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Calcium is necessary in the cell response to EM fields

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Abstract

Previous research showed that exposure of human HL-60 cells to extremely low frequency electromagnetic fields increases the steady-state levels of some mRNAs. Modifications in calcium flux have been suggested as a means of amplifying electromagnetic signals, and induced changes in calcium influx could hypothetically lead to gene activation. The present experiments tested the role of calcium in the response of cells to electromagnetic fields. Steady state transcript levels for c-*fos* and c-*myc* were determined under conditions of low extracellular calcium. The present study confirms that calcium plays a role in the response of cells to electromagnetic fields.

Key words: Extremely low frequency (elf) electromagnetic (EM) fields; Calcium; RNA transcript

1. Introduction

Steady-state levels of some mRNA transcripts are increased in cells exposed to extremely low frequency (elf) electric and magnetic (EM) fields [1,2]. It is still unclear which cell components mediate these or any field effects, but it is assumed that some type of initial interaction takes place at the level of the cell membrane [3–6]. One pathway by which EM field exposures could affect cells results from findings of changes in calcium flux patterns observed in cells exposed to EM fields [3–5; 7–11]. It has been proposed that EM field-induced changes in calcium influx leading to signal transduction provides an interaction mechanism which could ultimately lead to gene activation [5].

The hypothesis that extracellular calcium plays a pivotal role in the cell's response to EM fields was tested. In one set of experiments, EGTA was added to the media at a concentration adequate to actively deplete the extracellular calcium. It is difficult to assess, however, how much calcium is remaining in the media after treatment with EGTA, and secondary effects could result from the use of a chelator. In a second series of experiments, extracellular calcium was reduced to 1/1000 of normal levels (calcium reduction) which allowed determinations in a known, but highly reduced, quantity of calcium. In both sets of experiments, the effect of EM field exposures on the steady-state levels of c-*fos* and c-*myc* was negated. The results from these experiments are consistent with the hypothesis that the concentration of extracellular Ca^{2+} ions play a role in the response of the cell to EM fields, either at the membrane level, and/or in modulating the rate of calcium influx.

2. Materials and methods

2.1. Cells and media

HL-60 cells [12] were maintained in RPMI 1640 (Gibco) with 10% fetal calf serum. Prior to each exposure, cells in growth phase (~5×10^5 cells/ml) were concentrated by centrifugation and allocated into T-25 flasks at 1×10^6 cells/ml in 15 ml of the appropriate media as described previously [13]. Cells were washed twice with S-MEM minus calcium (5 min) and then pre-incubated one hour with Minimal Essential Media (S-MEM, Gibco), containing 10% dialyzed fetal calf serum, and adjusted to a calcium concentration of 10^{-6} M with $CaCl_2 \cdot 4H_2O$ (calcium reduction). Cells were also exposed in S-MEM adjusted to same calcium concentration as RPMI (100 mg/l) in order to test the effects of using different media. To test the effect of relative deprivation of calcium, 1.5 mM of ethyleneglycol-bis(β-amino-ethyl ether)*N'N'*-tetra-acetic acid (EGTA) was added to RPMI media [14]. The basic salt components of each media are compared in Table 1 [15].

2.2. Experimental design

The experimental design included: (i) measurements of different transcripts from the same RNA derived from cells of a single exposure; (ii) a series of exposures done on different days; and (iii) two different methods of determining the steady-state levels of the transcripts measured. The experimental series are summarized in Table 2.

2.3. Exposure conditions

HL-60 cells were exposed to a continuous sinusoidal 60 Hz field ($B = 8$ μT; calculated $E = \sim 10$ μV/m) for 20 min in all experiments. The exposure system consisted of a pair of Helmholtz coils (Electro-Biology Inc.), 13 × 14 cm. The coils were wound with 19-gauge magnetic wire 164 times around an approximate square frame. The space between the coils was 8 cm. The coils were energized by a Wavetek function generator. A calibrated probe was used to set the field strengths used. The coils were housed in a mu metal container within the incubator. Specific details of the exposure system have been described [13]. Control samples (unexposed cells) were run at the same time as the experimental samples (exposed cells) in a separate section

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Abbreviations: EM, electromagnetic; elf, extremely low frequency; *B*, magnetic field; *E*, electric field.

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of the incubator. Flasks used for control were also shielded in a mu metal container.

2.4. Isolation of RNA

At the conclusion of each exposure, the flasks were immediately plunged into an ice slurry for 10 min. Total cellular RNA was isolated using methods described previously [13]. Each RNA sample was tested by agarose gel electrophoresis to determine the integrity of the RNA based on the 5S, 18S and 28S bands following ethidium bromide staining of the gel.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

The RNA from four exposures done on different days was analyzed. PCR amplification was accomplished according to the method described in Broude et al. [6]. The first cDNA strand was synthesized using 1–2 μg of total RNA and random primer. This cDNA was then used in all subsequent amplification reactions by adding the corresponding specific primers. Conditions were selected to produce a proportional ratio between the amount of input mRNA and amplified product. Amplification was carried out in a COY thermocycler using 94°C (1 min), 60°C (1 min) and 72°C (1 min) for 35 cycles. The amplification products were analyzed by gel electrophoresis on 2% agarose gels containing 0.05% mg/ml ethidium bromide. Gels were photographed in UV-light and the negatives scanned using a computerized laser scanning system. The data were analyzed using software provided by the manufacturer. All RNA samples from each experimental series were analyzed in the RT-PCR reaction simultaneously and run on one gel. The PCR product was confirmed by Northern blots. Pictures on Polaroid 665 film were scanned on a laser scanner with a fixed plot window so that the areas of the peaks would reflect the density of the bands. Values are expressed as the ratio of density resulting from RNA extracted from exposed (E) and control (C) cells ± standard error of the mean (S.E.M.). Each RNA sample from a single exposure was analyzed by the RT-PCR reaction at least twice (see Table 2).

The following primers were used:

for c-*fos* specific:
5′-GGCTTCAACGCAGACTACGAGGCGT-3′(901–925) and
5′-CCTCCTGCCAATGCTCTGCGCTCG-3′(1996–1973) [16]

for c-*myc* specific:
5′-ACTGCGACGAGGAGGAGAAC-3′(4591–4610) and
5′-TACAAGCTGGAGGTGGAGCAG-3′(5050–5030) [17]

2.6. Hybridizations

Quantitative dot blot hybridizations were also used to measure steady-state levels of c-*myc* and β_2-microglobulin transcripts [18]. Northern blots were used to confirm the size of the transcripts under essentially the same conditions as the dot blot hybridizations. Hybridization conditions for dot blots and Northern blots have been described [19].

Human myc DNA probes were obtained from Oncor or by PCR amplification using the primer given above. β_2-Microglobulin DNA was obtained from Dr. I.B. Weinstein, Columbia University. DNA was labelled in vitro with [^{32}P]dCTP or [^{32}P]dATP (NEN) [20]; the specific activities obtained were between 5 and 10×10^7 cpm/μg. Radioactive measurements (cpms) were made by liquid scintillation counting of cut pieces of the nitrocellulose filters, as identified by the autoradiographic spot. Values are expressed as the mean ratio of the cpm of hybridized RNA from exposed cells (E) to that of unexposed cells (C) ± S.E.M.

3. Results

3.1. RT-PCR

Steady state levels of c-*fos* and c-*myc* mRNAs were measured initially by the RT-PCR technique. Although the accuracy of this method is insufficient to provide absolute values of copy numbers of transcript levels, it has adequate precision to detect changes in the levels of transcripts with minimum inter-experimental variability [6].

Relative steady state levels for each of the transcripts as determined by RT-PCR are illustrated in Fig. 1A and B. The data show that exposure of HL-60 cells to elf EM fields results in an increase in the steady state levels of c-*fos* and c-*myc* mRNAs. These results are consistent with other published data from this laboratory using the RT-PCR procedure [6] and dot blot hybridizations [1,13,19]. The effect of EM fields on the transcript increase, however, was negated under conditions of low extracellular calcium (see Fig. 1). The ratios of c-*fos* and c-*myc* mRNAs in exposed and unexposed cells were combined and calculated for all experiments. The values were 1.19 ± 0.01 (S.E.M.) for c-*fos* and 1.15 ± 0.04 for c-*myc* in experiments performed under conditions of normal calcium concentrations in the media. Under conditions of low calcium, the corresponding values were 0.92 ± 0.02 and 0.92 ± 0.02. In presence of EGTA, the corresponding values were 0.93 ± 0.02 and 0.79 ± 0.04.

3.2. Measurements of steady state levels of c-myc by dot blot hybridization

The results of experiments using RT-PCR were confirmed by using dot blot hybridizations on the same RNA samples (Fig. 2). Although variability in these experiments was greater than that observed using RT-PCR, the results were essentially the same. There was a single experiment (experiment 2, see Table 2), performed under conditions of calcium reduction, where the ratio of steady state mRNAs from exposed to control samples exceeded 1, and did not repeat results obtained by RT-PCR. The values, however, were significantly reduced over those obtained under conditions where normal levels of calcium were present in the media. The ratios of c-*myc* mRNAs in exposed and unexposed cells in all experiments were 1.28 ± 0.05 (normal calcium levels) and 0.94 ± 0.04 (reduced calcium). In experiments which used EGTA to reduce the calcium concentration, corresponding values were 0.9 ± 0.07 (presence of EGTA) and 1.5 ± 0.13 (no EGTA). The steady state levels of β_2-

Table 1

Component	S-MEM (mg/l)	S-MEM (mg/l)	RPMI 1640 (mg/l)	RPMI 1640 (mg/l)
	Low calcium		Low calcium	
$Ca(NO_3)_2 \cdot 4H_2O$			100	100
$CaCl_2 \cdot 4H_2O$	$\pm 1.5 \times 10^{-4}$	100		
EGTA			570	
KCl	400	400	400	400
$MgSO_4 \cdot 7H_2O$	97.6	97.6	100	100
NaCl	6800	6800	6000	6000
$NaHCO_3$	2200	2200	2000	2000
$NaH_2PO_4 \cdot H_2O$	1400	1400		
$NaHPO_4 \cdot 7H_2O$			1512	1512

microglobulin remained unchanged under all conditions, which is consistent with our previous results (see Fig. 3) [21].

The effects observed are independent of media type. In two experiments (6 and 7), calcium levels comparable to those in RPMI were added to S-MEM. The steady state levels of c-*myc* were compared between exposed and unexposed cells grown in RPMI and S-MEM with the same calcium concentration and cells in S-MEM with reduced calcium. The effect remains the same irrespective or whether RPMI or MEM is used as media, as long as the calcium concentration is not reduced (Fig. 3). There was no effect of EM fields on the steady state transcript levels of c-*myc* in calcium-deficient media. As expected, the levels of β_2-microglobulin remained constant after EM field exposure.

4. Discussion

There are good scientific reasons to believe that Ca^{2+} ions play a role in the initiation of a membrane-mediated response to EM fields [22–26]. The differential in calcium concentration between the extracellular and intracellular fluid results in an enormous concentration gradient on the plasma membrane [25]. The gradient is maintained

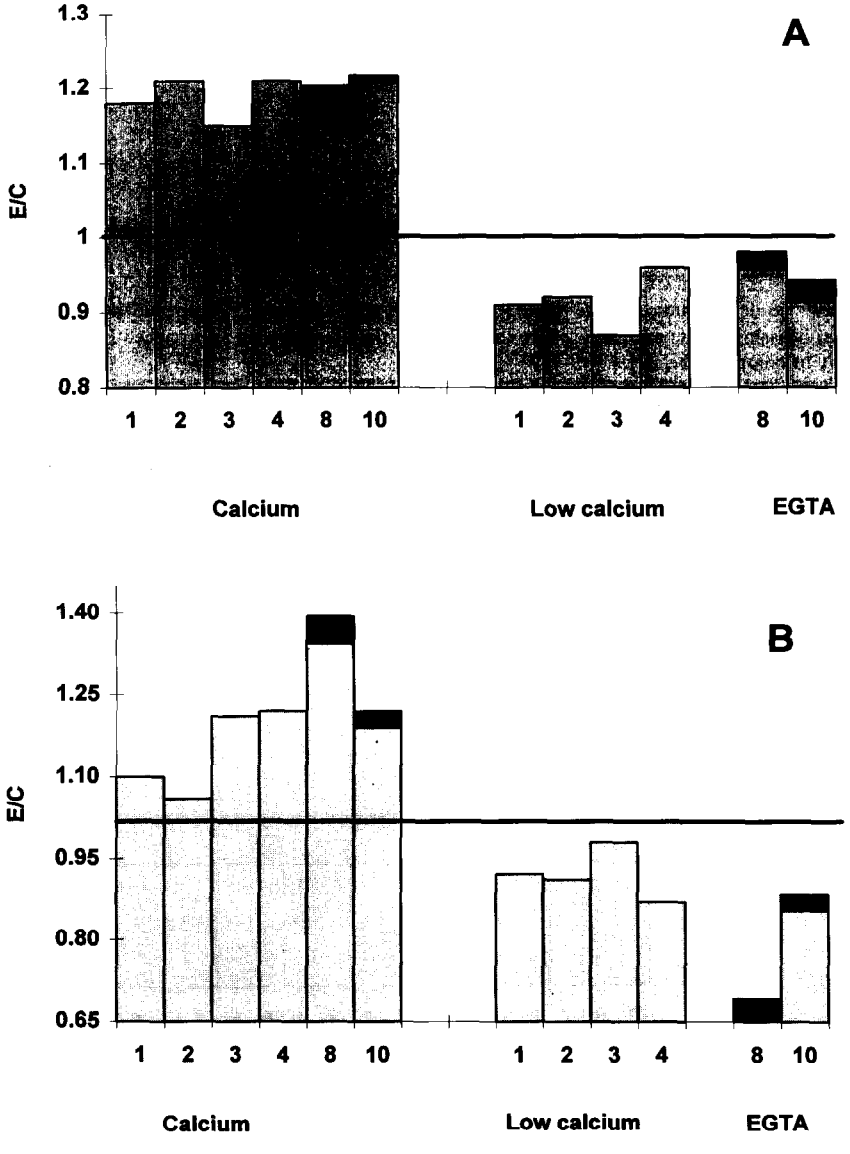

Fig. 1. RT-PCR measurements of the steady state levels of c-*fos* (Fig. 1A) and c-*myc* (Fig. 1B) transcripts in HL-60 cells following exposure to an EM field. The exposure was at 60 Hz (8 μT; ~10 μV/m) for 20 min. Calcium: determinations using cells grown under conditions using normal growth media (RPMI 1640). Calcium reduction: determinations in cells which were placed in S-MEM with dialyzed fetal calf serum and with the addition of 10^{-6} M calcium. Shaded EGTA: 1.5 mM EGTA was added to RPMI media. Standard errors of the mean are given where $n > 3$.

Table 2
Experimental design

Exposure	Probe	RT-PCR						Dot blots											
		Calcium		Calcium reduction		Presence of EGTA		Calcium (RPMI)			Calcium (MEM)			Calcium reduction (MEM)			Presence of EGTA (RPMI)		
		#RT*	#PCR	#RT	#PCR	#RT	#PCR	#blots	*n* cont	*n* exp	#blots	*n* cont	*n* exp	#blots	*n* cont	*n* exp	#blots	*n* cont	*n* exp
1	*myc*	2	2	2	2			3	9	17				2	11	11			
	fos	2	2	2	2														
2	*myc*	2	2	2	2			5	30	27				3	18	18			
	fos	2	2	2	2														
3	*myc*	2	3	2	3			lost						1	6	3			
	fos	2	3	2	3														
4	*myc*	2	2	2	3			1	6	6				1	4	4			
	fos	1	2	1	2			1	6	6				1	3	6			
	β_2-M							1	6	6				1	6	6			
5	*myc*							4	24	21				4	19	16			
6	*myc*							1	6	6	1	6	6	1	6	6			
	β_2-M							1	6	6	1	6	6	1	6	6			
7	*myc*							3	18	18	3	18	18	3	18	18			
	β_2-M							1	6	6	1	6	6	1	6	6			
8	*myc*	1	2			1	2	3	9	9							3	9	9
	fos	1	2			1	2												
9	*myc*	2	3			2	3	1	3	3							1	2	3
	fos	2	3			2	3												
10	*myc*	2	4			2	4	1	7	7							1	7	7
	fos	2	4			2	4												

**,# For RT-PCR measurements, two determinations were made for each PCR and averaged. For dot blots, each sample was repeated twice in a given blot in a dilution series (*n*). Missing points are those where the results of the final dilution approached background levels or where there was an inadequate quantity of RNA to complete the series.

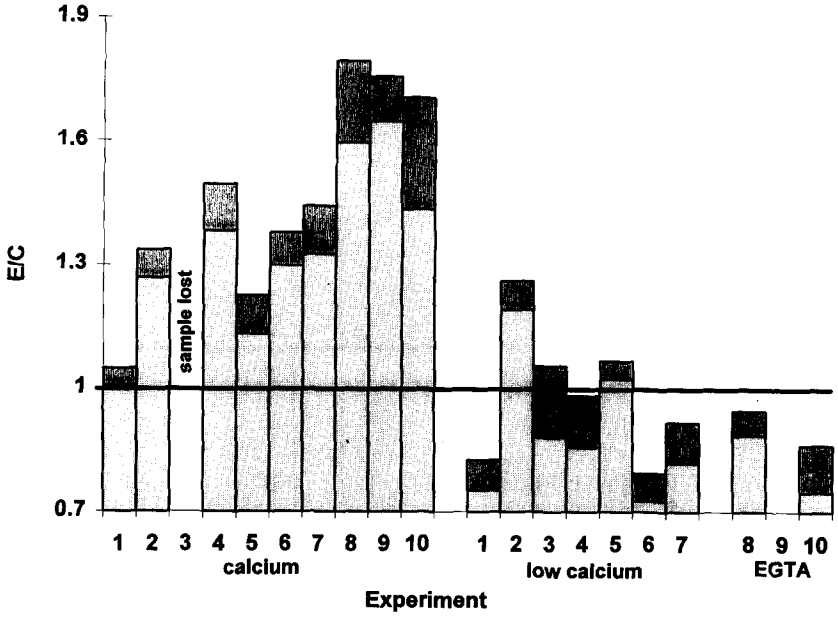

Fig. 2. Measurements of the steady state levels of c-*myc* transcripts using dot blot hybridizations. The experiments were performed under conditions of normal calcium concentration in the media, under conditions of calcium reduction, and in the presence of EGTA. The lower bar is the mean of multiple determinations as given in Table 2. The upper bar represents the standard error of the mean. The results from ten separate exposures done on different days are given (results of experiment 9 under EGTA are omitted since there was only two points). The levels of β_2-microglobulin were determined on RNA from exposures 4, 5 and 7. They remained relatively unchanged in the presence of EM fields under conditions of normal media or under conditions of low calcium. A portion of this data is presented graphically in Fig. 3.

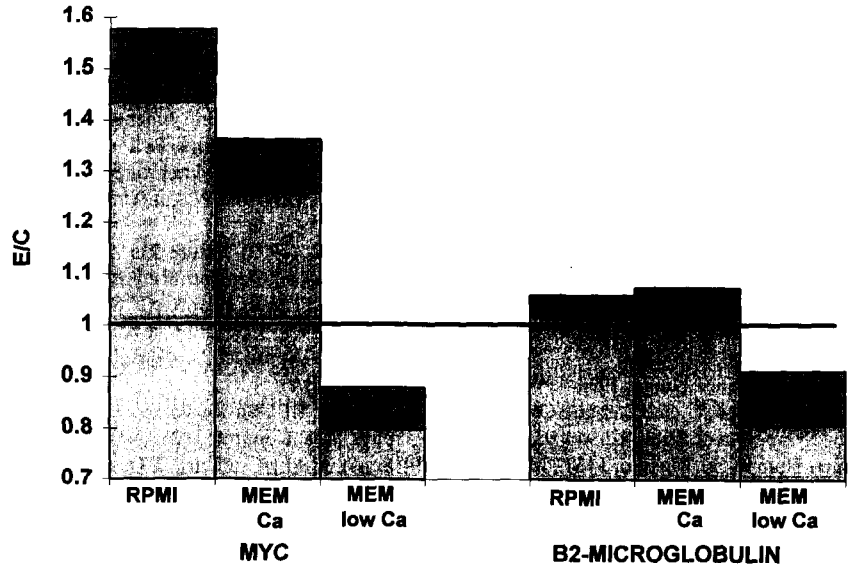

Fig. 3. The effect of composition of the media on the response of cells to EM fields. These experiments tested whether the basic composition of the media, rather than the calcium concentration, played a role in the differences observed under conditions of normal and reduced calcium levels. No significant difference was noted in when cells were placed in either of media containing 100 mg/l Ca $(NO_3)_2 \cdot 4H_2O$. There was a significant difference, however, when the calcium was reduced. The results given are mean values of hybridization data from two different exposures (numbers 6 and 7, see Table 2 and Fig. 2). The upper bar represents the standard error of the mean; $n = 12$ for determinations using *myc*; $n = 8$ for determinations using β_2-microglobulin.

in several ways [26], all of which could potentially be affected by the presence of EM fields. Calcium is an intracellular second messenger, and plays a key role in signal transduction processes [27]. Deprivation in calcium levels has been correlated with a loss of protein kinase C activity [28], as well as inhibition of TPA-promoted transformation [29] in some types of cultured cells.

A relationship between regulation of Ca^{2+} flux and exposure to EM fields has been previously made on the basis of several different types of studies (reviews in [4,5]). For example, direct measurement of intracellular calcium in HL-60 cells following exposure to relatively low magnetic fields for about 23 min shows a 30 nM increase in Ca^{2+} as measured by spectrofluorimetry [10]. Other studies have exposed lymphocytes to low frequency pulsed EM fields which enhances the response to mitogens and leads to a subsequent increase in calcium uptake [5,9,11]. Evidence that the effects of calcium influx could affect steady state levels of c-*myc* was shown by an increase in transcript levels following short exposures to EM fields that was coordinate with an increase in intracellular calcium in thymocytes stimulated with Con A [30].

A major problem is how elf EM fields are transmitted across the membrane to the interior of the cells, since cells have much higher endogenous electrical fields across their membranes than those induced by exposure to elf EM fields. It has been proposed that amplification via calcium flux could provide one means by which the membrane-mediated effect could be carried to the cell (reviewed in [4,5]).

Our results support the hypothesis that calcium concentration could directly or indirectly affect how a cell perceives EM fields. Previous data from other laboratories, as well as the data presented here, suggests that calcium could initiate signal transduction events in cells exposed to EM fields which could, in turn, be reflected in changes in transcript levels for inducible genes. A mechanism in which elf EM exposure affects signal transduction pathways, either directly or indirectly, would be consistent with activation of transcription. Proof of mechanism, however, will require demonstration of the pathway(s) from cell surface to the DNA in the nucleus.

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