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The phorbol 12-myristate 13- acetate (PMA)-induced oxidative burst in rat peritoneal neutrophils is increased by a 0.1 mT (60 Hz) magnetic field

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Abstract Magnetic fields (MF) may affect biological systems by increasing free radical concentrations. To test this, we have investigated whether low frequency (60 Hz) low intensity (0.1 mT) MF can modulate the phorbol 12-myristate 13- acetate (PMA) induced respiratory burst in primed rat peritoneal neutrophils, followed in real time using the dye 2′,7′-dichlorofluorescin (DCFH), which reacts with free radical-derived oxidants such as H_2O_2 (which is formed from the dismutation of superoxide) to become 2′,7′-dichlorofluorecein (DCF), a highly fluorescent compound. In the presence of the MF, a 12.4% increase in the fluorescence signal was observed in PMA-stimulated neutrophils ($n = 5$, $P < 0.02$, 18 pairs of measurements). We believe this represents the first experimental observation of MF influencing events involving free radical species generated during signal transduction in living cells.

Key words: Magnetic fields; Free radicals; Neutrophil respiratory burst

1. Introduction

A variety of biological effects has been associated with exposure to low frequency electromagnetic fields (EMF), and have been proposed to be due, at least in part, to magnetic field (MF)-induced increases in free radical concentrations [1,2]. MF effects on the rate of radical pair (RP) recombination is a theoretically sound mechanism by which MF interact with biological systems [2], and is well-established in vitro in organic-based media [3,4]. MF increase the probability that a free radical pair will remain in the triplet configuration (by decreasing intersystem crossing), thus increasing the probability that two free radicals will escape without termination. Because fewer terminations of radical pairs occur, the overall concentration of radicals increases, and a potentiation of free-radical induced effects in biological systems may be expected [2]; both time-varying and static magnetic fields can participate in such interactions [5].

We evaluated whether low frequency (60 Hz), low-intensity (0.1 mT) MF can modulate the PMA induced respiratory burst of free radical generation in primed rat peritoneal neutrophils. The generation of reactive oxygen species (ROS) including superoxide ($O_2^{\bullet-}$) by the plasma membrane NADPH oxidase of neutrophils is a major mechanism of bacterial killing [6]. The $O_2^{\bullet-}$ thus formed enzymatically dismutates to hydrogen peroxide (H_2O_2) through catalysis by superoxide dismutase, or is converted to other reactive oxygen intermediates such as hypochlorite by the myeloperoxidase reaction [7].

The neutrophil respiratory burst of oxidants was assayed in real-time using 2′,7′-dichlorofluorescin diacetate (DCFH-DA), which diffuses into cells, where it is cleaved by non-specific esterases to form 2′,7′-dichlorofluorescin (DCFH), which reacts with free radical-derived oxidants (e.g. H_2O_2, formed from the dismutation of $O_2^{\bullet-}$) and is converted to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF). Hence, a change in the fluorescent signal in the presence of a MF will indicate an influence of MF on oxidant generation [8].

2. Materials and methods

2.1. Preparation of primed neutrophils

For each batch of neutrophils, two Sprague–Dawley male rats (200–250 g b.w.) were anesthetized with ether and injected intraperitoneally (i.p.) with 30 ml of sterile 2% casein dissolved in phosphate-buffered saline, pH 7.4 (PBS). The rats were allowed to recover and were sacrificed 12–14 h after injection. Primed neutrophils were recovered from the peritoneal cavity by lavage with 60 ml of warm (37°C) PBS. Peritoneal bleeding was avoided. The cells were pooled following centrifugation for 5 min at $200 \times g$, washed three times with ice-cold PBS, resuspended in a volume of 1–2 ml of ice-cold PBS, and kept on ice prior to the assay. This procedure produces cell preparations which contain 80–95% neutrophils [9,10].

2.2. Respiratory burst assay with the DCFH-DA during exposure to 0.1 mT (60 Hz) electric fields

A stock solution of DCFH-DA (10 mM) in ethanol was prepared, purged with nitrogen and stored at −20°C. Neutrophils (10^6 cells/ml) were added to the reaction mixture (10 μM DCFH-DA in PBS) to a final volume of 1.036 ml. The reaction mixture, without PMA, was mixed in a cuvette, then placed in a thermojacketed cuvette-holder and brought to 37°C in a computer-controlled, LS-5B Perkin-Elmer LS-5B dual monochromator spectrophotofluorimeter. The temperature of this cuvette was maintained at 37 ± 0.05°C throughout. Fluorescence was recorded using two different excitation wavelengths (E_x, 480 or 502 nm; E_m 530 nm). PMA (50 nM) was added to the cuvette, and recordings were begun. An approximate 100–200 s lag time was noticed before an exponential increase in fluorescence was observed.

The cuvette was or was not exposed to a 0.1 mT (rms) sinusoidal 60 Hz magnetic field generated by a Helmholtz coil oriented so the applied magnetic field vector was parallel to the long axis of the cuvette. The 60 Hz signal was provided from a standard signal generator and fed unamplified into the Helmholtz coil. The MF treatments and control (no MF) treatments in five separate experiments were sequentially alternated over a total of 18 runs. For control exposures the coil was not energized and the 60 Hz magnetic field intensity along the long axis (vertical axis) of the cuvette, as well as for the two other orthogonal axes, was <0.02 mT. The vertical static magnetic field vector in the laboratory at the position of the cuvette was approximately 0.05 mT. The static magnetic field vectors in the other two orthogonal directions were 0.01 and 0.02 mT. Magnetic fields (AC and DC) were measured by an axial or a tranverse Hall effect probe (F. Bell Inc., Orlando, FL). The axial probe (model SAB4-1908) integrates over a cylindrical vol-

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ume of ~10 mm in length and 4 mm in diameter and was used for measurements along the cuvette long-axis. The tranverse probe (model FTG1-0415) integrates over a rectangular-shaped area of ~10 mm × 4 mm.

2.3. Single cell measurements of oxidant generation

A Delta Scan system (Photon Technologies Inc. New Brunswick, NJ) was used for single-cell fluorescence photometry. This system has a computer-controlled, dual excitation monochromator coupled via a fiber-optic light guide to a Nikon Diaphot inverted microscope. Cells were treated in a test tube at 37°C with DCFH-DA and to PMA as described above for the control cuvette experiments, then transferred into a 35 mm tissue culture plate and maintained at 37°C using a forced warm air curtain (Sage Inst., White Plains, NY) and a heating stage (Medical Systems Corp., Greenvale, NY). Single cells were visualized at 100 × using a thermostatted objective (37°C) and fluorescence was monitored using two excitation wavelengths (E_x 488 nm and 502 nm; Nikon dichroic cube with long-pass FITC filter) for a single cell over time. During the single-cell measurements, data acquisition was paused briefly to re-position the masking barriers to the photometer (the stage was not moved) to monitor the area immediately adjacent to, and within one cell diameter, of the original cell. Data acquisition was paused a final time to re-position the original cell for continued fluorescence monitoring. The static magnetic field vectors (3 orthogonal axes) at the position of the cells on the microscope stage immediately above the 100 × objective were approximately 0.02, 0.06 and 0.08 mT. The ambient magnetic fields, with all equipment on, in the same orthogonal positions were ≤0.02 mT.

2.4. Statistical analysis

The results of five groups of experiments were analyzed. On 5 separate occasions neutrophils were obtained; the number of pairs of ±MF stimulated respiratory burst for each experiment were n = 3, 5, 5, 2 and 3 for a total of 36 separate observations. The fluorescence was measured continuously following addition of PMA. The data from the first 200 s of 'lag time' were disregarded and the data from the remaining time points (every 6 s up to 500 s) were fitted to an exponential curve using a computer graphics program (Delta Graph Professional 3.5; DeltaPoint Inc.). The correlation coefficients were >0.9 for all of the 36 samples. The slopes of the fitted curves of the paired comparisons of +MF and −MF were compared using a computer statistics program (SuperAnova; Abacus Concepts; ANOVA 1-within factor). The average value for +MF and for −MF was calculated for each day's experiments; then these pairs (+MF and −MF) were used to evaluate the effect of MF on respiratory burst (degrees of freedom = 4).

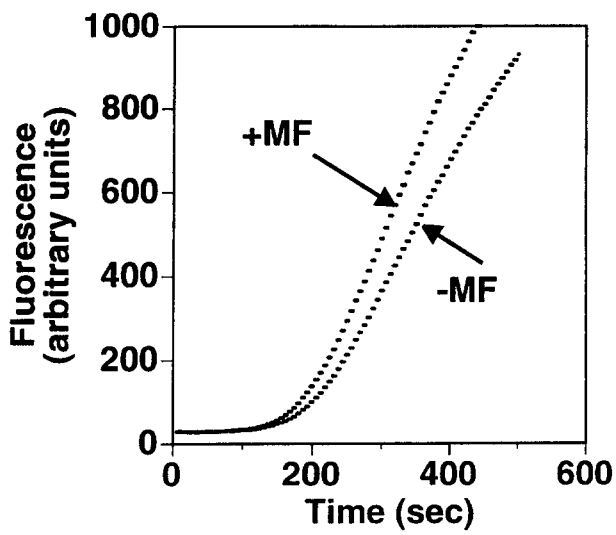

Fig. 1. Real-time assay of oxidant generation following PMA induced respiratory burst in neutrophils. The fluorescence signal of DCF in the presence or absence of MF (0.1 mT, 60 Hz). Conditions are described in section 2. One representative experiment of 18 pairs is shown.

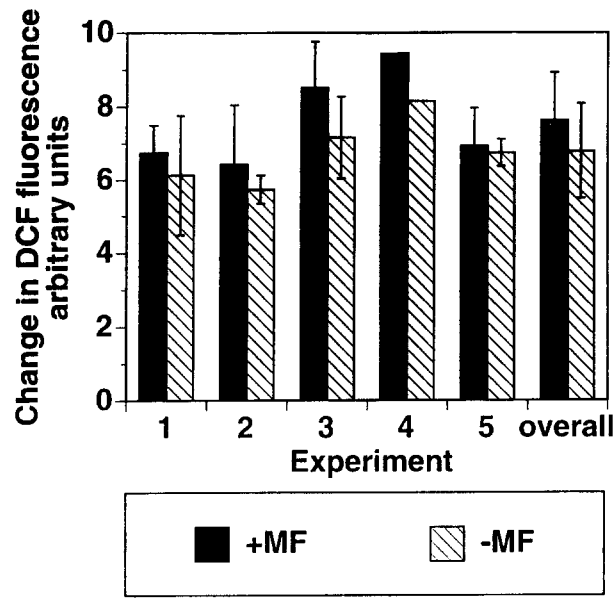

Fig. 2. Rate of change in the fluorescence signal of DCF in rat peritoneal neutrophils stimulated with PMA over time in the presence or absence of MF (0.1 mT, 60 Hz). The mean of each day's experiments ± S.D., as well as the overall mean ± S.D., are shown. n = 3, 5, 5, 2, and 3 for days 1 through 5, respectively.

3. Results

Various doses (0.01–500 nM) of PMA were tested in the reaction mixture in the absence of externally applied MF. The DCF fluorescence signal increased with increasing PMA up to 5 nM; no further increases were observed with greater doses of PMA (data not shown). A 10-fold greater final concentration of PMA was used in all MF experiments to produce a maximum oxidative burst. Therefore, the effects of MF on oxidative burst observed in our experiments are not due to variations in the amount of PMA added.

Fig. 1 illustrates a typical time course for DCF fluorescence signal following activation with PMA (50 nM) in the absence or presence of a 0.1 mT (60 Hz) MF. We observed a 12.4% ($P < 0.02$) increase in the exponential rate of increase in the fluorescence signal in the presence of the MF (Fig. 2).

To determine the location of oxidants which interacted with DCFH, we performed measurements in single cells. When fluorescence detection was focussed upon a single neutrophil, a PMA-induced increase in fluorescence was observed. However, the fluorescence of the buffer immediately adjacent to the cell (within one cell diameter) was similar to background fluorescence prior to PMA stimulation (Fig. 3). These data show that the events recorded in these experiments were intracellular in origin.

In control experiments, the effect of MF on the reaction of DCF and H_2O_2 alone was determined. The ester form of DCF (DCFH-DA) cannot react with H_2O_2; therefore, homogenates of neutrophils were prepared as a source of esterase activity to hydrolize the DCFH-DA to DCF. Using a concentration of H_2O_2 of 10 μM, which produced an optimum rate of fluorescence, we carried out three experiments in the presence of the homogenate in the absence or presence of the same MF used in our intact neutrophil experiments. There was no difference

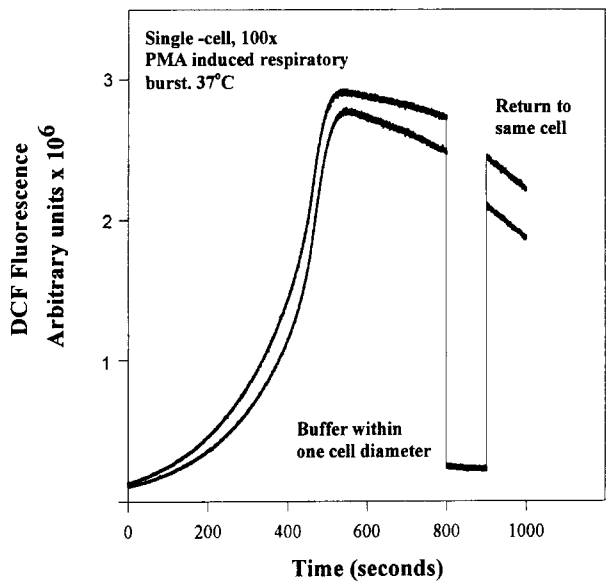

Fig. 3. Real time recording of oxidant generation following PMA induced respiratory burst in neutrophils. The fluorescence signal of DCF is shown for a single cell (0–800 s and 900–1000 s) and for the buffer in the immediate vicinity of the cell (within one cell diameter; 800–900 s). The two lines represent fluorescence produced from two excitation wavelengths, 480 and 502 nm.

in the rate of fluorescence increase in the presence or absence of MF (data not shown), indicating that MF do not affect the reaction of H_2O_2 and DCF alone.

In addition, no significant differences were observed in the presence or absence of the MF from neutrophils that were not induced with PMA (data not shown).

4. Discussion

This study demonstrates a statistically significant 12.4% increase in the exponential rate of fluorescence in the presence of MF during the PMA-induced respiratory burst of primed rat neutrophils. This increased production of fluorescence suggests that a greater concentration of radicals accumulated in the presence of MF. The dye DCFH reacts with a variety of reactive oxygen species which are either free radicals (e.g. peroxynitrite) or derived from free radicals (e.g. H_2O_2, which is formed from the dismutation of $O_2^{\bullet-}$).

Increased oxidant generation in our PMA-induced rat peritoneal neutrophils in the presence of MF may be due to an increased lifetime (and hence concentration) of free radicals in the cells directly caused by the MF. Such an effect has been observed in chemical systems [11–13], including chemical systems in which the biologically important vitamin E (tocopheroxyl) radical was shown to be affected by MF [14]. Recently, MF have been observed to modulate intersystem crossing rates of free radical driven enzyme reactions (e.g. B12 ethanolamine ammonia lyase) [15]. Nevertheless, alternative explanations cannot be eliminated by our results; for example, MF may affect activation of the respiratory burst by PMA. Indeed, Ca^{2+} is a second messenger in PMA-induced neutrophil oxidative burst [16], and electromagnetic fields have been shown to have effects on intracellular calcium increases in rat thymocytes and human T-lymphocytes [17,18]. An MF effect on Ca^{2+} seems unlikely, however, as the concentrations of PMA used were great enough to cause a saturating change in Ca^{2+}, but effects on other steps in the pathway from activation to release of reactive oxygen species cannot be ruled out.

Beneficial effects of MF in corticosteroid-inhibited intestinal wound healing have been reported [19]. This may be partially due to the increased concentration of free radicals/oxidants produced during the respiratory burst of neutrophils at the wound site, which would increase bacterial killing and thus accelerate wound healing. This finding is in accordance with our observation of an MF-induced increase in the neutrophil respiratiry burst.

Our findings demonstrate an interaction between MF- and PMA-induced oxidant generation in rat peritoneal neutrophils. We believe this represents the first observation of MF influencing events involving free radical species generated during signal transduction in living cells.

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References

[1] Scaiano, J.C., Cozens, F.L. and McLean, J. (1994) Photochem. Photobiol. 59, 585–589.
[2] Grissom, C.B. (1995) Chem. Rev. 95, 3–24.
[3] Bittl, R., Schulten, K. and Turro, N.J. (1990) J. Chem. Phys. 93, 8260–8269.
[4] Steiner, U.E. and Ulrich, T. (1989) Chem. Rev. 89, 51–147.
[5] Scaiano, J.C., Mohtat, N., Cozens, F.L., McLean, J. and Thansandote, A. (1994) Bioelectromagnetics 15, 549–554.
[6] Babior, B.M., Curnutte, J.T. and McMurrich, B.J. (1976) J. Clin. Invest. 58, 989–996.
[7] Suematsu, M., Schmid-Schonbein, G.W., Chavez-Chavez, R.H., Yee, T.T., Tamatani, T., Miyasaka, M., Delano, F.A. and Zweifach, B.W. (1993) Am. J. Physiol. 264, H881–H891.
[8] Vowells, S.J., Sekhsaria, S., Malech, H.L., Shalit, M. and Fleisher, T.A. (1995) J. Immunol. Methods 1778, 89–97.
[9] Horii, T., Satouchi, K., Kobayashi, Y., Saito, K., Watanabe, S., Yoshida, Y. and Okuyama, H. (1991) J. Immunol. 147, 1607–1613.
[10] Cunningham, F.M., Smith, J.H., Ford-Hutchinson, A.W. and Walker, J.R. (1979) J. Pathol. 128, 15–20.
[11] Turro, N.J. and Kraeutler, B. (1980) Accoc. Chem. Res. 13, 369–377.
[12] Cozens, F.L. and Scaiano, J.C. (1993) J. Am. Chem. Soc. 115, 5204–5211.
[13] Scaiano, J.C., Abuin, E.B. and Stewart, L.C. (1982) J. Am. Chem. Soc. 104, 5673–5679.
[14] Evans, C., Ingold, K.U. and Scaiano, J.C. (1988) J. Phys. Chem. 92, 1257–1262.
[15] Harkins, T.T. and Grissom, C.B. (1994) Science 263, 958–960.
[16] Ruotsalainen, M., Naarala, J. and Savolainen, K.M. (1995) Toxicol. Lett. 78, 195–205.
[17] Liburdy, R.P. (1992) FEBS Lett. 301, 53–59.
[18] Lindstrom, E., Lindstrom, P., Berglund, A., Mild, K.H. and Lundgren, E. (1993) J. Cell Physiol. 156, 395–398.
[19] Dindar, G., Renda, N., Barlas, M., Akinay, A., Yazgan, E., Tincer, T., Cakmak, M., Konkan, R., Gokcora, I.H. and Yucesan, S. (1993) Tokai J. Exp. Clin. Med. 18, 49–55.