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Extracellular Currents Alter Gap Junction Intercellular Communication in Synovial Fibroblasts

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We studied the effect of extremely low frequency (ELF) currents on gap junction intercellular communication (GJIC) mediated by connexin43 protein. Confluent monolayers of synovial fibroblasts (HIG-82) and neuroblastoma cells (5Y) were exposed in bath solution to 0–75 mA/m^2 (0–56 mV/m), 60 Hz. Single channel conductance, cell membrane current-voltage (I-V) curves, and Ca^{2+} influx were measured using the nystatin single and double patch methods. The conductances of the closed and open states of the gap junction channel in HIG-82 cells were each significantly reduced (by 0.76 and 0.39 pA, respectively) in cells exposed to 20 mA/m^2. Current densities as low as 10 mA/m^2 significantly increased Ca^{2+} influx in HIG-82 cells. No effects were seen in 5Y cells. The I-V curves of the plasma membranes of both types of cells were independent of 60 Hz electric fields and current densities, 0–75 mA/m^2, indicating that the effect of the 60 Hz fields on GJIC in HIG-82 cells was not mediated by a change in membrane potential. We conclude that ELF electric fields can alter GJIC in synovial cells via a mechanism that does not depend on changes in membrane potential, but may depend on Ca^{2+} influx. The results open the possibility that GJIC mediated responses in synovial cells, such as for example, their secretory responses to proinflammatory cytokines, could be antagonized by the application of ELF electric fields. Bioelectromagnetics 24:199–205, 2003.

Key words: connexin43; nystatin; membrane potential; neuroblastoma

INTRODUCTION

Exposure to low frequency electromagnetic fields (EMFs) has been reported to ameliorate pain perception in patients [Stone and Wharton, 1997; Pridmore and Oberoi, 2000; Hurley et al., 2001; Lefaucheur et al., 2001] and in various animal models [Leem et al., 1995; Prato et al., 1995; Jeong et al., 2000; Ma and Sluka, 2001], but little is known about the responsible mechanisms. One possibility is that the EMFs produced analgesia by directly affecting the cells of the nervous system. However, EMFs did not alter the resting membrane potential in an in vitro model of an excitable cell [Sonnier et al., 2000], and it seems unlikely that the weak EMFs typically used in the pain studies could alter the properties of the ion channels that produce the action potential [King, 1999]. However, specialized neuroepithelial cells could mediate the effect of fields, as is the case in some elasmobranch and teleost fishes [Fessard, 1974; Kalmijn, 2000]. Another potential explanation is that the EMFs interact with nonexcitable cells such as fibroblasts or macrophages that produce and respond to proinflammatory cytokines. In principle, a reduction in inflammation could account for the reduced perception of pain associated with exposure to EMFs.

Gap junctions are protein structures that electrically and metabolically connect the interiors of adjacent cells [Brink, 1996]. The functional state of gap junction

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channels can be modified dynamically (''gating''), resulting in restriction or augmentation of gap junction intercellular communication (GJIC). Gating mechanisms include phosphorylation of connexin protein, elevation of cAMP and cGMP levels, acidification, and changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [Bruzzone et al., 1996; Kumar and Gilula, 1996]. We showed that gap junctions occurred between synovial fibroblasts in vitro and between synovial cells that line the inside of the joint capsule [Kolomytkin et al., 1997, 2000].We also showed that GJIC was necessary for the cells to secrete proteins in response to stimulation with IL-1β [Kolomytkin et al., 1997, 1999, 2002]. A change in GJIC is therefore a critical process in the proinflammatory secretory pathway of these important cells.

A possible role for GJIC in explaining the effects of EMFs was suggested previously, based on the idea that gap junctions could permit cells to integrate an external electric field, leading to changes in membrane potential (V_m) and ultimately in cellular expression [Pilla et al., 1994]. A difficulty with this view of a link between EMFs and GJIC is that cell aggregates as large as 1–10 mm would result in V_m changes of only about 1 mV. Even then, the changes would be expected only if the aggregate was oriented along the direction of the field and would occur in only a relatively few number of cells at the edges of the aggregate.

If GJIC were involved in the process by which cells respond to fields, it seemed reasonable to us to expect that changes in GJIC would occur even in small aggregates and in the absence of changes in V_m. This hypothesis was tested here by studying the effects of weak extracellular currents on GJIC, using the double patch technique. We observed changes in GJIC due to EMFs and then studied how the changes might be related to the fields; we found evidence against the involvement of ΔV_m and in favor of a role for Ca^{2+} influx.

METHODS

Cells

HIG-82 cells (ATCC, Manassas, VA), a fibroblast line derived from rabbit synovium, were grown at 37 °C, 5% CO_2, without antibiotics in 25 ml polystyrene flasks containing F12 medium with 10% fetal bovine serum. For passage of cells, confluent cultures were trypsinized (1 ml, 0.08%), 3–5 min after which 4 ml of medium was added and the suspended cells were centrifuged, resuspended, and then seeded (10^6 cells) into 4 ml of medium. For electrophysiological measurements, 10^5 cells were added to 35 mm petri dishes and treated with hyaluronidase to facilitate patch formation [Kolomytkin et al., 1997]. All measurements were made in bath solution at 25 °C.

The human post-ganglionic neuroblastoma cell line SH-SY5Y (5Y) (ATCC) was used as a control for the effect of low frequency current on GJIC. The 5Y cells were grown under the same conditions as the HIG-82 cells, except for the addition of dibutyryl cAMP (50 μM, Sigma, St. Louis, MO) to the growth media to produce the electrically excitable phenotype [Toselli et al., 1996]. We showed previously that 5Y cells were connected by gap junctions [Sonnier et al., 2000].

Electrical Measurements

The nystatin perforated patch, whole cell recording method was used to measure currents under voltage clamp [Horn and Marty, 1988]. Because nystatin interfered with giga-seal formation, the tip of the pipette was filled with nystatin free solution prior to the addition of solution containing nystatin (0.3 μg/ml); the giga-seals were formed during the time needed for the nystatin to diffuse to the tip of the micropipette. Giga-seals (>10 GΩ) were formed under negative pressure (5–10 cm H_2O), typically within 0.5–5 min at a success rate greater than 50%. After giga-seal formation, the negative pressure was removed and nystatin channels were permitted to form. The giga-seals and nystatin channels remained stable for hours.

Single gap junction channel currents were measured between two aggregated cells by means of the double patch method [Neyton and Troutmann, 1985], using patch clamp amplifiers (Axopatch 10, Axon Instruments, Foster City, CA) (Fig. 1). A 20 kHz filter was used to reduce background current noise. Current-voltage (I-V) curves were obtained by the single patch method; currents were measured while applying a series of voltage steps to the cell membrane in increments of 10 mV and also by applying a voltage ramp. The initial voltage level was chosen close to the reversal

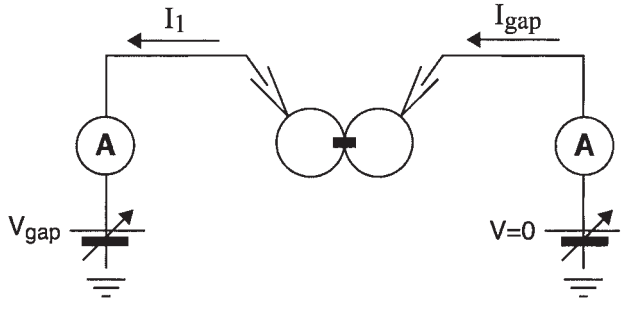

Fig. 1. Equivalent circuit for measurement of current through a gap junction channel (I_{gap}) between 2 patched cells in a 2 cell aggregate. I_1, sum of transmembrane and gap junction currents.

potential. The role of voltage dependent Ca^{2+} channels in the membrane was studied by measuring integral Ca^{2+} current, with 10 mM Ba^{2+} in the bath solution as the current carrier [Tateyama et al., 2001]. The data was stored on a hard drive and on magnetic tape and analyzed using commercial software (pClamp 8, Axon Instruments).

Electrodes and Solutions

Glass capillaries 1.0 mm in diameter were pulled in two steps (PB-7, Narishige) to obtain a tip diameter of ~1.0 μm, and then fire polished in a microforge (MF-9 Narishige). In the dual patch and I-V studies the pipette solution was (in mM) K-aspartate, 125; KCl, 30; NaCl, 4; $CaCl_2$, 1; HEPES-KOH, 10; pH, 7.2 (7–9 MΩ). The bath solution was (in mM) NaCl, 145; KCl, 5.4; $CaCl_2$, 1.5; $MgCl_2$, 1.0; HEPES-NaOH, 5.0; glucose, 5.0; pH, 7.3. In the Ca^{2+} studies, the pipette solution was (in mM) Cs acetate, 153; HEPES-CsOH, 10. The bath solution for the HIG-82 cells was (in mM) Ba acetate, 20; TEA-Cl, 135; glucose, 5; $MgCl_2$, 1; HEPES-TEA OH, 5; pH, 7.4. The bath solution for the neuroblastoma cells was (in mM) $BaCl_2$, 10; TEA-Cl, 145; glucose, 2; $MgCl_2$, 2; TTX, 0.001; HEPES-TEA OH, 5; pH, 7.4. The conductivity of the bath solutions was 1.3 S/m.

Application of 60 Hz Currents

Current was obtained from an autotransformer and measured with a multimeter (Model 175, Keithley, Cleveland, OH). The current was applied using silver/silver-chloride electrodes that were chemically isolated from the bath solution by salt bridges.

Statistics

Open and closed state probabilities were analyzed by fitting the data to a sum of two gaussian curves using the method of least squares (Matlab, Natick, MA). The data was evaluated using the *t*-test.

RESULTS

Intercellular Communication

Adjacent cells in 2 cell aggregates were patched to permit direct measurement of the intercellular current (Fig. 1). At $V_{gap} \cong -80$ mV only one gap junction channel remained open, thereby permitting the characteristics of a single channel to be measured. Typical results for the intercellular current between two cells in the presence and absence of 20 mA/m^2 (15 mV/m), 60 Hz in the bath solution are shown in Figure 2A. Multiple, apparently stochastic, openings and closings

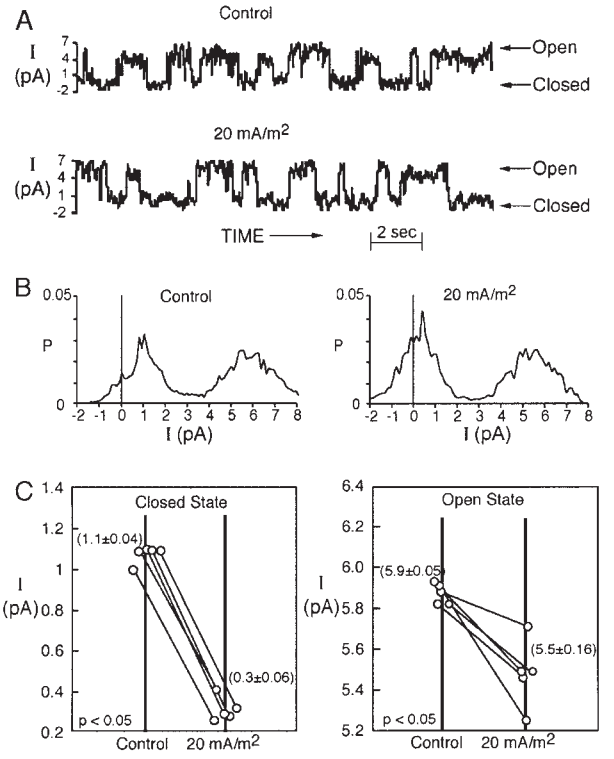

Fig. 2. Effect of 60 Hz current through the bath solution on gap junction intercellular communication between synovial cells. **A**: Single channel currents between one pair of cells. **B**: Corresponding probability densities. **C**: Effect of 60 Hz current on the location of the peak of the probability density (N = 5). The corresponding results for each pair of cells are shown joined by a solid line. The horizontal position of the points in each group (mean ± SD SD shown in parentheses) has been shifted slightly, for clarity.

of the channel were observed, corresponding to the position of the channel gate. When open, 5–6 pA passed through the channel; when the gate was closed, the current was 0–1 pA. The probability density of the two states for a particular cell pair is shown in Figure 2B. Application of 20 mA/m^2 to the bath solution significantly decreased the conductance of both the closed and open states, as determined from the shifts of the peaks in the probability density distributions (N = 5) (Fig. 2C). The same 60 Hz current density had no effect on intercellular communication between 5Y cells (Fig. 3).

Current-Voltage Curves

One of the cells in 2–4 cell aggregates was patched, and its I-V curve was measured in the absence and presence of 60 Hz current in the bath solution. For membrane voltages more negative than about −65 mV the transmembrane current was negative, indicating that the current flowed out of the cell. At about −65 mV, there was no net current flow. For membrane voltages

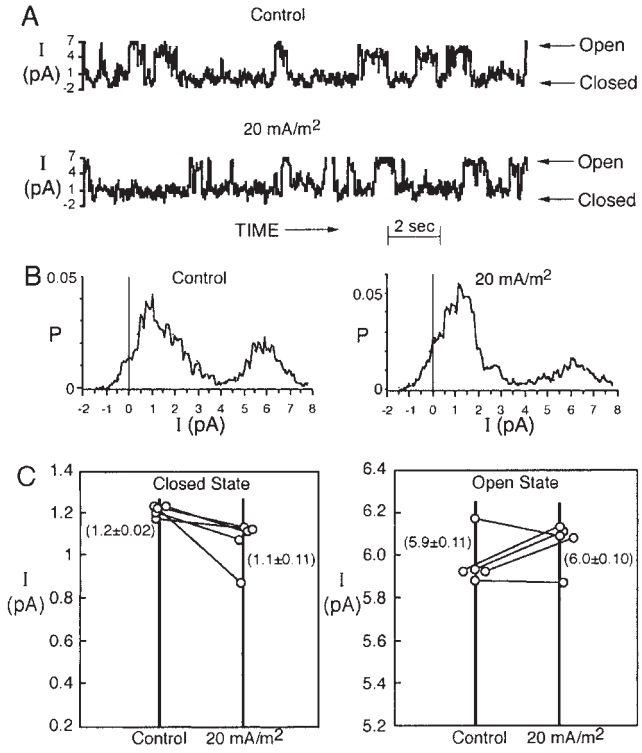

Fig. 3. Effect of 60 Hz currents through the bath solution on gap junction intercellular communication between neuroblastoma cells. **A**: Single channel currents between one pair of cells. **B**: Corresponding probability densities. **C**: Effect of 60 Hz current on the location of the peak of the probability density (N = 5). The corresponding results for each pair of cells are shown joined by a solid line. The horizontal position of the points in each group (mean ± SD SD shown in parentheses) has been shifted slightly, for clarity.

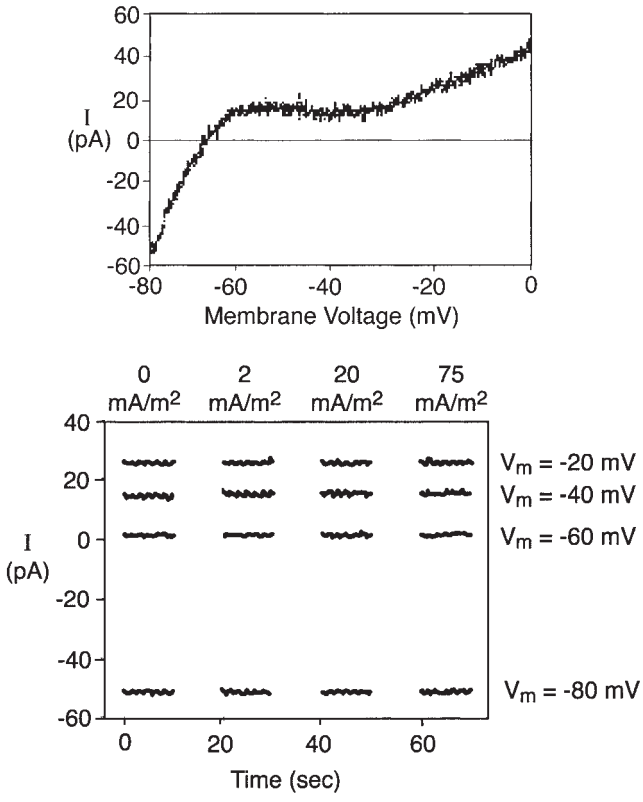

Fig. 4. Effect of 60 Hz currents through the bath solution on the I-V curve of synovial fibroblasts. **Top**: I-V curve of a cell in the absence of 60 Hz current. **Bottom**: effect of applied current. Negative current indicated flow into the cell.

more positive, the net current flow was into the cell. The I-V curve was unaffected by 0–75 mA/m^2 (0–56 mV/m) (Fig. 4). The experiment was repeated three times, and the result was the same in each instance.

Calcium Influx

To study the effect of 60 Hz currents on Ca^{2+} influx, the Na^+ and K^+ currents were eliminated by controlling the ionic composition of the bath and micropipette solutions, and Ba^{2+} was substituted for Ca^{2+} because Ca^{2+} channel inactivation occurs too quickly to be measured conveniently [Tateyama et al., 2001]. The membrane voltage was changed instantaneously from − 60 to + 50 mV in increments of 10 mV, and the corresponding changes in current were measured. As expected, the transmembrane current depended on the magnitude of the voltage step (Fig. 5); when 60 Hz currents were applied to the bath solution surrounding the cell, an increase in the flow of current through calcium channels was observed (N = 5). When the experiment was repeated using 5Y cells (N = 5), no effect on Ca^{2+} influx was seen (not shown).

DISCUSSION

In rabbit and human synovial cells, the gap junction protein is connexin43 [Capozzi et al., 1999; Waddell et al., 2002]. The open and closed state conductance of the connexin43 gap junction channel in rabbit synovial cells was consistently decreased during application of extracellular 60 Hz currents (Fig. 2). The average decrease (range) in conductance of the closed and open states were 0.76 pA (0.68–0.82 pA) and 0.39 pA (0.17–0.66 pA), respectively.

Our measurement method entailed the presence of a microelectrode and a high input impedance preamplifier in the immediate vicinity of the time varying field that created the currents in the bath solution. It might be suggested that the field directly affected the microelectrode or preamplifier, thereby accounting for the observed changes in current. For two reasons,

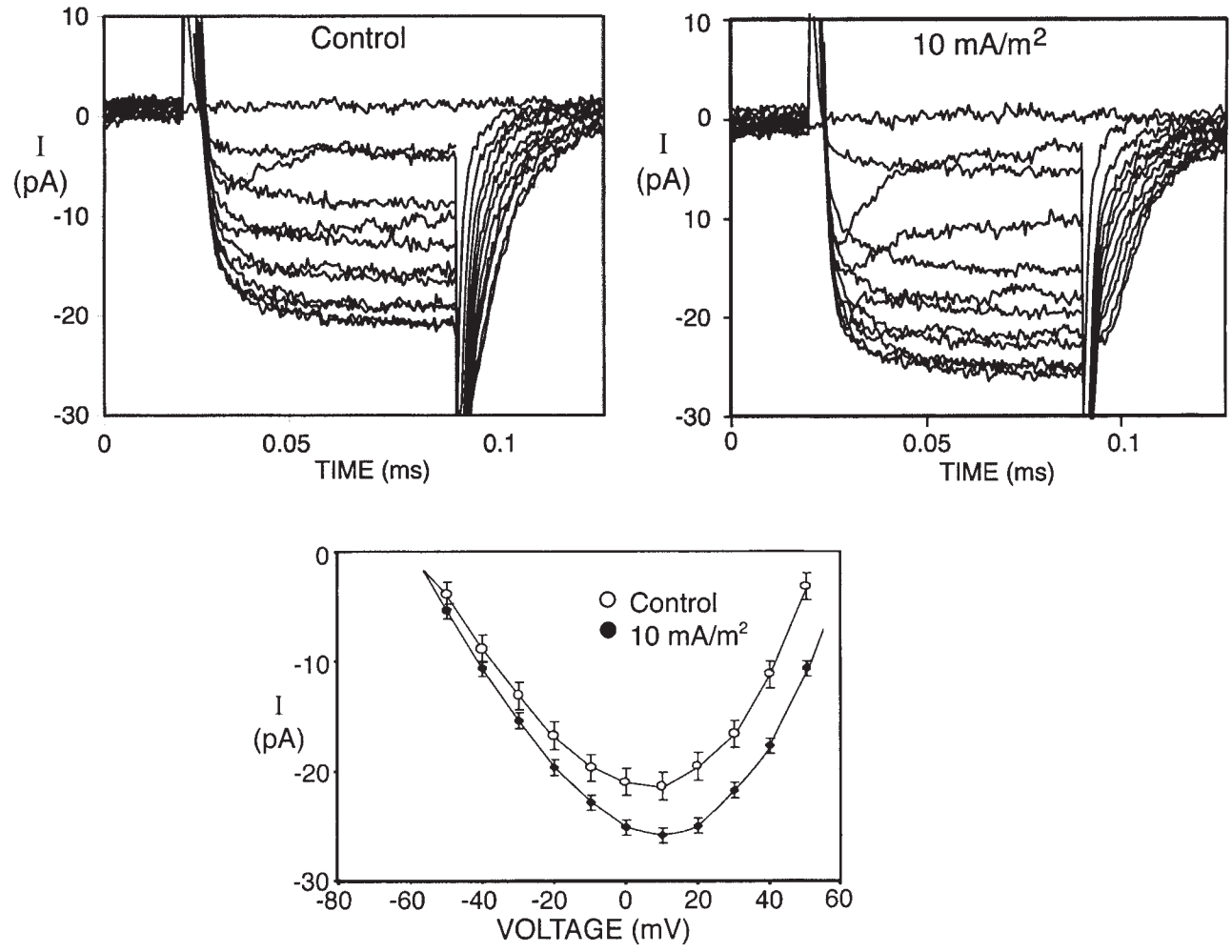

Fig. 5. Effect of 60 Hz currents through the bath solution on Ca^{2+} influx. **Top**: Transmembrane Ca^{2+} currents in HIG-82 cells. The current that flowed in response to a voltage step (-60 to $+50$ mV in increments of 10 mV) was measured (negative current into the cell). Each current trace was averaged over 5 equal voltage steps. Voltage peaks at the beginning and end of each current trace are artifacts. **Bottom**: Average results from 5 cells. Negative current indicated flow into the cell.

however, we think the explanation is unlikely. First, the change in closed state conductance varied from cell to cell. All geometrical relations and local field intensities were identical for a given cell during its open and closed state measurements. Consequently, the differential effect of the field on open state conductance, as evidenced by both the smaller magnitude of the change and its variability in relation to the closed-state change, are better explained under the assumption that the changes in current were physiological in origin.

Second, any putative effect on the measurement of channel current due to an interaction between the field and the measuring system should have also occurred during the measurements on the 5Y cells, whereas the contrary was observed (Fig. 3). Overall, therefore, we conclude that the changes in current that occurred during the presentation of the field were due to true changes in open and closed state channel conductances.

GJIC in neuroblastoma cells is also mediated by connexin43 [Carystinos et al., 2001], but only the synovial cells exhibited changes in GJIC in response to extracellular 60 Hz currents, showing that the interaction between the 60 Hz currents and the synovial cell membrane led to an intracellular signal that could regulate connexin43 in synovial cells, but not in neuroblastoma cells. This suggests that the effects of the 60 Hz currents on channel conductance were not simply nonspecific changes in cell physiology.

We found no evidence to support the hypothesis that the effect on GJIC between synovial cells was mediated by changes in V_m. Despite the application of 60 Hz currents almost 4 times those shown to affect GJIC, the I-V curve of the cell membrane was unaffected (Fig. 4). We estimate that we would have seen a change in the resting membrane potential (Fig. 4, voltage at zero current) of approximately 400 μV. We conclude, therefore, that the change in GJIC was probably not mediated by a change in V_m, as expected.

The presence of extracellular 60 Hz currents increased Ca^{2+} influx; the effect was seen for current densities as low as 10 mA/m^2 (Fig. 5). Ca^{2+} influx is an early process in many cell signaling cascades including the response of HIG-82 cells to stimulation with the proinflammatory cytokine interleukin-1β (IL-1β) [Kolomytkin et al., 1999]. Increased $[Ca^{2+}]_i$ regulates connexin43 in a variety of cell types. One possibility, therefore, is that the Ca^{2+} influx mediated the effect of the 60 Hz currents on GJIC.

It has long been suspected that the effects of EMFs were mediated by events that occurred at the cell membrane [Adey, 1981]. It has proved elusive, however, to establish what specific biological events at the membrane were critical in the transduction process. One theory is that a change in V_m was a critical step, and speculation about the role of GJIC was largely intended to answer the objection that EMF induced changes in V_m were negligible in comparison with stochastic fluctuations [Pilla et al., 1994]. Here, for the first time, we report evidence that changes in cellular physiology due to extracellular currents can occur in the absence of changes in V_m (at least above about 400 μV).

Changes in GJIC mediate the response of synovial cells to IL-1β [Kolomytkin et al., 2002]. The results here open the possibility that the antinociceptive effects of extracellular currents may arise indirectly via reduction in local inflammation.

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