Interferon-γ and Sinusoidal Electric Fields Signal by Modulating NAD(P)H Oscillations in Polarized Neutrophils

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ABSTRACT Metabolic activity in eukaryotic cells is known to naturally oscillate. We have recently observed a 20-s period NAD(P)H oscillation in neutrophils and other polarized cells. Here we show that when polarized human neutrophils are exposed to interferon-γ or to ultra-low-frequency electric fields with periods double that of the NAD(P)H oscillation, the amplitude of the NAD(P)H oscillations increases. Furthermore, increases in NAD(P)H amplitude, whether mediated by interferon-γ or by an oscillating electric field, signals increased production of reactive oxygen metabolites. Hence, amplitude modulation of NAD(P)H oscillations suggests a novel signaling mechanism in polarized cells.

INTRODUCTION

It is now generally accepted that information necessary for the control of cell function is often encoded in cytosolic calcium oscillations (Berridge, 1997; Corkey et al., 1988; De Koninck and Schulman, 1998; Goldbeter, 1996; Gu and Spitzer, 1995; Tsien and Tsien, 1990). However, other oscillating metabolites could also encode physiological information (Hess and Boiteux, 1971; Kindzelskii et al., 1997). In this report we examine the relationship between NAD(P)H oscillations and the control of cell function. We confirm previous findings that adherent and spontaneously polarized neutrophils, but not nonpolarized neutrophils, exhibit NAD(P)H oscillations ($\tau \approx 20$ s) (Kindzelskii and Petty, 2000). We show that the oscillation is strongly linked to and in fact appears to regulate reactive oxygen metabolite (ROM) production, an important neutrophil effector function (Babior, 1978). Treatment of neutrophils with interferon-γ (IFN-γ) increases both the amplitude of the NAD(P)H oscillation and the production of reactive nitrogen metabolites (Adachi et al., 1999). We now show that AC electric fields with proper frequency and phase characteristics resonate with cellular NAD(P)H. Remarkably, in a manner analogous to that of treatment with IFN-γ, resonating AC electric fields increase the NAD(P)H oscillatory amplitude, directly leading to concomitant increases in ROM production. Thus, ROM production is likely controlled by the amplitude of the NAD(P)H metabolic oscillation, which in turn can be regulated either by cytokine action or by the direct application of an external AC electric field.

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MATERIALS AND METHODS

Material

Reagents were obtained from Sigma Chemical Co. (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.

Cell chamber

Two Pt electrodes (0.25 mm in diameter) separated by 1.1 cm were attached to a glass microscope slide, and the space between the electrodes was filled with the cell suspension. A coverslip was placed over the electrodes to exclude air. The entire assembly was then sealed with silicone grease, forming a chamber with external dimensions of $0.254 \times 11.0 \times 25.4$ mm, with parallel electrodes running along the 25.4-mm dimension. The slide was placed coverslip down on an Axiovert inverted epifluorescent microscope (Carl Zeiss, New York, NY), equipped with a Zeiss temperature-controlled stage set to 37°C.

Fluorescence quantification

In most experiments, cells were individually illuminated, and single-cell NAD(P)H autofluorescence was characterized using 365DF20 and 430DF20 filters and a 405 long-pass dichroic mirror. Tetramethylrosamine (TMRos) fluorescence was detected using a 540DF20 and 590DF30 filter set with a 560 long-pass dichroic mirror. Fluorescence levels were quantified using a Hamamatsu (Bridgewater, NJ) photomultiplier tube held in a Products for Research (Danvers, MA) housing attached to the microscope and coupled to an amplifier (Photochemical Research Associates, London, ON, Canada) that was interfaced with a model 2010 multimeter (Keithley Instruments, Cleveland, OH). The multimeter was in turn interfaced with a computer through Test Point software (Capital Equipment Corp., Billerica, MA). In some experiments fluorescence was quantified using a D104 fluorescence microscope detection system interfaced with a computer running FeliX software (Photon Technology International, Monmouth Junction, NJ).

Application of electric fields

For square-wave voltage forms, an electrical stimulator (Grass Medical Instruments, Quincy, MA) was used to apply 1-s DC pulses across the cell

chamber. A model 2040 source meter (Keithly Instruments) was interfaced with a computer (programmed with Test Point software) to generate sinusoidal voltage forms.

Detection of reactive oxygen metabolites

Neutrophils were suspended in 2% gelatin incorporated with 100 ng/ml dihydrotetramethylrosamine (H_2TMRos) (Molecular Probes, Eugene, OR) at 45°C, before being loaded into the cell chamber and mounted on the microscope stage as described above. Reactive oxygen metabolites produced by the cells diffused into and were trapped within the gelatin matrix, where they oxidized the H_2TMRos to TMRos, which was detected by epifluorescence microscopy.

Single-cell gel electrophoresis assay

Neutrophils were directly incorporated into low-melting-point agarose before being loaded into the cell chamber and mounted on the microscope stage as described above. After the agarose solidified, cells were viewed under the microscope, and of the several neutrophils in the visual field, one adhered and spread neutrophil was selected. The frequency and phase of its NAD(P)H oscillation were determined by fluorescence microscopy as outlined above, and sinusoidal AC voltage was adjusted to double the NAD(P)H oscillatory frequency, applied across the Pt electrodes. The wave form was phase matched so that a null voltage would coincide with a NAD(P)H minimum. Metabolic resonance was thus established with the applied field and maintained for a period of 15 min. The target cell as well as control cells not in resonance were then removed from the scope and processed, using the single-cell gel electrophoresis (SCGE) assay for DNA damage as previously described (Kindzelskii and Petty, 1999).

RESULTS

NAD(P)H oscillations in spherical and polarized neutrophils

Fig. 1 *a* is a representative example ($n > 100$) of a differential interference contrast (DIC) image of a nonpolarized spherical neutrophil. After the DIC image was recorded, the cell was illuminated at 365 nm, and the resulting fluorescence was collected above 405 nm with epifluorescence microscopy. The fluorescence intensity of the entire cell is plotted as a function of time in Fig. 1 *b* ($t \approx 180$ s). It has been previously shown that under these conditions fluorescence is due almost exclusively to NAD(P)H (Hess and Boiteux, 1971; Kindzelskii et al., 1997; Petty et al., 2000). Because cytosolic NAD(P)H concentration is strongly coupled to cellular metabolism, epifluorescence microscopy of intracellular NAD(P)H opens a real-time window on metabolic activity at the single-cell level. In this case NAD(P)H oscillations with a period of ~3 min are suggestive of an underlying metabolic oscillation with a similar period.

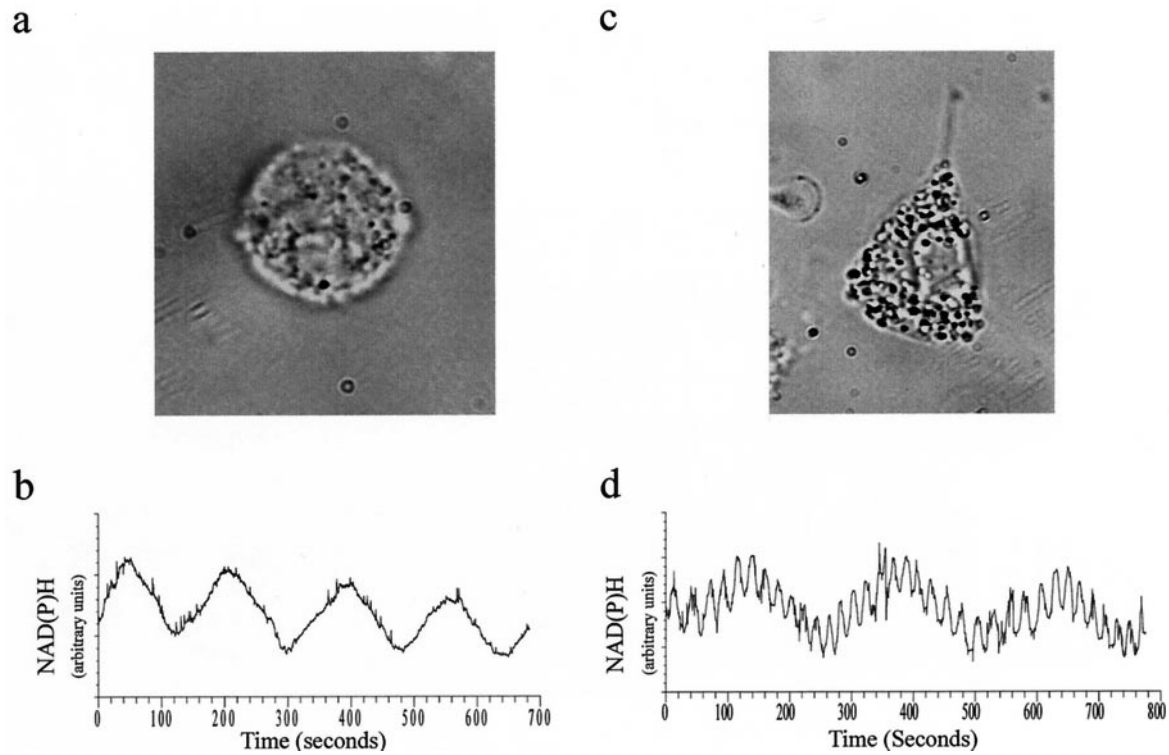

FIGURE 1 NAD(P)H oscillates in spherical and polarized neutrophils. Neutrophils were suspended in Hanks' balanced salt solution, placed on a slide, covered with a coverslip, and then mounted on a heated (37°C) microscope stage. (*a*) A spherical neutrophil was selected and imaged by differential interference contrast microscopy. (*b*) The time dependence of NAD(P)H within the spherical cell was then determined by monitoring NAD(P)H autofluorescence by epifluorescence microscopy. NAD(P)H oscillates with a period of ~180 s. (*c*) A polarized and adherent neutrophil was selected and imaged by differential contrast microscopy. (*d*) The time dependence of NAD(P)H within the polarized cell was determined by monitoring NAD(P)H autofluorescence by epifluorescence microscopy. NAD(P)H oscillates with a 21.6-s period oscillation superimposed over an underlying oscillation of 230 s.

Metabolic oscillations in polarized and adherent cells appear to be more complex than those of spherical cells, in that a second higher frequency oscillation mode appears. Fig. 1 *c* is a DIC image of a representative example ($n > 100$) of a polarized neutrophil that has adhered to a coverslip, and for which NAD(P)H fluorescence was assessed as in Fig. 1 *b*. In this instance we find (Fig. 1 *d*) that superimposed upon the "long" period oscillation (~230 s for this cell) is an oscillation with a period of 21.6 s. A statistical analysis of the frequency and amplitude properties of these two distinct oscillation modes over larger sample sizes is summarized in Table 1.

Interferon-γ increases the amplitude of the 20-s NAD(P)H oscillation

Neutrophils have receptors for and functionally respond to IFN-γ (Berton et al., 1986). Fig. 2 is a representative example of an experiment where we began monitoring NAD(P)H fluorescence in a polarized neutrophil 50 min after the addition of IFN-γ in Hanks' balanced salt solution. In this example, ~1 min after the recording started the amplitude of the NAD(P)H oscillation increased, confirming previous findings that the functional response to IFN-γ parallels an increase in the amplitude of the 20-s NAD(P)H oscillation in polarized neutrophils (Adachi et al., 1999). Statistical analysis over a larger sample size indicates that in neutrophils the time lag between IFN-γ addition and the increase in NAD(P)H oscillation amplitude is 47 ± 8 min ($n = 20$). (Control experiments where Hanks' balanced salt solution alone was added to the cells showed no increase in NAD(P)H oscillation amplitudes.) Furthermore, the increase in amplitude is persistent, as once it increased, it remained at the higher level for as long as we were technically able to continuously monitor the cells (~20–30 min).

In neutrophils reactive oxygen metabolite production takes place in discrete bursts, and the burst size is increased by IFN-γ

We have recently developed a new method to measure cellular ROM production. ROM-producing cells are first embedded in a gelatin matrix containing H_2TMRos. H_2TMRos is not fluorescent, but is rapidly oxidized by ROMs to TMRos, which is. Fluorescent emission, which can be taken as a measure of ROM concentration in the gel, is then monitored with epifluorescence microscopy (Kindzelskii et al., 1998).

TABLE 1 Characteristics of NAD(P)H oscillation modes in polarized neutrophils

Mode	Period (seconds)	Amplitude (%)*	$n^\dagger$
1	222 ± 77	±28	15
2	22.8 ± 2.9	±12	100

*The amplitude of each mode is expressed as a percentage modulation of the mean NAD(P)H signal.

†As cells were not routinely followed for long periods, the data set used to evaluate the characteristics of the first mode was smaller than that available for evaluation of the second mode characteristics.

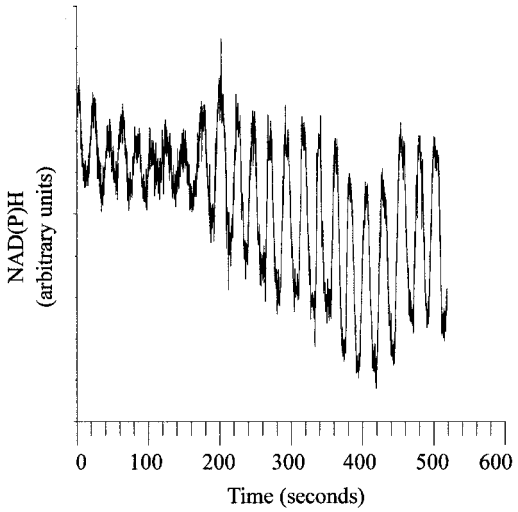

FIGURE 2 Interferon-γ increases the amplitude of NAD(P)H oscillations in polarized and adherent neutrophils. Neutrophils were suspended in Hanks' balanced salt solution, and IFN-γ was added to 25 ng/ml. The cells were then placed on a microscope slide, sealed with a coverslip, and placed on a heated microscope stage maintained at 37°C. After 50 min a polarized and adherent neutrophil was selected, and NAD(P)H within the cell was monitored by NAD(P)H autofluorescence. About 160 s after recording was initiated (time 0), the amplitude of the NAD(P)H oscillation increased.

Fig. 3 is an example of the technique conducted in the absence of cells. In this control experiment, exogenous H_2O_2 was added directly with a pipette to the edge of the coverslip on a slide containing (matrix) embedded H_2TMRos, and TMRos fluorescence was monitored. In this case, photomultiplier counts per second are directly plotted versus time. The counts from 0 to 22 s represent system background. However, at 22 s, a pulse of 0.3 μM H_2O_2 was directly delivered to the slide with a pipette. As a result TMRos fluorescence abruptly rises in a stepwise manner, as the H_2O_2 is rapidly consumed, and H_2TMRos is oxidized to TMRos. At 40 s a second pulse of 1.0 μM H_2O_2 was delivered to the slide. Again we see a stepwise increase in TMRos fluorescence, but the increase is larger than before, representing the greater concentration of H_2O_2 added.

Neutrophils produce a variety of ROMs, which are toxic to bacteria (Babior, 1978). IFN-γ primes unstimulated cells for ROM production and enhances ROM release from stimulated cells (Berton et al., 1986). In Fig. 4 *a* endogenous production of ROMs in a polarized and adherent neutrophil was assessed, by embedding the cell in a matrix containing H_2TMRos and measuring TMRos fluorescence as above. By focusing on and limiting the field of view to only the

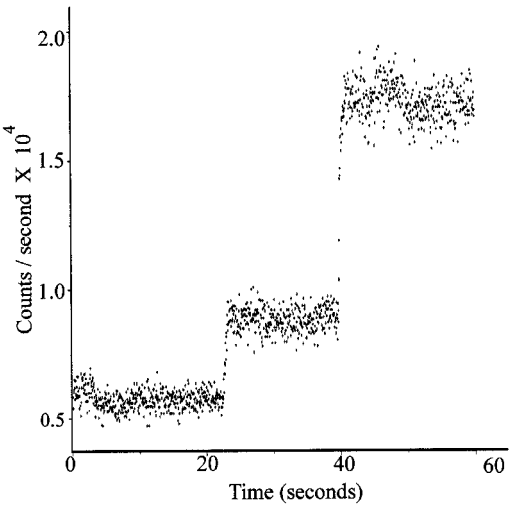

FIGURE 3 Reactive oxygen metabolites can be detected by monitoring the change in fluorescence after oxidation of dihydrotetramethylrosamine (H_2TMRos) to tetramethylrosamine (TMRos). A liquid gelatin matrix containing H_2TMRos was loaded onto a slide and sealed with a coverslip, and the gelatin was allowed to solidify. The slide was then mounted on the epifluorescence microscope stage, and fluorescence was monitored as a function of time with a photomultiplier counting system attached to the scope. H_2TMRos is not fluorescent but is oxidized to TMRos, which is. At 22 s 0.3 μM H_2O_2, and at 40 s 1.0 μM H_2O_2 were added directly to the slide with a pipette. Oxidant addition is accompanied by the formation of TMRos, which becomes trapped in the matrix, allowing oxidant addition to be followed by epifluorescence.

target cell, and then monitoring the increase in TMRos fluorescence with the epifluorescence microscope, we obtained in real time a direct measure of the oxidation of H_2TMRos in the cell vicinity. This is correlated with the cellular production of ROMs. In general, fluorescence increases as TMRos accumulates in a stepwise fashion (*arrows*), with each step following the previous one after a period equal to the NAD(P)H oscillatory period. However, at longer time points, the increase in fluorescence begins to fall off, likely because of quenching. These results support previous findings where we have reported that production of reactive oxygen metabolites in neutrophils is oscillatory (Kindzelskii et al., 1998).

Fig. 4 *b* shows a similar experiment, in which the neutrophil was exposed to IFN-γ 50 min before the recording was started. As in Fig. 4 *a*, ROM production was assessed by monitoring the accumulation of TMRos. For comparison purposes the data in Fig. 4 *a* have been replotted in Fig. 4 *b* on the same fluorescence scale to highlight and control for the difference between endogenous and IFN-γ-stimulated ROM production. It is quite clear that for IFN-γ-stimulated production, as for endogenous ROM production, ROMs periodically accumulate in the medium in discrete bursts. It is also clear that increased ROM production due to IFN-γ arises as a consequence of an increase in the burst size.

Metabolic oscillations can be controlled by externally applied pulsed DC electric fields with either uniform or alternating polarity

Previously it had been shown that when migrating neutrophils were exposed to low-voltage DC electric fields, the cells underwent an exaggerated length extension (Kindzelskii and Petty, 2000). However, the response did not take place under uniform field conditions, but only if the field was pulsed, and then only if each pulse was phased to coincide with a minimum in the 20-s NAD(P)H oscillation. Of relevance here was the additional observation that under these conditions a resonance was noted between the applied field and the NAD(P)H oscillation. However, in the aforementioned experiments, the field polarity, and so, presumably, the direction of any electrical force vector on the cells, was held constant. In Fig. 5 we investigated whether periodically reversing the polarity of a pulsed DC field (and so reversing the direction of any electrical force vector) would still permit resonance between the applied electric field and the NAD(P)H oscillation.

Accordingly, Fig. 5 shows a representative example (n = 100), where an individual neutrophil was allowed to adhere to and polarize on a glass coverslip, and NAD(P)H fluorescence was monitored as described above. After the first few NAD(P)H cycles, an electric field generated by a square-wave voltage source was synchronously applied across the cell at NAD(P)H minima. In the absence of an applied voltage, NAD(P)H concentration oscillates uniformly with a period of ~20 s. However, after application of only the second voltage pulse, NAD(P)H began to resonate with the electric field, in that the amplitude clearly began to increase. The NAD(P)H amplitude reached a maximum value after the sixth pulse. When the voltage pulses ceased, the NAD(P)H amplitude returned to baseline values. After a brief period, resonance was then reestablished as a series of additional synchronous voltage pulses was administered. However, in this case, while the applied voltage was increased by a factor of 5 over the initial pulses, the maximum NAD(P)H amplitude remained unchanged. When the field was terminated, the NAD(P)H amplitude again rapidly decayed to baseline values. A third series of synchronous pulses was then administered, but at 10 times the initial field intensity. Once again resonance was quickly established, with the NAD(P)H oscillations having the same maximum amplitude as before, indicating that the resonance is an all-or-none phenomenon. The NAD(P)H amplitude again decayed to baseline values when field pulses were discontinued. Finally, a fourth series of synchronized pulses was applied, but this time field polarity was reversed on alternate pulses. We found that resonance was established just as rapidly, and in fact it appears to be indistinguishable from the resonance induced by previous uniform pulse trains. Thus it appears that polarity is unimportant in the ability of an external pulsed electric field to resonate with neutrophil

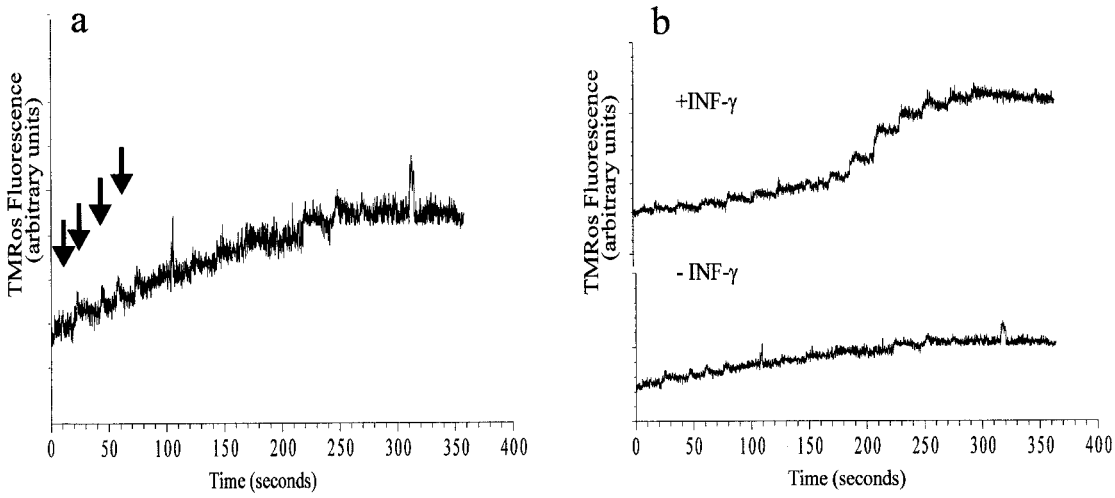

FIGURE 4 IFN-γ stimulates increased production of reactive oxygen metabolites (ROMs) in neutrophils. Neutrophils were suspended in a liquid gelatin matrix (with or without 25 ng/ml IFN-γ) containing H_2TMRos before being loaded onto a slide and sealed with a coverslip. Slides were mounted on a heated (37°C) microscope stage, and the gelatin was allowed to solidify. As in Fig. 3, oxidant generation is accompanied by the formation of TMRos, allowing cellular oxidant production to be followed by epifluorescence. In each instance ($\pm$ IFN-γ), a single polarized and adherent neutrophil was selected, and recording of TMRos fluorescence (arbitrary scale) in the vicinity of the cell was initiated 50 min after the slide was mounted on the microscope stage. (*a*) TMRos fluorescence in the absence of IFN-γ. The arrows indicate the position of abrupt increases in fluorescence, indicative of pulsed ROM production. (*b*) TMRos fluorescence after the addition of IFN-γ (+IFN-γ). The baseline data from *a* have also been replotted (−IFN-γ) but are now drawn to the same scale as the data from cells to which IFN-γ has been added, to emphasize the difference in ROM production between cells exposed or not exposed to IFN-γ.

metabolism, as long as each pulse is still timed to coincide with an NAD(P)H minimum.

Metabolic oscillations can be controlled by externally applied AC electric fields

Based upon Fig. 5, we hypothesized that sinusoidal AC electric fields would also resonate with NAD(P)H oscillations, provided that the period of the AC voltage form was double that of the NAD(P)H period, and that the AC sinusoid was phased so that the minimum and maximum voltages coincided with the NAD(P)H minimum. (It can be seen that these conditions satisfy the requirement that alternate electric field maxima and minima are in phase with successive NAD(P)H minima.) The prediction is verified in Fig. 6, where an experiment similar in design to that of Fig. 5 is shown. Fig. 6 is a representative example ($n = 23$) wherein electric fields generated by a series of three properly phase- and frequency-matched sinusoidal voltage forms, separated by periods of zero applied field, have sequentially established resonance with NAD(P)H oscillations in an adherent neutrophil. As in Fig. 5, resonance is an all-or-none phenomenon. Although the AC field amplitudes vary by 25-fold, the maximum amplitude of the resonating NAD(P)H oscillation remains constant. Once a field threshold has been reached and resonance established, increasing the strength of the electric field has little effect on the NAD(P)H oscillatory behavior. Also as in Fig. 5, after resonance has been established, but if the fields are then turned off, NAD(P)H oscillation amplitudes appear to rapidly return to baseline values. This was rigorously established in Fig. 6 by comparing the amplitudes of the four complete (20 s) NAD(P)H oscillations just before exposure to the first (9.0×10^{-2} V/m) electric field, with the amplitudes of the final four NAD(P)H oscillations shown in the figure, which were measured after exposure to the third (2.3 V/m) electric field had been terminated. In this case the baseline amplitude (in arbitrary units) of 3.85 ± 0.57 matches the final NAD(P)H amplitude of 3.85 ± 0.54. In independent experiments, we have established that for sinusoidal AC fields, the minimum intensity threshold necessary to establish resonance with human neutrophils lies between 5.9 and 6.8×10^{-2} V/m.

ROM production in neutrophils can be directly controlled by the application of an external electric field

Fig. 4 demonstrated that ROM production takes place in a series of discrete pulses that appear to be synchronized with NAD(P)H metabolic oscillations, and that IFN-γ-mediated increases in neutrophil ROM production arise as a consequence of increased pulse size. On the other hand, Fig. 2 demonstrates that IFN-γ also increases the amplitude of the NAD(P)H oscillation, so that increases in ROM pulse size are at least concomitant with an IFN-γ-modulated increase in the NAD(P)H amplitude. We hypothesized that the ROM pulse size is generally controlled by amplitude modulation of the NAD(P)H oscillation, so that IFN-γ-mediated in-

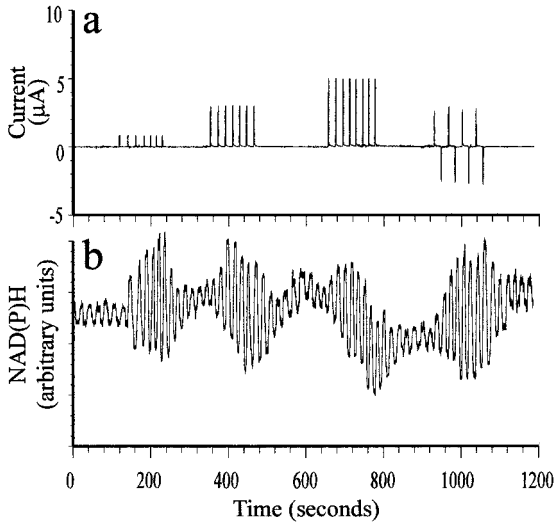

FIGURE 5 Pulsed DC electric fields resonate with naturally occurring NAD(P)H oscillations in human neutrophils. Neutrophils were placed in the cell chamber described below and allowed to settle and adhere to the coverslip for 20–30 min. The chamber was mounted on a heated 37°C microscope stage, a neutrophil was selected, and the autofluorescence arising from NAD(P)H was measured. Square-wave DC pulses were then applied across the chamber. Each pulse was timed to coincide with a successive NAD(P)H minimum. (*a*) Currents resulting from successive application of pulse chains of 1, 5, and 10 V of uniform polarity, followed by a fourth pulse chain of 5 V with alternating polarity, are plotted as a function of time. (*b*) The corresponding trace for the cellular NAD(P)H autofluorescence is plotted along the same time scale.

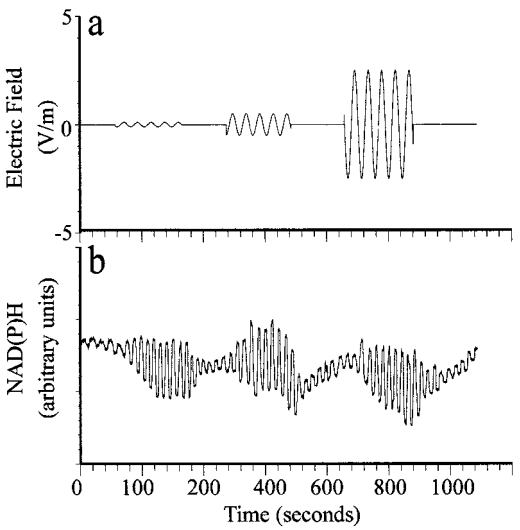

FIGURE 6 AC electric fields resonate with naturally occurring NAD(P)H oscillations in human neutrophils. As in Fig. 1, neutrophils were placed in the cell chamber and allowed to adhere to the coverslip, and an individual adherent neutrophil was selected. After the period of the NAD(P)H oscillation was measured, a sinusoidal AC voltage form with double the NAD(P)H period was applied across the cell chamber. Initial application of the AC voltage was begun at a NAD(P)H minimum, with a maximum amplitude of 1 mV. After five cycles, the applied voltage was turned off. This was followed by application of a 5-mV AC voltage for five cycles, followed by application of a 25-mV AC voltage. Voltage forms of 1, 5, and 25 mV gave rise to alternating electric fields characterized by maximum field intensities of 9.0×10^{-2}, 4.5×10^{-1}, and 2.3 V/m, respectively. The applied voltages plotted as a function of time are displayed in *a*, and simultaneous NAD(P)H autofluorescence is displayed in *b*.

creases in ROM pulse size are dependent upon the ability of IFN-γ to increase in the NAD(P)H amplitude. Because Figs. 5 and 6 demonstrate that external resonant electric fields increase the NAD(P)H amplitude in the absence of IFN-γ, it follows that if ROM production is strictly regulated by NAD(P)H amplitude, then resonant electric fields should be able to increase ROM production in the absence of IFN-γ. We find this to be true in Fig. 7, where production of ROMs in neutrophils exposed to resonating AC electric fields was assessed by fluorescently monitoring H_2TMRos oxidation to TMRos. In this representative experiment ($n = 15$) an adherent neutrophil was selected, and an AC field, which was frequency and phase matched to the NAD(P)H oscillation, was applied. By monitoring TMRos fluorescence, we find that ROMs clearly increase over baseline values under resonant conditions.

Chronic exposure to resonating AC electric fields damages cellular DNA

We suspect that heightened NAD(P)H concentrations may drive NADPH oxidase to produce excess superoxide or, alternatively, that excessive oxidation and reduction of cytoplasmic metabolite pools leads to spurious superoxide production. In either case, it is expected that superoxide anions will yield downstream reactive oxygen species, which are known to damage DNA (Cross et al., 1987). We have previously reported that pulsed DC fields can damage DNA in neutrophils (Kindzelskii and Petty, 2000; Petty, 2000). To determine whether neutrophils that have been in metabolic resonance with weak AC fields show signs of DNA damage, we performed SCGE or COMET assays on such cells. The SCGE assay is a convenient and sensitive technique for detecting DNA damage, including strand breakage, at the single-cell level (Singh et al., 1988). Fig. 8 is a representative experiment ($n = 12$) where several neutrophils were subject to an AC electric field characterized by a maximum field intensity of 7.9×10^{-2} V/m for 15 min. However, the field was phase and frequency matched to only one of these cells, which established and maintained metabolic resonance with the field throughout the experiment. The other cells were not in resonance with the field. After exposure to the electric field, all cells were subject to SCGE analysis. Notably, it is only the cell that was in resonance with the electric field, that produced a comet tail indicating DNA damage. Over the sample population of 12 independent experiments, the average comet tail length from a resonating cell was 24 ± 9 μm, excluding the nucleus.

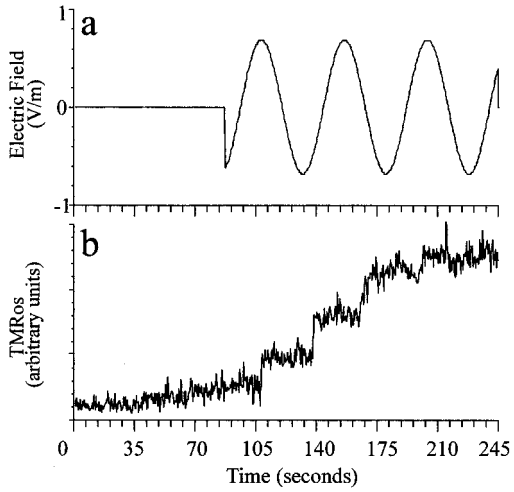

FIGURE 7 Neutrophils in resonance with sinusoidal electric fields produce excessive quantities of reactive oxygen metabolites. Neutrophils were suspended in a gelatin matrix containing H_2TMRos before being loaded into the cell chamber described in Fig. 6 and mounted on the microscope. ROM production was followed by monitoring of the formation of TMRos by epifluorescence microscopy. In this experiment, a polarized and adherent neutrophil was selected, and after an initial analysis of ROM generation, an AC electric field (0.38 V/m) that was frequency and phase matched to the NAD(P)H oscillation was applied. Field strength (*a*) and TMRos fluorescence (proportional to oxidant production) (*b*) are plotted with respect to the same time scale.

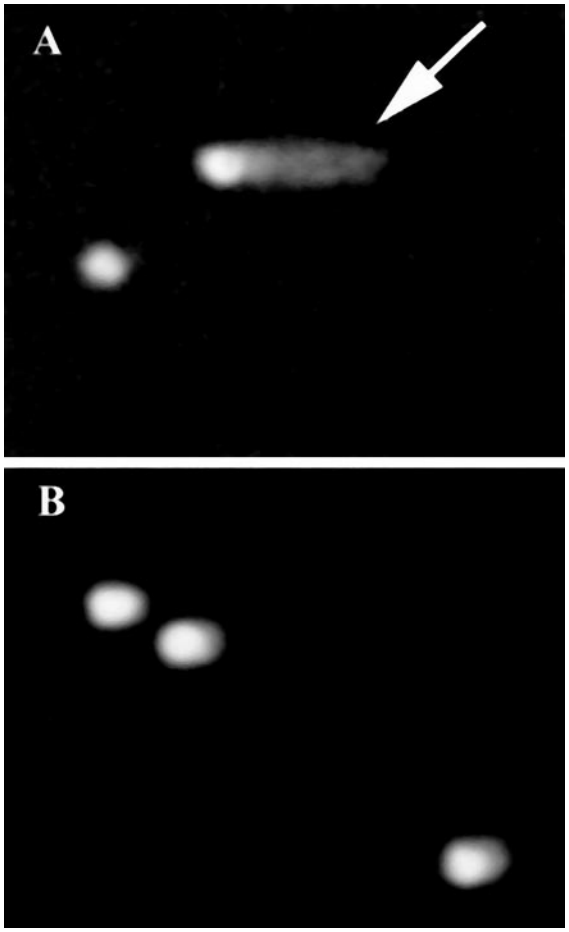

FIGURE 8 Neutrophils subject to extended metabolic resonance with low-intensity alternating electric fields exhibit signs of DNA damage. To detect DNA damage at the single-cell level, a comet assay was performed. Accordingly, neutrophils were directly incorporated into low-melting-point agarose (Sigma, St. Louis, MO) before being loaded into the chamber. After the agarose solidified, the slide was viewed by epifluorescence microscopy. Of the several neutrophils in the visual field, one adhered and a spread neutrophil was selected. The frequency and phase of its NAD(P)H oscillation were determined, and, as in Fig. 2, a sinusoidal AC voltage was applied across the Pt electrodes. The wave form was adjusted to double the NAD(P)H oscillatory frequency and phase matched so that a null voltage would coincide with a NAD(P)H minimum. Applied voltages and NAD(P)H concentrations were monitored as in Fig. 2. The maximum electric field intensity to which the cells were exposed was determined to be 7.9×10^{-4} V/m. For the chosen cell, metabolic resonance was quickly established with the applied field and maintained for a period of 15 min. The slide was removed from the scope and processed for comet analysis of DNA damage. (*a*) Two cell nuclei are visualized in the same visual field. The cell to the upper right (*arrow*) was the neutrophil that had established metabolic resonance with the electric field. For this cell we find signs of significant DNA damage, as indicated by the appearance of a "tail." The other cell in the visual field was a neutrophil that was exposed to the same electric field, but which was not in resonance. (*b*) Three other cell nuclei from neutrophils that adhered to another part of the same slide are visualized. These cells were also exposed to the electric field but were not in metabolic resonance with it.

DISCUSSION

Although it has been known that for some time that both excitable and nonexcitable cells often display regular oscillations in cytoplasmic Ca^{2+}, it is only recently that it has been shown how frequency modulation (FM), as well as amplitude modulation (AM) of Ca^{2+} oscillations could be responsible for signaling differential gene expression (Dolmetsch et al., 1997, 1998; Li et al., 1998). In a similar manner, it has been known that NAD(P)H oscillates in most cells, but the functional significance of this oscillation has not been apparent (Goldbeter, 1996). This work demonstrates that in neutrophils the important effector function of production of reactive oxygen metabolites is associated with amplitude modulation of the NAD(P)H oscillator. We have shown that the oscillator amplitude is modulated by IFN-γ signaling, suggesting that it is through AM modulation of NAD(P)H that the well-known ability of IFN-γ to upregulate neutrophil ROM production operates. That the effect is due to modulation of the time-varying NAD(P)H signal is evident because we have also shown that properly phased electric fields will modulate the amplitude of the NAD(P)H oscillator without any apparent involvement of IFN-γ receptor–IFN-γ interactions, and in so doing will increase ROMs in the absence of IFN-γ. Furthermore, Fig. 6 makes it clear that ROM production cannot simply be dependent on the immediate concentration of NAD(P)H reaching some threshold, as during resonance with an AC

field the maximum concentration of NAD(P)H is independent of the modulated 20-s oscillation. In engineering terms this would be akin to saying that the information content of the signal is independent of the amplitude or power of the carrier.

Thus it appears that amplitude modulation of metabolic signals is a normal intracellular signaling mechanism that can be directly accessed by external electric fields. This suggests that it may be possible in some circumstances to substitute for chemical (i.e., cytokine) control of cell metabolism and function by the direct intentional application of ultra-low-frequency electric fields. However, it also seems possible that under some circumstances, ultra-low-frequency environmental electric fields may inadvertently tap into the metabolic signaling pathway we describe and, as a consequence of enhanced ROM production, induce DNA damage.

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