Real-Time Control of Neutrophil Metabolism by Very Weak Ultra-Low Frequency Pulsed Magnetic Fields

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ABSTRACT　In adherent and motile neutrophils NAD(P)H concentration, flavoprotein redox potential, and production of reactive oxygen species and nitric oxide, are all periodic and exhibit defined phase relationships to an underlying metabolic oscillation of ~20 s. Utilizing fluorescence microscopy, we have shown in real-time, on the single cell level, that the system is sensitive to externally applied periodically pulsed weak magnetic fields matched in frequency to the metabolic oscillation. Depending upon the phase relationship of the magnetic pulses to the metabolic oscillation, the magnetic pulses serve to either increase the amplitude of the NAD(P)H and flavoprotein oscillations, and the rate of production of reactive oxygen species and nitric oxide or, alternatively, collapse the metabolic oscillations and curtail production of reactive oxygen species and nitric oxide. Significantly, we demonstrate that the cells do not directly respond to the magnetic fields, but instead are sensitive to the electric fields which the pulsed magnetic fields induce. These weak electric fields likely tap into an endogenous signaling pathway involving calcium channels in the plasma membrane. We estimate that the threshold which induced electric fields must attain to influence cell metabolism is of the order of 10^{-4} V/m.

INTRODUCTION

We have previously reported that NAD(P)H levels vary periodically in time in neutrophils, macrophages, and certain tumor cells. In all of these cells NAD(P)H oscillates in an approximate sinusoidal pattern with a period between 3 and 4 min. When they become adherent and motile, there is a transition from an essentially spherical morphology to one that is polarized, and accompanying the morphological transition, is a transition in NAD(P)H dynamics. We find that, superimposed upon the 3–4 min oscillation, is an additional oscillation of shorter period. This shorter period oscillation is a function of temperature, but is initially in the vicinity of 20 s at 37°C (Kindzelskii and Petty, 2000; Rosenspire et al., 2002, 2000). However, when the cells are exposed to various cytokines, or bacterial metabolites such as lipopolysaccharide, the period of this oscillation decreases to ~10 s (Adachi et al., 1999; Rosenspire et al., 2002). The higher frequency NAD(P)H dynamics is absolutely determinant for the polarized and motile state, in that we have never found spherical or non-adherent cells to exhibit 10- or 20-s period NAD(P)H oscillations.

The higher frequency NAD(P)H dynamics displayed by morphologically polarized neutrophils is intimately connected with cell function. For example, we have found that production of reactive oxygen species (ROS) and nitric oxide (NO) is directly linked to NAD(P)H oscillations. Neutrophils, in turn, utilize ROS and NO for a variety of purposes, including microbial killing, control of pericellular proteolytic activity, and paracrine signaling. We have found that in neutrophils and other polarized motile cells, ROS and NO production takes place in discrete bursts, the timing and amplitude of which are phase-linked with the NAD(P)H oscillation. Furthermore, we have shown that cytokines and microbial byproducts, which are known to stimulate an increase in neutrophil ROS and NO production, appear to do so by triggering an increase in the amplitude and/or frequency of the NAD(P)H oscillation (Rosenspire et al., 2002, 2000; Adachi et al., 1999).

We have also found that if a polarized cell is exposed to extremely low intensity pulsed or sinusoidal electric fields which are properly phased to its (10 or 20-s period) NAD(P)H oscillation, that the cell will resonate with the applied field, in the sense that the amplitude of the NAD(P)H oscillation will be increased along with the production of ROS and NO. In this respect electric fields appear to function in an analogous manner to cytokines such as interferon γ (IFNγ). The implication is that exogenous electric fields may be able to tap into an endogenous cytokine signaling pathway, affecting cell functions such as ROS and NO production. The ability of electric fields to access these pathways so as to substitute for IFNγ in the control of ROS and NO synthesis suggests that, in principle, it may be possible to control cell behavior by the application of properly designed exogenous ultra-low frequency electric fields, as a substitute for biochemical interventions or traditional drug therapy (Rosenspire et al., 2000).

Electric fields could be applied directly, but this would likely entail the invasive placement of electrodes. Alternatively, electric fields could be applied by capacitive

coupling. However, a possible drawback is that, as a consequence of relatively high conductivities, low frequency electric fields are severely attenuated in biological tissues. This means that the application of capacitatively coupled electric fields would likely entail high voltages. Time-varying magnetic fields, on the other hand, easily penetrate biological tissues, enabling them to be applied in a completely non-invasive manner by the placement of external coils. Unfortunately even less is understood about the effects of weak magnetic fields on biological systems than is known about electric fields, although weak magnetic fields are clearly bioreactive. In a particularly striking example, although the mechanism is not understood, low-intensity pulsed magnetic fields in the vicinity of 10–20 Hz are well known to stimulate bone growth, and FDA-approved electromagnetic devices are now routinely used in the clinic to aid in nonunion bone fractures (Bassett, 1984; Brighton, 1981; Brighton and Pollack, 1985; Lavine and Grodzinsky, 1987). With this in mind, we decided to investigate the behavior of neutrophil metabolism, and ROS and NO production in response to the application of ultra-low frequency magnetic fields.

MATERIALS AND METHODS

Material

Reagents were obtained from Sigma Chemical (St. Louis, MO), unless otherwise noted.

Cells

Neutrophils were purified from the blood of healthy individuals by step-gradient centrifugation over Histopaque 1077 and 1119, and suspended in Hanks Balanced Salt Solution (HBSS).

Application of electric fields

A HBSS solution containing purified neutrophils was placed onto a coverslip, in such a way so that the fluid was retained on the coverslip by surface tension. The neutrophils were then allowed to settle and adhere to the coverslip at room temperature for ~15 min. Two Pt electrodes (0.25 mm in diameter) separated by 1 cm were attached to a glass microscope slide. The coverslip with the adhered neutrophils was then inverted and placed over the electrodes. The quantity of fluid was sufficient to ensure that the space between the electrodes was filled with the cell suspension and excluded air. The entire assembly was then sealed with silicone grease, forming a chamber with external dimensions of $0.254 \times 10 \times 25.4$ mm, with the parallel electrodes running along the 25.4-mm dimension. The slide was placed coverslip-down on an Axiovert inverted fluorescent microscope (Carl Zeiss, New York, NY) equipped with a temperature-controlled stage. An electrical stimulator (Grass Medical Instruments, Quincy, MA) was used to apply 1-s DC pulses across the electrodes.

Application of pulsed magnetic fields

Cells suspended in HBSS were placed onto a coverslip, and allowed to adhere as described above. The coverslip was then placed onto a slide, and supported and adhered on the two edges parallel to the short dimension of the slide by silicone vacuum grease. Alternatively cells were placed into a

50-mm diameter tissue culture dish equipped with a 0.17-mm optical glass bottom (World Precision Instruments, Sarasota, FL). The slide or the tissue dish was then placed onto a heated stage attached to an Axiovert inverted fluorescent microscope (Carl Zeiss). A 100-mm diameter coil (7.5 mm in thickness) was positioned parallel to, and 2 cm above, the microscope stage. When a current was applied to the coil, a magnetic field essentially normal to the plane of the slide or tissue culture dish was produced. The input current to the coil was controlled by a custom-designed controller, engineered to allow the coil current to rise and fall in a linear fashion over 20 ms (kindly supplied by EBI, LP, Parsippany, NJ). This ensured that the magnetic pulse shape was trapezoidal in nature. The length of time that each pulse persisted once maximum current was achieved was variable, but usually a value of ~100 ms was chosen. The maximum magnetic field that the coil produced was variable from zero to 4 Gauss. Fig. 1 is a schematic representation of the setup utilized for application of the magnetic pulses when cells were placed into a culture dish, but the coil setup and its placement was the same if cells were adhered to a coverslip and mounted to a slide.

Microscopy and fluorescence quantification

Cells were individually illuminated, and examined using a Zeiss Axiovert 35 fluorescence microscope. Variable wavelength excitation illumination was provided by a xenon lamp coupled to model 101 monochromator (Photon Technology, Lawrenceville, NJ). NAD(P)H autofluorescence was excited using a wavelength of 365 nm and its emission was detected using a 405DF35 filter and 405 longpass dichroic mirror combination (Omega Optical, Brattleboro, VT). Flavoprotein autofluorescence was excited using a wavelength of 450 nm, and its emission detected using a 520DF40 filter and 495 longpass dichroic mirror combination. Tetramethylrosamine (TMRos) fluorescence was excited at 540 nm, and its emission detected using a 590DF30 filter with a 560 longpass dichroic mirror. Diamino-fluorescine-diacetate (DAF-2A) fluorescence was studied using 485 nm for
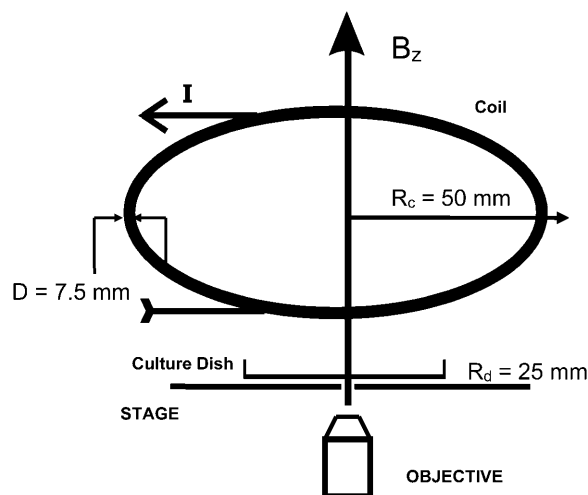
FIGURE 1 Schematic representation of setup utilized to apply magnetic pulses to cells. Cells were either adhered to a coverslip mounted on a slide or placed in a tissue-culture dish equipped with an optical glass bottom. In either case, the slide or the culture dish was placed on a heated microscope stage. A coil was positioned parallel to, and 2 cm above, the microscope stage. When current was allowed to flow through the coil, the magnetic field produced was essentially perpendicular to the plane of the microscope stage. The figure represents the case when a tissue culture dish was used. R_d is the radius of the tissue culture dish; R_c is the radius of the coil used to produce the magnetic field. B_z is the magnetic field induced by the current (I) flowing through the coil, and seen by the cells.

excitation, and a 520EFLP filter with a 505 longpass dichroic mirror for emission. Fluorescence intensity was measured and analyzed using a photomultiplier tube (Hamamatsu, Bridgewater, NJ) housed in a model 104 fluorescence microscope photometer system, interfaced with a Pentium III computer running FeliX software (Photon Technology).

Fluorescence assay for ROS release from neutrophils

Pericellular release of ROS from single cells was monitored as previously described (Kindzelskii and Petty, 2000). Briefly, neutrophils suspended in HBSS (Life Technologies, Grand Island, NY) were allowed to adhere to a glass coverslip. HBSS containing 2% fluid-phase low melting gelatin and 100 ng/ml dihydrotetramethylrosamine (H_2TMRos; Molecular Probes, Eugene OR) at 45°C was placed on the coverslip. The gelatin on the coverslip was then allowed to quickly cool and gel. Next, the coverslip was mounted on a slide, and supported and adhered by silicone vacuum grease just along the two edges parallel to the short dimension of the slide. The slide was then placed on a temperature-controlled microscope stage (Zeiss, New York, NY) held at 37°C. ROS released by the cells diffused into, and were trapped within the gelatin matrix, so that H_2TMRos was oxidized to tetramethylrosamine (TMRos). TMRos detection was accomplished by fluorescence microscopy.

Fluorescence assay for NO release from neutrophils

Single-cell pericellular NO production was monitored in exactly the same manner as single-cell production of ROS, with the exception that the gelatin was mixed with 15 mM diaminofluoresceine-2 diacetate (DAF-2DA; Daiichi Kagaku Yakuhin, Tokyo, Japan) in place of H_2TMRos. DAF-2DA has been previously shown to become fluorescent upon exposure to NO, but not to ROS (Kojima et al., 1998).

RESULTS

NAD(P)H in polarized motile neutrophils responds to pulsed magnetic fields

In Fig. 2, neutrophils were suspended in HBSS, and allowed to adhere to a coverslip, which was then mounted on a slide and placed on a temperature-controlled stage held at 37°C. We have found that under these conditions most neutrophils will become morphologically polarized after ~10–20 min in contact with the glass. Approximately 30% of the polarized neutrophils at any given time are also motile. Fig. 2 *a* is an example of a representative experiment where a single motile neutrophil was selected, and internal NAD(P)H concentration as a function of time measured by monitoring NAD(P)H autofluorescence as previously described (Kindzelskii and Petty, 2000; Rosenspire et al., 2000). In this cell, NAD(P)H autofluorescence is seen to fluctuate in a cyclical manner with a period of ~25 s. As we continued observing the cell for ~4 min, the NAD(P)H oscillations remained steady. Next, magnetic pulses were applied to the cell utilizing the setup and coil described in Fig. 1 at times corresponding to local minima in the NAD(P)H oscillation (indicated by *arrows*). The magnetic pulses were trapezoidal in shape. For each pulse, the magnetic field increased in a linear fashion over
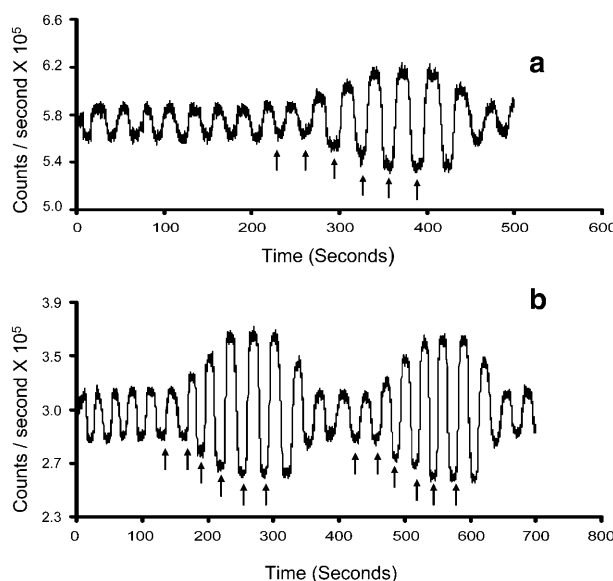

FIGURE 2 NAD(P)H concentration in motile neutrophils is oscillatory, and the amplitude of the oscillation can resonate with externally applied pulsed magnetic fields. NAD(P)H autofluorescence was monitored with a photomultiplier, and photomultiplier counts plotted as a function of time. (*a*) Representative example of a motile neutrophil. The concentration of NAD(P)H oscillates, and the oscillation is observed to be stable for >200 s. It increases in amplitude only when pulsed magnetic fields are applied at local minima (*arrows*). The amplitude then decreases to control value when pulses cease. (*b*) Representative example of a second motile neutrophil. NAD(P)H concentration oscillates, and amplitude of the oscillation increases when magnetic pulses are first applied synchronously with local minima of the NAD(P)H oscillation (*arrows*). When pulses cease, the amplitude returns to baseline value. However, reapplication of magnetic pulses at NAD(P)H minima again leads to an increase in NAD(P)H oscillation amplitudes.

20 ms, reaching a maximum field-strength of 2 Gauss. The 2-Gauss field was maintained for ~100 ms, at which point the field decayed in a linear fashion to zero-strength over the next 20 ms. After application of only the second magnetic pulse, the amplitude of the NAD(P)H oscillation clearly began to increase, reaching a maximum value after the fourth pulse. The increased amplitude of the NAD(P)H oscillation is dependent upon the continued application of the magnetic pulses at local NAD(P)H minima, since after the pulses ceased, the amplitude returned to its baseline value.

This is perhaps clearer in Fig. 2 *b*. Fig. 2 *b* is a representative example of an independent experiment performed with different cells on a different day from Fig. 2 *a*. As in Fig. 2 *a*, neutrophils were allowed to adhere to a glass coverslip, which was then mounted on a slide, and placed on a heated microscope stage. An individual motile neutrophil was selected and NAD(P)H autofluorescence monitored. At the points indicated by the arrows, magnetic pulses were then delivered to the cell. Once again application of magnetic pulses led to an increase in the amplitude of the NAD(P)H oscillation, which then rapidly returned to baseline after the pulses ceased. However, in this figure, after the initial

sequence of magnetic pulses ended and the NAD(P)H amplitude returned to its baseline value, a second series of magnetic pulses was applied to the same cell. The cell responded to these pulses in an identical manner as to the first series of pulses, clearly demonstrating that the increase in the amplitude of the NAD(P)H oscillation is dependent upon a continuous application of magnetic pulses.

We have repeated these experiments with at least 50 different cells. Although only ~30% of the adhered neutrophils on a slide are motile at any given time, and so exhibit the 20-s oscillation, we have found that in every case where the oscillation is present, the cells respond to pulsed magnetic fields delivered at the NAD(P)H minima by increasing the amplitude of the NAD(P)H oscillation, exactly as in Fig. 2. This behavior of NAD(P)H dynamics in response to pulsed magnetic fields is reminiscent of NAD(P)H behavior during neutrophil exposure to pulsed electric fields, where we have referred to the phenomena as *metabolic resonance* (Kindzelskii and Petty, 2000).

It is important to point out that NAD(P)H resonance is clearly dependent on the stimuli being synchronized with the endogenous metabolic oscillation. We find that when magnetic pulses are applied randomly, there is no biological response. This is clearly shown in Fig. 3. The figure is a representative example of an experiment ($n = 3$) where neutrophil NAD(P)H metabolism was first measured by autofluorescence, and then as in Fig. 2, the cells exposed to magnetic pulses. However, instead of the pulses being synchronized with the NAD(P)H minimum, the timing of each pulse was controlled by a computer, to ensure that they were random. In particular, the period between successive pulses was adjusted to a random fraction of the time equal to double the NAD(P)H period, so that although the pulses were random, if one waited long enough, the average period between pulses would approach the NAD(P)H period. Under these conditions, NAD(P)H metabolism appears unaffected by the application of magnetic pulses.

Redox oscillations of flavoprotein are sensitive to pulsed magnetic and electric fields

NAD(P)H is clearly an important element of intermediate metabolism, and Fig. 2 demonstrates that NAD(P)H dynamics is sensitive to pulsed magnetic fields. Flavoprotein redox potential is another element of intermediate metabolism. We have previously demonstrated that changes in cellular flavoprotein redox potential can be observed in real-time on the single level by measuring the autofluorescence of cellular flavoproteins, much as we measure cellular NAD(P)H levels. In particular, we have found in neutrophils that flavoprotein redox potential varies periodically with the same frequency as does NAD(P)H (Kindzelskii and Petty, 2004). This is perhaps not surprising, since NAD(P)H and flavoprotein redox potential are known to be linked in a number of metabolic pathways. However, this finding would also suggest that changes in the NAD(P)H oscillation, consequent to application of a pulsed magnetic field, should be reflected in similar changes to the amplitude of flavoprotein redox oscillations.

Fig. 4 is a representative example ($n = 8$) of an experiment where we directly determined the effect of pulsed magnetic fields on flavoprotein redox potential. As in Fig. 2, peripheral blood neutrophils were allowed to adhere to a coverslip, which was then mounted on a microscope slide. An individual motile neutrophil was selected, and the time course of flavoprotein autofluorescence determined. Under these conditions, flavoprotein autofluorescence levels are seen to fluctuate in a cyclical (although non-sinusoidal) manner with a period of ~20 s. After several cycles, the cell was exposed to a pulsed magnetic field (of the type described in Fig. 2), at local maxima in the redox pattern (times indicated by the *arrows*). Only oxidized flavoproteins are autofluorescent, so autofluorescence peaks correspond to periods when the ratio of oxidized/reduced flavoprotein is maximal (Kindzelskii and Petty, 2004). Fig. 4 shows that application of pulsed magnetic fields applied at times of maximally oxidized flavoprotein will, after two cycles, trigger an increase in the amplitude of the flavoprotein redox pattern.
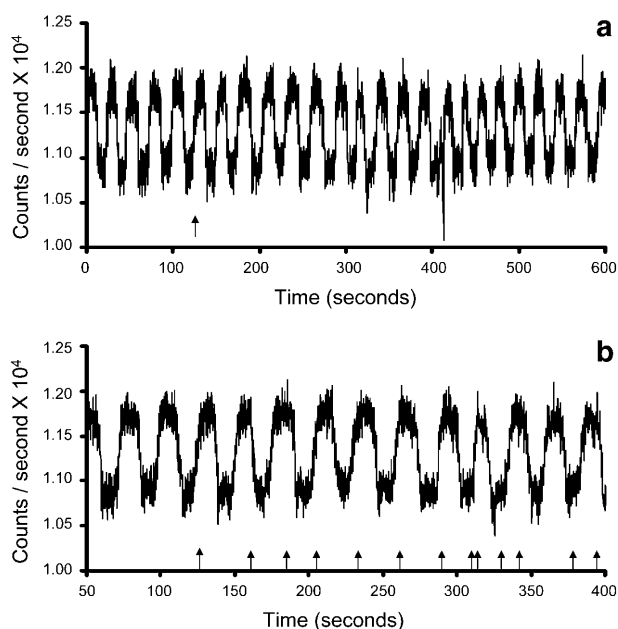
FIGURE 3 Non-synchronized magnetic pulses have no effect on neutrophil NAD(P)H oscillations. (*a*) NAD(P)H levels in a representative adherent neutrophil ($n = 3$) were monitored over a 10-min period, and seen to oscillate. The period of the NAD(P)H oscillation was measured, and beginning at 125 s (indicated by the *arrow*), magnetic pulses were delivered to the cell. Pulse generation was computer-controlled, and arranged so that each pulse arrived at a random time, but on average, the period between pulses was equal to the NAD(P)H period. (*b*) The same data set as *a*, but the scale of the figure is magnified, and includes only timepoints between 50 and 400 s. On this scale, the timing of each individual magnetic pulse is indicated by a well-resolved arrow.
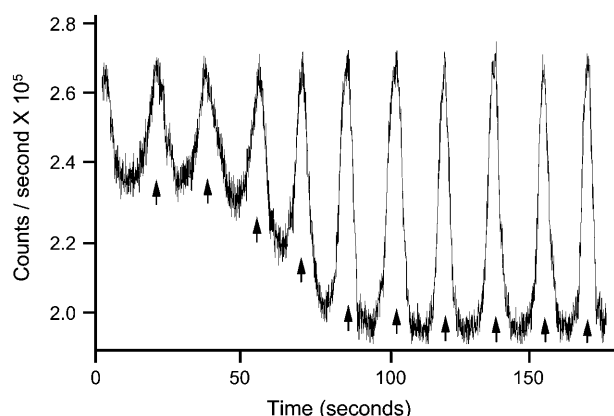
FIGURE 4 Neutrophil flavoprotein redox potentials are sensitive to applied magnetic fields. Neutrophils were suspended in HBSS, and allowed to adhere to a glass coverslip as in Fig. 2. The coverslip was mounted on a slide, which was then mounted on a heated (37°C) microscope stage. A magnetic coil was positioned over the slide. A single neutrophil was selected, and flavoprotein autofluorescence monitored with a photomultiplier. Photomultiplier counts are plotted as a function of time. Magnetic pulses were delivered to the neutrophil at the times indicated by the arrows. Each pulse was timed to coincide with maximum flavoprotein autofluorescence.

Because NAD(P)H dynamics can be affected by either pulsed electric or magnetic fields, Fig. 4 suggests that the dynamics of flavoprotein redox potential should be effected by pulsed electric fields in a manner similar to that in pulsed magnetic fields. Fig. 5 is a representative example ($n = 10$), where we directly investigated whether the application of pulsed weak electric fields would affect the amplitude of flavoprotein oscillations. Accordingly, in Fig. 5 peripheral blood neutrophils were allowed to adhere to a coverslip, which was then mounted over two platinum wires serving as

electrodes placed 1-cm apart on a microscope slide. The volume between the wires was filled with HBSS, and the entire assembly sealed with vacuum-grease to form a chamber (Kindzelskii and Petty, 2000). After placing the slide on a heated microscope stage, an individual motile neutrophil was selected, and the time course of flavoprotein autofluorescence determined. As in Fig. 4, flavoprotein autofluorescence levels are seen to fluctuate in a cyclical manner with a period of ~20 s. Next, a 20-mV potential was applied in (300-ms) pulses across the electrodes, so as to coincide with the peaks of the flavoprotein autofluorescence pattern. Fig. 5 shows that pulsed electric fields, in an effect similar to that found for pulsed magnetic fields, will, after two cycles, trigger an increase in the amplitude of the flavoprotein redox pattern.

Production of reactive oxygen species responds to pulsed magnetic fields

We have previously shown that increases in the production of reactive oxygen species (ROS) in polarized neutrophils is associated with increases in the amplitude of the NAD(P)H oscillation. Therefore, production of ROS increases when electrical pulses are applied at NAD(P)H minima and metabolic resonance is established (Kindzelskii and Petty, 2000; Rosenspire et al., 2000). Fig. 6 is a representative example of a series of experiments where we examined the production of ROS in polarized motile neutrophils in response to magnetic pulses delivered at NAD(P)H minima. Single-cell pericellular ROS production was measured by first allowing the cells to adhere to a coverslip, and then covering the adhered cells in a gel matrix containing dihydrotetramethyl-rosamine (H_2TMRos). The fluorescent signal generated as H_2TMRos was reduced to tetramethylrosamine (TMRos) by
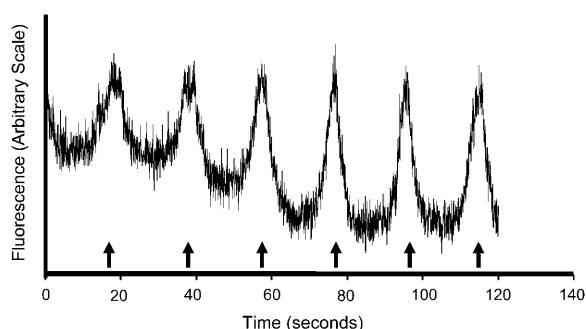

FIGURE 5 Neutrophil flavoprotein redox potentials vary periodically, and are sensitive to applied electric fields. Neutrophils were suspended in HBSS, and allowed to adhere to a glass coverslip. The coverslip was mounted on a slide over two parallel platinum wires, and sealed with vacuum-grease so as to form a chamber. The chamber was then mounted on a heated microscope stage held at 37°C. A neutrophil was selected and flavoprotein autofluorescence, an indicator of flavoprotein redox potential, monitored as a function of time. DC pulses were then applied across the chamber at the times indicated by the arrows. Each pulse was timed to coincide with maximum flavoprotein autofluorescence.
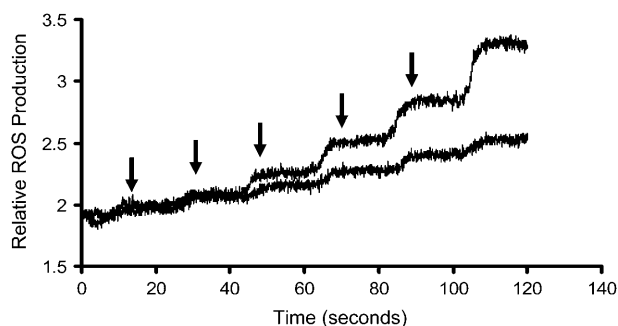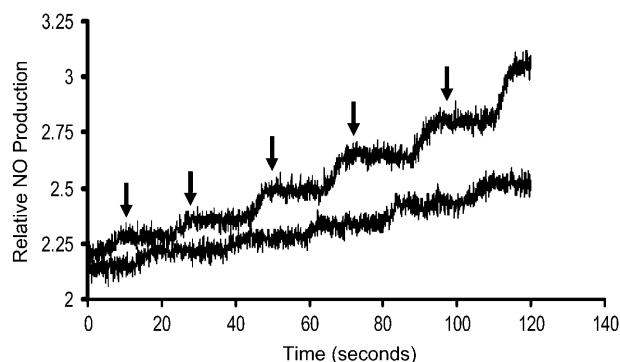

FIGURE 6 Production of ROS can be increased in response to pulsed magnetic fields. Neutrophils were allowed to adhere on a glass coverslip, and then covered with a warm liquid gelatin matrix containing H_2TMROS. When the gelatin had solidified, the coverslip was placed on a slide that was mounted on a heated (37°C) microscope stage, beneath a magnetic coil. In the lower curve, pericellular ROS production in the vicinity of a single neutrophil was monitored by following fluorescence of TMRos, which was produced due to oxidation of H_2TMRos. The upper curve represents the (TMRos) fluorescence record of a second neutrophil that was similar to the first, but with the exception that magnetic pulses were delivered to the cell at the times indicated by the arrows.

the action of ROS, is proportional to the ROS, and was recorded as previously described (Rosenspire et al., 2000).

In Fig. 6, the lower plot represents ROS production from a control neutrophil which has not been exposed to magnetic pulses. It is seen that the TMRos fluorescence pattern is characterized by a series of uniformly timed steps, at ~20-s intervals. We have previously shown that this pattern can be interpreted to imply cellular ROS production occurring in pulses that are synchronized with the (20-s) NAD(P)H oscillation (Rosenspire et al., 2000). In comparison, the upper plot represents ROS production in a neutrophil as it is exposed to magnetic pulses. Magnetic pulses (at times indicated by the *arrows*) were phased to coincide with the portion of the ROS fluorescence plot at points which have previously been shown to be coincident with NAD(P)H minima. The result is that the size of each step is clearly seen to increase, indicating an increase in the quantity of ROS produced for each pulse. We have examined over 100 neutrophils in this manner, and each and every time production of ROS increases in response to pulsed magnetic fields in exactly the same manner. This result is similar to other findings, where we have reported identical effects of pulsed electric fields on ROS production in polarized neutrophils (Rosenspire et al., 2000).

Production of nitric oxide responds to pulsed magnetic fields

We have also found that in polarized neutrophils, the production of nitric oxide (NO) closely parallels the production of ROS. In particular, NO production is increased by

FIGURE 7 Production of NO can be increased in response to pulsed magnetic fields. Adherent neutrophils were covered with a warm liquid gelatin matrix on a coverslip. After the gelatin had cooled, the coverslip was held on a heated microscope stage as in Fig. 6. However, instead of the H$_2$TMRos used in Fig. 6, the gelatin was impregnated with DAF-2DA, which fluoresces in the presence of NO. The lower plot was generated when a single neutrophil was selected, and pericellular NO production examined by monitoring DAF-2DA fluorescence. The upper curve represents the (DAF-2DA) fluorescence record of a second neutrophil that was similar to the first, with the exception that magnetic pulses were delivered to the cell at the times indicated by the arrows.

the application of pulsed electric fields, in a similar manner to production in ROS (Rosenspire et al., 2000). Fig. 7 is a representative example of a series of experiments where the effect of pulsed magnetic fields on production of nitric oxide was examined. Neutrophil pericellular NO production was measured from a single cell by using a fluorescence assay similar to the ROS assay used in Fig. 6, with the exception that the NO-sensitive dye, diaminofluorescein-2-diacetate (DAF-2DA), was substituted for HTMRos. The lower plot represents NO production from a control neutrophil that has not been exposed to magnetic pulses. As was the case for ROS production, the fluorescence pattern is uniformly stepped at ~20-s intervals, indicating pulsed NO production that is also synchronized to NAD(P)H. The upper plot represents NO production from a neutrophil that has been exposed to magnetic pulses synchronized to the NAD(P)H oscillation, and delivered at the times indicated by the arrows corresponding to NAD(P)H minima. In Fig. 6, NO production is seen to increase in a manner similar to that in ROS production. This is perhaps not surprising, since the NO synthetase substrate is NADPH. We have examined over 100 different neutrophils with results found to be similar to those seen in Fig. 6.

Neutrophils respond indirectly to pulsed magnetic fields through the induction of pulsed electric fields

Figs. 2–7, taken in the context of our previous findings, show that in polarized adherent neutrophils, flavoprotein activity, NAD(P)H, ROS production, and NO production all respond to pulsed magnetic fields in exactly the same manner as to pulsed electric fields. However, because electromagnetic theory necessarily implies the induction of an electric field in response to a time-varying magnetic field, the question arises as to whether the neutrophils in Figs. 2–7 are responding directly to the magnetic field, or to the induced electric field instead.

To answer this question, neutrophils were suspended in HBSS, and allowed to adhere and polarize on a 50-mm diameter circular tissue culture dish, which was equipped with a 0.17-mm glass bottom. The dish was then mounted on a temperature-controlled stage held at 37°C, which was attached to an inverted fluorescence microscope. As in Fig. 2, NAD(P)H autofluorescence was monitored, and magnetic pulses were delivered to the cells by transiently energizing a coil mounted above the stage.

For each pulse, by varying the input current, the maximum magnetic field produced by the coil was variable from 0 to 4 Gauss. However, regardless of the maximum magnetic field-strength achieved, the coil was energized and de-energized in a linear fashion over 20 ms. Fig. 8 shows representative oscilloscope plots indicating the electromagnetic characteristics of the pulses. In the upper plot, a magnetometer placed in the center of the coil was used to directly measure the
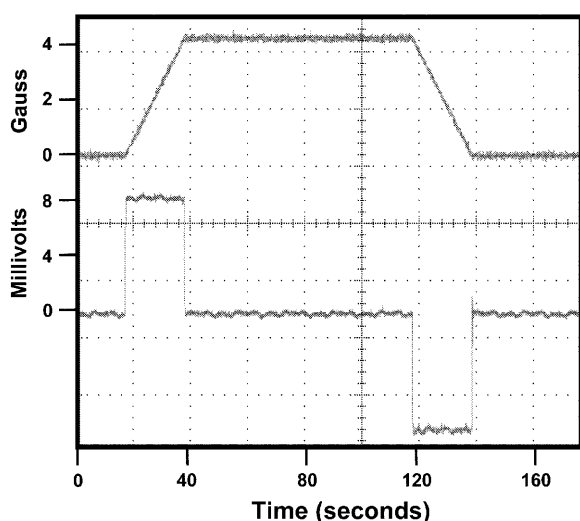
FIGURE 8 Electric and magnetic characteristics of the coil and actuator utilized in Figs. 3–7. (*Upper plot*) Magnetic characteristics. Magnetic field has a 20-ms rise, and 20-ms fall; increasing and decreasing field strength is linear in both instances. The maximum field strength equals 4 Gauss. (*Lower plot*) Square-wave electric field induced by the magnetic wave form in the upper trace, as measured by a coil probe.

minima as in Fig. 2. However, the steady-state current through the coil was varied, so that the maximum magnetic field produced by the coil could be adjusted. Initially the maximum magnetic field produced by each pulse was in the vicinity of 1 Gauss. We then noted whether resonance was established between the pulsed magnetic fields and NAD(P)H after a series of several magnetic pulses had been administered. If the cell was non-responsive, the maximum magnetic field-strength was increased slightly, and another series of pulses delivered to the cell. This was repeated until, as in Fig. 2, the cell began to resonate with the applied field. For each cell, and for each series of magnetic pulses, the induced electric field in the vicinity of the cell was calculated, and plotted against the maximum magnetic field produced by the coil, and an indication of whether resonance was established was noted.

The results are plotted in Fig. 9, where it is immediately apparent that the establishment of resonance is independent of magnetic field-strength. Resonance is only established if the induced electric field surpasses a threshold of ~50 mV/m. Thus it would seem that in neutrophils, pulsed magnetic fields interact with the metabolism of NAD(P)H through their induction of electric fields, rather than through a mechanism that depends on a direct magnetic interaction.

magnetic field produced by the coil under test-bench conditions. The field rises linearly from 0 to 4 Gauss over 20 ms, remains at 4 Gauss for 80 ms, and then linearly decays back to 0 over 20 ms. Because an induced electric field is proportional to the time-rate of change of the magnetic field, a magnetic field with these characteristics will induce a pulsed square-wave electric field. This is directly demonstrated by the lower plot in Fig. 8, where a probe has been positioned at the center of the coil, and the electric field induced in the probe by the time-dependent magnetic field depicted in the first plot directly measured.

The coil depicted in Fig. 8 was placed parallel, and 2 cm above, the plane of the tissue culture dish. The center of the coil was co-linear with the center of the culture dish. Under these conditions, the pulsed magnetic field produced by the coil will induce an electric field within the culture dish, which, because of the radial symmetry of the setup, is easy to calculate. Also, because the diameter of the culture dish is small enough compared to the diameter of the coil, only magnetic field components perpendicular to the plane of the culture dish need be considered. In this case, the induced electric field that we consider will essentially be entirely in the plane of the culture dish. In particular, if $\varepsilon_{x,y}$ is the induced field in the culture dish plane in the vicinity of any cell, $\varepsilon_{x,y} = \frac{dB_z}{dt} X R/2$, where B_z is the magnetic field perpendicular to the plane of the dish, and R is the radial distance of the cell from the center of the dish.

A series of adherent neutrophils was selected, and for each the radial distance from the center of the dish measured. Next, for each selected cell, NAD(P)H autofluorescence was monitored, and magnetic pulses delivered at NAD(P)H
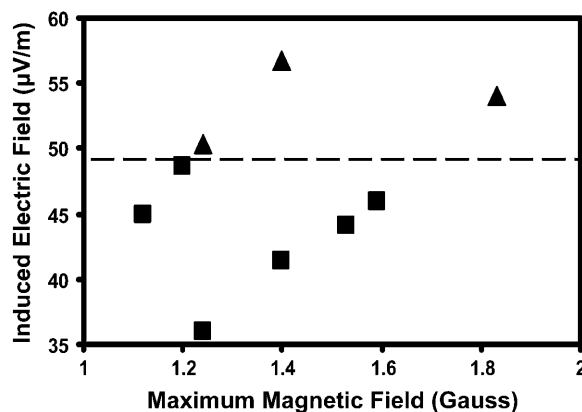

FIGURE 9 Neutrophil metabolism indirectly responds to pulsed magnetic fields as a consequence of magnetically induced electric fields. NAD(P)H autofluorescence was monitored in individual neutrophils, as magnetic pulses were applied to the cell at NAD(P)H minima, conditions conducive to metabolic resonance. For each series of pulses, the maximum magnetic field was adjusted from 1 to 4 Gauss. Based on the magnetic field rate of change and the position of the cell, the induced electric field in the vicinity of the cell was calculated. It was then noted whether or not the cell was responsive (resonated) to the magnetic field. Responsive cells were identified by the (▲) symbol, whereas non-responsive cells were identified by the (■) symbol. For each cell the (responsive, or non-responsive) symbol was placed on a two-dimensional plot of induced electric field versus maximum applied magnetic field. It is immediately obvious that whether cells respond to magnetic pulses is independent of maximum applied magnetic field. However, cells respond to the pulsed magnetic fields so long as the induced electric field is above a threshold (indicated by the *dotted line*) of just under 50 μV/m.

Pulsed magnetic fields control cell metabolism through a mechanism involving external calcium

Having established that pulsed magnetic fields control cell metabolism through their induction of electric fields, the question arises as to mechanism. Previous investigators have suggested that DC and extremely low-frequency electric fields can interact with cells through mechanisms that involve extracellular calcium channels (Trollinger et al., 2002; Cho et al., 1999, 2002; Kenny et al., 1997; Lorich et al., 1998). We also explored this possibility here. Fig. 10 is a representative example ($n = 5$) where we have determined the effect of extracellular calcium on the ability of pulsed magnetic fields to resonate with NAD(P)H in motile neutrophils. In the figure, neutrophils were initially suspended in 0.5 ml HBSS, and allowed to adhere and polarize in a 3-cm-diameter tissue culture dish (Corning, Corning, NY) that we had modified with a coverglass bottom. The dish was placed on the heated stage of the microscope and magnet setup described in Fig. 9. An individual adherent neutrophil was selected, and NAD(P)H autofluorescence was then monitored. Magnetic pulses (at times indicated by the *small arrows*), inducing an electric field >50 mV/m, were then continually applied at local NAD(P)H minima, beginning at the 75-s mark. As expected, the amplitude of the NAD(P)H oscillation increased. At the 215-s mark (indicated by the *first large arrow*), the media was carefully removed from the culture dish with a Pasteur pipette, and quickly replaced with 4 ml of calcium-

free HBSS. NAD(P)H continued to oscillate with the identical frequency, but by 300 s, the amplitude of the oscillation had returned to control values. The amplitude of the oscillation remained at control values despite the fact that magnetic pulses were continually applied at NAD(P)H minima.

At 545 s (indicated by the *second large arrow*), the calcium-free media was removed from the culture dish, and replaced with 4 ml of HBSS containing normal calcium. Within one cycle the amplitude of the NAD(P)H oscillation began to increase. Within three cycles, the amplitude had returned to the level obtained before external calcium was reduced, demonstrating that external calcium is necessary for pulsed electric fields to resonate with a cell.

Magnetic pulses can inhibit flavoprotein redox oscillations

We have previously reported that, for polarized neutrophils, electric field pulses delivered at NAD(P)H maxima lead to depolarization and collapse of NAD(P)H oscillations (Kindzelskii and Petty, 2000). However, NAD(P)H maxima should correspond to times of minimal flavoprotein redox potential. Therefore, in light of Fig. 9, application of magnetic pulses synchronized to minimal flavoprotein fluorescence should also lead to collapse of NAD(P)H oscillations, and by extension, collapse of the flavoprotein oscillation in polarized neutrophils.

This is confirmed in Fig. 11, where flavoprotein autofluorescence from a polarized neutrophil was monitored in response to the application of pulsed magnetic fields as initially described in Fig. 4. As in Fig. 4, neutrophils were allowed to adhere to a coverslip, and flavoprotein autofluor-
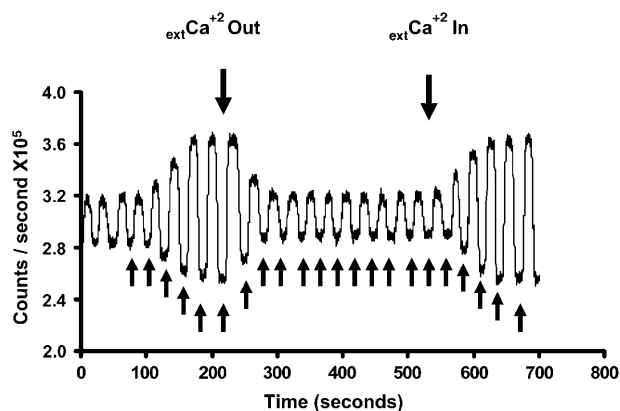

FIGURE 10 The ability of motile neutrophils to sense pulsed magnetic fields is dependent upon external calcium. Neutrophils were suspended in media containing normal amounts of calcium. An adherent motile neutrophil was selected, and changes in internal NAD(P)H concentration monitored by measuring NAD(P)H autofluorescence with a photomultiplier. Photomultiplier counts are plotted as a function of time. Pulsed magnetic fields were applied at local minima in NAD(P)H (*arrows*), and the NAD(P)H amplitude increased in response. At the point indicated, the media was replaced by calcium-free media. The amplitude of the NAD(P)H oscillation returned to control values despite the fact that magnetic pulses continued to be applied to the cell. At the timepoint indicated, the calcium-free media was then removed, and replaced with normal calcium-containing media. The NAD(P)H oscillation amplitude once again increased in response to applied magnetic pulses.
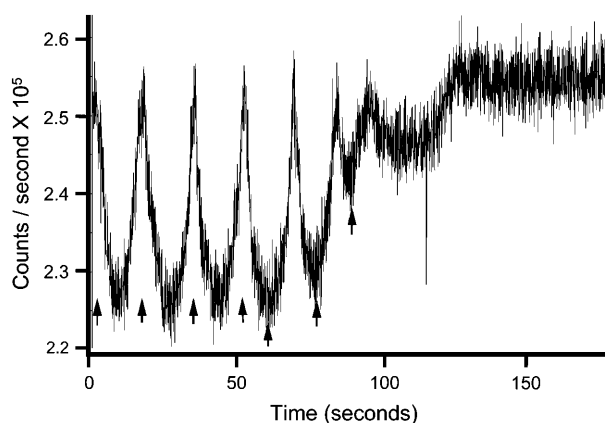

FIGURE 11 Flavoprotein redox oscillations are inhibited by pulsed magnetic fields timed to coincide with minimal flavoprotein autofluorescence. Neutrophils were allowed to adhere to a coverslip, and flavoprotein autofluorescence from an individual cell monitored as in Fig. 4. As in Fig. 4, magnetic pulses (at times designated by the *arrows*) were delivered to the cell, but timed to coincide with periods of either peak, or minimal autofluorescence. (Count rate from the photomultiplier tube is proportional to the flavoprotein fluorescence signal, and is plotted versus time.)

escence from a single cell monitored. Magnetic pulses (at times depicted by the *arrows*) were initially delivered to the cell synchronously at the peaks in flavoprotein autofluorescence. This led to enhancement of the flavoprotein redox oscillation amplitude. However, the phase of the magnetic pulse delivery was then shifted so that the pulses were synchronous with the minima of the redox waveform. After the third pulse, the redox oscillation collapsed. The results for this cell are representative of similar results obtained for 15 independent cells examined.

Production of ROS and NO can be terminated by weak pulsed magnetic fields

As we have mentioned, electric field pulses delivered at NAD(P)H maxima will lead to the morphological depolarization of neutrophils, and the collapse of their NAD(P)H oscillation. The association of ROS and NO production with oscillating NAD(P)H levels, and the demonstration that neutrophils respond to pulsed magnetic fields through the induction of pulsed electric fields, suggests that properly timed magnetic field pulses should terminate ROS and NO production in neutrophils. This is explicitly demonstrated in Fig. 12. In Fig. 12 *a*, adherent neutrophils were embedded in a gelatin matrix containing H$_2$TMRos, and pericellular release of ROS was monitored in real-time from a single motile neutrophil by observing TMRos fluorescence from the immediate vicinity of the cell. As previously discussed, the stepped fluorescence pattern implies pulsed production of ROS synchronized to cell metabolism. Magnetic pulses designed to induce square-wave voltage pulses (Figs. 8 and 9) were delivered to the cell (*arrows*) at points in the fluorescence pattern previously associated with maxima in the NAD(P)H oscillation. The result is that after the third pulse, the fluorescence intensity becomes constant, indicating that the pulsed production of ROS has ceased. Fig. 12 *a* is representative of the results obtained from 15 different cells that were observed under identical conditions.

In a similar manner, Fig. 12 *b* demonstrates that magnetic pulses can be utilized to terminate neutrophil NO production. In Fig. 12 *b*, neutrophils were treated identically to those in Fig. 10 *a*, with the exception that the gelatin matrix within which they were embedded contained the indicator dye DAF-2DA, which fluoresces upon exposure to NO, in place of H$_2$TMRos. The figure shows the results for NO synthesis obtained from a single neutrophil, which is representative of the results found for 15 independent cells studied. Here too, magnetic pulses delivered at points where NAD(P)H is maximal, lead after the third pulse to the cessation of NO production.

DISCUSSION

We have previously found that morphologically polarized cells (including neutrophils, macrophages, and tumor cells)
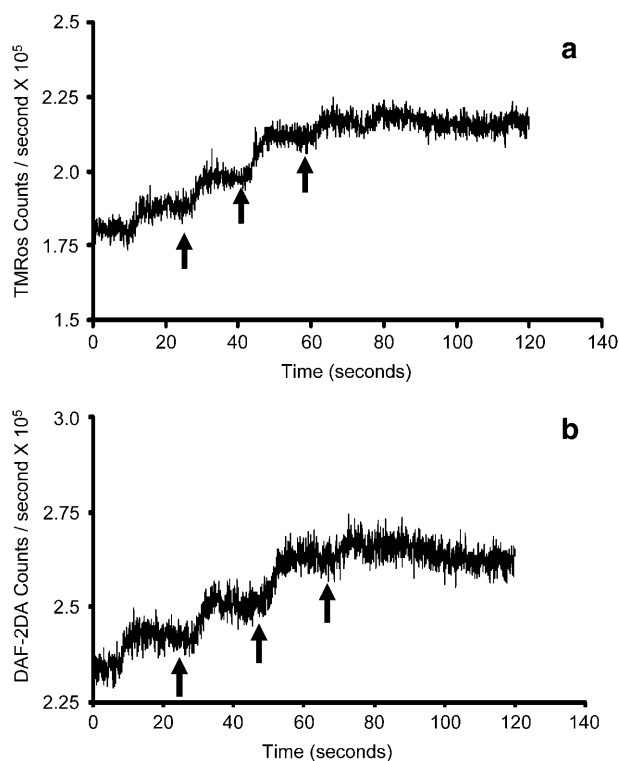
FIGURE 12   Production of ROS and NO by neutrophils can be inhibited by pulsed magnetic fields. (*a*) Neutrophil production of ROS was monitored by measuring TMRos fluorescence, as H2TMRos was oxidized. (Count rate from the photomultiplier tube which is proportional to the fluorescence signal, is plotted versus time.) The stepped pattern is reflective of ROS production taking place in discrete bursts. Magnetic pulses were applied at the times indicated by the arrows, leading to cessation of ROS bursts. (*b*) Neutrophil production of NO was monitored by measuring DAF-2DA fluorescence, and the count rate plotted as a function of time. As is the case for ROS, a stepped fluorescence pattern is reflective of burstlike NO production. Magnetic pulses were applied at the times indicated by the arrows, also leading to cessation of NO bursts.

are uniquely characterized by ultra-low frequency metabolic processes. In each cell NAD(P)H concentration and global flavoprotein redox potentials vary periodically in time, while maintaining a defined phase relationship and a period of some 10–25 s. In neutrophils and macrophages, pericellular ROS and NO synthesis (which is dependent upon the available cellular reducing potential and flavoprotein electron transport) is also cyclical. In this study we confirm our earlier findings that ROS and NO are synthesized in short bursts, with the burst frequency matching the frequency of the metabolic oscillation. Furthermore, ROS and NO production are proportional to the amplitude of the metabolic oscillation. It seems that these cells exhibit nonlinear substrate-level control of ROS and NO, where production is regulated not by the average levels of NAD(P)H and other electron donors of intermediate metabolism, but by the frequency and amplitude of their oscillations instead. In fact, we have previously demonstrated in these cells that cytokine

and bacterial metabolite-induced increases in ROS and NO production are correlated with increases in metabolic oscillation frequencies and amplitudes, but not with increases in average NAD(P)H levels (Adachi et al., 1999; Rosenspire et al., 2002).

We have also previously reported that in motile neutrophils and tumor cells, the amplitude of these metabolic oscillations will increase in magnitude, so as to resonate with directly coupled pulsed or sinusoidal electric fields when the pulses or local maxima of the sinusoidal fields are phased to coincide with the local minima of the fundamental NAD(P)H oscillation. As expected, increasing the amplitude of the metabolic oscillation increases the production of ROS and NO (Kindzelskii and Petty, 2000; Rosenspire et al., 2000, 2001). In this report, we have found that, in motile neutrophils, metabolic processes will resonate with external pulsed magnetic fields when the pulses are synchronous with the local minima of the NAD(P)H, or equivalently the reduced flavoprotein metabolic oscillation. NAD(P)H and flavoprotein redox oscillatory amplitudes, as well as pericellular ROM and NO production, remain at elevated levels as long as magnetic pulses continue to be delivered but return to normal when the pulses cease. On the other hand, if magnetic pulses coincide with local NAD(P)H maxima, or alternatively the minima of the oxidized flavoprotein oscillation, the metabolic oscillations quickly collapse. This is accompanied by a dramatic reduction in pericellular ROS and NO production.

There have been numerous previous reports in the literature examining the effect of static or time-varying weak magnetic fields on a variety of biological systems, with both positive and negative results (Lacy-Hulbert et al., 1998). However, there are several factors that set our study apart from earlier studies of weak magnetic effects on biological systems. Importantly, we are observing magnetic effects on single cells in real-time. Most previous investigators have worked with asynchronous populations of cells, measuring parameters such as cell growth or gene expression, parameters which are not measured in real-time. Positive results, when obtained at all, are only apparent after extensive statistical analysis of the data. The second distinguishing characteristic of our experiments is that we have been looking at frequencies (~0.05 Hz) far lower than other investigators. Many, if not most, previous investigations have focused on the frequency domain of 50–60 Hz. In our case, investigations in the 0.05-Hz frequency domain has been driven by the underlying biology of the neutrophil system.

Several investigators have considered that biological effects of extremely low frequency electromagnetic fields might be explained by magnetic effects on chemical reactions involving free radicals (Lacy-Hulbert et al., 1998). However, in the experiments reported here, such esoteric explanations need not be invoked—since it is quite clear that the cells respond to induced electric field pulses, rather than the magnetic fields per se. In all cases, the cellular responses to phase- and frequency-defined pulsed electric fields with respect to NAD(P)H and flavoprotein oscillations, as well as to ROS and NO production, mirror the responses obtained with magnetic pulses. Furthermore, Fig. 9, aside from explicitly demonstrating that it is the induced electric field to which the cells respond, allows the calculation of the minimum induced field intensity necessary to see an effect. We find this to be 5×10^{-5} V/m, which is in good agreement with previous results where the sensitivity of neutrophils and tumor cells to applied pulsed electric fields was directly determined to be $\sim 9 \times 10^{-5}$ V/m (Kindzelskii and Petty, 2000; Rosenspire et al., 2000, 2001).

As magnetic fields penetrate the cell volume, magnetic pulses will induce electric fields at the cell membrane, as well as inside the cell. However, in the experiment outlined in Fig. 9, magnetically induced electric fields inside the cell are independent of cellular position within the culture dish. Only the induced electric field at the membrane surface is a function of distance from the center of the dish. Because the ability of a pulsed magnetic field to induce a resonance depended upon cell position, the implication is that it must be the electric field induced at the cell membrane that is responsible for the effect.

At physiological pH, many of the macromolecular constituents of cells are charged, so it is not surprising that non-ionizing electromagnetic fields here and elsewhere have been shown to directly influence cell function. It is well established at high (gigaHertz and above) frequencies that non-ionizing electromagnetic fields interact with biological tissues primarily through thermal effects. Although there have been persistent reports that at these frequencies, as well as at lower power line frequencies, weak-to-moderately strong electric or magnetic fields can specifically interact with biological systems through nonthermal mechanisms (Czyz et al., 2004; Macri et al., 2002; Mileva et al., 2003; Hadjiloucas et al., 2002), generally speaking this is an area of controversy (Lacy-Hulbert et al., 1998). In contrast, there is ample and well-accepted evidence that at selected frequencies below 50 Hz, electromagnetic fields interact with biological tissues in a decidedly nonthermal manner (Aaron et al., 2004a,b).

It has been established that endogenous physiological DC electric fields of ~1 volt/cm are generated during embryonic development and wound healing in a wide variety of systems. These DC fields are necessary to, and control the directed cell movement of, epithelial and other cells, which is characteristic of these processes (Nuccitelli, 1992). The phenomena of directed cell movement in response to an electric field has been termed *Galvanotaxis*. Although many details of the Galvanotactic response remain to be elucidated, experiments have shown that endogenous, as well as similar-strength exogenous DC fields, stimulate cell movement by impinging upon elements of established signal transduction pathways, such as growth factor phosphoprotein kinases and calcium channel activity in target cells (Fang et al., 1998, 1999; Trollinger et al., 2002).

By far, the most thoroughly studied work concerning nonthermal biological effects of low-frequency electromagnetic fields has been done in bone. Low-frequency pulsed electric fields in the vicinity of 10–30 Hz were shown more than two decades ago to stimulate bone growth, and FDA-approved electromagnetic devices are now routinely used in the clinic to aid in nonunion bone fractures (Bassett, 1984; Brighton, 1981; Brighton and Pollack, 1985; Lavine and Grodzinsky, 1987). Although clearly a different phenomena than Galvanotaxis, the concept of applying electromagnetic stimuli to aid bone fracture healing originally followed similar lines of thought as in the analysis of Galvanotaxic phenomena. In particular, it was first reported some time ago that dehydrated (Bassett and Becker, 1962) as well as living bone (Cochran et al., 1968) generate internal electric fields when deformed by the application of mechanical forces. These observations led to the proposal that endogenous stress generates electrical signals that are necessary for bone to remodel and adapt to a changing mechanical environment. From this hypothesis, it was further extrapolated that exogenously applied, physiological levels of electric signals may augment the process of fracture healing. This mechanoelectric hypothesis seems to be generally correct. However, it is still not clear whether the biologically relevant potentials generated in vivo in stressed bone arise as a result of a piezoelectric effect, or alternatively are electrokinetic in origin—that is, whether they arise as a result of so-called streaming potentials due to the entrainment of ions due to fluid movement through stressed bone (Otter et al., 1998).

Nevertheless, a large body of evidence has continued to show that artificially applied electric fields affect bone cell activity. In culture, bone cells have been shown to be affected by DC electric fields (Ferrier et al., 1986; Bodamyali et al., 1999), as well as sinusoidal (Noda et al., 1987) and pulsed fields (Guerkov et al., 2001; Tepper et al., 2004; Lohmann et al., 2003; Fredericks et al., 2000). Most importantly, electric fields have been shown to influence the behavior of living bone tissue in animal studies. Here again, DC fields (France et al., 2001; Friedenberg et al., 1974; Dejardin et al., 2001), as well as low-frequency sinusoidal (McLeod and Rubin, 1990) and pulsed fields (Fredericks et al., 2000; Inoue et al., 2002) in the vicinity of 10–30 Hz, have all been shown to affect bone activity.

Unfortunately, the problem with most of the studies addressing the effect of ultra-low frequency electric fields on bone is that although the basic experimental observations are clearly reproducible, there is very little understanding of the underlying biophysical mechanism involved. However, what is clear is that the response of bone or other cells to electric or magnetic fields is sensitive to frequency, suggesting that there may be windows of biologically relevant frequency domains. Different windows likely correspond to different underlying biophysical mechanisms, which could potentially be cell-specific. For instance, Galvanotactic phenomena in

epithelial-like cells as well as bone cells are characterized by responses to DC signals, but bone, for unknown reasons, also seems to be very sensitive to electrical signals in the vicinity of 15 Hz (Bassett, 1984).

The effects we are reporting on here, in neutrophils, involve induced electrical signals pulsed in the frequency range of 0.05 Hz. However, the fields themselves contain higher frequency components. Fig. 13 shows the calculated normalized Fourier power-spectrum for the electric signal induced by the pulsed magnetic fields utilized in our experiments. Surprisingly, although the applied field is pulsed at 0.05 Hz, most of the energy actually delivered to the cells lies at frequencies between 2 and 30 Hz, with the energy peak at 4.5 Hz. It may be coincidence, but it is noteworthy to recall that this is just the frequency range where bone cells have been shown to be most responsive to time-varying electromagnetic fields.

Fig. 10 demonstrates that external calcium is needed for neutrophils to sense the 0.05-Hz pulsed magnetic (or electrical) fields utilized in our experiments, suggesting that membrane calcium channels are involved in the reception apparatus. In fact, a number of previous investigators have proposed that calcium channels are operated on by ELF electromagnetic fields (reviewed in Lacy-Hulbert et al., 1998). Generally speaking, most of these studies have used stronger electric field strengths (in the vicinity of ~100 V/m) than the fields we have utilized in our experiments, which in some instances are much weaker. It may very well be that different calcium channels are responsible for the weaker electric field effects we describe. However, there have been reports where alterations in internal calcium levels in cultured T-cell lines were seen in response to exposure to 50 Hz, 0.15 mT magnetic fields (Lindstrom et al., 1993; Mattsson et al., 2001). Interestingly, in these experiments it could not be ruled out that the effects were due to induced electric fields at or below 50 mV/m.

Nevertheless, the real problem, though, is the exquisite sensitivity that we find. We find that neutrophils are sensitive to electric fields of $\sim10^{-4}$ V/m, by any measure an extraordinarily low field strength, especially considering that
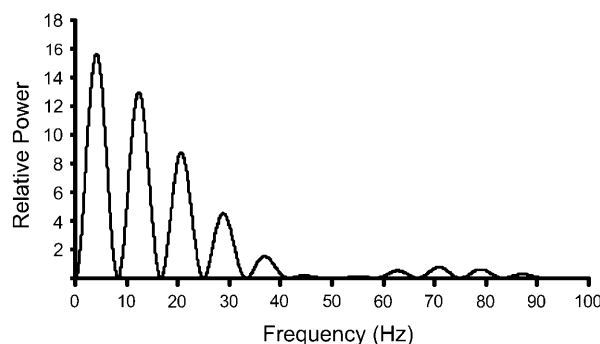
FIGURE 13 Normalized power spectrum for the induced (pulsed) electric fields in Figs. 2–4, 6, 7, and 9–11.

the dimension of a neutrophil is of the order of 10 μm. This is much more sensitive than straightforward theoretical considerations seem to suggest. For instance, in a thermodynamic perturbation-response analysis of the physical limits imposed on a cell sensing an oscillatory electric field, the field-sensing mechanism was modeled generally as a collection of independent voltage-sensitive membrane transport channels. The receptors were modeled as classical transmembrane ion channels whose gating characteristics each independently obeyed Boltzmann statistics. In this case, the channels were shown to operate essentially as a field rectification mechanism, by giving rise to DC transport of an arbitrary signaling molecule in response to the external AC field. Using this model, it was concluded that under the best case scenario, as a result of molecular shot noise decreasing the signal/noise ratio, a 100 μm spherical cell should be capable of detecting electric fields no smaller than 10^{-1} V/m. And this was only after a signal integration time of 10^4 s (Astumian et al., 1995). A similar analysis on elongated cells (10^{-3} m $\times$ 5 $\times$ 10^{-5} m) showed that, at best, sensitivity could be expected to increase an order of magnitude to $\sim 10^{-2}$ V/m (Weaver et al., 1998). Neutrophils, of course, are much smaller and not nearly as elongated, but nevertheless, in our hands are still capable of detecting pulsed electrical signals as low as 10^{-4} V/m in $\sim$40–60 s. Thus we find neutrophils to be more sensitive to oscillating electric fields, by at least a factor of 10^3, than these theories would predict.

So how might our experimental results be reconciled with physical theory? First we would argue that the neutrophil electric field detection system, whatever it is, cannot be modeled simply as individual membrane-embedded protein channels. Our results, taken in context with the theoretical studies, strongly imply that the electrical sensing system utilized by neutrophils and other motile cells has to be more complicated than a collection of independent ion channels. This is not to say that ion (i.e., calcium) channels cannot be involved, but rather that individual channels probably must be considered part of a larger system. For instance, in another theoretical analysis of the amplification of electromagnetic signals by ion channels, it was concluded that the signal/noise ratio increases with the square-root of the total number of channels (Gailey, 1999). Furthermore, a separate analysis concluded that signal/noise increases not only with the number of channels, but also with the frequency response of the intracellular sensing system (Galvanovskis and Sandblom, 1997). Thus when using signal/noise considerations to estimate the limits of cellular sensitivity to weak electric fields, it is probably insufficient to consider the biophysical properties of membrane transport channels in isolation, and independent of any consideration of how a transported moiety couples with higher order metabolic processes. Secondly, we consider it quite likely that the channels themselves are not independent, but actually influence each other through at least nearest-neighbor interactions. Inspired

by observations that eukaryotic and prokaryotic receptor proteins often form two-dimensional clusters in plasma membranes, the effects of allowing nearest-neighbor interactions to influence conformational rate constants in individual receptor proteins was analyzed in a computer simulation model. Because of the resulting cooperativity, it was concluded that such a coupled system showed greatly enhanced sensitivity to external signals compared with a corresponding set of uncoupled receptors (Duke and Bray, 1999).

There are two other points to make. First, it is important to emphasize that our system is not the first in which very low intensity electric fields have been associated with biological effects. For example, electric fields of 10^{-5} V/m have previously been shown to stimulate human bone cell proliferation and increase IGF-II secretion (Fitzsimmons et al., 1992). More importantly, in the final analysis, inconsistencies between experimental and theoretical articles concerning cellular electric field sensitivities is not necessarily an experimental problem. Experimental results have to be considered the ultimate check on the validity of any theoretical analysis.

Based on our results, the model that we propose for the neutrophil electric field detector is illustrated in Fig. 14. We envision that a collection of cooperatively coupled, electrically sensitive membrane complexes, including calcium channels, initially sense the electric fields. We assume that the complexes can be characterized by an intrinsic relaxation constant of $\sim$40–1000 ms, and so respond optimally to fields of between 1 and 25 Hz or so. In response to the fields, the complexes transmit an intermediate signal (probably calcium pulses), which, in a second level of signal processing
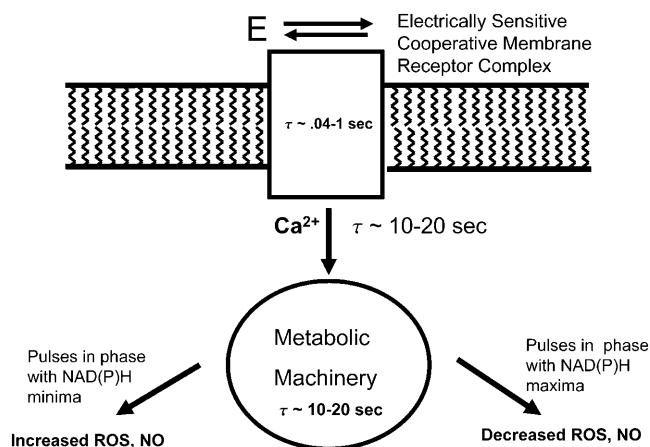

FIGURE 14 Model of the electrically sensitive receptor system in neutrophils. The system is envisioned to contain two components. A primary membrane receptor complex responds to electrical signals in the frequency range of 1–25 Hz by transmitting calcium pulses. These pulses then interact with the metabolic machinery of the cell. If the pulses are of the proper frequency ($\sim$0.05 Hz), the cell will respond with one of two different outcomes, depending on the phase relationship of the pulses to the neutrophil NAD(P)H oscillation.

dependent upon the metabolic oscillations, interacts with, and is then decoded by, the metabolic machinery of the cell. The frequency of the calcium pulses must be compared with the fundamental 0.05 Hz metabolic oscillations, so that, in a sense, the intermediate metabolism of the cell functions as a biochemical bandwidth filter centered at 0.05 Hz. In this way, the 0.05-Hz electrical-pulse-frequency domain of interest is seen to arise quite naturally. Moreover, the physiological response of the cells to the pulsed fields must also depend upon the phase relationship of the pulses with the metabolic oscillation. This is quite clear in that we have demonstrated that, depending on the phase relationship of induced electrical pulses to the NAD(P)H oscillation, production of ROS and NO is either enhanced or suppressed.

In summary, the experiments outlined in this report confirm that, in vitro, the metabolism of neutrophils dramatically responds to extremely low intensity and ultra-low frequency (~0.05 Hz) pulsed electric fields, whether the fields are directly applied or induced by time-varying magnetic fields. When the field pulses are timed so as to coincide with periods of maximal NAD(P)H (or equivalently maximally reduced flavoproteins), production of ROS and NO is increased. Alternatively, if the field pulses coincide with periods of minimal NAD(P)H (or equivalently maximally oxidized flavoprotein), production of ROS and NO are greatly diminished. In effect, very weak induced electric fields can be utilized to signal and control cellular function much as do cytokines or drugs. Because time-varying magnetic fields easily penetrate tissue, these experiments suggest that ultra-low frequency pulsed magnetic fields may be useful to control cellular metabolism in vivo, and could have potential therapeutic value.

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