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PP1/PP2A phosphatases inhibitors okadaic acid and calyculin A block ERK5 activation by growth factors and oxidative stress

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Abstract Okadaic acid is an inhibitor of the protein Ser/Thr phosphatases PP1 and PP2A, which blocks the activation of extracellular signal-regulated protein kinase 5 (ERK5), a member of the MAP kinase family activated by growth factors and several types of stressors. The blocking of ERK5 activation by okadaic acid was observed in HeLa cells exposed to epidermal growth factor and H_2O_2 as well as in PC12 cells stimulated by nerve growth factor and H_2O_2. Calyculin A, another PP1 and PP2A inhibitor, behaved similarly although these compounds are not structurally related. This suggests that either PP1 or PP2A or both are necessary for ERK5 activation. Protein kinase C (PKC) acts as a negative regulator of the ERK5 activation pathway, however our data suggest that the effects of PKC and the phosphatase are unrelated. **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Extracellular signal-regulated protein kinase 5; Okadaic acid; Calyculin A; Protein kinase C; Epidermal growth factor

1. Introduction

Mitogen activated protein kinase (MAPK) cascades are organised in modules that contain three protein kinases activated by sequential phosphorylations. MAP kinases are activated by dual-specificity kinases [MAPK/extracellular signal-regulated protein kinases (ERK) or MEK] which in turn are activated by phosphorylation in Ser/Thr residues by MEK kinases. ERK5 is a member of the MAPK family which is activated in response to mitogens and several types of stress [1,2]. ERK5 activation requires dual phosphorylation in a TEY motif, which makes this kinase more similar to ERK1/2 than to the other members of the MAPK family. However ERK5 has a unique C-terminal sequence that is not present in other members of the family [3,4]. The role of ras in ERK5 activation seems to be cell dependent. In HeLa cells the overexpression of mutant forms of ras have no effect on ERK5 activation by epidermal growth factor (EGF) [1], whereas in rat pheochromocytoma cell line (PC12) and COS7 cells the activation of ERK5 by EGF and nerve growth factor (NGF) seems to be ras dependent [5].

MEK5 has been reported to be the only kinase able to activate ERK5 and experiments using dominant negative forms of MEK5 suggest that this kinase is selective for the ERK5 pathway [1]. Raf, mos and MEKK1, specific MEK kinases of the ERK and JNK pathways, are unable to activate MEK5 [6]. In contrast, MEKK2 [7], MEKK3 [8] and the cot oncoprotein [9] activate ERK5 in a MEK5 dependent manner, and experiments using a dominant negative mutant of MEKK3 suggest that in some cell types this kinase is a mediator of EGF and H_2O_2 activation of ERK5 [7,8]. A role for c-src in H_2O_2 mediated ERK5 activation has also been described [10]. In contrast to the positive effect of these protein kinases, protein kinase C (PKC) has been described as a negative regulator of ERK5 activation [11].

The function of protein phosphatases in the regulation of MAP kinase pathways has been mainly associated to the down-regulation of these pathways. The dual-specificity phosphatases are responsible for selective dephosphorylation of the critical phosphothreonine and phosphotyrosine residues present in the different members of the MAP kinase family. However PP2A and a uncharacterised tyrosine phosphatase have been involved in the early inactivation of EGF stimulated MAP kinase in PC12 cells [12]. Moreover PP2A can act at multiple levels of the cascade, as suggested by the observations that this enzyme can dephosphorylate MEK1/2 in vitro [13] and that this kinase is activated by the treatment of cells with okadaic acid [14]. In this paper we describe the effect of okadaic acid and calyculin A, two Ser/Thr phosphatase inhibitors specific for protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), on the ERK5 pathway. Our results show that, opposite to what is observed in other MAP kinase pathways, ERK5 activation requires the activity of a PP1/PP2A-like phosphatase to become activated.

2. Materials and methods

2.1. Reagents and antibodies

NGF and EGF were purchased from Life Technologies. Okadaic acid, calyculin A, cyclosporine A, GF109203X and U0126 were from Calbiochem. Anti ERK5 (C-20) (1/100) was from Santa Cruz, anti-p42 ERK2 (1 μg/ml) from Upstate Biotech, anti-phospho ERK5 (3 μg/ml) from Calbiochem and anti phospho-MAPK (1/2000) from New England Biolabs. Enhanced chemiluminescence reagent (ECL), protein G-Sepharose and [γ-^{32}P]ATP were from Amersham-Pharmacia. Tissue culture reagents were from Gibco.

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Abbreviations: EGF, epidermal growth factor; ERK1/2, extracellular signal-regulated protein kinases 1/2; ERK5 extracellular signal-regulated protein kinase 5; FBS, foetal bovine serum; NGF, nerve growth factor; PC12, rat pheochromocytoma cell line; PKC, protein kinase C; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A

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2.2. Cell culture and preparation of lysates

HeLa cells were cultured at 37°C in a 95/5 air/CO_2 water saturated atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat inactivated foetal bovine serum (FBS) and PC12 cells in DMEM containing 10% heat inactivated horse serum, 5% heat inactivated FBS, both containing 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. For treatments, the cells were transferred to 60 mm dishes and after 48 h starved overnight in DMEM. The cells were incubated with the inhibitors for the indicated times and then stimulated with EGF (100 ng/ml), NGF (50 ng/ml) or H_2O_2 (1 mM). Cells were harvested, washed with cold phosphate-buffered saline and lysed with 0.2 ml of buffer A (50 mM Tris/HCl, pH 7.5, 0.27 M sucrose, 1 mM EGTA, 1 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate, 10 mM sodium β-glycerophosphate, 5 mM PPi, 1% Triton X-100, 1 mM benzamidine, 1 mM phenylmethylsulfonylfluoride (PMSF), and 0.1% (v/v) β-mercaptoethanol). After 15 min on ice the lysate was removed from the dishes and centrifuged at 13 000×*g* for 15 min at 4°C. Supernatants were used for Western blotting and kinase assays.

2.3. Immune complex ERK5 kinase assay

To assay ERK5 activity, 5 μl of protein G-Sepharose equilibrated in buffer A were incubated with 5 μg of anti ERK5 during 30 min at 4°C. The beads were washed twice with buffer A and 500 μg of cell extract protein was added and incubated for 90 min at 4°C. The immunocomplexes were washed twice with buffer A plus 0.5 M NaCl and twice with kinase assay buffer (50 mM Tris–HCl pH 7.5, 0.1 mM EGTA, 0.1% β-mercaptoethanol).

The assay was done at 30°C for 30 min in a 50 μl final volume of kinase assay buffer containing 0.1 mM ATP (5 μCi per assay), 5 mM Mg acetate and 0,33 mg/ml of myelin basic protein (MBP) as substrate. Reaction was stopped with Laemmli sample buffer and proteins separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) in a 12% gel and the corresponding band detected by autoradiography and quantified by densitometry.

2.4. Western blot analysis

The protein content of cellular extracts was quantified by Bradford assay [15]. For ERK5 band-shift assay 100 μg of protein were loaded on 7% SDS–PAGE. For ERK1/2, 50 μg of protein were loaded in 10% gels. To detect phospho-ERK5, 500 μg of cell extracts were immunoprecipitated as described above and separated in a 10% gel. The proteins were transferred onto PVDF membranes and incubated with the corresponding antibodies at the indicated concentrations. Proteins were detected using the ECL.

3. Results

3.1. PKC and classical phosphatases PP1/PP2A inhibitors do not cooperate on ERK5 activation

PKC has been described as a negative regulator of ERK5 activation in response to G-CSF [11]. In agreement with this notion we observed that preincubation of HeLa and PC12 cells with the PKC inhibitor GF109203X potentiated the activation of ERK5 by EGF and NGF respectively, as measured as a shift in the mobility of ERK5 in SDS–PAGE (Fig. 1).

We asked the possibility that a protein phosphatase would reverse the effects of PKC, and as a first approach we decided to test the possible involvement of a PP1- or PP2A-like phosphatase. We reasoned that if PKC and one of these phosphatases had the same target, combinatorial treatments of the

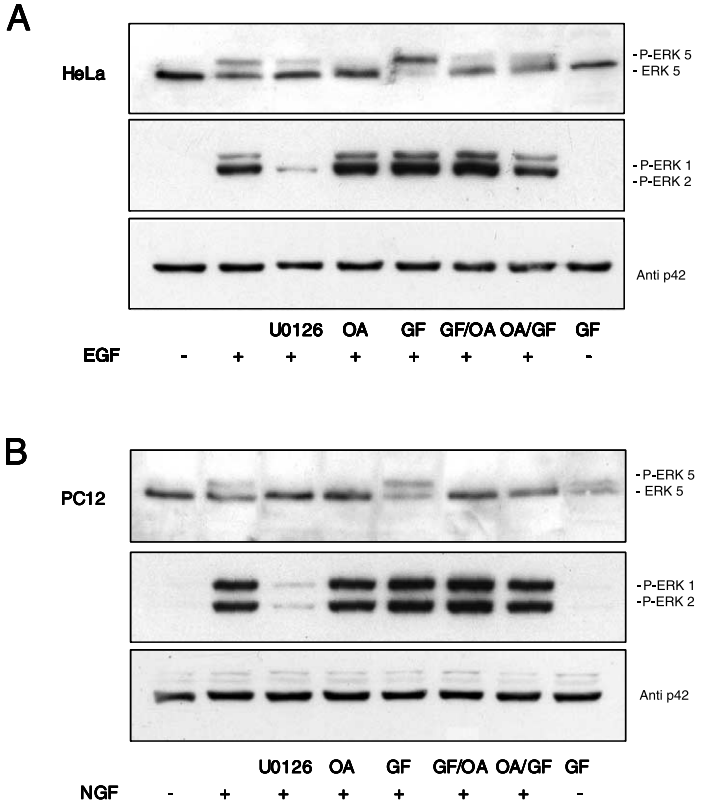

Fig. 1. Effect of some kinase and phosphatase inhibitors on ERK5 activation. HeLa and PC12 cells were starved overnight and then incubated for 30 min with 10 μM U0126 (U0126), 1 μM okadaic acid (OA, OA/GF) or 5 μM GF109203X (GF, GF/OA). In OA/GF and GF/OA, 5 μM GF109203X and 1 μM okadaic acid were added respectively after the first treatment, and the cells were further incubated for 30 min. Finally the cells were stimulated with 100 ng/ml EGF for 15 min (A) or 50 ng/ml NGF for 30 min (B). ERK5 activation was detected as a shift in its mobility. ERK1/2 phosphorylation was detected using an antibody against their dual phosphorylated forms. In order to check that the same amount of protein was loaded an antibody against p42-ERK2 was used.

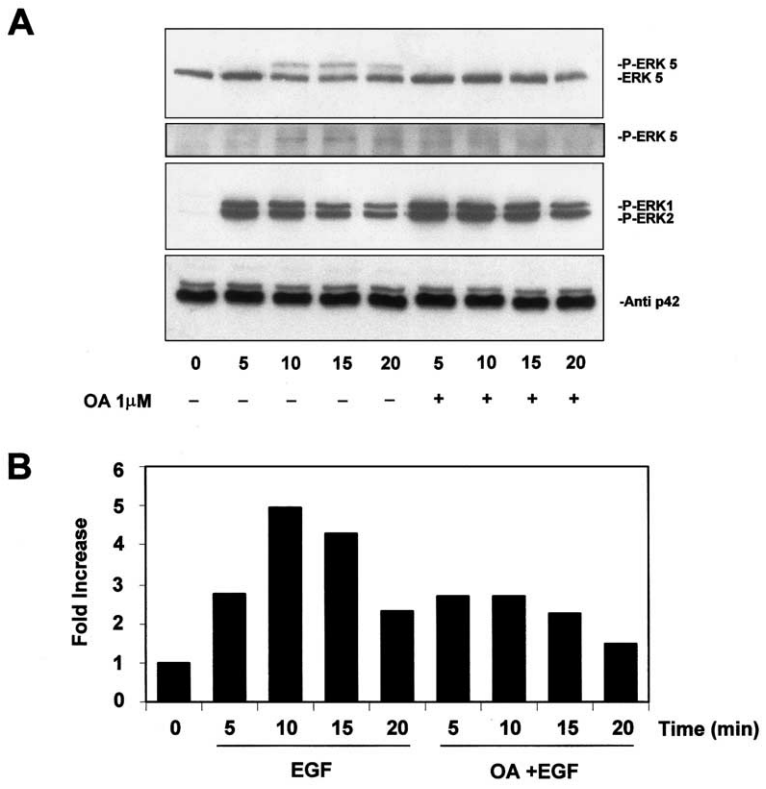

Fig. 2. EGF stimulated ERK5 activation in HeLa cells is blocked by okadaic acid. HeLa cells were serum starved overnight, treated with 1 μM okadaic acid (OA) or DMSO for 30 min, and then stimulated with 100 ng/ml EGF for the indicated times. Cells were lysed and the extracts processed as indicated under materials and methods. ERK5 activation was detected as (A) a shift in its mobility or with specific antibodies against the phosphorylated form of the enzyme, and (B) by its activity in an immunocomplex kinase assay. ERK1/2 phosphorylation was detected using an antibody against their dual phosphorylated forms. In order to check that the same amount of protein was loaded an antibody against p42-ERK2 was used.

cells with GF109203X and okadaic acid might lead to different phosphorylation levels of their target, what would influence the strength of ERK5 activation by growth factors. Unexpectedly, treatment with okadaic acid either prior to or after the addition of GF109203X resulted in a blockage in ERK5 activation by growth factors. The previous data could be considered as indicative that a PP1- or PP2A-type phosphatase acted as a positive regulator of the ERK5 pathway activation in response to EGF or NGF, what would be in contrast with that described for the ERK1/2 pathway. In fact, treatment of the cells with okadaic acid alone blocked ERK5 activation in a way similar to U0126, a compound shown to block ERK5 as well as ERK1/2 activation. No effects of okadaic acid or GF 109203X were observed on ERK1/2 phosphorylation.

3.2. *Protein phoshatases PP1 and PP2A inhibitors block the EGF stimulated ERK5 activation in HeLa cells*

In HeLa cells, we observed that EGF-promoted ERK1/2 phosphorylation reached a maximum at 5 min and was potentiated by inhibition of phosphatases with okadaic acid. In these experiments, EGF also stimulated ERK5 reaching a maximal activation at 15 min (Fig. 2). The effect of okadaic acid on ERK5 was a blockage of the activation, measured either as a shift in ERK5 mobility, with antibodies against phosphorylated ERK5 or by direct assay of its activity on MBP. To check if the effect of okadaic acid on ERK5 activation was unspecific, we performed similar experiments using calyculin A, another protein phosphatase inhibitor specific for PP1 and PP2A but with a completely different chemical structure. Both inhibitors blocked the activation of ERK5 in a concentration dependent manner (Fig. 3A–C), but they did not block ERK1/2 phosphorylation. In contrast to this, treatment of the cells with 4 μM cyclosporine A, a potent inhibitor of PP2B did not block ERK1/2 or ERK5 activation in response to EGF in HeLa cells (Fig. 3C).

3.3. *Okadaic acid and calyculin A also block ERK5 activation in response to oxidative stress*

In addition to its response to EGF and NGF, ERK5 pathway is also activated by some stressors, what distinguishes it from the ERK1/2 pathway. To check if okadaic acid also affected ERK5 activation by other stimulus, HeLa and PC12 cells were stimulated with H_2O_2 either directly or after pretreatment with okadaic acid or with calyculin A (Fig. 4). In both cell types activation of ERK5 was inhibited by these phosphatase inhibitors.

4. Discussion

The function of protein phosphatases on MAP kinase pathways has been mainly associated to their down-regulation. Dual-specificity phosphatases act directly on MAP kinases and cause their inactivation, whereas the classical Ser/Thr phosphatases may also act on different components located upstream in the activation cascade. Keeping this in mind, it was unexpected to observe that inhibition of the classical PP1/

PP2A phosphatases with okadaic acid completely blocked the activation of ERK5 instead of potentiating its activation, as occurs with ERK1/2. It must be emphasized that the effect of okadaic acid on other MAP kinase pathways has always been an increase in MAP kinase activation [14,16]. Interestingly, calyculin A mimicked okadaic acid effects on ERK5 activation and the effects of both compounds were concentration dependent in a range that agrees with their potencies as phosphatase inhibitors. This argues against an unspecific effect of okadaic acid and reinforces the idea that a PP1- or PP2A-type phosphatase must be involved.

The effects of okadaic acid and calyculin A on ERK5 were not restricted to its stimulation by growth factors but were also observed in its response to oxidative stress. Although it is known that calyculin A shows the same specificity for PP1 and PP2A whereas okadaic acid is a better inhibitor of PP2A than of PP1, our experiments in vivo do not allow to discriminate between these two phosphatases. Nevertheless, our results indicate that a PP1/PP2A-like phosphatase activity is mandatory for ERK5 to become activated in response to several stimuli.

It is known that PKC negatively regulates the activation of ERK5 [11]. The possibility that PKC and the phosphatase involved in ERK5 activation targeted the same component of the activation cascade is not supported by our results on the combinatorial effects of the inhibitors. It is worth noting that the effect of okadaic acid blocking ERK5 activation always prevailed over that of GF109203X, what could be interpreted as a prominent role of the PP1/PP2A-type phosphatase on the regulation of ERK5 pathway.

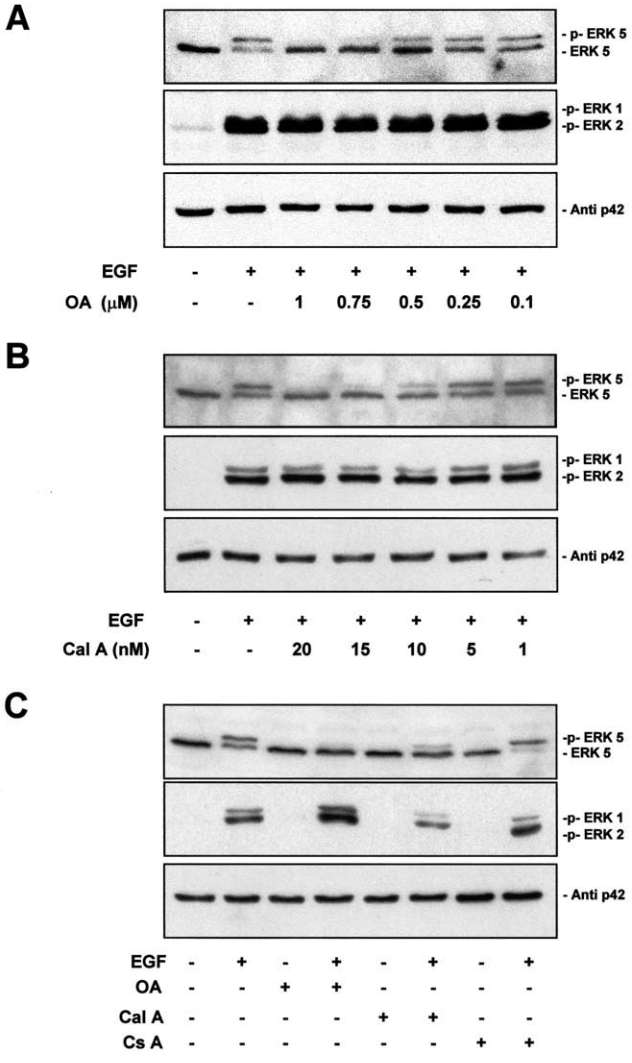

Fig. 3. Okadaic acid and calyculin A block ERK5 activation by EGF in HeLa cells in a concentration dependent manner. HeLa cells were starved overnight, incubated for 30 min with the indicated concentrations of okadaic acid (OA) (A) and calyculin A (Cal A) (B) and then treated for 15 min with EGF. Controls without inhibitors were incubated with DMSO. HeLa cells were incubated with 1 μM okadaic acid, 20 nM calyculin A or 4 μM cyclosporin A (CsA) and stimulated with or without 100 ng/ml of EGF for 15 min (C). Activation was measured as a shift in the mobility of ERK5 or with antibodies against the phosphorylated forms of ERK1/2. To ensure that the same amount of protein was loaded an antibody against the p42-ERK2 was used.

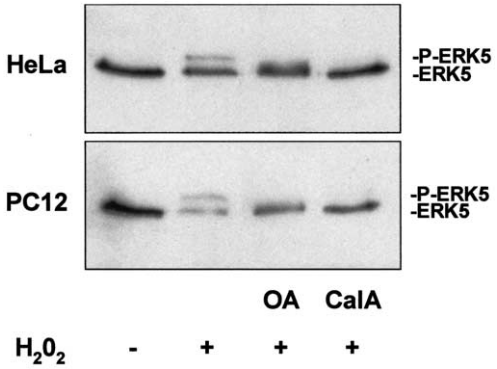

Fig. 4. ERK5 activation by H_2O_2 in HeLa and PC12 cells is blocked by okadaic acid. HeLa and PC12 cells were starved overnight and then treated with either 1 μM okadaic acid (OA), 20 nM calyculin A (Cal A) or DMSO for 30 min. Afterwards, HeLa and PC12 cells were stimulated with 1 mM H_2O_2 for 30 min and 1 h respectively. ERK5 activation was detected as a shift in its mobility.

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