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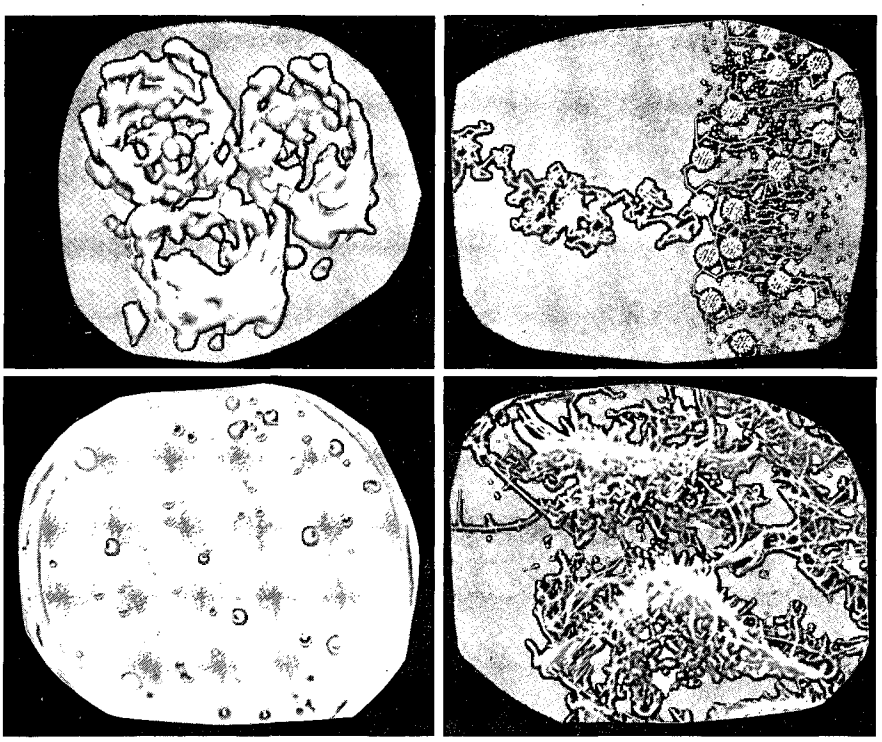

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ABSTRACT The first real-time measurements of $[Ca^{2+}]_i$ during exposure to a 60 Hz electric field (1.7 mV/cm) reveal an increase in $[Ca^{2+}]_i$ detected 100 seconds after mitogen activation [1]. This implicates the plateau phase and calcium influx across the cell membrane, rather than the release of calcium from intracellular stores during the early phase, in the interaction. This hypothesis was tested by separating these two phases of Ca^{2+} signaling in time. 60 Hz fields only exerted influence on the calcium influx during the plateau phase of calcium signaling. We hypothesize that such alterations in Ca^{2+} signaling will be propagated down the signal transduction (ST) pathway [1-3]. This was tested by performing the first simultaneous measurements of Ca^{2+} influx (early ST marker) and mRNA transcripts for the proto-oncogene c-MYC (mid-stage ST marker) in lymphocytes exposed to a 60 Hz field (1.7 mV/cm). Both ST markers are enhanced indicating linkage between a field effect acting at the cell membrane involving calcium and subsequent ST pathway processes relevant to gene activation.

INTRODUCTION. We tested two hypotheses in these studies. First, we asked is the cell membrane, and specifically the calcium ion channel, directly involved in ELF field interactions? This is important since receptor sites and ion channels are located in the cell membrane and these are the first structures involved in the signal transduction process. Second, we asked whether alterations in calcium ion flux are propagated down the signal transduction cascade to alter events such as gene activation? This question follows from our interaction model in which ELF fields alter calcium ion flux and, thereby, influence subsequent cellular events in the signal transduction cascade such as gene activation [1-3]. Results presented here indicate that (1) the cell membrane is a site of ELF field interaction, that (2) the early signal transduction markers of calcium ion influx and intracellular calcium are altered by ELF fields, and, importantly, that (3) these changes in calcium ion second messenger are associated with induction of c-MYC mRNA, a mid-stage signal transduction marker. Our observation of increased mRNA transcripts confirms the pioneering work of Goodman and Henderson [4]; they employed transformed HL60 leukemia cells, while we used mitogen-activated normal lymphocytes. Taken together our calcium ion transport, $[Ca^{2+}]_i$, and c-MYC mRNA

findings provide the first evidence supporting a field interaction in which changes in intracellular calcium are propagated down the signal transduction cascade to alter gene activation events.

EXPERIMENTAL PROCEDURES. Preparation of rat thymic lymphocytes, the $^{45}Ca^{2+}$ influx assay, the temperature-regulated, solenoidal exposure system and dosimetry procedures, details of Fura-2 loading and real-time measurements of intracellular calcium during field exposures are recently described in [1-3]. Northern analyses were as described [5].

RESULTS AND DISCUSSION. To evaluate early calcium responses to 60 Hz electric fields the first real-time measurements of $[Ca^{2+}]_i$ during field exposure were performed. **Figure 1** illustrates a typical time course for $[Ca^{2+}]_i$ during Con-A activation in the absence or presence of a 1.7 mV/cm 60 Hz electric field. When Con-A (1μg/ml) was added at 200 seconds $[Ca^{2+}]_i$ increased during the <u>early phase</u> of calcium signaling to a value of approximately 300 nM at 800 seconds. In the presence of the 60 Hz field when Con-A was added at 200 seconds and an increase in $[Ca^{2+}]_i$ was observed that was identical to that shown for unexposed cells over the first 100 seconds. However, $[Ca^{2+}]_i$ increased at a greater rate than for cells in the absence of the field. By 800 seconds the <u>plateau phase</u> of $[Ca^{2+}]_i$ reached approximately 380 nM. Alteration of the <u>plateau phase</u> of calcium signaling implicates the calcium ion channel of the outer plasma membrane as a site of field interaction, in contrast to structures associated with calcium release from intracellular stores.

To test the above hypothesis we separated the <u>early</u> and <u>plateau</u> phase of calcium signaling in time by placing cells in calcium-free media, and then adding calcium to the buffer. **Figure 2** shows at approximately 200 seconds Con-A was added and $[Ca^{2+}]_i$ increased to approximately 200 nM at 600 seconds corresponding to the <u>early phase</u> with release of calcium from intracellular stores. When extracellular calcium was added to the cells at approximately 670 seconds the <u>plateau phase</u> of $[Ca^{2+}]_i$ reached 390 nM at 800 seconds. In the absence of Con-A when the 60 Hz electric field was applied no change in $[Ca^{2+}]_i$ was detected; this is consistent with observations presented in

Figure 1. In **Figure 2**, when Con A was added to control cells in calcium-free buffer there followed an increase in $[Ca^{2+}]_i$, and an essentially identical increase was observed for Con-A treated cells exposed to the 60 Hz electric field. Thus, the electric field does not influence the <u>early phase</u> of calcium signaling. When extracellular calcium was added to thymocytes during field exposure an increase in $[Ca^{2+}]_i$ was observed to levels of approximately 490 nM at 800 seconds. These findings demonstrate that the ELF field influences calcium movement across the outer cell membrane which implicates the cell membrane as a site of field interaction.

We hypothesize that such alterations in early signal transduction will influence subsequent "downstream" cellular events in the signal transduction pathway such as gene activation and cell proliferation [1-3]. To test this we performed the first experiments to simultaneously quantitate Ca^{2+} influx (early ST marker) <u>and</u> c-MYC mRNA (mid-stage ST marker) in the same cell preparation exposed to 60 Hz ELF fields (1.7 mV/cm, 37°C, 60 minutes).

Figure 3 shows three separate Northern analyses of the <u>same</u> sample that were imaged using a cooled-CCD camera. There are three bands for each Northern blot which correspond to: -Con-A, +Con-A (1 μg/ml), and +Con-A plus 60 Hz (left to right). **Panel A** shows the flat-fielded image of the Northern blots, **Panel B** shows a binary image indicating regions of interest (ROI), **Panel C** shows a pixel-by-pixel subtraction of the ROIs minus a thresholded value, and **Panel D** depicts a 3D-contour plot representing the quantitated intensity of each band. We performed a multivariate analysis of variance for mean pixel intensity of each band across the three Northern analyses: -Con-A = 332 ± 128; +Con-A = 398 ± 48; +Con-A plus 60 Hz = 864 ± 181. In the presence of Con-A we detect a significant 2-fold enhancement of c-MYC mRNA during exposure to the 60 Hz field ($p = 0.0114$). This <u>same sample</u> was assessed for $^{45}Ca^{2+}$ influx: (CPM/10^7cells) -Con-A = 9,878 ± 749; +Con-A = 10,014 ± 1,046; +Con-A plus 60 Hz = 15,713 ± 1,403. A statistically significant 1.6-fold enhancement was detected at the $p = 0.029$ level for 60 Hz field treatment plus Con-A vs. Con-A alone. These calcium results are consistent with mRNA findings presented in **Figure 3**. Taken together they provide the first experimental support for an ELF interaction mechanism involving the

cell membrane and Ca^{+2} signaling triggering "downstream" ST pathway processes such a gene activation [1-3].

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FIGURE LEGENDS

Figure 1: Real-Time Measurements of Intracellular Calcium in Mitogen-Activated Thymic Lymphocytes During 60 Hz Field Exposure. See text and [1].

Figure 2 Real-Time Measurements of Intracellular Calcium in Mitogen-Activated Thymic Lymphocytes During 60 Hz Field Exposure: Separation of Early and Plateau Phase of Calcium Signaling. See text and [1].

Figure 3 Northern Analyses of c-MYC mRNA: Quantitation by Digital Image Analysis Using a Cooled CCD-camera.

Figure 1

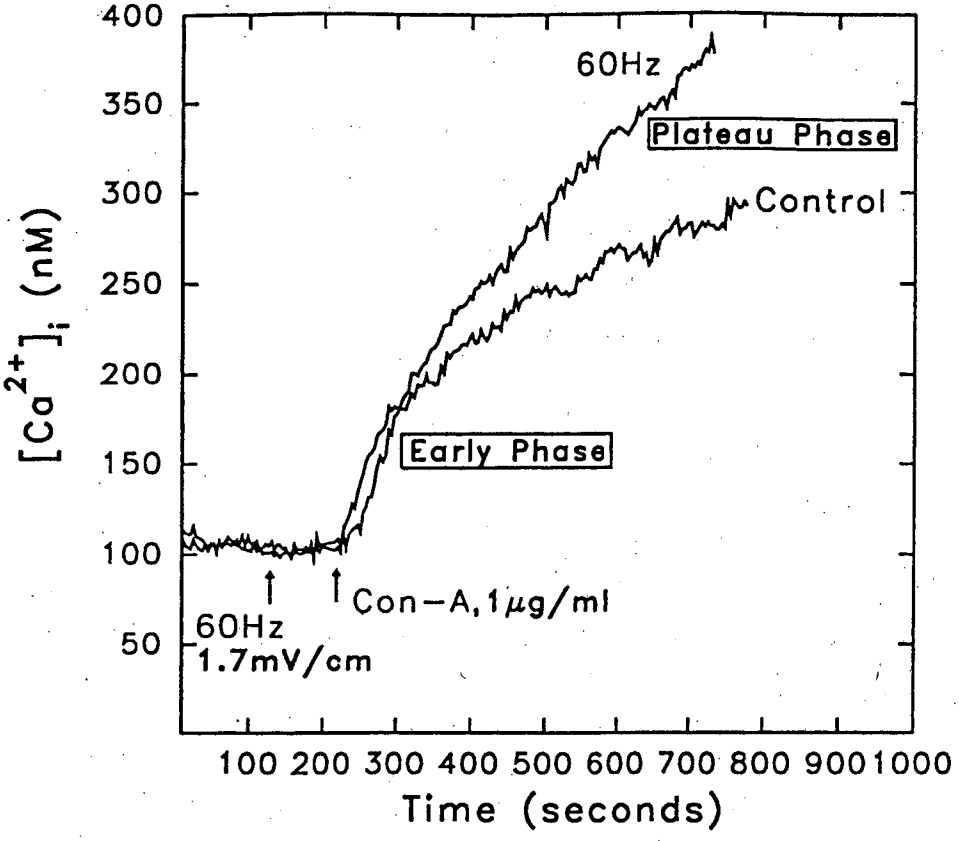


Figure 2

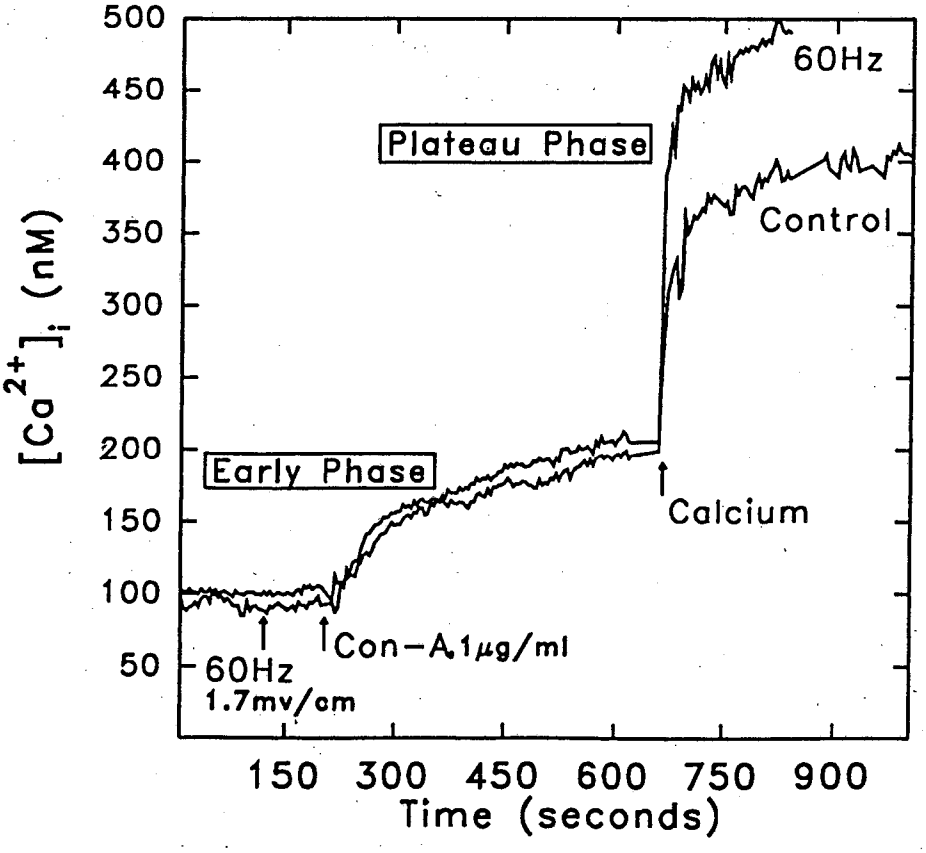
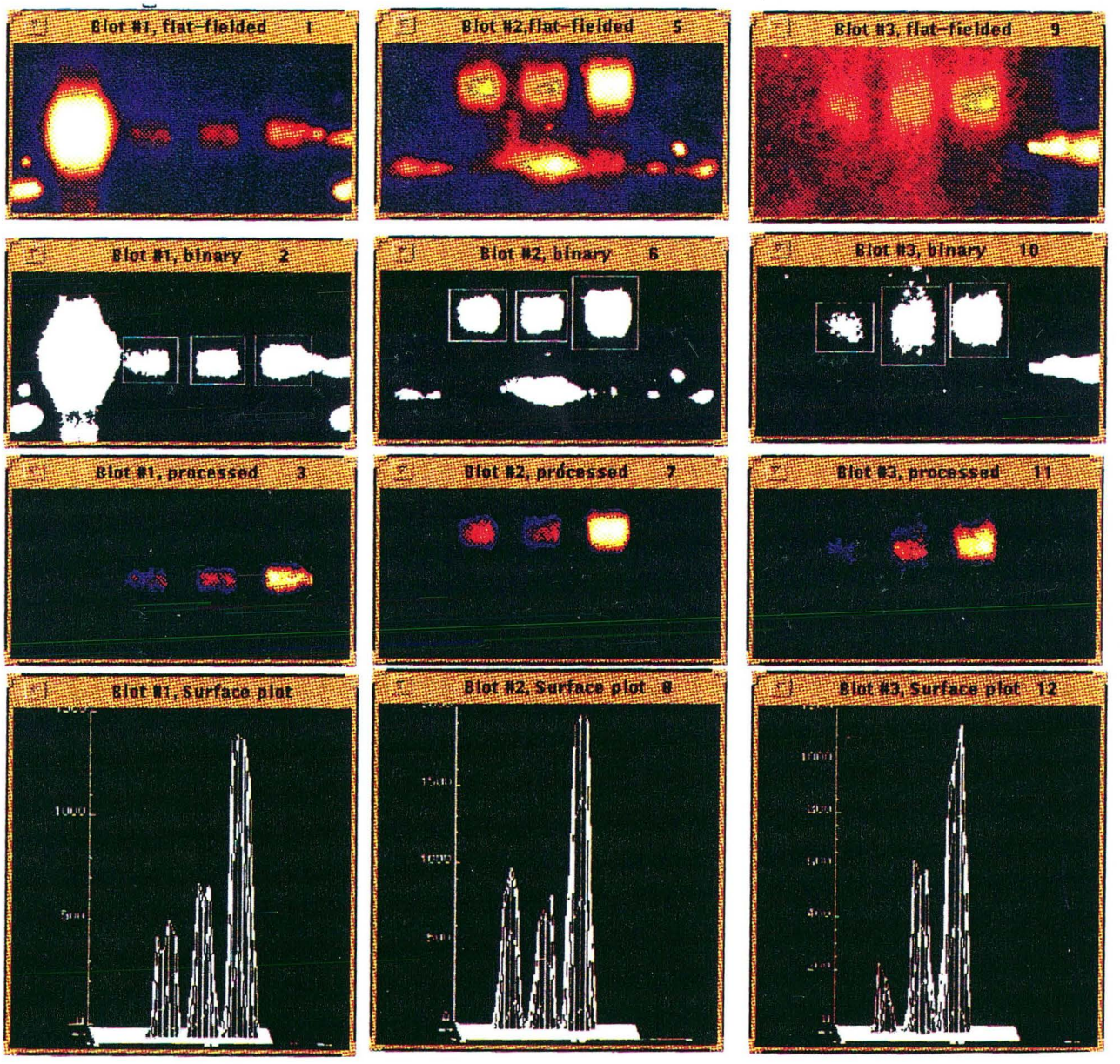


Figure 3

QUANTITATIVE CCD-CAMERA IMAGING OF NORTHERNS
c-MYC mRNA Samples Analyzed on 3 Different Blots

Samples: | -Con-A | | +Con-A | | +Con-A + 60Hz |



Pane

A

B

C

D

MULTIVARIATE ANALYSIS OF VARIANCE FOR 3 NORTHERNS

	-Con-A	+Con-A	+Con-A+60Hz
Pixel Intensity (Mean ± SD):	332 ± 128	398 ± 48 *	864 ± 181 *

* p = 0.0114

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