Transient Ca^{2+}-dependent activation of ERK1 and ERK2 in cytotoxic responses induced by maitotoxin in breast cancer cells

Claudia Malaguti[a], Takeshi Yasumoto[b], Gian Paolo Rossini[a,*]

[a] *Dipartimento di Scienze Biomediche, Sezione di Chimica Biologica, Università di Modena e Reggio Emilia, Via Campi 287, I-41100 Modena, Italy*
[b] *Japan Food Research Laboratories, Tama Laboratories, 6-11-10 Nagayama, Tama, Tokyo 206-0025, Japan*

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Abstract Treatment of MCF-7 breast cancer cells with the marine toxin maitotoxin (MTX) induces cell death. The cytotoxic effects are clearly detectable within 2–4 h after cell treatment with 10^{-10}–10^{-9} M concentrations of MTX. The response was found to depend on extracellular Ca^{2+}, inasmuch as cell death was prevented when culture dishes received MTX, following addition of EGTA. MTX caused transient phosphorylation of extracellular signal-regulated kinase isoforms 1 and 2 (ERK1 and ERK2) mitogen-activated protein kinase isoforms in MCF-7 cells, which was maximal 15 min after toxin addition to culture vessels. The effect was dependent on influx of extracellular Ca^{2+}, as it was abolished by EGTA, and was induced by ionophores, such as A23187 and ionomycin. Our findings show that signaling pathways involving Ca^{2+} ions may cause activation of ERK1 and ERK2 in cell death responses.

Key words: Ca^{2+} ionophore; Mitogen-activated protein kinase; Phosphorylation; Cytotoxic response

1. Introduction

Extracellular signal-regulated kinase isoforms 1 and 2 (ERK1 and ERK2) represent major members of the mitogen-activated protein kinase (MAPK) family of serine/threonine protein kinases [1,2]. While activation of ERK1 and ERK2 is usually linked to stimulation of cell proliferation, evidence obtained in several experimental systems indicates that these kinases can perform more complex regulatory functions. Thus, kinase activity may be associated with inhibition of cell proliferation and with cell death [1–10]. In these latter cases, ERK activation has been found to occur through multiple signaling pathways [1,2,5,6,8,9] or as a consequence of inhibition of phosphoprotein phosphatases 1 and 2A [3,4,7,10], suggesting that it might participate to death responses induced by a variety of agents, and attained by different molecular mechanisms. Information regarding ERK activation in cytotoxic responses involving increased intracellular levels of Ca^{2+} ions, however, are lacking.

Maitotoxin (MTX) is a large (above 3 kDa) water-soluble polyether compound produced by dinoflagellates, such as *Gambieridiscus toxicus* [11]. The toxic effects of this agent depend on its capacity to induce influx of extracellular Ca^{2+} in both excitable and non-excitable cells [12]. Although a detailed description of the mechanism of action of MTX is still lacking, there is a substantial agreement on the contention that stimulation of Ca^{2+} influx would be mediated by both voltage-dependent and receptor-operated Ca^{2+} channels [12–16].

Several events have been shown to occur as a consequence of cell treatment with MTX, including increased phosphoinositide turnover [17–19], translocation of protein kinase C from the cytosolic to membrane compartment [19] and stimulation of arachidonic acid release [20]. In different experimental systems, this type of signaling mechanism involves phosphorylation and consequent activation of MAPKs [21–23]. In keeping with these observations, we have hypothesized that ERK activation might participate to death responses induced by MTX. In this report, we show that MTX is toxic in MCF-7 breast cancer cells, where it induces a transient, Ca^{2+}-dependent activation of ERK1 and ERK2 isoforms.

2. Materials and methods

2.1. Materials

Peroxidase-linked anti-mouse and anti-rabbit Ig antibodies and the ECL detection reagents were from Amersham. Tissue culture media and reagents were from Life Technologies. MTX was prepared as previously described [24]. The anti-ERK1 and anti-ERK2 antibodies were from Santa Cruz. The anti-phospho-p44/42 MAPK antibodies were obtained from New England Biolabs. The anti-E-cadherin antibody was a product from Zymed Laboratories. Pre-stained molecular mass markers were obtained from Sigma. The nitrocellulose membrane Protran B83 was obtained from Schleicher and Schuell. All other reagents were of analytical grade.

2.2. Cell culture conditions and treatments

MCF-7 cells were grown in 5% carbon dioxide in air at 37°C, in 90 mm diameter Petri dishes, with a culture medium composed of Eagle's MEM containing Earle's salts, 1% non-essential amino acids and 10% fetal calf serum, as previously described [25].

Stock solutions of MTX were prepared by dissolving the toxin in 50% ethanol. Cells in logarithmic growth received the tested compound and paired cell dishes received vehicle. Cell processing to prepare and analyze extracts was carried out at the end of individual treatments at 37°C, as specified below.

2.3. Preparation of cell extracts

Cells were harvested, washed three times with 20 mM phosphate buffer, pH 7.4, 0.15 M NaCl (PBS) and were lysed by the addition of either a hypotonic or a solubilization buffer. In the first case, cells were dispersed in 1 ml of 20 mM Tris-HCl, pH 7.5, at 2°C, 1 mM EDTA and were lysed by sonication at 2°C with two 8 s bursts, at an output of 15 W. This total lysate was used to determine the DNA content of cell suspensions, using the procedure of Labarca and Paigen [26]. Cell lysis by a solubilization buffer was performed by dispersion in 0.5 ml PBS, containing 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 50 mM NaF, 10 mM $Na_4P_2O_7$, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 30 μg/ml aprotinin, and by two 10 s bursts of vortexing. Cell lysates were then subjected to centrifugation for 30 min at $16\,000 \times g$. The supernatants of this centrifugation, operationally defined cytosoluble extracts, were used for colorimetric determinations of the protein content with bicinchoninic acid [27].

*Corresponding author. Fax: (39) (59) 428524.
E-mail: rossini@unimo.it

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2.4. Fractionation of proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Cytosoluble extracts were brought to 2% SDS and 5% β-mercaptoethanol, before being fractionated by SDS-PAGE, according to Laemmli [28]. Samples containing the same amounts of protein were fractionated using a 10% separating gel and a 3% stacking gel. After completion of electrophoresis, proteins were electrophoretically transferred onto a nitrocellulose membrane, which was treated with 20 mM Tris-HCl, pH 7.5, at 25°C, 0.15 M NaCl, 0.05% (v/v) Tween 20 (immunoblotting buffer) containing 3% non-fat dry milk for 1 h at room temperature. Membranes were then incubated for 1 h at room temperature with immunoblotting buffer containing 1% non-fat dry milk and primary antibody. When E-cadherin was analyzed, 1 mM $CaCl_2$ was included in every buffer employed in immunoblotting, up to the incubation with the secondary antibody. After incubation, membranes were washed five times with immunoblotting buffer and were incubated for 1 h at room temperature with a peroxidase-linked secondary antibody at a 1:3000 dilution in immunoblotting buffer containing 1% non-fat dry milk. After washing, the membrane was developed by the ECL detection system.

3. Results and discussion

Preliminary experiments were carried out to define the MTX concentration to employ in our study. To this end, we exploited the capacity of this compound to trigger cell death and we then evaluated the effect of increasing doses of MTX on the cell content in culture dishes of proliferating MCF-7 cells. The results we obtained showed that a 6 h treatment with 5×10^{-10} M MTX was sufficient to cause death of about 50% of the cultured cells (Fig. 1).

We then checked whether Ca^{2+} chelation, by addition of EGTA to culture vessels, could inhibit the cytotoxic response induced by MTX. This aspect was evaluated by measuring the DNA content of cell cultures and by comparing the results obtained with samples of MTX-treated cultures which had, or had not, received EGTA before the addition of the toxin. The results we obtained are reported in Fig. 2 and show that preincubation of MCF-7 cells with EGTA actually prevented cell losses from culture vessels, detected after 4–8 h of MTX treatment.

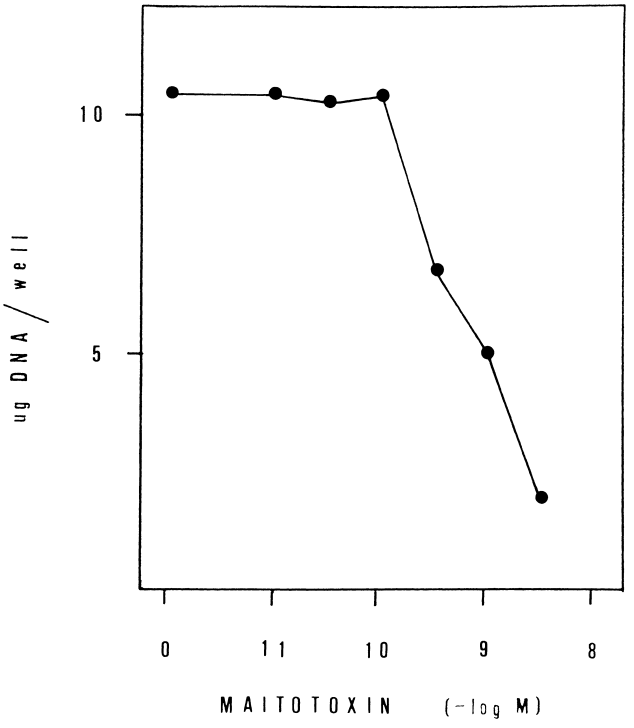

Fig. 1. Effect of MTX on proliferating MCF-7 cells. Cells in logarithmic growth were treated with increasing concentrations of MTX and cultures were maintained for 6 h at 37°C before being processed and used for measurements of the DNA content, as described under Section 2.

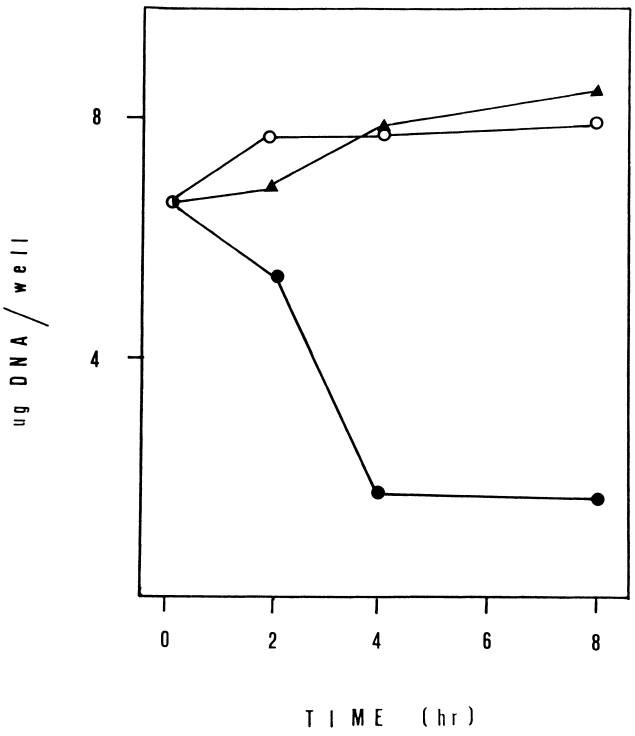

Fig. 2. Effect of EGTA on the cytotoxic response induced by MTX in MCF-7 cells. Cells in logarithmic growth were pre-incubated for 15 min at 37°C with 5 mM EGTA (triangles) or without any addition (circles) before receiving 0.5 nM MTX (closed symbols) or vehicle (open circles) and the incubation was continued for the indicated times at 37°C. At the end of the incubations, cells were processed and used for measurements of the DNA content, as described under Section 2.

The effect of MTX on activation of MAPKs was next analyzed by evaluating their phosphorylation state [1,2]. To this end, we measured the time-course of the cellular content of ERK1 and ERK2 isoforms, both as phosphorylated enzymes and total protein, after MTX addition to MCF-7 cells. The results we obtained by immunoblotting using specific antibodies (Fig. 3) showed that MTX treatment caused phosphorylation of ERK1 and, to a lesser extent, of ERK2, which was maximal after 15 min of compound addition and decreased thereafter, to become barely detectable after 1 h (upper panel). Parallel measurement of ERK1 and ERK2 proteins in the same extracts revealed that the levels of these enzymes were not appreciably altered in the first 30 min of MTX treatment, but a loss of these kinases could be detected by 1 h after toxin addition (Fig. 3, center panel). In line with the toxic response detected under our experimental conditions, involving cell detachment from culture vessels, the measurement of a relevant component involved in cell-cell adhesion of MCF-7 cells, such as E-cadherin [29], revealed that MTX treatment induced cell loss of this protein, which became undetectable after a 2 h treatment with the toxin (Fig. 3, lower panel).

The role of extracellular calcium in the induction of ERK1 and ERK2 phosphorylation by MTX was next ascertained by analyzing the effect of EGTA addition to cultured cells. As it is shown in Fig. 4 (lane 2 of the upper panel), pre-incubation of MCF-7 cells with EGTA did not affect the basal phosphorylation state of ERKs 1 and 2, but abolished the MTX-induced increase of phosphorylated forms found 15 min after toxin addition (Fig. 4, lanes 3 and 4 of the upper panel). Furthermore, an increased phosphorylation of ERK1 and ERK2 was observed when MCF-7 cells were treated with other Ca^{2+} permeants, such as ionomycin and the calcium ionophore A23187 (Fig. 4, lanes 5 and 7 of the upper panel), and this response could be abolished when MCF-7 cells received EGTA before the addition of those compounds (Fig. 4, lanes 6 and 8 of the upper panel). As the cell content of ERK1 and ERK2 protein was not appreciably affected by those treatments (Fig. 4, lower panel), our results showed that induction of increased intracellular calcium concentrations was the proximal trigger of ERK1 and ERK2 phosphorylation by MTX in MCF-7 cells.

Detection of MAPK activation after MTX treatment has not been reported so far. Our observations that increased phosphorylation of ERK1 and ERK2 in MCF-7 cells is triggered by other Ca^{2+} permeants, and is prevented by EGTA, indicate that the MTX-induced response is due to its function as activator of Ca^{2+} channels [12–16] and that an initial rise in intracellular Ca^{2+} would be sufficient to cause ERK phosphorylation in our experimental system. These findings are similar to those reported for MAPK activation upon membrane depolarization in PC12 cells [30]. In that experimental system, the Ca^{2+}-dependent response was transient, whereas a sustained activation was found after cell treatment with nerve growth factor and it was shown that two different pathways of MAPK activation exist in these excitable cells [30]. The time-course we have measured for ERK phosphorylation showed that the response is an immediate and transient event also in MCF-7 breast cancer cells (Fig. 3). Thus, our findings support the notion that Ca^{2+} transiently activates MAPK both in excitable and non-excitable cells [30–33].

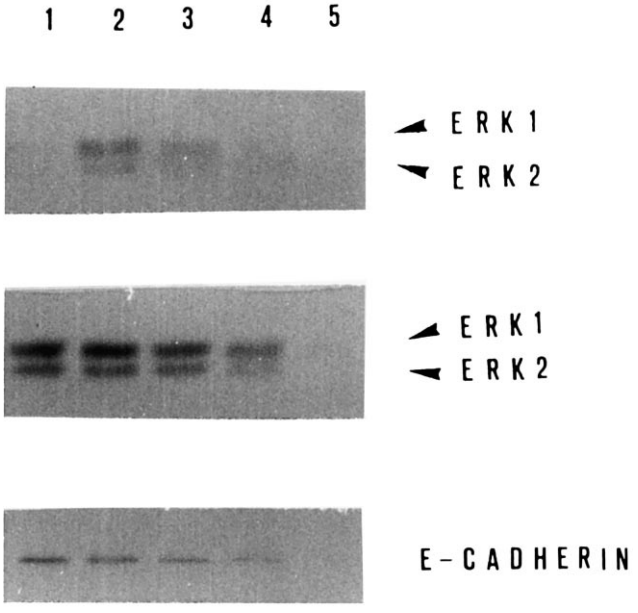

Fig. 3. Time-course of MTX effects on ERK1, ERK2 and E-cadherin in MCF-7 cells. Cytosoluble extracts were prepared from MCF-7 cells which had been treated with 0.5 nM MTX for 0 (lanes 1), 15 (lanes 2), 30 (lanes 3), 60 (lanes 4), 120 (lanes 5) min at 37°C. Aliquots of those extracts containing 30 μg protein were loaded onto each lane and proteins were fractionated by SDS-PAGE, before being subjected to immunoblotting, using antibodies recognizing phosphorylated forms of ERK1 and ERK2 (upper panel), a 1:1 mixture of anti-ERK1 and anti-ERK2 antibodies (center panel) or an anti-E-cadherin antibody (lower panel), as described under Section 2.

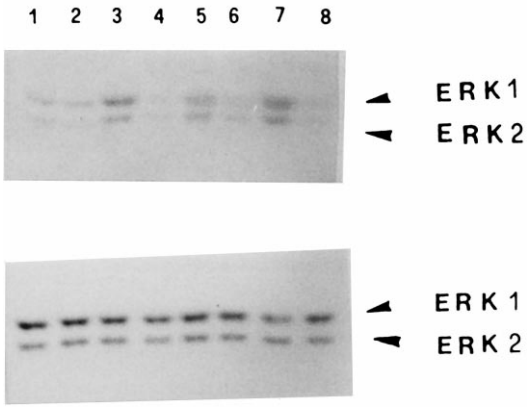

Fig. 4. Phosphorylation state of ERK1 and ERK2 in MCF-7 cells treated with EGTA, MTX, ionomycin and A23187. Cytosoluble extracts were prepared from MCF-7 cells which had (lanes 2, 4, 6 and 8) or had not (lanes 1, 3, 5 and 7) been pre-incubated with 5 mM EGTA, before being treated with vehicle (lanes 1 and 2), 0.5 nM MTX (lanes 3 and 4), 4 μM ionomycin (lanes 5 and 6) or 4 μM A23187 (lanes 7 and 8), for 15 min at 37°C. Aliquots of those extracts, containing 40 μg of protein, were loaded onto each lane and proteins were fractionated by SDS-PAGE, before being subjected to immunoblotting, using antibodies recognizing phosphorylated forms of ERK1 and ERK2 (upper panel) or a 1:1 mixture of anti-ERK1 and anti-ERK2 antibodies (lower panel), as described under Section 2.

In excitable cells, Ca^{2+} activation of MAPK has been related to mechanisms controlling normal functioning, such as neuronal plasticity [30]. The significance of the phenomenon in non-excitable cells, instead, is presently unclear. In our experimental system, ERK phosphorylation preceded cell death (Fig. 2), indicating that it might play a role in toxic responses induced by MTX in cultured cells. Furthermore, as this response was elicited by other Ca^{2+} permeants, and was blocked by EGTA, our findings indicate that signaling pathways involving Ca^{2+} ions may cause activation of ERK1 and ERK2 in some cell death responses.

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