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A weak pulsed magnetic field affects adenine nucleotide oscillations, and related parameters in aggregating *Dictyostelium discoideum* amoebae

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Abstract

A model eukaryotic cell system was used to explore the effect of a weak pulsed magnetic field (PMF) on time-varying physiological parameters. *Dictyostelium discoideum* cells (V12 strain) were exposed to a pulsed magnetic field (PMF) of flux density 0.4 mT, generated via air-cored coils in trains of 2 ms pulses gated at 20 ms. This signal is similar to those used to treat non-uniting fractures. Samples were taken over periods of 20 min from harvested suspensions of amoebae during early aggregation phase, extracted and derivatised for HPLC fluorescent assay of adenine nucleotides. Analysis of variance showed a significant athermal damping effect ($P < 0.002$, $n = 22$) of the PMF on natural adenine nucleotide oscillations and some consistent changes in phase relationships. The technique of nonlinear dielectric spectroscopy (NLDS) revealed a distinctive effect of PMF, caffeine and EGTA in modulating the cellular harmonic response to an applied weak signal. Light scattering studies also showed altered frequency response of cells to PMF, EGTA and caffeine. PMF caused a significant reduction of caffeine induced cell contraction ($P < 0.0006$, $n = 19$ by paired *t*-test) as shown by Malvern particle size analyser, suggesting that intracellular calcium may be involved in mediating the effect of the PMF.

Keywords: Weak magnetic fields; Dictyostelium discoideum; Adenine nucleotide oscillations and phase relationships; Nonlinear dielectric spectra; Cellular frequency response

1. Introduction

Many studies over the last 15 years have shown that biological systems are affected by weak electromagnetic fields (EMFs). Much evidence suggests the cell membrane as a major site for interaction [1]; its constituent phospholipids and proteins have special oscillatory and dielectric properties that make it capable of interacting with even very weak electric fields.

Adenylate cyclase, a membrane enzyme, and its product, 3′5′ cAMP have been shown to be affected by weak PMFs, and is implicated in the therapeutic application of weak PMFs in orthopaedic medicine and in physiotherapy [2–5]. cAMP mediates a wide range of physiological changes, including nerve transmission [6], cell proliferation and growth [7], aggregation and motility [8], all of which have been shown to be affected by weak PMFs. The activity of cAMP is synergistic with calcium activity, which has also been shown to be affected by weak PMFs [4,9,10]. For these experiments a simple organism, *Dictyostelium discoideum*, was used to investigate the hypothesis that a weak PMF affects membrane adenylate cyclase activity, by monitoring its adenine nucleotides and related cell parameters.

The unicellular organism *D. discoideum*, a Slime Mould amoeba, has a pronounced and well characterised oscillatory activity of adenylate cyclase [11]. *D. discoideum* is acknowledged as a model signalling system [12] for phagocytic cells of the mammalian immune system [13], and in some respects it may serve as a model for osteoclastic cells of the bone system. At a certain stage in their life cycle, free living *D. discoideum* amoebae begin to secrete regular pulses of cyclic AMP, as a chemotactic attractant signal to other amoebae to aggregate. The key event of interest to

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our research is the oscillatory secretion of cAMP which, in this system, acts autocatalytically, as first and second messenger in a way analogous to hormonal cell control in higher eukaryotes. The amoebae respond in a refractory way to a gradient of extracellular cAMP, and in order to maintain responsiveness, extracellular phosphodiesterases are expressed to degrade the cAMP [14].

Among the many positive and negative feedback loops in the cellular transduction of this cAMP signal, are movement of calcium ions, and the formative and degradative products, ADP, ATP, 5′ AMP and adenosine. Because of the amplification of oscillatory elements of this system at this stage in its life cycle, we felt that periodic perturbation from a time varying magnetic field and the induced electric field might show some interesting and observable reactions on the natural oscillations of adenine nucleotides.

It has been shown that the spectrophotometric light scattering signal at 500 nm displayed in a particular axenic strain of aggregating *D. discoideum*, has a regular low frequency sinusoidal waveform of approximately 3–4 mHz [15], which correlates with periodic oscillations of adenylate cyclase activity [16]. This waveform is superimposed on a higher frequency signal, of spiked oscillations, ranging from 4–10 mHz [15]. These higher frequency oscillations may correlate with the natural plasma membrane fluctuations observed by other researchers [17]. It has been suggested that plasma membrane fluctuations may be important in determining PMF effects [17]. These fluctuations have been observed by the light scattering effects of membrane displacement, and registered with a frequency of between 0.3 and 15 Hz [18].

In these experiments, caffeine and EGTA were used to investigate the effect of weak PMF on the morphologically induced light scattering correlates of adenylate cyclase activity. Caffeine is acknowledged as an intermediary in the metabolic action of intracellular calcium release in blocking adenylate cyclase function in *D. discoideum* [16]. EGTA is a compound that preferentially binds calcium; it remains in the extracellular matrix. In these experiments it was used to monitor the extracellular and membrane-bound calcium mediated component of the interaction of PMF with adenylate cyclase activity.

To support the hypothesis that membrane proteins are important for the effects of weak PMF, nonlinear dielectric spectroscopy (NLDS) has been employed. NLDS, a technique developed over the last seven years, by Woodward and Kell [19–21] and Woodward et al. [22] monitors the nonlinear response of membrane enzymes, in real time, to an applied weak sinusoidal electric field (typically about 360 mV/cm). This is applied through a cell suspension, via the outer electrodes of a four-terminal gold electrode system and sensed by the inner electrodes, operated by computer driven data control and acquisition. It is then subjected to analysis at many frequencies other than the fundamental in order to detect the presence of harmonics due to any nonlinearity in the dielectric response of the sample. This technique is used here to provide further evidence of the membrane involvement in weak PMF modulated effects. NLDS is shown for the first time to be capable of indicating subtle biochemical changes in the cell caused by prior exposure to a weak magnetic field, and the specific frequency and voltage windows involved in the electroconformational coupling [22] of the exposed cells with the applied AC field.

2. Materials and methods

Reagents were supplied by BDH, and were Analar grade, unless otherwise specified. Nucleotide standards were obtained from Sigma. Chloroacetaldehyde was supplied by Aldrich. *D. discoideum* spores were obtained from the Imperial Cancer Research Fund laboratories. The PMF generator used was supplied by the Department of Medical Electronics, St. Bartholomew's Hospital, London.

2.1. Magnetic fields

The magnetic field was produced by 1 A current passing through air-cored coils from a generator. The coils of 11 cm diameter were spaced 11 cm apart (in non-Helmholz configuration) and consisted of 200 turns of enamelled copper wire. The signal from the generator comprised trains of 2 ms pulses gated at 20 ms. The time derivative of the individual pulse as monitored by search coil is shown in Fig. 1. Magnetic flux density was determined using a hall probe (linear hall effect IC sensor), a search coil (97 turns, 2.2 cm^2) an oscilloscope and a calibration solenoid (992 turns, 30.6 G/A centre coil). The peak magnetic flux density was 0.4 mT. The current induced in the agar medium of a plate held midway between the coils and measured at right angles to the axis of the coils, was less than 1 μA. A rise of 2°C was observed in the exposed samples in these experiments. As a control for this, experi-

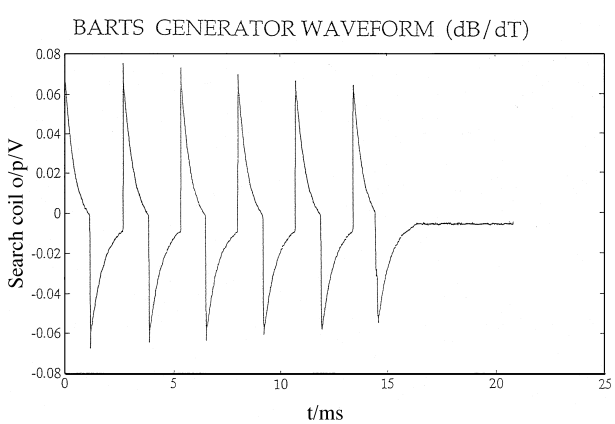

Fig. 1. Search coil output showing change in magnetic flux density with time, (dB/dT) at midpoint between the coils for the duration of part of one pulse train.

ments concerning adenine nucleotide oscillations were repeated using counterwound coils ('dummy coils' in which each coil was counterwound, with half the turns in one direction and the other half reversed) which generated heat with only marginal magnetic field of less than 0.01 mT peak flux density. Experiments were also repeated using matched incubators at different temperatures. Temperature was monitored with a thermocouple (sensitivity 0.1°C). No special screening was used, as the incubator itself acted as a screen for ambient local field perturbations, being metal lined. For experiments involving light scattering and NLDS, the dummy coils and PMF coils were used simultaneously at room temperature, providing the control and exposed setting, respectively.

2.2. Cultures

D. discoideum V12 strain spores were cultured as follows. A loopful of spores was transferred asceptically to a sterile petri dish and well-mixed by pipetting in 5 ml sterile distilled water. A loopful of log phase *E. coli*, kept on agar slopes at 4°C, was added as the bacterial food supply. This suspension was well mixed by pipetting and 0.3 ml spread asceptically onto Petri dishes containing solid agar. The agar was constituted as follows: D-glucose 10 g, bacteriological peptone 10 g, yeast 1 g, $MgSO_4 \cdot 7H_20$ 1 g, Na_2HPO_4 1 g, agar (Oxoid, Technical No. 3) 18 g, dissolved and autoclaved in one litre of double distilled water. The cultures were grown for 46 h at 22°C in an incubator. After 46 h growth, when small round clear plaques (the signs of bacterial lawn clearing) were apparent on the agar plates, the plates were removed from the incubator, and harvested by flooding them with double distilled water at 22°C, and scraping and pipetting the suspended amoebae and bacteria into centrifuge tubes. The mixture was centrifuged at 4°C for 1 min at 3000 rpm and the bacteria decanted with the supernatant. The amoebal pellet was resuspended in potassium phosphate buffer (KK_2) at 22°C consisting of KH_2PO_4 0.02 M, K_2HPO_4 0.004 M, $MgSO_4$ 0.001 M, pH 6.3, and the centrifugation process repeated 5 times, until the supernatant was clear of bacteria. The amoebae were then resuspended in KK_2 buffer 22°C, mixed thoroughly in a vortex mixer, counted under a microscope using a haemocytometer and the cell count adjusted to 1×10^7 cells ml^{-1}.

2.3. HPLC analysis of adenine nucleotides

For the experiments involving HPLC analysis of adenine nucleotides, the agar plates containing the growing cultures, were placed between the magnetic coils (for the exposed position) and just outside the measurable fringe field alongside them (for the control position) throughout 46 h growth. The incubator in which they were grown had a background ac magnetic field of 1.69 μT, as determined by Emdex Mate (Enertech) fluxmeter, and the static magnetic field at the exposure and control site was the geomagnetic field, as determined by compass.

After 46 h, the plates were removed from their experimental positions for harvesting, as described above. After harvesting, 12 ml of the cell suspension was dispensed into a glass petri dish and the dish was replaced in the experimental position in the 22°C incubator, midway between the magnetic coils, for 2 h. A similar control sample was placed in position alongside the PMF-exposed dish, for 2 h, and run simultaneously for each experiment, in the same incubator but out of measurable range of the magnetic field.

2.4. Derivatization and detection of adenine nucleotides

In all experiments, following the 2-h incubation in the experimental positions, 1 ml samples were taken every 2 min for 20 min and immediately dispensed into eppendorf tubes containing 0.3 ml perchloric acid to inhibit further phosphodiesterase activity. The samples were centrifuged and neutralized with 5 M KOH, and adjusted to pH 4.5 with acetate buffer (50% acetic acid, 1 M sodium acetate 20:160). 158 μl 1 M chloroacetaldehyde was added, and samples were mixed well for 5 s in a vortex mixer, and placed in a heating block at 95°C, for 15 min to derivatize. Samples were then cooled, centrifuged and filtered (HPLC Technology 0.45 um, 4 mm filters) and assayed by HPLC, using a 10 cm 5 μm ODS column with a 2 cm guard column (HPLC Technology). The derivatized adenine nucleotides were detected on a Perkin Elmer L5 fluorescence spectrophotometer with the detector set at 234 nm excitation and emission at 380 nm. Mobile phase consisted of 0.02 M KH_2PO_4 and HPLC grade methanol (250:40) pH 5.5, with flow rate of 1 ml/min.

2.5. Measurement of cell diameter

The Malvern laser diffraction particle size analyser was used to determine mean cell diameter (a correlate of cell volume). The Malvern particle size analyser works on the assumption of a spherical cell. It nevertheless produces data that corresponds to the observed size (diameter) and aggregation stage of the amoebae, as determined by light microscopy. A refractive index measure was set for the aggregating amoebal cells, as median to a range that yielded repeatable observations. Harvested and suspended samples were taken from their experimental positions, diluted to a concentration of 10^5 cells ml^{-1} in 50 ml KK_2 buffer and immediately sampled using the KK_2 buffer as the transfer vector. A computerized Malvern programme calculated the results of the laser light scattering measurements. For experiments involving caffeine, caffeine was added to the cell suspensions to give a final concentration of 6 mM, after harvesting, and 2 h prior to sampling. A haemocytometer was used to count cells.

2.6. Light scattering measurements

Cells were grown as indicated above. Immediately after harvesting, the cells were divided evenly and half were resuspended in KK_2 buffer (pH 6.3), the other half in KK_2 buffer containing the appropriate concentration of caffeine (6 mM) or EGTA (50 mM) with pH adjusted to 6.3. 6 cc aliquots of cell suspension were pipetted into plastic petri dishes, two dishes containing control samples and two containing either caffeine- or EGTA-treated samples. One dish each of control and treated sample was placed equidistant between dummy coils (control samples) and one dish each of control and treated sample placed equidistant between the adjacent active PMF coils. The samples were left for 1 h before sampling. Samples were then taken for spectrophotometric light scattering analysis. The method was as follows: the cell suspension was thoroughly homogenised by gentle pipetting, and 100 μl amoebal cell suspension (10^7 cells ml^{-1}) was withdrawn and pipetted into an optical glass cuvette containing 3 cc of the appropriate salt suspending medium (KK_2 buffer or buffer with caffeine/EGTA). Control and test cell suspensions were monitored by continuous scanning in two paired Shimadzu 1201 UV/vis light spectrophotometers at 500 nm for a minimum of 20 min, simultaneously. The kinetic data were recorded from all samples over a period of 2 h, and later downloaded into a PC and windowed (Hamming with side-lobe reduction) for fast Fourier transform analysis. All harmonics were recorded and subjected to multivariate analysis, using principal components analysis operated via the UNSCRAMBLER 5 software package (Camo A/S, Olav Tryggvasongst. 24, N-7011, Trondheim, Norway). The frequency and amplitude of oscillations were also estimated by manual measurement of the printed output.

2.7. Nonlinear dielectric measurements

Cells of the strain V12 were grown and prepared as previously described. The harvested cells were suspended in potassium phosphate buffer (KK_2) pH 6.3 at a density of 1×10^7 cells ml^{-1} and 1 ml cell suspension placed in the electrode well for analysis. The cells were scanned at a range of frequencies from 10–1000 Hz to establish any key area of harmonic response. Similarly, a range of voltages was used, from 0.5–1.5 V, in order to establish a possible threshold voltage for any harmonic response. The most effective frequency range was discerned to be 10–50 Hz, and the optimum voltage range from 1.2–1.4 V, this being the maximum advisable voltage to avoid electrode fouling [19]. In subsequent experiments, using coated electrodes [23], the voltage range was increased to 1.25–1.75 V as the coating reduced sensitivity, such that a larger applied voltage passed less current through the interfacial layer, albeit increasing stability. Therefore five frequencies, 10, 20, 30, 40 and 50 Hz, and three voltages: 1.2, 1.3, 1.4 V and latterly 1.25, 1.5 and 1.75 V were selected for the signal sweep. Original work with *Dictyostelium* used a sampling frequency of 25 × frequency of the fundamental. A data block, consisting of 512 samples, was recorded for the response at each combination of voltage and frequency. The mean was subtracted from the block, which was Blackman windowed and fast Fourier transformed retaining the first five harmonics. Thirty data blocks were recorded thus, and the spectra were mean averaged to reduce noise. As the only biologically relevant information was previously found to be contained in the harmonics generated, inter-bin noise was zeroed [22].

A reference spectrum was obtained for each cell suspension, using the supernatant from the sample with conductivity matched by adding sufficient distilled water to compensate for the missing cell volume. A difference spectrum was obtained by dividing the sample power spectrum by the reference power spectrum. This deconvolution revealed harmonics due solely to the cell biology, and ideally free of artefactual non-linearities from electrode and medium.

Multivarate analysis was used to detect differences in the very subtle changes in harmonics over a period of time between control and treated samples of *D. discoideum*. Chemometric analysis of treated cells required multiple spectra of treated and control suspensions alternately. To speed this data collection, a faster logmedian data collection programme was used [22] to record only the harmonic values of interest with very short data blocks and resulting Fourier transforms. This had, in concert with multivariate analysis, proved capable of providing meaningful spectra without the requirement of a reference spectrum. This method proved valid for detection of PMF effects, as PMF without the presence of cells had no effect on supernatant. However for detection of effects of caffeine and EGTA, difference spectra were still necessary, as the compounds themselves gave distinctive harmonics. These harmonics were filtered out by subtracting the supernatant reference spectrum from the sample spectrum, and in the final analysis disregarding any harmonic parameters that were common to both the cell suspension and the compound. A complete spectral voltage/frequency sweep of a sample could be collected in 1.5 min. In the experiments reported here, a conductive polymer coating [23] applied to the electrodes helped to stabilise fluctuating interfacial impedance and allowed collection of 70 samples of 75 variables each over a 2-h exposure without any significant electrode drift or instability. Any consistently changing parameters in the NLDS response between control and test sample could be extracted and highlighted and identified. Cells were grown and prepared as previously indicated, and 10 ml of cell suspension placed in a flask between coils for PMF exposure. At the same time, an identical flask containing 10 ml from same stock suspension of cells was placed in an adjacent control position, between dummy coils. Cells treated with metabolic modifiers, were treated identically, and were from the same batch of cells as their

controls, except that after harvesting, 10 ml were suspended in KK_2 buffer, (control sample) and 10 ml were suspended in KK_2 buffer containing appropriate concentration of metabolic modifier (treated sample), with pH adjusted to 6.3. After 30 min, a 1-ml aliquot was withdrawn from a flask containing the control cells, after the flask had been given a gentle stir, and placed in the electrode well and scanned. It was then withdrawn by pipette and returned to the flask. The flask was again given a gentle stir, and a fresh aliquot withdrawn for sampling. This procedure was repeated 10 times for the control samples. The same procedure was immediately executed 10 times with the treated sample. The whole procedure was repeated to give 4–7 blocks of 10 spectra per block. The four or seven blocks represented control and treated samples alternately, beginning with control samples, and ending with control samples. In each experiment, the treated samples had been exposed to treatment for 45 min prior to the first sampling. To check that electrode characteristics had not changed during the 2-h experiment, a difference spectrum of both standard resting yeast suspension, and of *Dictyostelium* cell suspension, was taken before and after the experiment, and the stability, position and amplitude of harmonics noted and compared. The coated electrode proved remarkably stable, giving good standard difference spectra before and after experiments, and confirming reliability of the data acquired.

The matrix of 70 samples × 75 variables thus obtained, was subjected to PCA analysis, using the UNSCRAMBLER 5 software package, (Camo A/S, Olav Tryggvasonsgt. 24, N-7011, Trondheim, Norway).

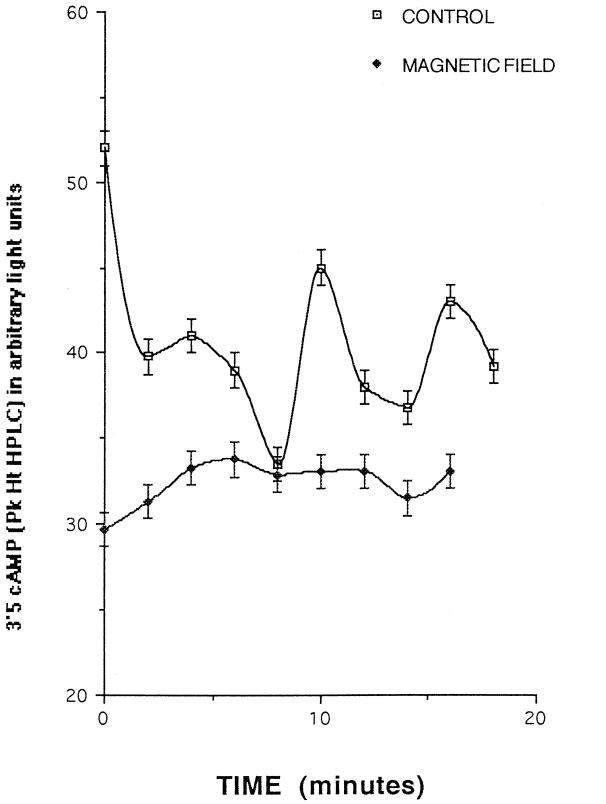

Fig. 2. Typical biological oscillation of 3′5′ cAMP, as revealed by HPLC fluorescent assay of derivatives of cell extracts of aggregating *D. discoideum* taken at 2-min intervals from two live suspensions (10^7 cells/cc). The control sample was grown simultaneously with the magnetic field exposed sample (mf) over 48 h. The error bars show the standard error of the mean for sampling.

3. Results

3.1. Adenine nucleotide oscillations

A typical nucleotide oscillation is displayed graphically in Fig. 2, showing a damping effect of the magnetic field, as well as a reduction in nucleotide level. The coefficient of variation was used as a quantitative measure of this oscillation. The validity of this technique in monitoring authentic biological oscillations is demonstrated by comparing the standard error of the mean of the technique (S.E.M. = 1.06, $n = 6$), as shown by simultaneous sampling, with the standard error of the mean for biological oscillation (S.E.M. = 4.5, $n = 10$), as shown by samples taken at 2-min intervals. The coefficient of variation of each nucleotide level over 20 min, using levels measured at 2 min intervals, was used as an estimate of oscillation with time in each experiment. The mean nucleotide concentration (level) was also determined. The means of these results from 22 experiments were analysed by comparing the data from the control samples with the PMF-exposed samples, for all adenine nucleotides using ANOVA. There were no significant differences in levels of nucleotides between control and exposed samples. However, there was a significant difference between the coefficient of variation in PMF-exposed samples and their controls ($P < 0.002$, $n = 22$) as shown in Fig. 3. There was some variation in the coefficients of variance between individual adenine nucleotides, and in their concentrations, and a difference in the degree of significance of PMF effect for individual adenine nucleotides as shown by *t*-test, (see Table 1). Control experiments for thermal effects, using counter-wound dummy coils showed no significant changes, in any of the nucleotides in either coefficients of variation or in levels, suggesting the effect to be athermal (data not shown). This finding was further supported by an experiment, in which matched incubators were used to monitor the effects of temperature. There were no significant effects of a 4°C temperature difference on either the coefficient of variance or the level of adenine nucleotides in this experiment (data not shown).

3.2. Phase relationship of adenine nucleotide oscillations

The phase relationships of various adenine nucleotides in one representative experiment is shown in Fig. 4a,b. The

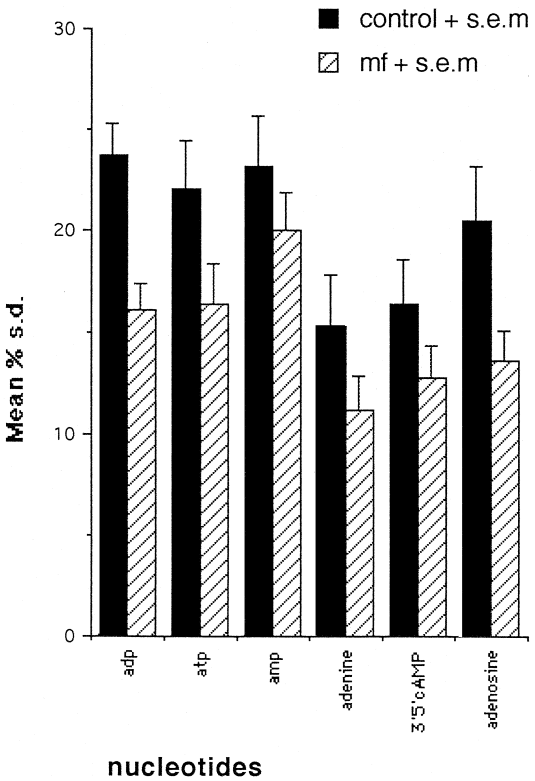

Fig. 3. Relative mean coefficients of variation (% standard deviation) of adenine nucleotides in HPLC fluorescent derivatized extracts of live aggregating *D. discoideum* cells (10^7 cells/cc) taken over 20 min at 2-min intervals, showing a significant ($P < 0.002$, $n = 22$ by ANOVA) difference between coefficients of variation of the magnetic field exposed samples and their contemporary controls.

mean level of oscillation of each nucleotide is given in Table 1, and the coefficient of variation. In 5 out of 7 experiments, the ATP peak was shown out of phase with the AMP peak. In 5 out of 7 experiments, ADP was found to be in phase with AMP. In 6 out of 7 experiments, the cAMP peak was shown out of phase with the adenosine peak. cAMP was found to be out of phase with ATP in 7 out of 7 experiments, and out of phase with AMP in 6 out of 7 experiments.

3.3. Effect of PMF on phase relationships of adenine nucleotides

In 6 out of 7 experiments, there was no change in the phase relationship of ATP and AMP between PMF-exposed samples and their controls. However, in 5 out of 7 experiments, there was a change of phase relationship of ADP and AMP between PMF-exposed samples and their controls. There was a change in the phase relationship of cAMP and AMP, and cAMP and ATP, between PMF-exposed and control samples, in 6 out of 7 and 5 out of 7 experiments, respectively.

3.4. The effect of PMF and caffeine on cell morphology

There was no significant difference between the cell counts for those samples grown in PMF and their controls (1.8158×10^7 cells ml^{-1} and 1.8916×10^7 cells ml^{-1}, $n = 58$, respectively, $P = 0.493$). The PMF did not show any significant effect on cell diameter. There was a significant effect of caffeine in reducing cell diameter, as shown by paired *t*-test ($P = 0.025$, $n = 19$). The PMF was shown to significantly reverse this effect of caffeine ($P = 0.0006$, $n = 19$) by paired *t*-test. The tendency of the magnetic field is to reverse the significant caffeine effect and restore the diameter of the cell to its normal status. These results are displayed in Table 2.

3.5. The effect of PMF, caffeine and EGTA on spectrophotometric light scattering

A typical light scattering plot is shown in Fig. 5. Perchloric acid reduced the amplitude of oscillation completely. The key frequencies detected by visual inspection of measurement of data, and also by fast Fourier transform (FFT) analysis are shown in Table 3.

3.6. Visual estimations of frequency of light scattering

Two key frequency components were detectable: a lower frequency (0.2–5 mHz) oscillation, modulated by a higher frequency (15–25 mHz) oscillation. Taking the pooled results of three series of experiments, conducted over a period of 15 months, the PMF did not have an overall

Table 1
Concentrations and coefficients of variation of adenine nucleotides detected by HPLC fluorescence analysis, using derivatised cell extracts of *D. discoideum* (V12 strain, 10^7 cells/cc) at early aggregation stage of development

	Control	Magnetic field	Paired *t*-test
Nucleotide levels			
ADP	11.58 μM	10.4 μM	$P = 0.36$ ($n = 22$)
ATP	47.5 μM	55.5 μM	$P = 0.53$ ($n = 20$)
AMP	5.17 μM	4.5 μM	$P = 0.29$ ($n = 22$)
Adenine	28.5 μM	20.3 μM	$P = 0.11$ ($n = 16$)
3′5′ cAMP	0.459 μM	0.412 μM	$P = 0.42$ ($n = 10$)
Adenosine	4.64 μM	4.03 μM	$P = 0.26$ ($n = 20$)
Coefficients of variation			
ADP	23.69	16.1	$P = 0.0001$ ($n = 22$)
ATP	22.1	16.3	$P = 0.017$ ($n = 20$)
AMP	23.2	20.02	$P = 0.14$ ($n = 22$)
Adenine	15.2	11.2	$P = 0.01$ ($n = 16$)
3′5′cAMP	16.3	12.7	$P = 0.08$ ($n = 10$)
Adenosine	20.5	13.6	$P = 0.01$ ($n = 20$)

The figures are shown for cultures exposed for 48 h in magnetic field and for their simultaneous controls, and the comparison by statistical analysis using paired *t*-test is shown alongside.

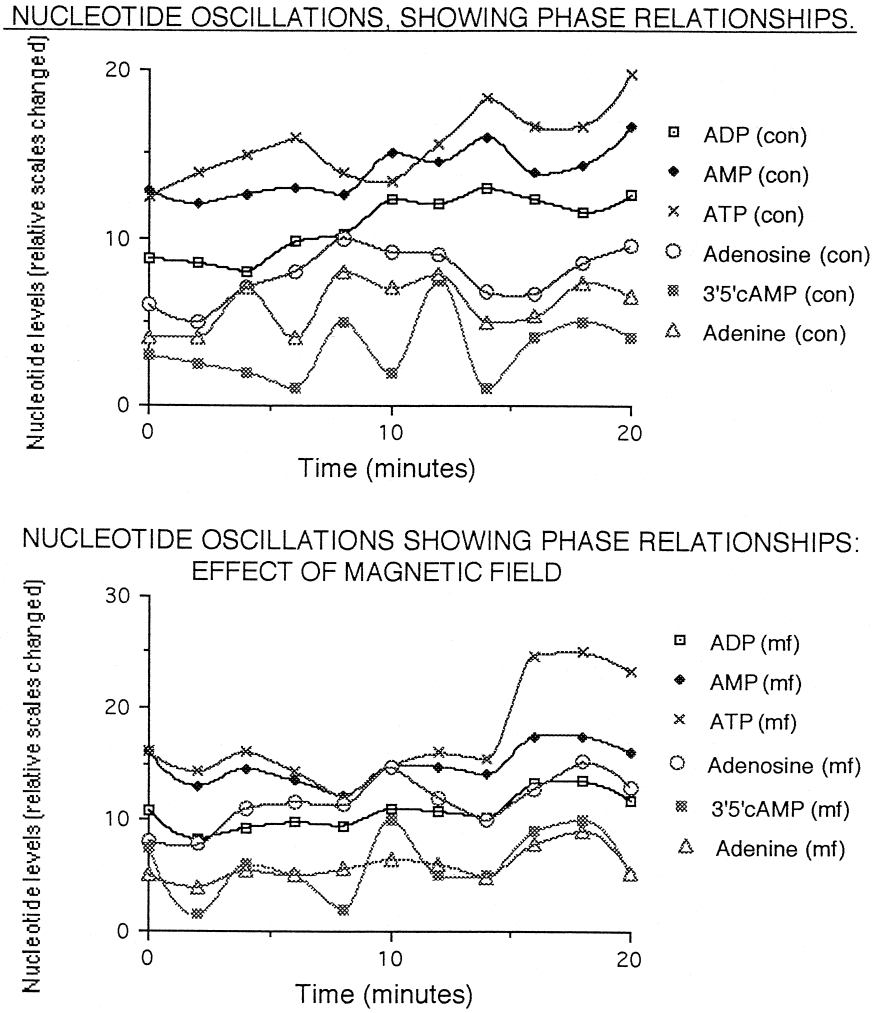

Fig. 4. Oscillations of adenine nucleotides showing phase relationships, in control and PMF-exposed samples, as revealed typically by fluorescent HPLC assay of derivatised extracts of live aggregating *D. discoideum*, sampled at 2-min intervals over 20 min. The phase relationship of cAMP and ATP is reversed in the PMF-exposed sample.

significant effect on modulating the frequency of the higher frequency component of light scattering over 2 h exposure ($P < 0.2$, $n = 96$ by paired *t*-test). However, in one of the three series of experiments, the frequency of light scattering was significantly increased by PMF ($P < 0.01$, $n = 28$) from 15 to 16 mHz.

Caffeine did not have a significant effect on the higher frequency component of light scattering. However, caf-

Table 2
Results of Malvern laser diffraction particle size analysis of *D. discoideum* amoebae at aggregation stage of development, showing effects of treatment in the control sample (CON), caffeine (CAFF), and pulsed magnetic field (MF) on cell diameter

	Control	S.D.	MF	S.D.	Caffeine	S.D.	Caffeine + MF	S.D.
Diameter (μm)	12.84	4.4	12.89	3.5	11.36	2.79	12.52	2.86
	(CON) vs. (MF)		(CON) vs. (CAFF)		(CAFF) vs. (CAFF + MF)		(CON) vs. (CAFF + MF)	
Significance ($n = 19$) by paired *t*-test	$P = 0.92$		$P = 0.025$		$P = 0.0006$		$P = 0.64$	

Paired *t*-test was used to assess any significant difference between treatments, and the results are displayed, showing a significant effect of caffeine in reducing cell diameter ($P < 0.025$, $n = 19$), and a significant effect of MF in reversing this effect ($P < 0.0006$, $n = 19$).

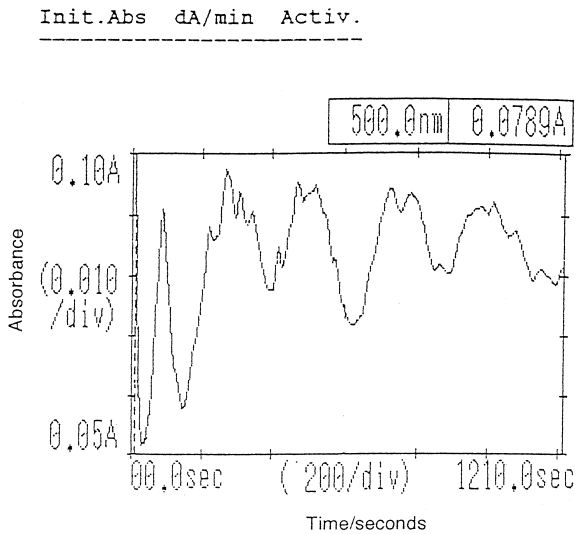

Fig. 5. Plot of spectrophotometric light scattering of aggregating *D. discoideum* cells in suspension (3.3×10^5 cells/cc), showing oscillations relating to the metabolic activity that characterises chemotaxis, using Shimadzu 1201 UV/vis spectrophotometer, scanning at 500 nm.

feine combined with the PMF gave a significant increase in the higher frequency component of light scattering from 15 mHz in the control samples to 19 mHz in the caffeine + PMF-treated samples ($P < 0.005$, $n = 46$, by paired *t*-test.)

EGTA increased the frequency of higher frequency component of light scattering from 15 mHz in the control samples to 18/19 mHz in EGTA-treated sample. ($P < 0.0062$, $n = 48$ by paired *t*-test.) EGTA also combined additively with PMF to give a highly significant increase in the higher frequency component of light scattering from 15 mHz in the control samples to 20 mHz in the samples treated with EGTA + PMF ($P < 0.002$, $n = 48$, paired *t*-test.)

3.7. Visual estimation of amplitude of light scattering

PMF did not affect the amplitude of the lower frequency (0.2–5 mHz) component of light scattering. Caffeine and EGTA significantly increased the amplitude of the lower frequency (0.2–5 mHz) component of light scattering ($P < 0.01$, $n = 51$, $P < 0.01$, $n = 27$), respectively.

3.8. Fast Fourier transform analysis of light scattering oscillations

PCA analysis of the Fourier derived harmonics did not show a well defined distinction between control (dummy coils) and Barts PMF-exposed samples. In one series of experiments, a very weak distinction was evidenced on the

Table 3
Key cellular frequencies shown to be modulated in *D. discoideum* aggregating cell suspensions (10^7 cells/cc) after treatment with caffeine (CAF) and PMF

(A) Light scattering frequencies (mHz) (FFT and PCA analysis)					(B) Light scattering frequencies (mHz) Manual measurement					
PMF	CAF	EGTA	CAF + PMF	EGTA + PMF	CON	PMF	CAF	EGTA	CAF + PMF	EGTA + PMF
	8		8	8						
	9		9							
10										
12										
14	14			14						
15					15					
16			16			16				
	17			17			17			
	18									
19		19						19	19	
20		20	20	20						20
21										
22		22								
	23		23	23						
25	25			25						
	26	26	26							
		27		27						
28				28						
30	30		30							
33				33						
		40	40							

Cellular frequency responses are shown monitored by spectrophotometric light scattering at 500 nm, using FFT with principle components analysis and manual measurement.

7th principal component, comprising only 2% of the data variance, based on the following frequencies (mHz): 12, 14, 16, 19, 20, 28. In another series of experiments, a weak distinction between control (dummy coils) and Barts

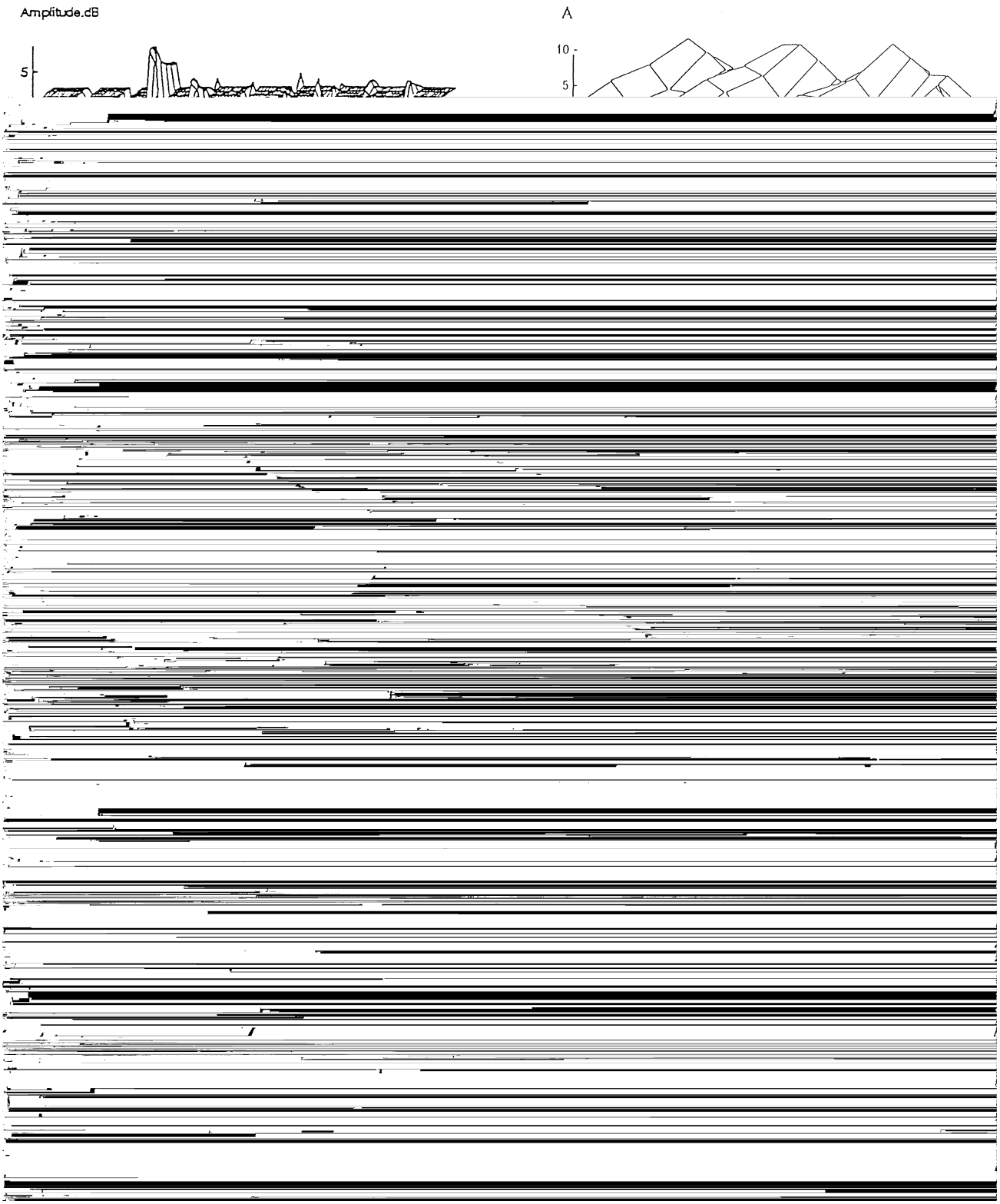

Fig. 6. (1) NLDS frequency profile of aggregating *D. discoideum* cells (10^7 cells/cc) at 1.2, 1.3, and 1.4 V, showing amplitude (*Y* axis) of characteristic 2nd and 3rd harmonics (*X* axis), coherent over 5 different fundamental frequencies (*Z* axis); (11) NLDS temporal profiles of aggregating *D. discoideum* cells, as above, showing second harmonic amplitude (*Y* axis) at 15 different combinations of voltage and fundamental frequency (*X* axis) over 20–25 min (*Z* axis). The profiles for the various treatments are: (a) control (b) EGTA (50 mM) (c) PMF (d) EGTA + PMF.

PMF-exposed samples showed up on the 9th principal component, comprising 3% of the data, based on the following frequencies (mHz): 20, 22, 15, 25. 20 mHz was the only frequency common to the two series. A possible weak distinction between control and EGTA-treated samples was shown, based on the 11th principal component, comprising only 2% of the data variance, key frequencies (mHz): 12, 19, 20, 22, 26, 27, 40. The combination of EGTA + PMF showed a weak distinction in harmonic profile between control and treated samples, based on the fifth principal component, which comprised 7% of the variance, key harmonics (mHz): 8, 14, 17, 20, 23, 25. Caffeine showed a possible damping effect on certain harmonics, based on the evidence of a 12th principle component, comprising 2% data, key frequencies (mHz) in this response being: 8, 9, 23, 25,26, 30, 50, 60. The combination of caffeine and Barts PMF was distinguished fairly well from control samples, based on 9th principle component comprising 3% data variance, with key frequencies (mHz): 8, 14, 17, 25, 26, 30, 60.

Table 3 gives an overview of the various frequency responses as evidenced by light scattering.

3.9. The effect of PMF on frequency of nucleotide oscillations

The only significant difference in the frequency of nucleotide oscillations between control and PMF-exposed samples, by visual estimation, was shown in ADP ($P < 0.03$, $n = 7$ by paired *t*-test), in which the effect of the PMF was to increase the frequency of the nucleotide oscillation.

3.10. The effect of PMF, caffeine and EGTA on the nonlinear dielectric response

Early experiments revealed that *D. discoideum* showed a nonlinear dielectric response characterised by 2nd and 3rd harmonics, coherent over the range of signalling frequencies (see Fig. 6(1)). A 3-dimensional plot of frequency/voltage, vs. harmonic number, vs. amplitude of harmonic (decibels) was able to visualise the NLDS spectrum peculiar to *Dictyostelium*, at three specific voltages, as seen in Fig. 6(1). Further programming allowed information to be displayed showing the temporal variation of a harmonic during 20 min of biological oscillatory activity, for the whole range of voltage and frequency combinations. Fig. 6(11) shows the effect of EGTA (50 mM) and a PMF on the second harmonic over 20 min.

The plots in Fig. 7 show strong separation between treated and control cells. It is clearly possible to distinguish the effect of Barts PMF (a) and (b) EGTA, (c) Caffeine (d). The principle components that distinguish these effects comprise a high percentage of the data variation. The loadings on the discriminating components reveal

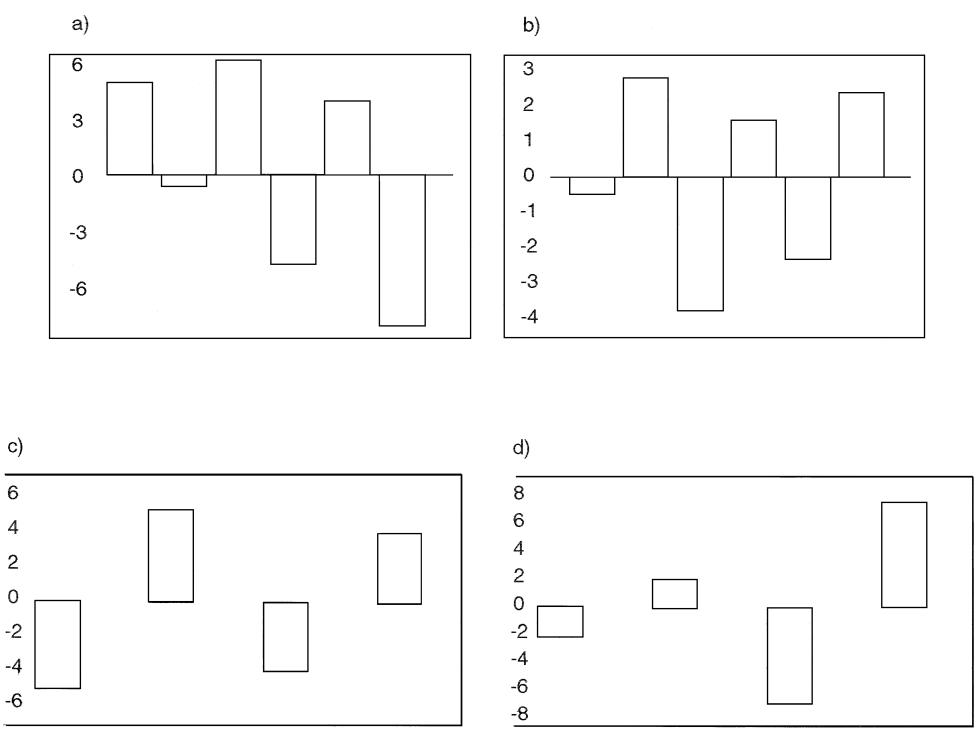

Fig. 7. Plots showing mean PCA scores of harmonic parameters discriminating between the contol suspension of *D. discoideum* (10^7 cells/cc in KK_2 buffer) and (a) and (b) PMF-exposed cells; (c) EGTA (50 mM in KK_2 buffer)-treated cells; (d) caffeine (6 mM in KK_2 buffer)-treated cells. The scores cover a 3-h period, in which series of 10 sequential spectra of control samples alternate with 10 sequential spectra of treated/exposed samples. This allows clear separation of these alternating blocks of data in the selected PCA score on the *Y* axis. Cells had been subjected to 45 min of exposure/treatment before their sampling began.

the key combinations of voltage, frequency and harmonic number, responsible for the differences.

In two separate experiments, PCA showed a very clear distinction between Barts PMF-exposed cells and dummy field exposed cells, on first and second principle component, respectively, comprising 45% and 30% of the data variance, respectively (Fig. 7a,b). In one of these experiments, 49% of the data variance on PC 1 showed a very clear temporal effect, with certain key harmonics changing their amplitude smoothly over time, indicating the harmonic changes due to underlying developmental metabolism, independent of any treatment. Table 4 shows the specific fundamental frequencies and harmonics characterising the altered NLDS response of *D. discoideum* aggregating amoebae to the PMF.

A clear distinction between EGTA-treated cells and control cells is shown on principle component 1, comprising 67% of the data variance (Fig. 7c). Loadings on this component show the particular parameters on which a clear distinction between control and test samples can be made. Table 4 shows the specific fundamental frequencies and harmonics characterising the altered NLDS response of *D. discoideum* aggregating amoebae to EGTA.

A clear distinction between caffeine and control samples was evidenced on 1st principle component comprising 55% data variance (Fig. 7d). The most universal response of the cells to caffeine treatment is shown by loadings on the key principle component. Table 4 shows the specific fundamental frequencies and harmonics characterising the altered NLDS response of *D. discoideum* aggregating amoebae to caffeine.

Table 4
Fundamental frequencies and harmonic numbers characterising altered nonlinear dielectric response of treated/exposed Dictyostelium discoideum amoebae during early aggregation

F (Hz)	Harmonic	PMF			CAF			EGTA		
		V_1	V_2	V_3	V_1	V_2	V_3	V_1	V_2	V_3
10	2		+							
	3			+						
	4							−		
	5	+	+ +	−	−	−	−	−	−	
20	2	−		+						
	3	−		−		−	−			−
	4			−						
	5	−		+ +		−	−			
30	2	−		−					−	
	3						−	−		
	4		−					−	−	
	5	+		−	−	−	−			
40	2					−		−		
	3								−	−
	4			+		−	−			
	5	+		+	−	−			−	
50	2				−	−	−		−	
	3							−		
	4			−				−	−	−
	5		+							

+ = Higher amplitude of harmonic in control sample.
− = Lower amplitude of harmonic in control sample.
$V_1 = 1.25$ V, $V_2 = 1.5$ V, $V_3 = 1.75$ V.

4. Discussion

4.1. PMF modulation of adenine nucleotide oscillations in Dictyostelium discoideum

Many researchers working with *D. discoideum* have noted that nucleotide oscillations coincide with other metabolite oscillations during aggregation, and that these oscillations show phase relationships, often entrained by pulsing with 3′5′ cAMP [24]. These oscillations have been shown to vary in amplitude and pattern, depending on the strain of *Dictyostelium* used [25]. However, it has been found that cAMP oscillations are coupled but not intrinsic to oscillatory behaviour in *Dictyostelium* and that there may be two or more oscillating systems, one of which involves cAMP [11]. These are the first experiments to monitor their interaction with PMF.

There seems to be some controversy among researchers as to the oscillation of ATP. Although some researchers working with the strain NC4 [24] have concluded that there is no detectable oscillation of total cellular ATP associated with spontaneous periodic signals, other researchers [25] have pointed out that the data shows possible variations of as much as 15% during the period of the signal. This is close to the mean coefficient of variation of 22.1 (see Table 1), calculated in the experiments reported here; any difference could be due to the different strain (V12) used here. Reported levels of ATP, using strain Ax-2, are 0.18 $mM/10^8$ cells ml^{-1}, (or 18 uM for a cell density of 10^7 cells ml^{-1}) detected by enzymatic spectrophotometric assay [24]. This value is slightly lower than the mean value detected by the method in these experiments, 55.5 $uM/10^7$ cells ml^{-1}, for strain V12.

It has been suggested that the generated cAMP signal, on spiking the sample with an extracellular aliquot of cAMP, always follows or coincides with the ADP wave, although its exact position shifts from one experiment to another [25]. The experiments reported here show a varying pattern of phase relationship between ADP and cAMP during the natural, rather than forced oscillatory cycle, in which ADP is more often out of phase with cAMP. It was hypothesized that oscillations of ADP as an oxidative metabolite could serve to initiate the spontaneous periodic signalling observed in *Dictyostelium* [25]. This could be effected by possible guanylate cyclase sensitivity to ADP, and adenylate cyclase sensitivity to cGMP and ATP, effected by changes in calcium binding [25]. The changes

observed in PMF-exposed samples, with respect to the phase relationship between ADP and AMP, AMP and ATP, 3′5′ cAMP and AMP, and the damping in the coefficient of variation of adenine nucleotide oscillations could thus be caused, indirectly, by modulation of calcium binding.

Some researchers have suggested that *D. discoideum* adenylate cyclase is activated by AMP, in the presence of an associated ATP pyrophosphohydrolase, which produces AMP from ATP, and is activated by 3′5′ cAMP [26]. They suggest that the interaction between two enzymes could provide a feedback loop which could generate an oscillation in cAMP. This would predict an oscillation in AMP and would imply an AMP wave preceding the cAMP signal. Our experiments would support this prediction, insofar as the cAMP wave is shown to be consistently out of phase with the AMP wave (see Fig. 4).

It could be that PMF causes a damping of adenine nucleotide oscillations in *Dictyostelium* by modulation of calcium binding and alteration of the time constants (K_m) of enzymes involved in the delicate feedback mechanisms involved in the metabolic processes during aggregation, and consequently in the phase relationship of various adenine nucleotide metabolites. The experiments presented here, coupled with other experiments in our laboratory showing PMF induced changes in the kinetics of membrane bound phosphodiesterase in aggregating *D. discoideum* [27], would support such a hypothesis.

4.2. PMF modulation of adenine nucleotide oscillations in Dictyostelium discoideum via cell calcium

Adenylate cyclase activity is known to be modulated by intracellular calcium, which in turn is modulated by extracellular calcium and potassium via voltage-gated ion channels [28,29]. EMF effects on cell calcium and cAMP have been shown in a number of different cells [1,10,30]. Many researchers have suggested that these effects are mediated predominantly by cell membrane dynamics [17,31]. Adenylate cyclase activity in aggregating *D. discoideum* cells has been shown to correlate with transient increase in intracellular calcium concentration [32]. When extracellular cAMP binds to the membrane receptor of a responsive *D. discoideum* cell, a brief influx of calcium ions ensues, followed by a calcium efflux. It has been shown that these adenylate cyclase modulating ion fluxes are part of cell membrane signalling responses, involved in messenger/receptor molecule binding at the membrane. These cell signalling events are related in our cell system to cell motility and shape changes [33].

Many researchers have shown that light scattering or optical density changes in aggregating *D. discoideum* amoebae, correlate with adenylate cyclase activity [16,24,25,34]. It has been shown that metabolic and shape change activity correlate with amplitude of spectrophotometric light scattering responses [16]. Jaffe et al. [35] have shown, using apaequorin transfected cells, that in aggregating *Dictyostelium* amoebae, calcium waves of up to 100 mHz are seen, along with calcium spikes of 8 mHz. These periodicities are similar to periodicities in the light scattering experiments reported here, in which two frequency ranges are notable, 0.2–5 mHz and 8–40 mHz. The former, lower frequency sinusoidal type waveform showed some amplitude modulation by caffeine and EGTA, and the higher frequency oscillation showed frequency modulation by caffeine and EGTA in combination with PMF. Table 3 shows our own experiments confirming this. We have shown, using Malvern particle sizing, that PMF interacts with a caffeine modulated shape-changing cellular parameter, that correlates with adenylate cyclase activity. So it may be hypothesised that in the light scattering experiments and in the nucleotide oscillation experiments, PMF is interacting with a calcium signalling pathway that is connected with adenylate cyclase enzyme activity.

It has been shown that caffeine blocks adenylate cyclase activity and suggested that its effect is via increasing intracellular calcium release [16] by acting on the membranes of endoplasmic or sarcoplasmic reticulum [32]. It has been shown that high levels of intracellular calcium result in total inhibition of adenylate cyclase [36]. The experiments reported here show that a caffeine mediated cell-volume change is blocked by PMF. It appears therefore, that the PMF may be affecting intracellular calcium concentration, counteracting the caffeine effect of cell volume reduction. Calcium is important in actin–myosin activity, and hence cytoplasmic gel/sol transformations, especially during chemotactic cell movements, and is thus important in cell shape change. Changes in refractive index of the cell, caused by changes in cytoplasmic viscosity, are an important correlate of light scattering activity [37,38]. As high level of cytoplasmic calcium is known to inhibit adenylate cyclase activity [12], and the PMF in these experiments is shown to damp the adenine nucleotide oscillations that correlate with adenylate cyclase activity, and also events mediated by intracellular calcium, it may be suggested that PMF is causing these two effects by modulation of calcium activity. It has been shown that a PMF of different signal parameters changes membrane fluidity in *Paramecium*, and induces changes in membrane voltage [8] and that adenylate cyclase in *Paramecium* is directly activated by membrane voltage, via a potassium voltage-gated channel [18]. Therefore, it could be hypothesised that PMF causes changes in adenylate cyclase activity in *Dictyostelium* via changes in membrane voltage-gated channels, and subsequent intracellular calcium release. It is reported that 21 mM EDTA has a 40-fold stimulation of cellular cAMP [39], and by implication, stimulation of adenylate cyclase. EGTA, which is more specific than EDTA in binding extracellular or membrane-bound calcium, is shown by these experiments to affect the light scattering and nonlinear dielectric profile of aggregating *D. discoideum* cells. EGTA, caffeine and PMF all

affect the NLDS profile in aggregating *D. discoideum*, with some harmonic parameters in common. The nonlinear dielectric response is related specifically to activity of membrane proteins. The effects of both caffeine and EGTA are enhanced, in the case of light scattering, by the addition of the PMF. This suggests that PMF effect is involved with calcium activity, perhaps by modulation of the influence of extracellular calcium, via a calcium binding protein, on the release of intracellular calcium. It has been shown that AC fields can either increase or decrease ATPase activity, depending on the level of enzyme activity [43]. Ca^+-ATPases have been found to be involved in calcium efflux from *D. discoideum* cells [39–41]. H^+-ATPases in the plasma membrane have been found to be involved in the NLDS response of yeast cells [19]. It is also possible that PMF may be acting on calcium fluxes via Ca^+-ATPase in *D. discoideum*.

It is likely that a series of interacting systems with delicate feedback mechanisms are responsible for the overall effects observed with *D. discoideum* in these experiments. Therefore it is not possible and may not be valid to pinpoint one particular aspect as a cause of these results. However, an effect on membrane enzymes and membrane structure with resulting changes in both degree and rate of biochemical activity could be a possible explanation, supporting hypotheses already proposed by other workers. It is well known that both frequency and amplitude of the modulating EMF are critical for many of the effects observed in different cells and cell systems [42,43] and a better understanding of the amplitude and frequency windows is required to be able to characterise both detrimental and beneficial effects of EMFs. Similarly, it is also important to determine the frequency and amplitude windows for many naturally occurring metabolic processes within the cell, as it seems that these parameters may well be interacting with any applied EMF [44]. The technique of NLDS seems well suited to this exploration, and it is used for the first time in these experiments to monitor the modulation by PMF of the harmonic response of living cells to an applied AC signal.

5. Conclusion

These are the first reported experiments, to our knowledge, to establish that a weak electromagnetic field has any effect on the designated parameters of *D. discoideum*. Although Neumann et al. [45] showed that high pulsed fields used in electroporation affect Dictyostelium aggregation morphology, the electric field used by his group was more than three orders of magnitude greater than the electric field induced in these experiments. In these experiments, a weak PMF, incapable of producing such electroporation, is shown to have a significant effect of damping the biological oscillation of adenine nucleotides during the signalling phase of aggregating *D. discoideum* amoebae. This effect is shown to be athermal by the absence of significant effect in cells exposed to dummy coils, and to higher temperature. The sensitivity of *D. discoideum* to weak PMF is further supported by the NLDS profiles. There is a strong indication that weak PMF acts at the membrane level through a calcium-mediated metabolic pathway.

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