 3).

### Discussion

The results in Part A on the effect of electrical stimulation on cell proliferation indicated that all three electrical signals produced a significant increase in total bone-cell DNA per dish compared with that in the controls. However, the fact that capacitive-coupling stimulation resulted in a significant, ever-increasing DNA production at each time-period up to twenty-four hours, while combined-electromagnetic-fields and inductive-coupling stimulation produced only minimal increases in DNA production beyond the first thirty minutes, supported the concept that the dose-response of capacitive coupling is different from that of the other two signals. This encouraged us to proceed with Part B of the study, in which various metabolic inhibitors were used to block specific signal transduction pathways in order to determine the mechanism of signal transduction for each form of electricity used in the study.

The results in Part B indicated that transduction of a capacitively coupled electrical signal is by means of Ca<sup>2+</sup> ion translocation through voltage-gated calcium channels (blocked by verapamil) leading to an increase in phospholipase A<sub>2</sub> (blocked by bromophenacyl bromide) and to an increase in cytosolic Ca<sup>2+</sup>. The increase in phospholipase A<sub>2</sub> leads to an increase in prostaglandin E<sub>2</sub> synthesis (blocked by indomethacin), and the increase in cytosolic Ca<sup>2+</sup> leads to an increase in activated (cytoskeletal) calmodulin (blocked by W-7) (Table IV). These findings are in agreement with those in our previous report<sup>30</sup>. Activated calmodulin is known to promote nucleotide synthesis and cellular proliferation<sup>31,32</sup>. Prostaglandin E<sub>2</sub> acts as an autocrine and/or paracrine factor to stimulate bone-cell proliferation and possibly to increase intracellular calcium<sup>33</sup>.

The results in Part B also showed that the transduction of combined electromagnetic fields and inductively coupled signals is by means of the intracellular release of Ca<sup>2+</sup> from in-

tracellular stores (blocked by TMB-8) leading to an increase in cytosolic  $\text{Ca}^{2+}$  that, in turn, leads to an increase in activated calmodulin (blocked by W-7) and a subsequent increase in bone-cell proliferation. Thus, although the initial transduction site with capacitively coupled stimulation (voltage-gated calcium channel  $\text{Ca}^{2+}$  influx into the cell) is different from that with stimulation with combined electromagnetic fields and with inductive coupling (intracellular release of  $\text{Ca}^{2+}$ ), all three methods of stimulation have a common final pathway—that is, an increase in cytosolic  $\text{Ca}^{2+}$  and an increase in activated calmodulin. However, the precise mechanism by which the electrical and electromagnetic fields are transduced at these sites is not yet understood in terms of a rigorous model. Also, one should be cautioned that an *in vitro* state, in which isolated bone cells are grown under exacting conditions, is an artificial environment; such conditioned cells may respond only in a limited way by following limited biochemical pathways in response to limited stimulation. The same cells in their natural setting *in vivo* are exposed to a myriad of different upregulating and downregulating signals and thus may respond differently from those described in the present study.

It is interesting to note that bone cells also respond to mechanical strain with an increase in intracellular  $\text{Ca}^{2+}$ . We demonstrated that bone cells subjected to a biaxial, cyclic mechanical strain of 0.17% showed an increase in intracellular  $\text{Ca}^{2+}$  through a release from intracellular stores that was due to activation of the inositol phosphate cascade (blocked by neomycin) in the cell membrane<sup>34</sup>. An increase in inositol triphosphate stimulated an intracellular  $\text{Ca}^{2+}$  release that, in turn, led to an increase in activated calmodulin (blocked by W-7) and a subsequent increase in cellular proliferation (Fig. 4)<sup>34</sup>. More recently, a group of investigators showed that a fluid shear-induced mechanical signal in osteoblasts leads to increased expression of cyclooxygenase-2/c-Fos through a mechanism that involves reorganization of the cytoskeleton<sup>35</sup>. The same group later showed that these fluid shear-induced responses were due to inositol-triphosphate-mediated intracellular  $\text{Ca}^{2+}$  release that was blocked by neomycin<sup>36</sup>. Thus, all three forms of electrical stimulation as well as mechanical strain led, within the limitations of these experiments at least, to the same common pathway, an increase in cytosolic  $\text{Ca}^{2+}$  and an increase in activated cytoskeletal calmodulin.

It is apparent from the above discussion that the initial transduction of a capacitive coupling signal is at or within the bone-cell membrane, whereas the initial transduction of either combined electromagnetic fields or inductive coupling is within or upon intracellular calcium stores (for example, the endoplasmic reticulum). The time-varying electromagnetic fields of the inductive-coupling and combined-electromagnetic-fields signals pass through the bone-cell membrane to set up a time-varying electrical field within the cytosol that, in turn, brings about the release of intracellular  $\text{Ca}^{2+}$ . One possible explanation for the differences seen in the dose-response curves of capacitive coupling compared with those of combined electromagnetic fields and inductive coupling is that the intracellular store of  $\text{Ca}^{2+}$  is limited compared with the infinite

amount of  $\text{Ca}^{2+}$  ions in the extracellular fluid available to enter the bone cell by means of activation of voltage-gated calcium channels in the cell membrane.

Several other investigators have studied bone-cell second messengers that are activated by various electrical fields. Studies have shown an increase in cAMP, little change in cAMP, or even a decrease in cAMP when bone cells have been exposed to various electrical fields<sup>37-39</sup>. An increase in ornithine decarboxylase following electrical stimulation of bone cells has also been recorded<sup>37</sup>. However, the most telling evidence to date is the increase in prostaglandin  $\text{E}_2$ <sup>38</sup> and cytosolic calcium<sup>30,40,41</sup> as the predominant second messengers in electrically induced osteogenesis. The current study certainly supports those findings.

Other investigators have looked farther downstream in the metabolic pathway of electrically stimulated bone cells to assess the influence of electricity on growth factors. Fitzsimmons et al.<sup>42-46</sup> showed that low-amplitude, low-frequency capacitively coupled signals or combined-electromagnetic-fields signals led to an increase in insulin-like growth factor (IGF)-II mRNA accumulation, IGF-11 secretion, and IGF-11 receptor number as well as a net calcium flux in TE-85 osteosarcoma cells. In a previous study, we showed that capacitively coupled electrical fields increased transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ) mRNA in MC3T3-E1 bone cells and that this increase was blocked by verapamil and W-7<sup>47</sup>. This result suggests that electrical stimulation delivered by capacitive coupling induces an increase in TGF- $\beta_1$  mRNA in osteoblastic cells by a mechanism involving the cytosolic  $\text{Ca}^{2+}$ /calmodulin pathway.

The above studies provide insight into the biochemical events that occur in the transduction of electrical signals used to stimulate healing of fracture nonunions and to enhance spinal fusions. Our data support the hypothesis that differences in the dose-response (bone-cell proliferation) of the various forms of electrical stimulation are due to differences in signal transduction.

There is now solid evidence that there are distinct transduction pathways for mechanical stimulation and that electrical stimulation with capacitive coupling, inductive coupling, and combined electromagnetic coupling leads to a proliferative response of bone cells. Moreover, the pathways are complementary in that they all lead to an increase in cytosolic  $\text{Ca}^{2+}$  and activated calmodulin. Electrical stimulation is finally moving beyond the “black box” image that it has had for so many years. These studies provide a theory of basic cellular mechanisms to augment the clinical reports of the efficacy of electrical stimulation. ■

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