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CD45 phosphatase in Jurkat cells is necessary for response to applied ELF magnetic fields

Ewa Lindström[a,*], André Berglund[b], Kjell Hansson Mild[b], Per Lindström[c], Erik Lundgren[a]

[a]*Department of Cell and Molecular Biology, Umeå University, S-90187 Umeå, Sweden*
[b]*National Institute of Occupational Health, Umeå, Sweden*
[c]*Department of Histology and Cell Biology, Umeå University, Sweden*

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Abstract Oscillations of free intracellular calcium $[Ca^{2+}]_i$ were seen in individual Jurkat cells as response to a 50 Hz, 0.15 mT magnetic field (MF). In contrast, a CD45-deficient Jurkat cell line was unable to respond to MF stimulation. The phosphatase activity of CD45 has been implicated to regulate $p56^{lck}$ tyrosine kinase activity by removing an inhibitory phosphate. By using Jurkat cells that expressed a chimeric molecule, comprising the cytoplasmic phosphatase domain of CD45, the MF induced calcium response was restored. This showed the necessity for an intact signal transduction pathway leading to a calcium increase as a result of stimulation of cells by MF. Thus, our data suggest that the target for the applied MF are molecules involved in early events in the signalling pathway from the T cell antigen receptor.

Key words: Signal transduction; Jurkat T-cell line; CD45 phosphatase; Intracellular calcium; Low frequency magnetic fields

1. Introduction

A diversity of biological effects has been attributed to exposure of low frequency magnetic fields to cells of the immune system (reviewed in [1]), and epidemiological studies have attracted public concern, as they suggest a relationship between residential MF exposure from high-voltage transmission lines and childhood cancers [2]. In vitro studies on molecular and cell biological parameters such as increased calcium influx and induction of c-*myc* transcripts upon MF exposure has been reported [3]. Analysis of transcript levels for c-*fos* and c-*myc* suggests an incrase due to MF exposure [4,5]. However, yet so far no molecular targets have been identified through which MF exert these effects and the biophysical mechanism of interaction remains similarly obscure. The main problem is the lack of understanding which parameters of the field and of the biological system that are critical and responsible for the interactions.

To identify candidate molecular targets, we have set up an experimental system with the human T cell leukemic line Jurkat, in which the signal transduction pathway — from the T cell receptor to activation of transcription of several genes — is known in considerable detail. The molecules of the T cell antigen receptor (TCR) complex have no intrinsic tyrosine kinase or phosphatase activities, but phosphorylation and de-phosphorylation are important immediate/early events in T cell activation. The antigen-binding subunits form a complex with the invariant chains of CD3: the ε, γ and δ chains and the ζ dimers. The cytoplasmic domains of those are responsible for coupling the Ti subunit to the intracellular signalling machinery. Gene products involved in these signalling pathways include the tyrosine kinases ZAP-70 and Syk and members of the *src* family of protein tyrosine kinases (PTKs), $p56^{lck}$ and $p59^{fyn}$, as well as the protein tyrosine phosphatase (PTPase) CD45. Lck interacts in Jurkat cells with the cytoplasmic domain of the co-receptor CD4 which upon interaction with the MHC complex are brought in close contact with the CD3 complex, resulting in phosphorylation of the antigen recognition activation motif (ARAM) amino acid sequences at the CD3 cytoplasmic chains [6,7,8]. ZAP-70 is associated with the phosphorylated ζ-chains through SH2-phosphotyrosine interactions [9]. The protein-tyrosine phosphorylation can be induced by stimulating the function of PTKs, or by inhibiting the PTPases. The tyrosine phosphorylation of phospholipase-Cγ1 (PLCγ_1) is evident upon TCR stimulation and requires an intact CD45 PTPase activity [10]. PLCg_1 is an enzyme that hydrolyses phospahtidyl-inositol bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). When IP_3 binds to the specific receptor (IP_3R) it causes a rise in $[Ca^{2+}]_i$ in Jurkat cells [11].

In a previous study we have registered an oscillatory $[Ca^{2+}]_i$ increase (up to ten times the basal levels) in single Jurkat cells. By using EGTA in the perfusion buffer, influx of extracellular calcium was found to be the predominant reason for the elevated levels of calcium within the cell [12]. The $[Ca^{2+}]_i$ oscillations have further been characterised with respect to frequencies between 5 and 100 Hz and flux densities up to 0.3 mT and found to be maximal for 50 Hz, 0.15 mT [13]. The observed calcium response pattern was similar to that measured for an anti-CD3 monoclonal antibody, both in amplitude and the lag time before the Ca-oscillations started. We have also measured the generation of the phosphatidylinositol second messenger IP_3 in Jurkat cells and found the maximal concentration appeared 2 min after the start of the MF exposure [14]. These findings exclude that depolarisation of the plasma-membrane, or direct interaction with calcium ions are the primary targets for the MF.

To elucidate if MF has targets along a pathway upstream of PLCγ_1 we have used the mutant Jurkat cell line with diminished CD45 activity, J45.01, to examine the requirement for the

*Corresponding author. Fax: (46) (90) 77-1420.

Abbreviations: ELF, extremely low frequency; $[Ca^{2+}]_i$, cytoplasmic free calcium concentration; K_d, dissociation constant; Mab, monoclonal antibody; MF, magnetic field; protein tyrosine kinase; PTK; protein tyrosine phoshpatase, PTPase; T, Tesla; TCR, T cell receptor complex; Ti, T cell receptor α and β chains.

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CD45 phosphatase activity for MF induced increase in $[Ca^{2+}]_i$. CD45 is a family of glycoproteins ranging from 180 to 220 kDa, present on all nucleated hematopoietic cells and is the major PTPase in T cells. Loss of CD45 expression in cells results in spontaneous tyrosine hyper-phosphorylation of a number of proteins, such as the TCR ζ subunit and the *Src* family PTK $p56^{lck}$ [7,15]. It has previously been shown that expression of the extracellular domain of CD45 is not required for coupling of the TCR to its signalling machinery [16,17]. On the other hand, expression of the cytoplasmatic phosphatase domain of CD45 was shown to restore TCR signalling in a $CD45^-$ cell line [18]. The suggested role of CD45 in regulating TCR signalling includes dephosphorylation of the regulatory C-terminal tyrosine (505) of Lck, allowing an open active conformation of Lck which enables it to phosphorylate protein tyrosine kinase substrates and to keep the kinase active for participation in the signal transmission [19,20,21]. CD45 PTPase has been shown to affect early events in TCR-mediated signalling, and CD45 negative cells fails to couple to the phosphatidylinositol second messenger pathway [10].

In this study we show that Jurkat cells only responds to the MF if the cells have intact expressed the intracellular domain of CD45.

2. Materials and methods

2.1. Measurements of intracellular calcium

Measurements of $[Ca^{2+}]_i$ after pre-incubation with the calcium binding probe fura-2/AM, 2 μM, on poly-L-lysine coated glass (0.01%) has been described previously [13]. Briefly, attached cells were placed in a perfusion chamber and real-time fluorescence was measured at 37°C. The $[Ca^{2+}]_i$ in single cells was calculated from the ratio of emitted light at 510 nm, measured with a photomultiplier when the cells were illuminated with 340 nm or 380 nm excitation light, respectively. The calibration of the fura-2 signal has been described [13], using a K_d of 231 nM. We had one criterion for selection of individual cells to test of the effect of MF: the cell should not show fura-2 fluctuations 4 min before start of MF exposure. Cells that fluctuated more than 20% in their basal values were sorted out and from previous experience we know that such cells only rarely respond to antibodies or other stimuli. Only one experiment per cover glass was done and the recording time was less than 15 min. The figures shown are representative for at least seven individual experiments. A positive response to MF is defined as at least a threefold increase of the maximal peak value in $[Ca^{2+}]_i$.

2.2. Jurkat cell lines

The human leukemic T cell line Jurkat and derivative clones were obtained from Dr. Gary Koretzky, University of Iowa, USA. The following clones of Jurkat were used in this study: Jurkat (clone E6–1); wildtype, J45.01; Jurkat clone with diminished phosphatase activity [10], J45/CH11 chimera; Jurkat clone with reconstituted phosphatase activity (transfected with cytooplasmic part of CD45 and fused with the extra cellular and the transmembrane sequences from plasmid A2, encoding the MHC class I [18]), J45/A2; Jurkat $CD45^-$ MHC I^+ control clone (J45.01 transfected with the A2 plasmid). Cells used for experiments were in logarithmic phase in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine and cultured in a Forma-incubator, 5% CO_2 in air, rebuilt to keep the internal ELF stray fields less than 0.2 μT.

2.3. MF exposure system

A homogenous vertical magnetic field was generated by a pair of Helmholtz' coils mounted on the objective stage. The coils had a diameter of 10 cm and were made of 20 turns of 0.6 mm diameter enamelled copper wire. The distance between the coils was 5 cm. The coils were fed from a signal generator and an amplifier and the current was switched on by closing the circuit [13]. The magnetic field at the centre between the coils (i.e. at the place where the cells were located) were both calculated from the current in the coils, as well as measured with a Bell 640 Gauss meter equipped with a 3-axis probe and a small pickup coil (200 turns, 3.5 cm diameter) connected to a voltmeter. The current in the coils was adjusted to give a flux density of 0.15 mT. When the same current was applied to the coils in a test set outside the microscope a slightly higher reading was obtained, thus indicating a small distortion due to the metallic structure of the objective stage of the microscopes. Measurements at the place of the cells gave a total flux density 64 μT and the vertical components of the geomagnetic field at the site of the cells was 48 μT. The environmental stray ELF fields in the room was below 0.2 μT.

2.4. Fluorometric analysis

Flow cytometry was performed with a FACScan fluorcytometer (Becton Dickinson, Mountain View, CA, USA). Analysis of 10^6 cells stained on ice for 35 min was done with the following monoclonal antibodies (Mabs): goat anti-mouse-FITC, anti-CD19, -CD2, -CD4, -CD45 and anti-TCR, all from Becton Dickinson. Anti-CD3 Mab UCHT-1 from Dako A/S (Copenhagen, Denmark). Cells were washed and then stained 35 min on ice with secondary Mab-FITC. Fluorescence data of 10,000 cells was collected, using logarithmic amplification and the fluorescence for each antibody was quantified using the single histogram statistics program setting the marker at fixed position.

3. Results

Jurkat cells exposed to 50 Hz, 0.15 mT, responded with an increase in $[Ca^{2+}]_i$ that started within 120 s after the onset of the MF and lasted during the exposure time. The oscillating Ca^{2+} spikes in the different single cells examined reached a maximum of 500–900 nM (Fig. 1A). In contrast, testing J45.01 cells, a mutant Jurkat clone with markedly reduced surface expression of CD45 (Fig. 2), we found no increase in $[Ca^{2+}]_i$ when these cells were exposed to 50 Hz, 0.15 mT, sinusoidal MF (Fig. 1B). These cells were unresponsive also upon stimulation with an anti-CD3-ε Mab (data not shown), confirming what others earlier have demonstrated using an anti-TCR-β Mab as stimulus [10]. These data suggest that CD45 is required for coupling to the inositol signalling pathway resulting in $[Ca^{2+}]_i$ increase upon stimulating cells with MF, in analogy with the need for CD45 to transduce the signal upon stimulation by anti-CD3 antibodies.

To decide if the response to MF was determined by the phosphatase activity and/or if also the extracellular part of CD45 was necessary, we tested the Jurkat clone J45/CH11. This clone expresses only the cytoplasmic domain of CD45 including

Table 1
Staining characteristics of the Jurkat clones used for exposure to MF, expressed as percentage positively stained cells of the total number of cells analysed.

Jurkat clone	Second antibody	Irrel. antibody	a-CD2	A-CD3	a-CD4	a-CD45	a-TCR
Jurkat	5	4	70	86	30	99	91
J45.01	3	4	94	86	15	21	96
J45/A2	2	2	26	10	3	21	40
J45/CH11	2	4	98	80	60	21	73

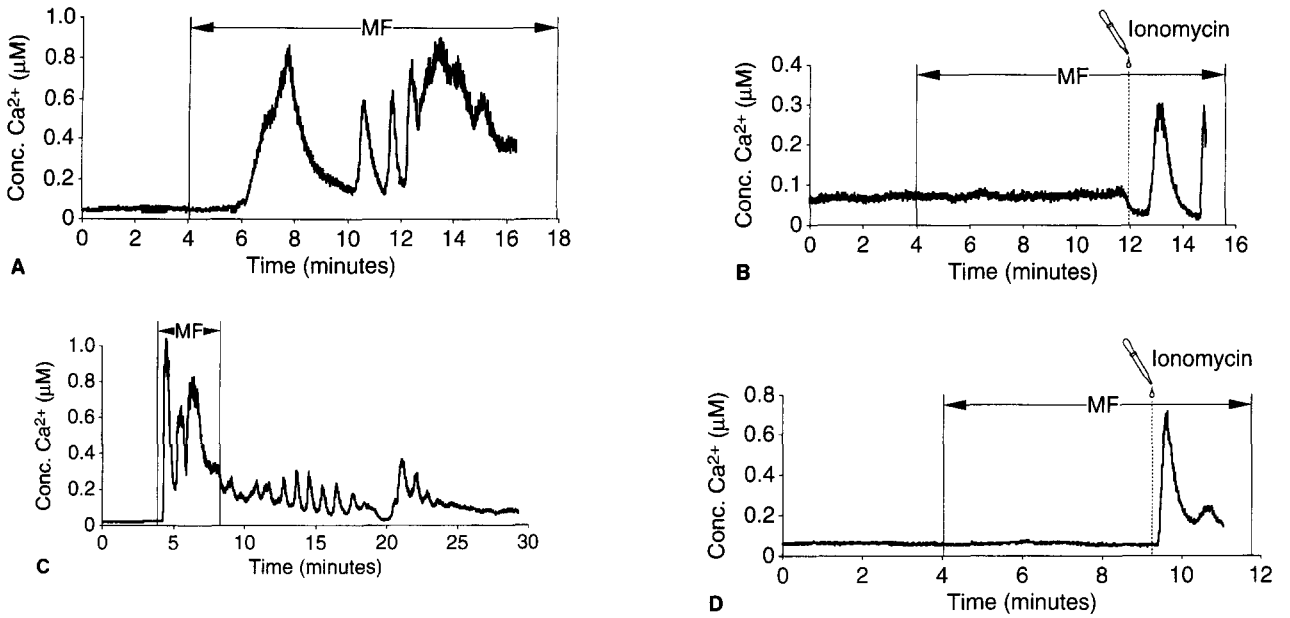

Fig. 1. $[Ca^{2+}]_i$ measurements in Jurkat cell clones. 50 Hz, 0.15 mT MF was applied at time indicated and at the end of the experiment ionomycin (1μg/ml) was added, as a positive control. (A) Jurkat (clone E6-1) (B) J45.01, Jurkat clone with diminished phosphatase activity [10] (C) J45/CH11 chimera, Jurkat clone with reconstituted phosphatase activity [18]. (D) J45/A2 without phosphatase activity, control to J45/CH11 (see section 2 for details).

the two subdomains of the PTPase and the extracellular part HLA-A2 has no similarity to CD45. Fig. 1C shows that this Jurkat clone responded in the same way as the wild type Jurkat, which indicates that the cytoplasmic part of CD45 containing the phosphatase domain was necessary for coupling to $PLC\gamma_1$ and the ensuing increase in $[Ca^{2+}]_i$ as a response to MF stimulation. As a negative control we used the Jurkat clone J45/A2, which has the same construct as the chimera but lacks the cytoplasmic part of CD45. No response, to either MF or anti-CD3, was observed in this clone (Fig. 1D).

Flow cytometry was performed to analyse the expression of TCR accessory molecules. The different clones of Jurkat stained equally well for control secondary Mab and for the irrelevant anti-B cell marker (Table 1). The two $CD45^-$ clones J45.01 and J45/A2, stained much less than wild type Jurkat cells for CD45. Additionally, J45/A2 stained also less for CD2, CD3, CD4 and Ti compared to wild type Jurkat cells (Table 1).

4. Discussion

The T cell receptor complex generates signals essential for T cell activation upon recognition of antigen. The degree of activation by receptor ligation is greatly enhanced when coreceptors are engaged at the same time. This modulation results from the interplay between molecules associated with the cytoplasmic tails of the TCR-associated CD3, the ζ proteins and CD4 [22]. T lymphocytes express abundantly a transmembrane tyrosine phosphatase, CD45 that is required for effective signalling. It appears that the TCR is a flexible signalling complex of proteins that can produce variable effects depending upon how it is ligated (reviewed in [23]). In the intricate molecular balance that exists to activate the phosphatidyl-inositol second messenger pathway, it is difficult to pinpoint the role for individual proteins.

The CD45 extracellular domain is 391–552 amino acids in length, depending on the isoforms generated by alternate mRNA splicing. One isoform is recognised by the monoclonal antibody CD45R0, and is preferentially expressed on memory and helper T-cell subsets. One ligand is identified as the B-cell specific adhesion molecule CD22 [24]. It has been proposed that interaction between extracellular CD45 and accessory molecules on T cells may participate in modulation of the signalling cascade [25,26]. We have found that MF had a stimulatory effect of $PLC\gamma_1$ activation, measured as increased $[Ca^{2+}]_i$, irrespective of whether the extracellular part of CD45 was expressed or not (compare Fig. 1B and C). The requirement for CD45 in T cell activation seems to reside at a very early stage in the signal transduction cascade, since in the absence of CD45, the rapid receptor-triggered tyrosine phosphorylation of cellular proteins is severely reduced and results in impaired activation of phospholipase C [21]. Although formally not excluded, we do not think that CD45 is the prime target for the applied MF. However, our experiments shows that MF exerts its effects on the signalling cascade from the TcR. The necessity of an intact PTPase activity hints to the possibility of interference with phosphorylation events as a possble mechanism. In the CD45 deficient cells, also the levels of accessory molecules were reduced to different degrees (Table 1). The $CD45^-$ clone J45.01 was low in CD4 molecule density, an important molecule in the regulatory network that controls the influx of extracellular calcium during T cell activation [27]. We do not know whether low CD4 level is related to the reduced CD45 expression or represent a clonal variation. The association between CD45 and Lck has been shown not to require the expression of, or activation of the TCR [28], thus making CD4 or Lck as possible target candidates for the effect of MF.

This report demonstrates that the possible targets for the effect of weak MF in Jurkat cells are initial molecules along the pathway from the TCR complex, with its accessory molecules, down to events regulating $[Ca^{2+}]_i$ which was used as a readout

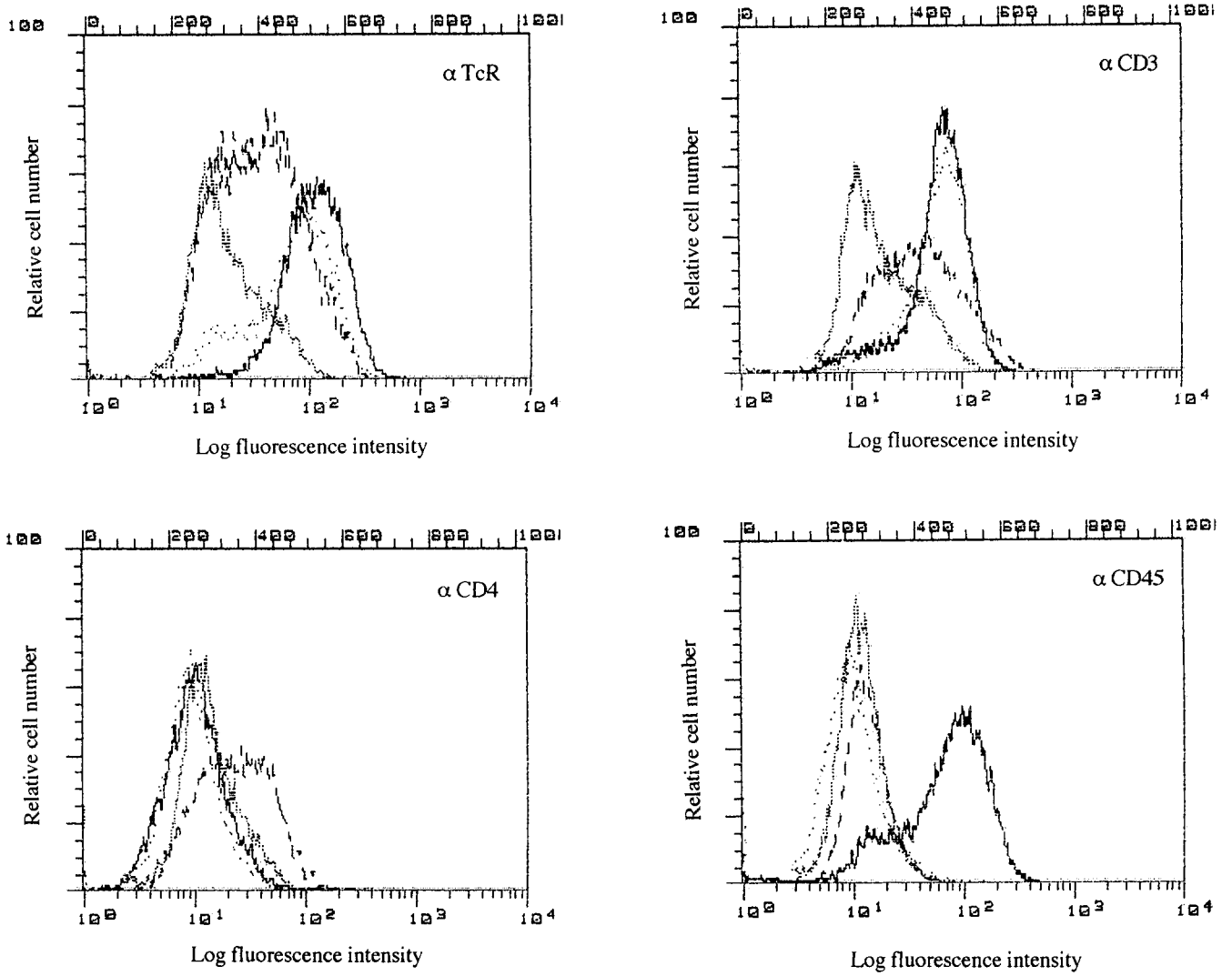

Fig. 2. Immunofluorescence staining of TcR Ti, CD3, CD4 and CD45 surface expression characteristics of the Jurkat clones: E6-1 (solid lines), J45.01 (dotted lines), J45/A2 (shaded lines) and J45/CH11 (dashed lines).

in these experiments. As the effect of MF here seen is of the same order as that seen upon crosslinking the TCR complex, we think it would be possible to analyse changes in phosphorylation pattern of molecules involved in the early signal transduction events. Thus, the strategy is to identify target molecules for later mutational analysis in order to set up a biophysical explanation of our observation.

There exists a controversy about the mechanism for the biological effects of weak MF. One argument is that the effects of weak fields will be masked by thermal noise energies [29]. Others have shown that when the limit between an applied field and the thermal noise is small, the weak fields cannot be dismisssed [30]. It is premature to speculate how weak MF may act before a target molecule or target molecule complexes for MF are identified.

The approach used demonstrates the necessity for expression of the cytoplasmic domain of CD45 including its phosphatase domain, suggesting that MF uses the same signalling pathway to induce $[Ca^{2+}]_i$ oscillations as that used during anti-CD3 stimulation.

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