

Intracellular Calcium Activity in Isolated Bovine Adrenal Chromaffin Cells in the Presence and Absence of 60 Hz Magnetic Fields

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This study examined whether 60 Hz magnetic field (MF) exposure alters intracellular calcium levels ($[Ca^{2+}]_i$) in isolated bovine adrenal chromaffin cells, a classic model of neural responses. $[Ca^{2+}]_i$ was monitored by fluorescence video imaging of cells loaded with the calcium indicator fluo-4 during exposures to magnetic flux densities of 0.01, 0.1, 1.0, 1.4, or 2.0 mT. MFs generated by Helmholtz coils constructed from bifilar wire allowed both 60 Hz field and sham exposures. Following a 5 min monitoring period to establish baseline patterns, cells were subjected for 10 min to a 60 Hz MF, sham field or no field. Reference calcium responses and assessment of cell excitability were obtained by the sequential addition of the nicotinic cholinergic receptor agonist dimethylphenylpiperazinium (DMPP) and a depolarizing concentration of KCl. Throughout an 8 day culture period, cells exhibited spontaneous fluctuations in $[Ca^{2+}]_i$. Comparisons of the number of cells exhibiting transients, the number and types of calcium transients, as well as the time during monitoring when transients occurred showed no significant differences between MF exposed cells and either sham exposed or nonexposed cells. With respect to the percentage of cells responding to DMPP, differences between 1 and 2 mT exposed cells and both nonexposed and sham exposed cells reached statistical significance during the first day in culture. No statistically significant differences were observed for responses to KCl. In summary, our data indicate that $[Ca^{2+}]_i$ in chromaffin cells is unaffected by the specific 60 Hz MF intensities used in this study. On the other hand, plasma membrane nicotinic receptors may be affected in a manner that is important for ligand–receptor interactions. *Bioelectromagnetics* 23:557–567, 2002. © 2002 Wiley-Liss, Inc.

Key words: neural-type cells; fluo-4 fluorescence imaging; intracellular calcium; calcium transients; acute field exposure

INTRODUCTION

Considerable research has been directed at determining how and to what extent electromagnetic fields (EMFs) produced by 50/60 Hz electric power affect living systems. Regarding the mammalian nervous system, exposure to 50/60 Hz EMFs has been implicated in a wide range of effects [Paneth, 1993]. In humans, for example, an increased incidence of headaches and depression has been reported for individuals residing near transmission lines [Poole et al., 1993]. In animal studies, alterations in central neurotransmitter turnover and pineal function have been observed after 60 Hz MF exposure [e.g., Kato et al., 1993; Lai et al., 1993]. These reports suggest that neural cells may be capable of transducing power line frequency EMFs into physiological responses.

Because calcium plays an important role in regulating a variety of cellular processes in a wide

range of cell types [Berridge et al., 1999], one way that MF exposure might cause biological effects is by altering intracellular calcium levels ($[Ca^{2+}]_i$). Indeed,

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several in vitro studies utilizing different types of cells and different methodologies for analyzing cellular calcium dynamics have provided evidence that changes in $[Ca^{2+}]_i$ occur in response to 50/60 Hz MF exposure. For example, 60-min exposure of mitogen-stimulated rat thymic lymphocytes to a 22 mT 60 Hz MF caused a significant increase in $^{45}Ca^{2+}$ influx compared to nonexposed cells [Walleczek and Liburdy, 1990]. In a series of studies by Lindstrom et al. [1993, 1995], application of a 50 Hz MF at magnetic flux densities of 0.1 or 0.15 mT during fluorescence imaging of $[Ca^{2+}]_i$ in the human T-lymphocyte cell line Jurkat resulted in pronounced increases in $[Ca^{2+}]_i$ in a subpopulation of the cells. In endocrine cells isolated from rat anterior pituitary, fluorescence imaging of $[Ca^{2+}]_i$ revealed an increase in $[Ca^{2+}]_i$ for up to 3 h after exposure of the cells to a 50 μ T 50 Hz MF [Barbier et al., 1996].

Although effects of 50/60 Hz EMFs on $[Ca^{2+}]_i$ have been reported for several types of cells, studies to date have not included an analysis of powerline frequency EMF effects on $[Ca^{2+}]_i$ in neural-type cells. The present study was undertaken to begin filling this gap. The neural cell type chosen for the analysis, chromaffin cells isolated from the adrenal medulla, share fundamental similarities with sympathetic neurons of the peripheral nervous system. These include a common derivation from the neural crest and common mechanisms for the synthesis, storage, and calcium dependent exocytotic release of catecholamines in response to the binding of acetylcholine. Also, like sympathetic neurons, chromaffin cells are electrically excitable, with a resting membrane potential of approximately -75 to -80 mV [Lopez et al., 1995]. Of particular relevance to the present study is the well-established use of chromaffin cells to examine the mechanisms by which $[Ca^{2+}]_i$ is modulated in response to both receptor-mediated [e.g., O'Sullivan et al., 1989] and non-receptor-mediated [e.g., Lopez et al., 1995] stimuli.

We used the non-ratiometric, visible wavelength dye fluo-4 to monitor $[Ca^{2+}]_i$ in a population of cells during simultaneous 60 Hz MF exposure. Effects on $[Ca^{2+}]_i$ as well as the ability of chromaffin cells to respond to stimuli that increase $[Ca^{2+}]_i$ were assessed after exposure of the cells to magnetic flux densities of either 0.01, 0.10, 1.0, 1.4, or 2.0 mT. We show that a variety of spontaneous calcium fluctuations occur over the time period of MF exposure, but that no significant differences in the pattern of calcium activity occurred in response to any of the magnetic flux densities investigated. There was also no influence of MF exposure on the percentage of cells exhibiting calcium activity.

MATERIALS AND METHODS

Preparation of Chromaffin Cells

Chromaffin cells were isolated from fresh bovine adrenal medullas as described by Waymire et al. [1983] and seeded into 100 mm plastic Petri dishes (Baxter) in Ham's F-12 medium containing 10% (vol/vol) calf serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, 0.25 μ g/ml fungizone, and 32 μ M cytosine arabinoside. As assessed by neutral red staining, chromaffin cell homogeneity was greater than 90%. The cells were maintained in suspension culture at 36.5 °C in a humidified atmosphere of 95% air/5% CO₂. Under these culture conditions, cells retained their normal round morphology and large cell aggregates (up to 100 cells) often formed. Cells were cultured at low density (1×10^5 cells/ml) to prevent cell-cell contact mediated alterations in gene expression [Saadat et al., 1987] that could potentially decrease the likelihood of observing EMF effects.

Dishes of cells were always placed on the same two shelves in a tissue culture incubator where ambient AC magnetic flux densities (measured with an EFM Company EFM-131 MF measurement system) and static magnetic flux densities (measured with a Walker Scientific, Inc., Fluxgate FGM-3D1 magnetometer) were determined to be the lowest (≤ 1 and 75 μ T, respectively). In addition, ambient 60 Hz EMFs were mapped, monitored, and recorded at all sites within the laboratory where cell handling occurred so that exposure of the cells to incidental EMFs could be minimized [Valberg, 1995]. Because replacement of the culture medium was not required under our culture conditions, there was no manipulation of the cells prior to experimentation.

Monitoring $[Ca^{2+}]_i$ by Fluorescence Imaging

To monitor $[Ca^{2+}]_i$, cells were plated at a density of $3.9\text{--}4.9 \times 10^4$ cells/cm² in 35 mm culture dishes coated with 2% rat tail collagen the day before an experiment. Visual inspection of the cells after attachment showed the presence of both isolated cells (to the extent of 8–40%) and cells making contact with other cells in small clusters of up to nine cells (Fig. 1). Cells were incubated for 45–90 min at 30–32 °C with 2.8 μ M of the calcium fluorescent indicator dye, fluo-4/AM (Molecular Probes) that was dissolved in a balanced salt solution (BSS) (145 mM NaCl, 5 mM KCl, 1.2 mM NaH₂PO₄, 2 mM CaCl₂, 1.3 mM MgCl₂, 10 mM glucose, and 15 mM HEPES, pH 7.4, and 0.1% bovine serum albumin). Cells were washed free of the dye and equilibrated for 10–15 min at 32 °C in the presence of 2 μ M of the calcium channel agonist, Bay K-8644, before initiating experiments. These and all

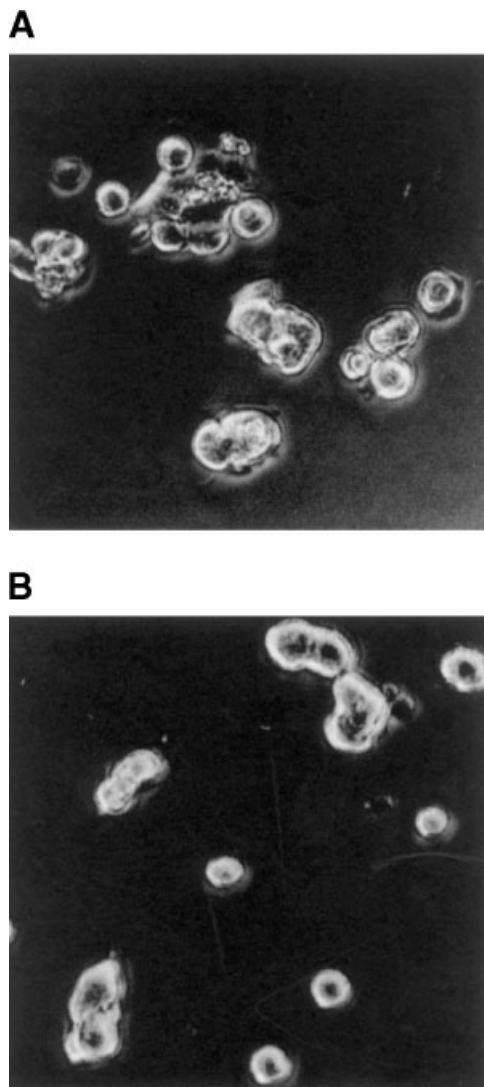


Fig. 1. Photomicrographs of typical imaged fields of chromaffin cells on day 1 and 5 in culture. In (A) (day 1), cells were attached to collagen immediately after isolation; in (B) (day 5), the large cell aggregates that formed in suspension culture were disrupted by incubation with the neutral protease dispase before collagen attachment (dispase has no effect on chromaffin cell viability, function or responsiveness to stimuli [Craviso, unpublished communications]). Note the slightly greater number of single cells in (B) vs. in (A). (Note: cells are 20 μ m in diameter.)

subsequent steps were carried out inside MU-metal shielding.

Dishes of fluo-4 loaded cells were placed on the stage of an inverted microscope (Nikon Diaphot) and [Ca²⁺]_i was monitored by illuminating cells at 470 ± 10 nm and collecting fluorescent emissions >510 nm using an intensified video camera. Video sequences were stored on a video cassette recorder and digitized by a frame grabber. Computerized analysis of

sequences on a frame-by-frame basis was performed by computing the average fluorescence of each cell using custom designed software [Publicover et al., 1999a]. Typically, 20 or more cells were monitored at one time for up to 25 min, the length of time found to be optimal for recording fluo-4 fluorescence without substantial dye photobleaching or intracellular dye loss. Background fluorescence was determined simultaneously in several cell free regions of the visual field to ensure data were collected during stable recording conditions (e.g., no fluctuations in lamp intensity). At the end of an experiment, chromaffin cell identity was verified in each visual field by neutral red staining. Cells were imaged in the presence of 2 μ M Bay K-8644 that was added during the equilibration period.

Experiments were conducted at 32 $^{\circ}$ C to help maintain cell viability, while minimizing photobleaching or loss of the dye. To ensure the absence of temperature gradients during imaging, temperature was controlled to within 0.1 $^{\circ}$ C throughout the culture dish by the combined effects of perfusing the cells with prewarmed BSS (1–2 ml/min) and blowing warm air under the dish. A thermistor probe (Yellow Springs, Inc.) attached to the inside edge of the dish continuously monitored temperature. To ensure also that minor differences in heating would not compromise comparisons of results across experiments, preliminary experiments established that a progressive temperature increase of up to 2 $^{\circ}$ C during imaging did not significantly alter the fluorescence intensity of the cells.

Experimental Protocols

Calcium imaging was performed for three experimental groups that comprised nonexposed cells, sham exposed cells, and MF exposed cells. Figure 2 depicts the protocol followed for each group. For nonexposed cells (absence of sham or field exposure), fluo-4 fluorescence was monitored for approximately 20 min (Fig. 2A). Reference calcium responses to externally-applied stimuli were then obtained by monitoring fluorescence after the addition of the nicotinic agonist dimethylphenylpiperazinium (DMPP) (10 μ M) and cell viability was assessed by the calcium response to the subsequent addition of high potassium (KCl; final concentration of 56 mM). For 60 Hz exposed cells, fluo-4 fluorescence was monitored for 5 min to establish baseline calcium activity. Sixty hertz MFs (sham or field) were then applied for 10 min and monitoring continued for another 4 min after the field was turned off (Fig. 2B). Reference calcium responses and assessment of cell viability were then obtained by addition of DMPP and KCl, respectively. Individual dishes of cells were used only once.

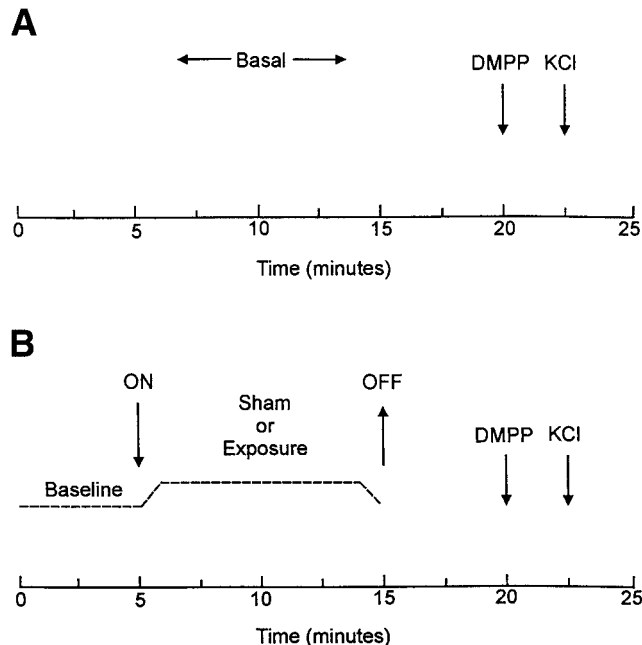


Fig. 2. Experimental protocol for real time monitoring of $[Ca^{2+}]_i$ in chromaffin cells in the presence and absence of 60 Hz MFs. For (A) nonexposed cells and (B) cells subjected to sham exposure or field exposure (continuous or pulsed), the time of addition of DMPP (10 μ M) and KCl (56 mM) to the cells is indicated by arrows. In (B), arrows also indicate the time that current was either turned on or turned off and the sloped segments of the sham/exposure interval represent ramping up and ramping down of the fields.

MF Exposure System

Cells were exposed to 60 Hz linearly polarized sinusoidal MFs generated by a pair of 16 cm diameter Helmholtz coils (8 cm distance between the coils) mounted on a nonmetallic framework positioned over the microscope stage [Publicover et al., 1999b]. Each coil had 25 turns of twisted enameled copper bifilar wire (16 gauge), using an optimal pitch of 0.3–0.5 twists/cm [Wilson et al., 1994]. The coils were wrapped with electrically grounded copper tape with a small circumferential gap to stop the flow of induced eddy currents and energized with a programmable function generator (Hewlett-Packard HP33120A) and amplifier (Bogen CT-100 with a 100W RMS power output; Bogen Communications, Inc.). A computer controlled relay in the circuit ensured that no current passed through the coils during the nonexposure state. The direction of the current through the two strands of bifilar wire was either parallel for field exposure mode or antiparallel for sham exposure. Fields were applied either continuously or in a pulsed mode. The pulsed mode consisted of applying 60 pulses, each of 5 s duration, with a 5 s duration between pulses (pulse repetition time of 10 s) and a 2-s rise and fall time.

Magnetic flux density at the position of the culture dish was checked at the start and at the end of each experiment using a EFM-140 MF sensor together with a digital multimeter (Fluke 8010A).

The uniformity of the vertically applied MF in the central region between the coils where the dish of cells was placed was constant to within 1.5%. Ambient MFs were eliminated by carrying out experiments inside MU-metal shielding. Perturbations in the applied MF were prevented by eliminating all metallic materials on the microscope stage and by viewing the cells with either a high resolution 10 \times objective custom fabricated entirely of plastic or a Nikon 20 \times objective that produced minimal field distortions [Publicover et al., 1999b]. To minimize vibrations transferred to the cells from the coils, the framework holding the coils rested on foam rubber and was isolated from the microscope stage. Exposure protocols were completely automated using custom software (written in Visual Basic) that controlled the function generator. During cell exposure, RMS current amplitude through the coils was continuously monitored using a multimeter and the waveform was continuously assessed using an oscilloscope. Turn on and turn off transients were eliminated by ramping MFs up and down (2 s period for the pulsed mode; 30 s period for the continuous mode).

Statistical Analysis

Videotapes containing the recorded data were analyzed under blind conditions. Dishes of cells in which less than 50% of cells responded to KCl (less than 20% of the total number of dishes examined) were excluded from data collection. Data for cells exposed to the 1 mT pulsed field, 2 mT pulsed field, and 1.4 mT continuous field were from single experiments. All other data represent the combined results of two or more replicate experiments using different cell preparations. Cells at different days in culture were not necessarily from the same cell preparation. All data were tested for statistical significance across experiments using the multivariate analysis of variance (MANOVA) program available in the software Systat 9 (SPSS, Inc.). Bonferroni *t*-tests were carried out to test for differences between individual means. Differences were considered significant when $P < 0.05$.

RESULTS

Spontaneous Fluctuations in $[Ca^{2+}]_i$ in Chromaffin Cells

The data reported here represent the analysis of the calcium activity of more than 1,000 chromaffin

TABLE 1. Frequency of Occurrence of Fluctuations in [Ca²⁺]_i in the Presence and Absence of 60 Hz MFs

Age of cells ^a	Exposure ^b	Total cells	Cells showing fluctuations ^c	Cells showing fluctuations after the first 5 min ^d	
				No.	% ± SE ^e
1 day	None	180	72	47	26 ± 3
	Sham	170	54	43	25 ± 4
	1.0 mT	64	20	18	28 ± 6
	1.0 mT ^{pulsed}	52	16	12	23 ± 6
	1.4 mT	20	11	7	35 ± 10
	2.0 mT	79	58	33	44 ± 5 ^f
	2.0 mT ^{pulsed}	48	21	13	27 ± 7
5 days	None	127	22	12	9 ± 3
	Sham	23	4	2	9 ± 7
	1.0 mT	92	20	12	13 ± 3
8 days	None	102	10	5	5 ± 3
	Sham	62	13	10	16 ± 3
	1.0 mT	99	19	13	13 ± 3

^aCells for each day in culture were not always from the same cell preparation.

^bNone-ambient was 0.2 μT; sham values ranged from 1.9–3.1 μT.

^cCells showing fluctuations in [Ca²⁺]_i at any time prior to DMPP addition.

^dCells showing fluctuations in [Ca²⁺]_i different from the 0 to 5 min baseline monitoring period.

^eMANOVA values.

^fSignificantly different from sham exposed cells ($P < 0.05$).

cells. On day one in culture, up to 40% of the cells exhibited spontaneous fluctuations in [Ca²⁺]_i (Table 1). Fluctuations occurred in both single cells and cells in aggregates, and as described in detail below, consisted of both increases and decreases in [Ca²⁺]_i. At later times in culture, the majority of cells were quiescent (Fig. 3) and rarely exhibited spontaneous calcium transients unless imaged in the presence of the calcium channel activator Bay K-8644 [Sorimachi et al., 1989]. Recognizing that the loss of spontaneous calcium activity over time in culture could compromise our ability to identify subpopulations of cells that may show a response to MF exposure (i.e., increase or decrease in activity), experiments were conducted in the presence of Bay K-8644 so that cells at all ages exhibited fluctuations in [Ca²⁺]_i. The concentration of Bay K-8644 that was used does not act as a stimulus per se, but instead has been reported to augment chromaffin cell responses to submaximal concentrations of cholinergic receptor agonists and elevated potassium [Garcia et al., 1984; Ladona et al., 1987].

Frequency of Occurrence of Fluctuations in [Ca²⁺]_i

Data in Table 1 show the number of cells in each experimental group that exhibited fluctuations in [Ca²⁺]_i on each day examined. Data also show how many of these cells exhibited fluctuations during the 10-min period corresponding to MF exposure and the 4-min period after the field was turned off (see Fig. 2).

Comparison of the frequency of calcium transients during this interval showed no significant differences between nonexposed cells and sham exposed cells on any day examined. In cells exposed for 10 min to 60 Hz magnetic flux densities of 0.01, 0.05, 0.1, and 0.8 mT 60 Hz MFs, frequencies were similar to both nonexposed and sham exposed cells (data not shown). At

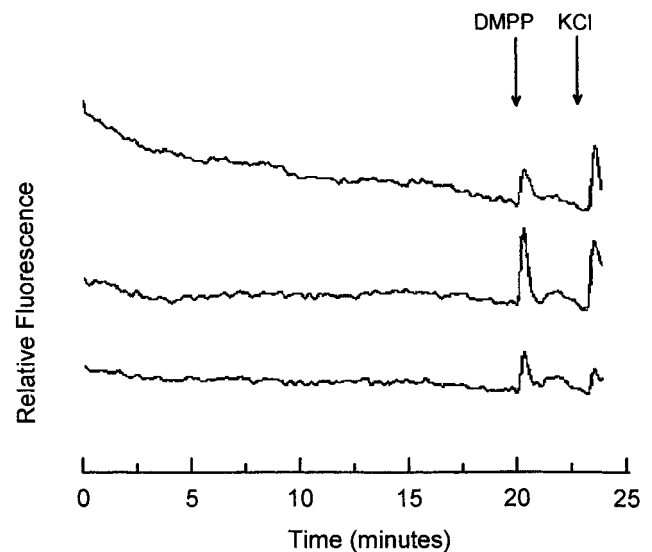


Fig. 3. Representative traces of fluo-4 fluorescence in quiescent chromaffin cells. Each trace represents an individual cell. The slight decline in fluorescence in the top trace is indicative of photobleaching that sometimes occurred during the monitoring period. Addition of DMPP and KCl are indicated by the arrows.

higher magnetic flux densities of 1 and 2 mT, there was one condition, day 1 cells exposed to a 2 mT continuous MF, when calcium transients occurred more often compared to both nonexposed and sham exposed cells. The difference, however, was significant only with respect to sham exposed cells ($P < 0.05$).

Classification of the Types of Fluctuations in $[Ca^{2+}]_i$

In chromaffin cells, spontaneous calcium fluctuations consisted of both increases and decreases in $[Ca^{2+}]_i$, with some cells exhibiting more than one type of calcium transient. To assess possible alterations in this heterogeneous behavior in response to 60 Hz MF exposure, a classification scheme was devised that takes into account all the types of changes in $[Ca^{2+}]_i$ observed in each experimental group, as well as their frequency of occurrence. The scheme is shown in Figure 4 together with examples of each type of time-varying calcium fluctuation.

The first level of classification was based on the duration of a calcium fluctuation where the left side of Figure 2 depicts activity lasting less than 1 min (short lived) and the right side depicts activity lasting more than 1 min (longer lived). The second level of classification characterized short-lived events as occurring either singly or in clusters (e.g., single spikes vs. multiple spikes) and longer lived events as either transient or sustained (in the latter case, prolonged changes in $[Ca^{2+}]_i$ that did not return to baseline). The third level of classification indicated the direction (increase or decrease) of the change in either $[Ca^{2+}]_i$ or calcium activity.

Table 2 shows the number of times that each type of calcium fluctuation or event was observed. The data presented are only for 1 day old cells that typically manifested the most frequent fluctuations in $[Ca^{2+}]_i$. In nonexposed cells, the majority of calcium events included single spike activity, increases and decreases in multiple spiking activity, and sustained increases

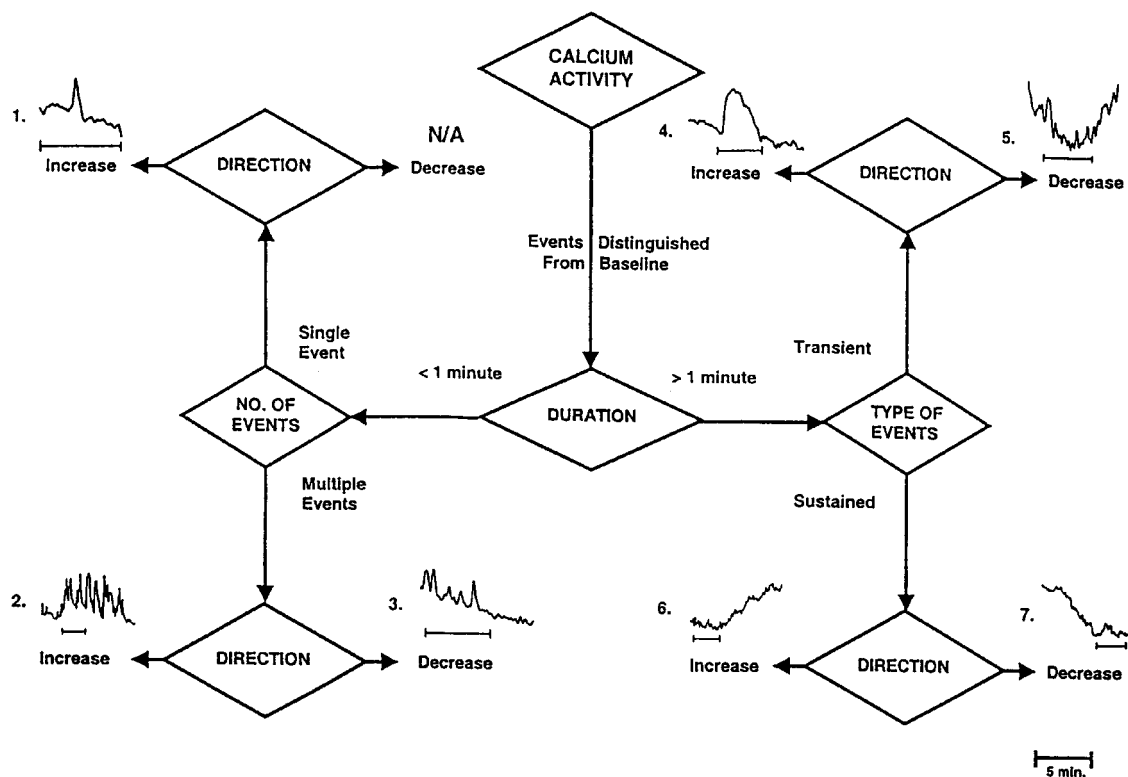


Fig. 4. Classification scheme of spontaneous calcium activity in chromaffin cells. Representative traces show each type of spontaneous calcium activity documented during the observation period. Traces on the left side of the diagram depict individual events lasting less than 1 min, whereas traces to the right depict individual events lasting more than 1 min. The bar under each trace represents 5 min. Calcium events characteristic of increases in $[Ca^{2+}]_i$ included single spikes (1), multiple spikes (2), broad spikes (4), and slow, progressive rises in $[Ca^{2+}]_i$ that never returned to baseline (6). Calcium events characteristic of decreases in $[Ca^{2+}]_i$ included the cessation of repetitive spiking activity (3) and a transient (5) or a progressive, sustained drop in $[Ca^{2+}]_i$ that did not return to baseline (7). N/A indicates a type of event that was never observed.

TABLE 2. Occurrence of Each Type of Calcium Activity* in the Presence and Absence of 60 Hz MFs

Exposure:	Number of events ^a						
	None	Sham	1 mT	1 mT ^{pulsed}	1.4 mT	2 mT	2 mT ^{pulsed}
Single spikes							
Increase	13	10	4	2	1	9	3
Multiple spikes							
Increase	11	5	3	4	3	8	1
Decrease	16	20	6	5	3	22	9
Transient events							
Increase	6	3	3	0	2	3	1
Decrease	4	1	0	0	0	0	0
Sustained events							
Increase	11	5	6	2	2	7	0
Decrease	7	8	1	1	2	2	1
Total of all events	68	52	23	14	12	51	15

*See Figure 4 for classification.

^aData from Table 1 for day 1 cells only.

in [Ca²⁺]_i. Calcium activity profiles were generally similar for sham exposed cells as well as for all MF exposed cells. There were no pronounced differences among experimental groups according to any of the three levels of classification. Thus, 60 Hz MF exposure had no apparent effect on the kind of spontaneous calcium activity that the cells exhibited.

Number of Fluctuations in [Ca²⁺]_i

In some cells, we had noted the occurrence of two or more distinct calcium transients during the interval between the start of MF exposure and DMPP application. Data in Table 3 demonstrate that the occurrence of multiple fluctuations in [Ca²⁺]_i during this interval was similar among experimental groups, indicating the lack of effect of 60 Hz MF exposure on how often cells exhibited more than one calcium transient.

Time of Occurrence of Fluctuations in [Ca²⁺]_i

Further evaluation of 60 Hz MF effects on [Ca²⁺]_i included a comparison of when calcium transients appeared after the 5 min baseline monitoring period. As shown by the data in Table 3, the occurrence of individual calcium events during each successive 5 min interval after baseline monitoring was similar for nonexposed, sham exposed, and 60 Hz MF exposed cells. Thus, 60 Hz MF exposure did not appear to accelerate or delay the time at which cells typically exhibited fluctuations in [Ca²⁺]_i.

Waveform Characteristics of Repetitive Calcium Spiking Activity

In cells that exhibited repetitive calcium spiking (condition 2 in Fig. 4), we determined whether 60 Hz MF exposure caused a change in the frequency or

TABLE 3. Time of Occurrence of Calcium Events in the Presence and Absence of 60 Hz MFs*

Exposure	Total ^a	Per cell ^b	5–10 min ^c	10–15 min ^c	15 min to DMPP ^c
None	68/47	1.5	34/68	21/68	13/68
Sham	52/43	1.2	2/52	15/52	15/52
1 mT	23/18	1.3	6/23	10/23	7/23
1 mT ^{pulsed}	14/12	1.2	6/14	3/14	5/14
1.4 mT	12/7	1.7	5/12	3/12	4/12
2 mT	51/33	1.6	21/51	23/51	7/51
2 mT ^{pulsed}	15/13	1.2	5/15	9/15	1/15

*Data from Table 1 for day 1 cells only.

^aTotal number of cells showing calcium events divided by the total number of calcium events.

^bAverage number of calcium events per cell.

^cNumber of calcium events at each 5 min interval after baseline monitoring divided by the total number of calcium events.

waveform characteristics of the activity [see Galvanovskis et al., 1996, 1999]. To explore this possibility, a power spectrum analysis of spiking activity was performed on five cells from each experimental group, using MATLAB (version 5.3). For nonexposed and sham exposed cells, more than 90% of the power was located between 50 and 120 mHz. The power spectrum of spiking in cells exposed to 1 and 2 mT MFs exhibited no significant differences compared to nonexposed or sham spectra, further indicating the lack of an effect of 60 Hz MFs on intracellular calcium dynamics.

Response of Chromaffin Cells to Nicotinic Receptor Stimulation

Figure 3 shows that addition of the nicotinic receptor agonist DMPP to chromaffin cells elicited a rapid and pronounced rise in $[Ca^{2+}]_i$. On average, 60% of the cells responded to DMPP over an 8 day culture period (Fig. 5), indicating no decline of receptor function as the age of the cells increased. However, differences between days were significant. Data in Table 4 compare responses to DMPP in nonexposed cells, sham exposed cells, and MF exposed cells. No significant differences were observed between nonexposed and sham exposed cells on day 1 and 5 in culture, whereas on day 8 in culture, differences between the two groups reached statistical significance. For day 1 cells exposed to either continuous or pulsed 1 and 2 mT MFs, DMPP responses were significantly different compared to both nonexposed and sham exposed cells, except for one condition (1 mT pulsed

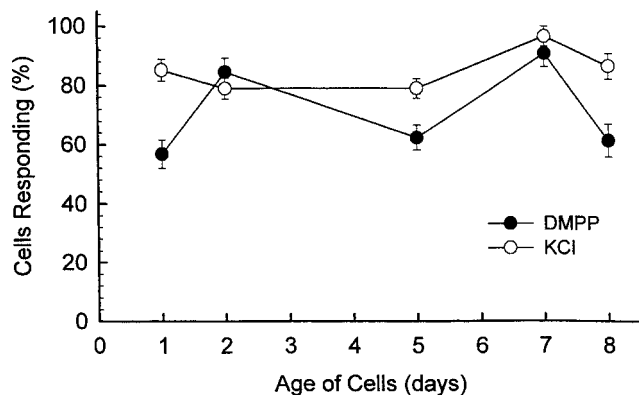


Fig. 5. Response of chromaffin cells to DMPP and KCl over an 8 day culture period. Data represent the percent \pm SE of cells responding to 10 μ M DMPP (\bullet) and to 56 mM KCl (\circ). The number of cells analyzed for each day are: 180 (day 1), 124 (day 2), 127 (day 5), 106 (day 7), and 102 (day 8). For cells responding to DMPP, differences are significant ($P \leq 0.001$) between day 1 and 7; day 2, and day 5 and 8; day 5 and 7; and day 7 and 8. For cells responding to KCl, differences are significant ($P \leq 0.005$) between day 1 and 5; day 2 and 7; and day 5 and 7.

MF) where the difference was significant only with respect to nonexposed cells. For day 5 and 8 cells exposed to 1 mT MFs, responses to DMPP were not significantly different from either nonexposed or sham exposed cells.

Response of Chromaffin Cells to KCl

A greater percentage of cells (a mean of approximately 86%) responded to depolarizing concentrations of KCl than to DMPP throughout the 8 day culture period and differences between days were significant (Fig. 5). However, there were no significant differences on any day examined between cells that were exposed to 60 Hz MFs and cells that were either nonexposed or sham exposed (Table 4).

DISCUSSION

In this study, we examined the effects of acute 60 Hz MF exposures on $[Ca^{2+}]_i$ in an in vitro model of a nontransformed neural cell type, chromaffin cells isolated from the adrenal medulla. Magnetic flux densities included specific field intensities reported to both alter $[Ca^{2+}]_i$ in other cell types [e.g., Lindstrom et al., 1993] and affect neural function at the whole organism level [e.g., Lai et al., 1993; Lai and Carino, 1999]. Rigorous comparisons of the calcium activity of chromaffin cells that were MF exposed cells, sham exposed, or nonexposed indicate that $[Ca^{2+}]_i$ is unaffected by 60 Hz magnetic flux densities of 0.01, 0.1, 1.0, 1.4, or 2.0 mT. However, because there may be window effects [Bawin and Adey, 1976; Blackman et al., 1982], we can not rule out the possibility that magnetic flux densities intermediate between those used in this study may elicit calcium responses.

Biological responses attributed to powerline frequency EMFs have generally been subtle and modest in magnitude [e.g., Galvanovskis et al., 1996, 1999]. This raises the question of whether the methodology employed to assess 60 Hz EMFs on $[Ca^{2+}]_i$ in chromaffin cells was sensitive enough to detect such responses. Our choice of fluo-4 as the fluorescent probe to monitor $[Ca^{2+}]_i$ directly addresses this issue. Fluo-4 has a high quantum yield, making it particularly sensitive and useful for documenting the dynamic aspects of spontaneous and stimulus induced calcium transients. It is excited by visible, rather than UV light, which is especially important since it obviates concerns of nonMF effects on calcium signaling pathways that could erroneously be attributed to MF exposure [Ihrig et al., 1997] and of phototoxic damage of cells over relatively long monitoring periods.

With respect to the latter, our observations of robust increases in $[Ca^{2+}]_i$ after addition of DMPP and

TABLE 4. Response of Nonexposed Cells and 60 Hz MF Exposed Chromaffin Cells to DMPP and High Potassium

Age of cells	Exposure	Total cells	DMPP + cells	DMPP response (% ± SE) ^a	KCl + cells	KCl response (% ± SE) ^a
1 day	None	180	111	62 ± 3	164	91 ± 2
	Sham	170	121	71 ± 3	162	95 ± 2
	1.0 mT	64	64	100 ± 5 ^b	63	98 ± 3
	1.0 mT ^{pulsed}	52	43	83 ± 5 ^c	46	89 ± 4
	1.4 mT	20	11	55 ± 9	16	80 ± 6
	2.0 mT	79	76	96 ± 4 ^b	73	92 ± 3
	2.0 mT ^{pulsed}	48	48	100 ± 6 ^b	47	98 ± 4
5 days	None	127	75	59 ± 4	100	78 ± 4
	Sham	23	17	74 ± 10	15	65 ± 9
	1.0 mT	92	45	49 ± 5	69	75 ± 5
8 days	None	102	63	62 ± 5	87	85 ± 3
	Sham	62	24	39 ± 6 ^c	61	98 ± 4 ^d
	1.0 mT	99	47	48 ± 5	93	94 ± 3

See Table 1 for experimental details.

DMPP + cells and KCl + cells indicate cells that respond to addition of DMPP and KCl, respectively.

^aMANOVA values.

^bSignificantly different from nonexposed cells ($P < 0.001$) and sham exposed cells ($P < 0.001$).

^cSignificantly different from nonexposed cells ($P < 0.05$).

^dSignificantly different from nonexposed cells ($P < 0.01$).

high potassium clearly indicate an unimpaired ability of chromaffin cells to respond to external stimuli. As an additional confirmation, preliminary experiments established that two other measures of cell responsiveness to nicotinic receptor stimulation, ⁴⁵Ca²⁺ influx and release of catecholamines, are unaffected by the presence of the dye within the cells. Thus, if chromaffin cells are sensitive to 60 Hz EMFs, alterations in [Ca²⁺]_i should have been observed in fluo-4 loaded cells unless some other condition used in this study did not allow an effect to be manifested. The latter is always a possibility since seemingly trivial details in how MF experiments are conducted might determine whether an MF effect is observed and whether the findings can be replicated by other laboratories [Ihrig et al., 1997; Boorman et al., 2000].

In characterizing spontaneous fluctuations in [Ca²⁺]_i in chromaffin cells, we documented episodes of both quiescence and activity during a 20 min observation period. When calcium activity occurred, it took many different forms that included transient or sustained increases and decreases in [Ca²⁺]_i, as well as suppression or induction of spiking activity (oscillations). Adding to the heterogeneity of spontaneous calcium activity were differences pertaining to when calcium fluctuations occurred during the observation period as well as the number of calcium fluctuations that individual cells exhibited. In the latter case, more than one type of calcium fluctuation was sometimes observed. Thus, it was against a high background level of heterogeneous calcium activity that the effects of MF exposure were gauged.

Some investigators circumvent this problem by preselecting cells on the basis of one particular type of calcium activity. For example, Lindstrom et al. [1993, 1995] used only quiescent cells in their analysis of 50 Hz EMF effects on [Ca²⁺]_i in Jurkat cells, whereas Galvanovskis et al. [1996, 1999] restricted their analysis of EMF effects to Jurkat cells that had been forced into spiking activity. Whether this experimental strategy provides a more reliable way of detecting powerline frequency EMF effects on [Ca²⁺]_i is open to speculation since attempts by different laboratories to replicate the findings of EMF-stimulated alterations in [Ca²⁺]_i in Jurkat cells have been unsuccessful [Lyle et al., 1997; Wey et al., 2000].

In the present study, only day 1 cells exposed to a 2 mT continuous MF show an apparent increase in fluctuations in [Ca²⁺]_i. However, we do not feel that the data provide convincing evidence of an effect due to MF exposure. First, the apparent increase in calcium activity was statistically significant only with respect to sham exposed cells ($P < 0.05$) and not nonexposed cells. Second, the total number of cells exhibiting alterations in [Ca²⁺]_i during baseline monitoring was, for an unexplained reason, much greater in this particular experimental group. Thus, the apparent increase in the number of cells exhibiting calcium events during the exposure period is likely a consequence of the increased calcium activity of the cells overall rather than a consequence of MF exposure. Not adequately taking details such as these into consideration, together with less rigorous analyses of spontaneous changes in calcium due to shorter exposure periods and smaller

sample sizes, may have been factors that contributed to preliminary reports from our laboratory indicating apparent increases in $[Ca^{2+}]_i$ in chromaffin cells during exposure to 0.8, 1.0, and 1.2 mT MFs [Craviso et al., 1998, 1999].

Our data showing an apparent increased responsiveness of day 1 cells to nicotinic receptor agonist stimulation after exposure to 1 or 2 mT MFs could mean that 60 Hz EMFs are capable of altering chromaffin cell plasma membrane receptors or properties of the membrane that are crucial for agonist receptor interactions. That the apparent increase in responsiveness to DMPP was observed only for cells early in culture could further imply that there is a critical time in which plasma membrane components are sensitive to 60 Hz EMFs. These are intriguing possibilities, especially in view of reports of powerline frequency EMF effects not only on extracellular receptors, such as β -adrenergic receptors [Luben, 1993] and serotonin 5-HT-1B receptors [Massot et al., 2000], but also on membrane fluidity [Santoro et al., 1997]. However, we are reluctant to say at this time that a true MF effect exists in our case since cells exposed to a 1.4 mT MF did not show a greater responsiveness to DMPP compared to either nonexposed or sham exposed cells. Also, there was sufficient variability in cell responsiveness to DMPP from day to day that drawing valid conclusions about EMF effects on nicotinic receptors on day 1 cells is not prudent. Obviously, additional studies using different methodologies to assess 60 Hz EMF effects on nicotinic receptor function in chromaffin cells will be needed to resolve this issue. Such studies are currently underway in our laboratory [Craviso et al., 2000].

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