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Magnetic fields promote a pro-survival non-capacitative Ca^{2+} entry *via* phospholipase C signaling

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ABSTRACT

The ability of magnetic fields (MFs) to promote/increase Ca^{2+} influx into cells is widely recognized, but the underlying mechanisms remain obscure. Here we analyze how static MFs of 6 mT modulates thapsigargin-induced Ca^{2+} movements in non-excitable U937 monocytes, and how this relates to the anti-apoptotic effect of MFs. Magnetic fields do not affect thapsigargin-induced Ca^{2+} mobilization from endoplasmic reticulum, but significantly increase the resulting Ca^{2+} influx; this increase requires intracellular signal transduction actors including G protein, phospholipase C, diacylglycerol lipase and nitric oxide synthase, and behaves as a non-capacitative Ca^{2+} entry (NCCE), a type of influx with an inherent signaling function, rather than a capacitative Ca^{2+} entry (CCE). All treatments abrogating the extra Ca^{2+} influx also abrogate the anti-apoptotic effect of MFs, demonstrating that MF-induced NCCE elicits an anti-apoptotic survival pathway.

1. Introduction

Positive correlations between exposure to MFs and development of several pathologies have emerged from epidemiological studies (Lacy-Hulbert et al., 1998; Comba and Fazzo, 2009), especially concerning promotion of malignancies such as leukemia and brain tumors (Feychting and Ahlbom, 1994; McNally and Parker, 2006; Roosli et al., 2007; Malagoli et al., 2010). MFs are not directly mutagens or carcinogens, but act as co-mutagenic and co-carcinogenetic agents (Fiorani et al., 1992; Morandi et al., 1996; Simko and Mattsson, 2004) thus behaving as tumor promoters rather than initiators. Suicide of damaged cells by apoptosis is the first barrier against carcinogenesis, and its inhibition is one key mechanism of tumor promotion (Wright et al., 1994). MFs inhibit damage-induced apoptosis (Fanelli et al., 1999), allowing survival of damaged, possibly mutated cells: this provides the logical framework for MFs as efficient tumor promoters.

The inhibition of apoptosis *via* MFs requires Ca^{2+} influx into cells from the extracellular environment (Fanelli et al., 1999). Ca^{2+} entry is a complex modulator of apoptosis, being either a pro-apoptotic (McConkey et al., 1989) or an anti-apoptotic (Galli et al., 1995) event.

Ca^{2+} entry is a recurring motif within the biological effects of MFs (Walleczek and Liburdy, 1990; Liburdy, 1992; Teodori et al., 2002), but the mechanisms involved remain unclear. Early biophysical theories such as the cyclotron resonance (Jacobson, 1991), hypothesizing that MFs affect cells and tissues by directly increasing Ca^{2+} ion energy (Liboff and McLeod, 1988; McLeod et al., 1992), were not confirmed by the experimental results (Sandweiss, 1990; Coulton and Barker, 1993). The alternative explanation is that MFs increase cellular Ca^{2+} intake by affecting intracellular signaling processes (Liburdy et al., 1993).

Ca^{2+} channels are highly regulated protein complexes, responding to membrane polarization (voltage-dependent channels) or to intracellular Ca^{2+} movements (store-operated channels) (Berridge, 2009). Excitable cells display both types of intake, whereas in non-excitable cells, like leukocytes, Ca^{2+} influx occurs only through store-operated channels, being secondary to intracellular Ca^{2+} mobilization (Salido et al., 2009). Thus, Ca^{2+} influx in non-excitable cells relies on stimulation of Ca^{2+} fluxes from the ER (Putney, 2001).

Physiological ER Ca^{2+} emptying is consequent to receptor engagement. Signal transduction cascades triggered by G-protein or tyrosine kinase-linked receptors activate β or γ isoforms of phospholipase C (PLC) (Clejan et al., 1995; Dibirdik et al., 1998; Kristupaitis et al., 1998; Dibirdik et al., 2000), which hydrolyzes membrane-bound 4,5-phosphoinositol-bis-phosphate (PIP_2) into 1,4,5-inositol-tris-phosphate (IP_3) and diacylglycerol (DAG). IP_3 then binds to IP_3 receptors on ER, stimulating the opening of Ca^{2+}

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channels and generating a reticular flux (Berridge, 2009); the partially emptied ER then promotes a capacitative Ca^{2+} entry (CCE) from outside to replenish ER Ca^{2+} (Putney, 2001). On the other side, DAG is a substrate of many processing enzymes, eliciting alternative signaling pathways including activation of protein kinase C (Sakane et al., 2008) or support to IP_3 functions (Berridge, 1987). In the presence of DAG lipase, DAG is converted into arachidonic acid, which stimulates nitric oxide synthase (NOS) to produce NO, which promotes a second type of Ca^{2+} influx, the non-capacitative Ca^{2+} entry (NCCE). CCE and NCCE differ in role (ER homeostasis or intracellular signaling, respectively), and pharmacological sensitivities (Moneer et al., 2005; Taylor and Moneer, 2004).

An alternative way of stimulating ER Ca^{2+} emptying is poisoning ER Ca^{2+} ATPases (the pumps that actively import Ca^{2+} against gradient from cytosol) with thapsigargin (THG) (Thastrup et al., 1990); the inhibition of Ca^{2+} uptake rapidly depletes ER Ca^{2+} and promotes a CCE (Putney, 2001). In some cells, THG-induced fluxes may activate PLC (Chakrabarti, 2006), causing a secondary ER Ca^{2+} emptying and CCE, which in this case can be accompanied by a NCCE (Bird et al., 2004).

MFs were shown to activate PLC (Kim et al., 2002; Piacentini et al., 2004). Since PLC promote Ca^{2+} influx, these findings provide an interesting basis to explore whether MFs-induced increase of Ca^{2+} influx may depend by a PLC-mediated signal transduction pathway.

In this study, we show that static MFs activate a G-protein/PLC-mediated signal transduction mechanism that stimulates a NCCE on human monocytic U937 cells and is responsible for the anti-apoptotic effect of MFs.

2. Materials and methods

2.1. Materials

U73122 and ionomycin were purchased from Calbiochem; fetal calf serum (FCS), L-glutamine, penicillin, streptomycin and RPMI 1640 were from Lonza; $CaCl_2$ from MP Biomedicals; Fluo-3 AM is from Invitrogen/Molecular Probes. Pertussis toxin (PTX), D(+)-glucose, puromycin (PMC), thapsigargin (THG), ethylene glycol-bis(beta-aminoethyl-ether)-N,N,N′,N′-tetraacetate (EGTA), 4,5-diaminofluorescein diacetate (DAF2DA), N (G)-nitro-L-arginine methyl ester (L-NAME), RHC 80267, SKF 96365, gadolinium (III) chloride exahydrate ($GdCl_3$), phytohemoagglutinin (PHA) were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Cell culture

U937 cells (human histiocytic lymphoma) and Jurkat T cells (acute lymphoblastic leukemia) were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin, and kept in a controlled atmosphere (5% CO_2) incubator at 37 °C. All experiments were performed with cells at the log phase growth, with a viability ≥98% (as determined by trypan blue staining). Jurkat T cells activation: 10^7 cells were resuspended in 200 μl of complete medium, incubated for 1 h with 5 μg/ml phytohemoagglutinin (PHA) at 37 °C, diluted to 0.5×10^6 cells/ml and kept 24 h before treatment.

2.3. Induction and detection of apoptosis

Apoptosis was induced with the protein synthesis inhibitor puromycin (PMC, 10 μg/ml) or etoposide (VP16; 50 μM) kept throughout the experiments; inhibitors were pre-incubated as described below. The fraction of apoptotic nuclei was estimated after 5 h of incubation for both treatments, by evaluating the fraction of cells presenting apoptotic nuclear fragmentation (Dini et al., 1996) with the cell permeant DNA-specific dye Hoechst 33342 as described (Fanelli et al., 1999; Cerella et al., 2009; Cerella et al., 2007). Apoptosis was confirmed by mitochondrial membrane potential analysis (data not shown) (Cerella et al., 2007).

2.4. Magnetic field exposure

Axial MFs were produced by metal magnetic disks (Teodori et al., 2002; Ghibelli et al., 2006; Fanelli et al., 1999). The actual field intensity on the cells (6 mT) was measured for control with a gaussmeter (Hall-effect Gaussmeter, GM04 Hirst Magnetic Instruments Ltd., UK). Magnets did not produce temperature variation. Magnetic disks were placed under Petri dishes prior to the apoptogenic treatments (Fanelli et al., 1999; Ghibelli et al., 2006); for intracellular Ca^{2+} measurements, magnetic disks were placed aside the flow cytometric vial just before measurements (Fanelli et al., 1999).

2.5. Cellular inhibitory treatments

G protein: cells were incubated with 100 ng/ml pertussis toxin (PTX) 24 h before other treatments. PLC: U73122 (10 μM) was added 30 min before treatments. NO synthase: 600 μM of the arginine analogue inhibitor L-NAME was added 30 min before treatments. DAG lipase: 50 μM RHC 80267 was added 30 min before treatments. Extracellular Ca^{2+} chelation: 650 μM ethylene glycol-bis(beta-aminoethyl-ether)-N,N,N′,N′-tetraacetate (EGTA) 15 min before other treatments. Ca^{2+} entry: 5 μM SKF 96365; and $GdCl_3$ (assayed at 1 μM and 100 μM) 30 min before treatments.

2.6. Inositol 1,4,5-tris-phosphate (IP_3) measurement

200 ml of 60% ice-cold perchloric acid were added to the cell suspension at different times after exposure to MFs, centrifuged and neutralized with 10 M KOH. IP_3 assay was performed with a commercially available kit (D-myo-inositol 1,4,5-trisphosphate (IP_3) [^{3}H] Biotrak Assay System, Amersham Biosciences UK Limited, Cardiff, UK). The bound IP_3 is separated from free IP_3. Measurement of the radioactivity in the tube enables the amount of unlabelled IP_3 in the sample to be determined by interpolation from a standard curve. The data represent the mean of 5 independent experiments.

2.7. NO measurements

U937 cells were incubated with 1 mM 4,5-diaminofluorescein diacetate (DAF2DA) at 37 °C for 1 h; loaded cells were immediately analyzed by flow cytometry (FL1 photomultiplier; bandpass 530 nm, bandwidth 30 nm), and data analyzed with WinMDI software (http://facs.scripps.edu/software.html); or observed at the fluorescence microscopy.

2.8. Ca^{2+} measurements

Fluo-3 AM staining: 1×10^7/ml cells were washed twice in Hank's balanced salt solution (HBSS) + 650 μM glucose + 650 μM $CaCl_2$ and loaded with 1 μM Fluo-3 AM in the dark at 20 °C for 40 min. After dye removal, cells were resuspended in HBSS + 650 μM glucose + 650 μM $CaCl_2$ at 2×10^6/ml (Cerella et al., 2007; Fanelli et al., 1999).

Flow cytometric analysis: [Ca^{2+}]c measurements were performed using a FACScalibur (Becton Dickinson, San Jose, CA), tuned at 488 nm, using the standard FL1 photomultiplier (bandpass 530 nm, bandwidth 30 nm). Data were recorded in list mode for further analysis with Cell Quest software (Becton Dickinson). Fluorescence values were converted in [Ca^{2+}]c according to (Grynkiewicz et al., 1985) (Fluo-3 K_d = 430 nM; F_{max} was measured