

A role for Ca^{2+} in the effect of very low frequency electromagnetic field on the blastogenesis of human lymphocytes

P. Conti*, G.E. Gigante, E. Alesse, M.G. Cifone, C. Fieschi, M. Reale* and P.U. Angeletti

Dipartimento di Scienze Biomediche dell'Università dell'Aquila, Collemaggio-67100 L'Aquila, Italy and Cattedra di Immunologia, Istituto di Medicina Sperimentale, Università di Chieti, 66100 Chieti, Italy

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The DNA synthesis of lymphocytes triggered by phytohemagglutinin or phorbol-myristate-acetate is strongly reduced by the externally applied electromagnetic field (ELF). Ca^{2+} uptake by stimulated lymphocytes is also reduced by ELF. The effect appears to be synergistic with that of the well-known calcium blocker agent, verapamil.

Ca²⁺ flux Electromagnetic field Lymphocyte blastogenesis Phytohemagglutinin
Phorbol-myristate-acetate Verapamil

1. INTRODUCTION

The interaction of ELF on several biological systems has been extensively studied [1]. In particular, ELF has recently been proven to increase the rate of healing of bone fracture in man [2,3]. Furthermore, ELF has been reported to affect the immune system [4], the cell differentiation [5] and the arachidonic acid cascade [6].

Though the mechanism of ELF action has not yet been established it seems that a role for Ca^{2+} can be envisaged. In fact, each of the biological phenomena affected by ELF requires Ca^{2+} in some step.

In [4] we showed that ELF reduces the extent of lymphocyte blastization induced by lectins and we proposed that the electromagnetic field could affect the movement of Ca^{2+} across the plasma membrane.

Abbreviations: ELF, very low frequency electromagnetic field; PHA, phytohemagglutinin; PMA, phorbol-myristate-acetate; CRPMI, complete medium RPMI 1640

Here we tried to confirm this hypothesis by studying the effect of ELF on PHA and PMA stimulated lymphocytes. A role for Ca^{2+} was supported by the effect of Ca^{2+} inhibitor verapamil and by the direct measurements of Ca^{2+} fluxes studied by ^{45}Ca .

2. MATERIALS AND METHODS

2.1. Isolation of human mononuclear cells

Heparinized blood samples were obtained from adult normal volunteers and PBMC were collected on Ficoll-Hypaque as in [7], washed twice with HEPES-buffered (10 mM) Hanks balanced salt solution and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated Fetal Calf serum, penicillin 6 (200 U/ml), gentamicin (10 $\mu\text{g}/\text{ml}$), L-glutamine (0.3 mg/ml) and 10 mM HEPES, henceforth referred to as complete medium (CRPMI).

These cells were cultured for 72 h at 37°C in a 5% CO_2 -humidified atmosphere, in 200 μl of CRPMI at a concentration of 1×10^6 cells/ml in multiwell plates in the presence and absence of phorbol ester (final concentration, 8 $\mu\text{g}/\text{ml}$) or

PHA-p Difco (final concentration, 20 $\mu\text{g/ml}$). In some wells, verapamil at final concentrations of 5 μg and 25 $\mu\text{g/ml}$ was also added.

2.2. Exposure to ELF

The ELF was generated by passing a current through a pair of concentric 966-turn coils, 10 cm radius, separated by 2 cm. The device in question was wired in parallel to a pulse generator which gave a train of pulses of variable form and intensity. Square pulses with a frequency of 3 Hz were used. The generated field had an intensity of ~ 60 G. The waveform of current passing in the coils was only weakly smoothed due to the inductance of the two coil system (249 mH). The magnitude and waveform of the field was checked by using a Hall-effect probe. The temperature between the coils was measured with a digital thermometer. The coils were placed in a tissue culture incubator held at 37°C, while the pulse generator unit was outside.

The cells were exposed to ELF in the wells of a microtiter plate. Only those wells which showed an ELF homogeneity better than 1% when placed in the central space between the coils were used.

2.3. Assay for Ca^{2+} influx

A standard radiolabeling technique was used to measure cell associated $^{45}\text{Ca}^{2+}$ in the cell samples [8]. A lymphocyte suspension (0.1 ml, 2×10^7 cells/ml) in Ca^{2+} free MEM was incubated with 10 μl lectin or buffer (1 μCi , specific activity 30 Ci/g, NEN-USA) at 37°C. After 1 h 0.4 ml of ice-cold 10 mM CaCl_2 in saline was added. The tubes were immediately centrifuged for 30 s, then washed twice with fresh ice-cold CaCl_2 -saline. The radioactivity of $^{45}\text{Ca}^{2+}$ incorporated into the cells was measured by scintillation spectrometry.

2.4. Experimental procedures

The first series of experiments were conducted by exposing the PHA and PMA stimulated lymphocyte cultures (quintuplicate) to ELF and verapamil alone and in combination. The cultures were exposed during the whole incubation time (72 h). The [^3H]thymidine (25 mCi/mM) was added to a final concentration of 2 $\mu\text{Ci/ml}$, 6 h before the end of the incubation. At the end of the culture the cell viability was evaluated by trypan blue exclusion, it was always over 90% both when exposed and when not exposed. No appreciable dif-

Table 1
Incorporation of [^3H]thymidine by lymphocytes in the presence and absence of ELF

Row no.	No field	Field ^a	No field	Field ^a
	1st Experiment		2nd Experiment	
1 Control	2818 \pm 832	3221 \pm 965	5928 \pm 784	6200 \pm 639
2 PHA	241045 \pm 10023	159020 \pm 12404	101576 \pm 9907	65343 \pm 5148
3 PMA	39880 \pm 6933	14293 \pm 5735	30225 \pm 11047	5791 \pm 2363
4 PHA + VRP.1	43216 \pm 8951	27586 \pm 6663	7236 \pm 1550	10743 \pm 850
5 PHA + VRP.2	179178 \pm 21616	145778 \pm 7737	35451 \pm 3327	26226 \pm 4584
6 PMA + VRP.1	1680 \pm 705	7073 \pm 1291	6786 \pm 2693	2413 \pm 583
7 PMA + VRP.2	9878 \pm 2017	22508 \pm 16621	18017 \pm 4521	15531 \pm 4232
	3rd Experiment		4th Experiment	
1 Control	6767 \pm 1661	3872 \pm 1292	5101 \pm 2486	4507 \pm 2210
2 PHA	264065 \pm 65159	68892 \pm 21571	213905 \pm 59276	67976 \pm 14887
3 PMA	30687 \pm 14351	7330 \pm 4116	83025 \pm 39629	16570 \pm 6590
4 PHA + VRP1	21265 \pm 4574	4277 \pm 1167	55970 \pm 15514	17932 \pm 9427
5 PHA + VRP2	88905 \pm 21368	31770 \pm 7485	111298 \pm 13892	47830 \pm 13734
6 PMA + VRP1	2950 \pm 684	386 \pm 73	3286 \pm 1548	1806 \pm 774
7 PMA + VRP2	8452 \pm 2395	3535 \pm 1669	10407 \pm 1816	5406 \pm 2368

^a A 3 Hz and ~ 60 G square waveform field

PHA, phytohemagglutinin; PMA, phorbolmyristateacetate; VRP, verapamil; 1 = 25 μM , 2 = 5 μM

ference in pH between the cultures incubated with and without field was observed, as measured with a digital pH-meter. At the end of the incubation, the cells were harvested with glass fiber filters using a semiautomatic multiple sample precipitator, air-dried and the radioactivity was determined with a counter. Significance of the difference between the measured radioactivity mean values in different culture conditions was tested by Student's *t*-test.

In another set of experiments we measured the Ca^{2+} influx in PHA- and PMA-stimulated lymphocytes after exposure to ELF and treatment with verapamil.

3. RESULTS

Table 1 shows the effect of ELF on the incorporation of thymidine into normal human lymphocytes stimulated by PHA (rows 2,4 and 5) or by PMA (rows 3,6 and 7) as such (row 1-3) or in the presence of verapamil (rows 4-7).

The mitogenic effect of PHA or PMA appears to be markedly reduced by the exposure to ELF and by treatment with verapamil. In fact, the differences between the incorporation of thymidine in the presence or absence of field and in the presence and absence of verapamil are always highly significant ($p < 0.01$). The two inhibitory effects seem to be additive.

Fig.1a shows that the percentage reduction of thymidine incorporation is linearly related, in a

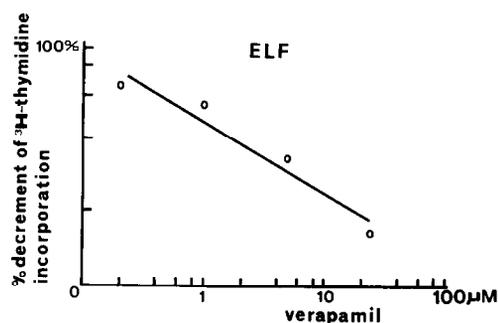
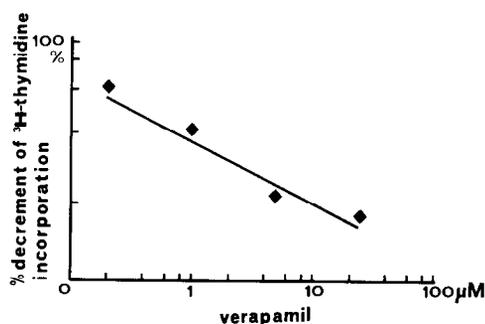


Fig.1. Dependence of percent inhibition of [3H]thymidine incorporation by verapamil concentrations: (a) no field present; (b) 3 Hz and ~ 60 G square waveform field applied. For experimental details see section 2.

log-log representation, to the verapamil concentration added to the cell cultures. A similar behaviour was obtained when the ELF was applied to the cell culture. This experimental evidence may indicate

Table 2
Calcium uptake by lymphocytes in the presence and absence of ELF

	No field	Field ^a	No field	Field ^a	No field	Field ^a
	1st Experiment		2nd Experiment		3rd Experiment	
Control	1062 ± 59	968 ± 35	2797 ± 120	3010 ± 62	4328 ± 302	4378 ± 62
PHA	1407 ± 94	838 ± 26	3298 ± 214	2586 ± 50	5076 ± 407	5076 ± 32
PHA + VRP (25 μM)	1020 ± 48	520 ± 40	2362 ± 108	2598 ± 51	3514 ± 201	3470 ± 45
Control	1212 ± 229	985 ± 20	3008 ± 245	3300 ± 275	2049 ± 49	2290 ± 137
PMA	3900 ± 700	896 ± 400	4200 ± 207	3275 ± 301	2774 ± 189	1761 ± 53
PMA + VRP (25 μM)	1800 ± 145	800 ± 112	3200 ± 275	3450 ± 190	1172 ± 113	2170 ± 207

^a A 3 Hz and ~ 60 G square waveform field

that ELF does not interfere with the verapamil effect on calcium.

Table 2 shows that more $^{45}\text{Ca}^{2+}$ is incorporated by cells after stimulation with PHA and PMA. However, both ELF and verapamil reduce the uptake of $^{45}\text{Ca}^{2+}$ to the value of controls. In this case a synergic effect of ELF and verapamil was not apparent.

4. DISCUSSION

In a previous paper we hypothesised that the exposure to ELF reduced the extent of lymphocyte blastogenesis due to a reduction of Ca^{2+} flux across the plasma-membrane, occurring within the first minutes after the addition of the lectins [5]. To support this idea in these experiments we have studied the combined effect of ELF and verapamil on lymphocytes after stimulation with PHA and PMA. Verapamil is a drug that blocks Ca^{2+} channels or pores then modifying the Ca^{2+} permeability of cell membranes [9,10].

Calcium may be a second intracellular messenger and indeed it has been considered a mediator of phorbol ester effects. Mobilization of Ca^{2+} from an intracellular pool is thought to trigger or regulate many PMA-induced biological responses. In addition, PMA was reported to induce changes in membrane potential and calcium influx and PMA stimulation of DNA synthesis was reported to be dependent on extracellular calcium [11]. Exogenous Ca^{2+} has been reported to play a role in the initiation of T cell activation [12,13] that eventually gives rise to DNA synthesis [14,15].

Here we have shown that the stimulation of DNA synthesis with PHA and PMA is accompanied by an increased $^{45}\text{Ca}^{2+}$ uptake by lymphocytes (table 2). Similar reports have already been published [16].

We have also shown that ELF and verapamil are strong inhibitors of PHA- and PMA-induced stimulation of human lymphocytes. The inhibition seems always to imply a reduced flux of Ca^{2+} into the cell as shown by the ^{45}Ca experiments (table 2).

The combined action of ELF and verapamil gives an inhibition greater than that produced by each factor. This additivity might be related either to the non-saturation condition used for each inhibitor or to different targets for the two agents. The second hypothesis might imply that ELF is ac-

ting only on the flux of extracellular Ca^{2+} , like verapamil that is also able to interfere with the mobilization of extracellular Ca^{2+} , but maybe with a different mechanism.

The results reported above appear to confirm the effect of ELF on plasma membranes already proposed [17]. Additional evidence is needed to determine whether the reduced flux of Ca^{2+} is the only (or the most important) effect underlying the action of ELF.

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