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Role of protein kinase B and the MAP kinase cascade in mediating the EGF-dependent inhibition of glycogen synthase kinase 3 in Swiss 3T3 cells[1]

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Abstract Epidermal growth factor (EGF), insulin-like growth factor 1 (IGF1) and phorbol myristate acetate (PMA) induce the inhibition of glycogen synthase kinase 3 (GSK3) by stimulating the phosphorylation of an N-terminal serine. Here, we show that protein kinase B (PKB) plays a key role in mediating EGF-induced inhibition of GSK3α and that the classical MAP kinase (MAPK) cascade has two functions in this process. Firstly, it makes a transient contribution to EGF-induced inhibition of GSK3α. Secondly, it shortens the duration of PKB activation and GSK3α inhibition. In contrast, PKB alone mediates the IGF1-induced inhibition of GSK3α, while the MAPK cascade mediates the inhibition of GSK3α by PMA.

Key words: Protein kinase B; Epidermal growth factor; Insulin; MAP kinase; Glycogen synthase kinase 3

1. Introduction

Glycogen synthase kinase 3 (GSK3) is a protein kinase that is thought to play multiple roles in cell regulation. For example, it phosphorylates and inhibits both glycogen synthase [1] and protein synthesis initiation factor eIF2B [2], which are likely to underlie, at least in part, the insulin-induced stimulation of glycogen synthesis [3–5] and protein synthesis [6]. Inhibition of GSK3 may also underlie the insulin or insulin-like growth factor 1 (IGF1)-induced dephosphorylation of the microtubule-associated protein Tau in cultured hippocampal neurones [7]. The inhibition of GSK3 has been suggested to underlie the phorbol ester-induced dephosphorylation of the transcription factor c-Jun, at sites that lie proximal to the DNA binding domain [8], and to play a role in growth factor-induced protection against apoptosis [9].

The inhibition of GSK3 by insulin [10], growth factors [11] and phorbol myristate acetate (PMA) [12] results from the phosphorylation of GSK3 at a N-terminal serine residue (Ser-21 in GSK3α, Ser-9 in GSK3β) and three protein kinases (protein kinase B (PKB) [10], MAP kinase (MAPK)-activated protein kinase 1 (MAPKAP-K1, also called p90rsk) and p70 S6 kinase (p70 S6K)) [13,14], that become activated in response to one or more of these signals, phosphorylate these residues in vitro. The insulin- or IGF1-induced inhibition of GSK3 is prevented by inhibitors of phosphatidylinositol (PI) 3-kinase (wortmannin or LY294002) that block the activation of PKB (also called c-Akt), but not by inhibitors of the activation of p70 S6K (rapamycin) or MAPKAP-K1 (PD98059) [10]. Moreover, cell transfection experiments using a dominant negative mutant of PKB support the view that PKB is likely to mediate the inhibition of GSK3 by insulin [15].

The inhibition of GSK3 by epidermal growth factor (EGF) is unaffected by rapamycin [16] and evidence has been presented that this effect is mediated by MAPKAP-K1. Thus, GSK3β becomes phosphorylated at Ser-9 when co-transfected with MAPKAP-K1 [12] and the EGF-induced inhibition of GSK3 is largely suppressed in a cell line overexpressing a catalytically inactive mutant of MAPK kinase 1 (MKK1, also called MEK) [17], an enzyme that lies 'upstream' of MAPKAP-K1 in the classical MAPK cascade. In addition, the EGF-induced inhibition of GSK3 is enhanced in cells expressing a MKK1 mutant which displays some constitutive activity [17]. Moreover, in cells overexpressing MAPKAP-K1, the phosphorylation of GSK3β at Ser-9 is stimulated by PMA, which is known to trigger activation of the MAPK cascade, but not the activation of PKB [18].

The acute regulation of GSK3 activity by EGF and PMA was studied before the advent of inhibitors that are capable of preventing activation of the MAPK cascade. The advantage of using such small cell permeant protein kinase inhibitors (reviewed in [19]) is that they can be used to study the roles of protein kinases without the need for overexpression of active or inactive forms of protein kinases, which can lead to erroneous conclusions. We have therefore used two structurally distinct inhibitors of the MAPK cascade, namely PD98059 (which prevents the activation of MKK1 [20]) and U0126 (which inhibits MKK1 [21]), in conjunction with two PI 3-kinase inhibitors (wortmannin and LY294002) and rapamycin, to identify the signalling pathways that mediate the EGF-induced inhibition of GSK3. In contrast to earlier reports, we find that a PI 3-kinase-dependent pathway, probably mediated by PKB, plays a major role in this process and that the MAPK cascade plays two distinct roles. Firstly, it contributes, albeit very transiently, to the inhibition of GSK3. Secondly, it controls the duration of inhibition of GSK3 by PKB.

2. Materials and methods

2.1. Materials

IGF1 and EGF were purchased from Gibco BRL (Paisley, UK),

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[1] This paper is dedicated to the memory of Dr. Yuji Saito who initiated work in our laboratory on the regulation of GSK3 by EGF.

Abbreviations: EGF, epidermal growth factor; IGF1, insulin-like growth factor 1; PMA, phorbol myristate acetate; MAPKAP-K1, MAP kinase-activated protein kinase 1; PKB, protein kinase B; MAPK, MAP kinase

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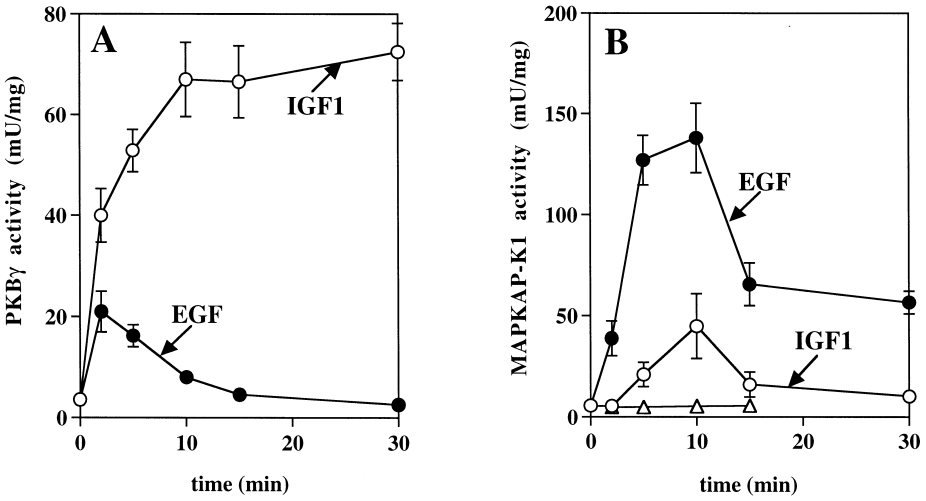

Fig. 1. Activation of PKBγ and MAPKAP-K1 by IGF1 and EGF in Swiss 3T3 cells. Cells were stimulated with 100 ng/ml IGF1 (open circles) or 100 ng/ml EGF (closed circles) for the times indicated and PKBγ (A) or MAPKAP-K1 (B) immunoprecipitated from the cell lysates and assayed as described in Section 2. The open triangles show further experiments in which the cells were incubated for 1 h with U0126 prior to stimulation with EGF. The results shown are presented as S.E.M. for five separate experiments. Immunoprecipitations were carried out in duplicate in each experiment.

PMA and wortmannin from Sigma Chemical (Poole, UK), rapamycin, LY294002, PD98059 and U0126 from Calbiochem (Nottingham, UK) and protein G-Sepharose from Pharmacia (Milton Keynes, UK). Immunoprecipitating antibodies specific for PKBγ [22] and MAPKAP-K1 [23] were raised in sheep and affinity-purified on CH-Sepharose columns to which the appropriate peptide antigen was coupled covalently. Further antibodies were raised in sheep that recognise GSK3α phosphorylated at Ser-21 and that recognise dephosphorylated and phosphorylated GSK3 equally well. These antibodies were raised against the peptides RARTSS*FAEPG (residues 16–26 of GSK3α, where * denotes a phosphorylated residue) and QAPDATPTLTNSS (residues 471–483 of human GSK3α) and affinity-purified as described above. All four antibodies can be purchased from UBI (Lake Placid, NY, USA).

2.2. Cell culture, stimulation and cell lysis

Mouse Swiss 3T3 fibroblasts were cultured to confluence [20] and incubated for 16 h in Dulbecco's modified Eagles medium from which foetal calf serum was omitted. Cells were stimulated for the times indicated with IGF1, EGF or PMA with or without prior treatment for 10 min with wortmannin (100 nM), LY294002 (100 μM) or rapamycin (100 nM), or for 1 h with PD98059 (50 μM) or U0126 (10 μM). In experiments where cells were pretreated with wortmannin and either PD98059 or U0126, wortmannin was added for the last 10 min of the pre-incubation only. The cells were lysed in 1.0 ml of 50 mM Tris-HCl (pH 7.5), 1% (w/v) Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 10 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 μM microcystin-LR, 0.1% (by volume) 2-mercaptoethanol, 1 mM benzamidine and 0.2 mM phenylmethylsulphonyl fluoride, frozen immediately in liquid nitrogen and stored at −80°C. Protein concentrations were determined according to Bradford [24] using bovine serum albumin as a standard.

2.3. Immunoprecipitation and assay of PKB and MAPKAP-K1

PKBγ [22] and MAPKAP-K1 [23] were immunoprecipitated from 100 and 50 μg of cell lysate, respectively, and assayed using the peptide GRPRTSSFAEG, termed Crosstide [10,21,25]. One unit of activity (U) was defined as that amount which catalysed the phosphorylation of 1 nmol substrate in 1 min.

2.4. Immunoblotting of GSK3α

Cell lysates (40 μg) were denatured in 1% (by mass) sodium dodecyl sulfate (SDS), electrophoresed on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes and immunoblotted with appropriate antibodies. Detection was performed using the enhanced chemiluminescence reagent (Amersham).

3. Results

3.1. IGF1- and EGF-dependent activation of PKB and the MAPK cascade in Swiss 3T3 cells

In order to study the potential roles of PKB and MAPKAP-K1 in the IGF1- and EGF-induced regulation of GSK3 activity, we first examined the time course of activation of these protein kinases after stimulation with each agonist. The activation of PKB and MAPKAP-K1 was measured after their immunoprecipitation from the cell lysates. In Swiss 3T3

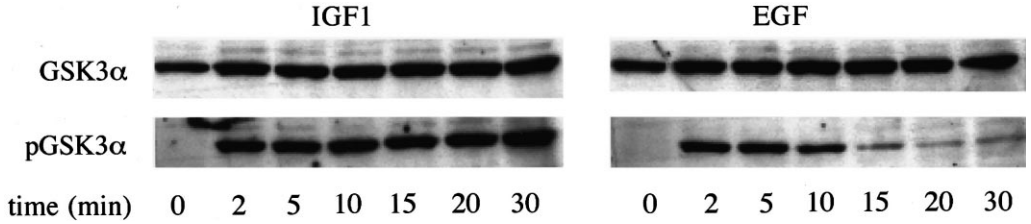

Fig. 2. Phosphorylation of GSK3α at Ser-21 in response to IGF1 or EGF in Swiss 3T3 cells. Cells were stimulated with 100 ng/ml IGF1 (A) or 100 ng/ml EGF (B) for the times indicated and the phosphorylation of GSK3α at Ser-21 was examined by immunoblotting with an antibody that only recognises the Ser-21-phosphorylated enzyme (pGSK3α) (see Section 2). The lysates were also immunoblotted with an antibody that does not distinguish between the phosphorylated and dephosphorylated proteins (GSK3). GSK3α migrated between bovine serum albumin (66 kDa) and ovalbumin (43 kDa) with an apparent molecular mass of 51 kDa as expected. Similar results were obtained in three separate experiments.

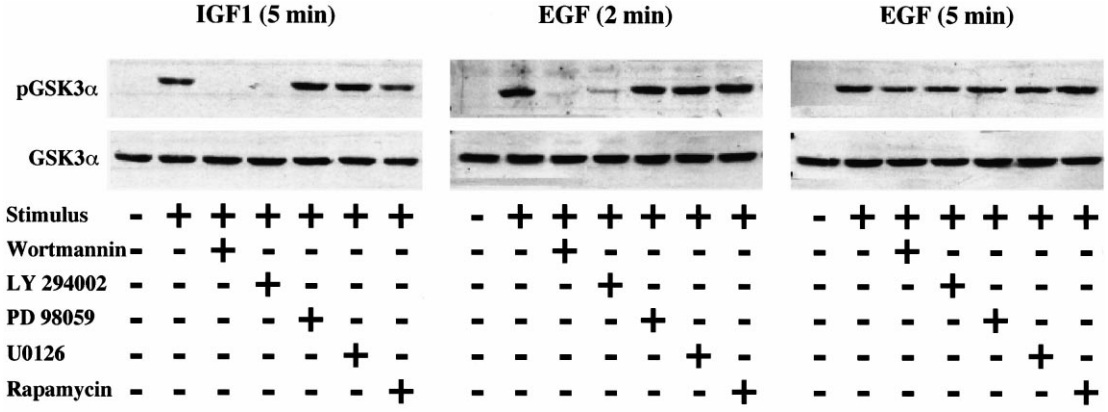

Fig. 3. Effect of specific kinase inhibitors on the IGF1- and EGF-induced phosphorylation of GSK3α at Ser-21 in Swiss 3T3 cells. Cells were stimulated for 5 min with 100 ng/ml IGF1 or for 2 or 5 min with 100 ng/ml EGF and the lysates were immunoblotted with antibodies that recognise the Ser-21-phosphorylated enzyme (pGSK3α) or with antibodies that do not distinguish between the phosphorylated and dephosphorylated forms of the enzyme (GSK3). Prior to stimulation with agonist, the cells were pretreated with wortmannin, LY294002, PD98059, U0126 or rapamycin as described in Section 2. Similar results were obtained in three separate experiments.

cells, we found that PKBα and PKBγ were both activated by IGF1. The activation of both isozymes occurred with a half time of about 1 min and was maximal after 5 min. There was no activation of PKBβ (data not shown). Since PKBγ accounted for almost 80% of the total PKB activity in the cell lysates, the activity of this isoform was measured in all subsequent experiments. The MAPKAP-K1 antibody used in our experiments immunoprecipitates both MAPKAP-K1 isoforms (MAPKAP-K1a/RSK1 and MAPKAP-K1b/RSK2).

IGF1 triggered a strong and sustained activation of PKBγ, whereas activation by EGF was weaker and very transient (Fig. 1A). As expected from studies in other cells, the activation of PKBγ by either agonist was abolished by pretreatment of the cells with the PI 3-kinase inhibitors wortmannin or LY294002 (data not shown). In contrast, both IGF1 and EGF triggered a transient activation of MAPKAP-K1, a downstream component of the classical MAPK cascade, although activation induced by IGF1 was much weaker (Fig. 1B). As expected, the activation of MAPKAP-K1 by either EGF (Fig. 1B) or IGF1 (data not shown) was abolished by U0126 (Fig. 1B) or PD98059 (data not shown), the specific inhibitors of the MAPK cascade. The immunosuppressant drug rapamycin, an inhibitor of mTOR that prevents the IGF- or EGF-induced activation of p70 S6K, had no effect on the activation of PKB by either agonist (data not shown).

3.2. *EGF- and IGF1-induced phosphorylation of GSK3α at Ser-21*

It has been shown previously that GSK3α and GSK3β are completely inactivated by the phosphorylation of Ser-21 and Ser-9, respectively [13,14], and that IGF1-induced inhibition in cells is accompanied by the phosphorylation of these and no other sites [10]. Moreover, IGF1 is unable to induce any inhibition of a GSK3β mutant in which Ser-9 has been mutated to Ala [26]. It is therefore possible to assess the inhibition of GSK3 simply by immunoblotting cell lysates with a phospho-specific antibody that recognises the Ser-21-phosphorylated form of GSK3α.

Previous work has shown that IGF1 [27] and EGF [11] both induce 50% inhibition of GSK3, which is maximal after

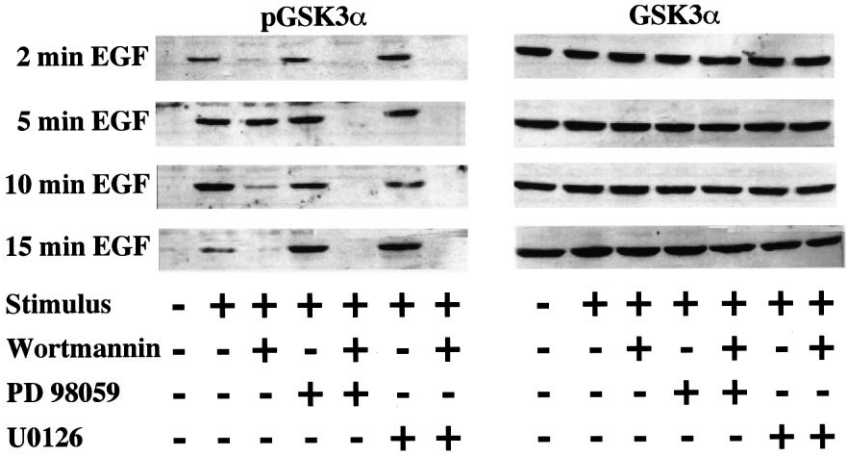

Fig. 4. Effect of combining inhibitors of PI 3-kinase and the MAPK cascade on the EGF-induced phosphorylation of GSK3α at Ser-21 in Swiss 3T3 cells. Cells were stimulated for the times indicated with 100 ng/ml EGF and the lysates immunoblotted with antibodies that recognise the Ser-21-phosphorylated enzyme (pGSK3α) or with antibodies that do not distinguish between the phosphorylated and dephosphorylated forms of the enzyme (GSK3). Prior to stimulation with agonist, the cells were pretreated as described in Section 2 with wortmannin or PD98059/U0126 or with combinations of these inhibitors as indicated. Similar results were obtained in three separate experiments.

about 5 min. In the present study, the phosphorylation of GSK3α at Ser-21 induced by either agonist was found to be nearly maximal after 2 min. However, while Ser-21 phosphorylation stimulated by IGF1 was sustained for at least 30 min, EGF-stimulated phosphorylation was transient and declined to near basal levels after 15 min (Fig. 2).

The time course of Ser-21 phosphorylation in response to EGF or IGF1 (Fig. 2) correlated with the activation of PKB rather than with the activation of MAPKAP-K1 (Fig. 1). Consistent with an important role for PKB, the IGF1-induced phosphorylation of GSK3α at Ser-21 after 5 min (or at any other time point) was prevented by prior treatment of the cells with wortmannin or LY294002, but not by PD98059 or U0126 (Fig. 3). The EGF-induced phosphorylation of Ser-21 after 2 min was also prevented by wortmannin or LY294002 and unaffected by PD98059 or U0126 (Fig. 3B). However, surprisingly, the EGF-induced phosphorylation of GSK3 at Ser-21 was hardly affected by inhibitors of PI 3-kinase or of the MAPK cascade after 5 min stimulation (Fig. 3). Statistical analysis of the immunoblotting data from three different experiments revealed that wortmannin inhibited EGF-induced inhibition of GSK3 by only 36 ± 2% after 5 min, while LY294002, PD98059 and U0126 had no statistically significant effect at this time point.

One possible reason for the very weak effect of PI 3-kinase inhibitors or MAPK cascade inhibitors on the EGF-induced phosphorylation of GSK3α at Ser-21 after 5 min was that activation of either pathway was sufficient for near maximal phosphorylation of Ser-21 at this time. If this was the case, then, blockade of both pathways would be required to suppress the effect of EGF. This explanation appears to be correct, because the combination of wortmannin and PD98059 or wortmannin plus U0126 completely prevented the EGF-induced phosphorylation of Ser-21 after 5 min (Fig. 4B).

Even more unexpectedly, the phosphorylation of Ser-21 was largely inhibited by wortmannin alone after stimulation with EGF for 10 or 15 min and pre-incubation with PD98059 or U0126 actually enhanced the EGF-induced phosphorylation of Ser-21 after 15 min (Fig. 4). After 15 min, the enhancement

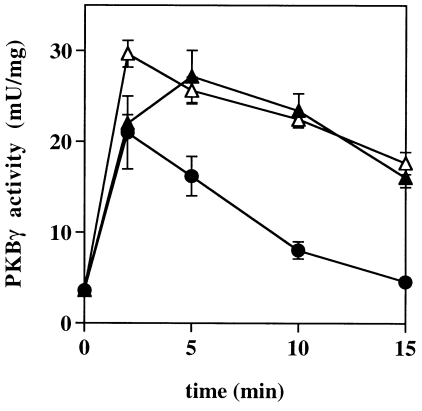

Fig. 5. Activation of PKBγ by EGF in the absence and presence of MAPK cascade inhibitors. Cells were stimulated with 100 ng/ml EGF (closed circles) for the times indicated and PKBγ was immunoprecipitated from the cell lysates and assayed as described in Section 2. The triangles show further experiments in which the cells were pre-incubated for 1 h with U0126 (open triangles) or PD98059 (closed triangles) prior to stimulation with EGF. The results shown are presented as S.E.M. for three separate experiments. Immunoprecipitations were carried out in duplicate in each experiment.

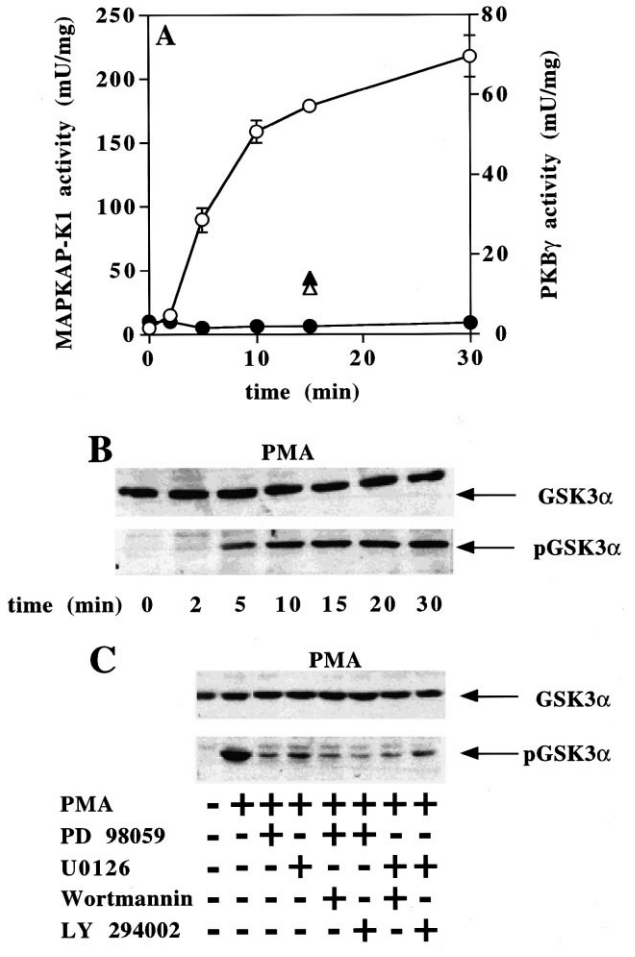

Fig. 6. Activation of MAPKAP-K1 and PKBγ and phosphorylation of GSK3α at Ser-21 by PMA in Swiss 3T3 cells. All experiments were repeated three times with similar results. (A) Cells were stimulated with 0.4 μg/ml PMA for the times indicated and PKBγ (closed circles) and MAPKAP-K1 (open circles) were immunoprecipitated from the cell lysates and assayed as described in Section 2. The triangles show further experiments in which the cells were pre-incubated for 1 h with U0126 (open triangles) or PD98059 (closed triangles) prior to stimulation with PMA. (B) The lysates from A were immunoblotted with an antibody that recognises the Ser-21-phosphorylated form of GSK3α (pGSK3α). (C) Lysates from cells that were stimulated for 15 min with 0.4 μg/ml PMA were immunoblotted as in B. Prior to stimulation with PMA, the cells were pre-incubated with PD98059, U0126, PD98059 plus wortmannin, PD98059 plus LY294002, U0126 plus wortmannin or U0126 plus LY294002 as described in Section 2.

of GSK3α phosphorylation at Ser-21 was 299 ± 13% and 373 ± 15% in the presence of PD98059 and U0126, respectively (± S.E.M. for three experiments). These observations led us to re-examine the EGF-induced activation of PKB in the presence of PD98059 or U0126. These experiments revealed that, in the presence of MAPK cascade inhibitors, the activation of PKB by EGF was not nearly so transient and remained at a much higher level after 15 min (Fig. 5). These unexpected observations are considered further under Section 4.

3.3. Effect of PMA on the phosphorylation of GSK3

As observed in other cells, PMA induced the activation of MAPKAP-K1 (but not PKB) and this was strongly, but not

completely, suppressed by pretreatment of the cells with PD98059 or U0126 (Fig. 6A). PMA also induced the phosphorylation of GSK3α at Ser-21 and this was partially suppressed by pretreatment with PD98059 or U0126 (Fig. 6B). Pretreatment with PD98059 plus wortmannin, or U0126 plus wortmannin, did not produce any further reduction in the PMA-induced phosphorylation of Ser-21 (Fig. 6C).

4. Discussion

The results described in this paper confirm that IGF1 triggers the phosphorylation and inhibition of GSK3 via a PI 3-kinase-dependent pathway that is unaffected by inhibitors of the MAPK cascade or rapamycin and which is presumably mediated by PKB. However, our results indicate that the signalling pathways that are rate-limiting for the EGF-induced phosphorylation of GSK3α at Ser-21 vary with the time of stimulation. A PI 3-kinase-dependent pathway (presumably mediated by PKB) is also responsible for the phosphorylation of GSK3α at Ser-21 after stimulation for 2 min. However, after 5 min, a MAPK-dependent pathway (presumably mediated by MAPKAP-K1), as well as the PI 3-kinase-dependent pathway, is also capable of phosphorylating Ser-21 maximally. Thus, after 5 min, it is necessary to block both pathways in order to observe significant inhibition of EGF-induced Ser-21 phosphorylation.

These observations are reminiscent of the tumour necrosis factor-induced phosphorylation of the transcription factor CREB at Ser-133 in HeLa cells. After 5 min stimulation, Ser-133 phosphorylation can be prevented by an inhibitor of stress-activated protein kinase 2/p38 (SAPK2/p38) but, after 15 min, CREB phosphorylation can only be blocked by inhibiting the classical MAPK cascade and SAPK2/p38 [28]. These observations highlight the need to use combinations of small cell permeant inhibitors in order to deduce which signalling pathways are rate-limiting in any particular situation.

A surprising observation was that the phosphorylation of GSK3α at Ser-21 was again largely blocked by inhibitors of PI 3-kinase alone, after stimulation with EGF for 10 min, even though the activation of MAPKAP-K1 was still quite high and the activation of PKB was declining (Fig. 1). These observations indicate that, although active, MAPKAP-K1 loses the ability to phosphorylate GSK3 after 10 min. One possible explanation for this finding is that MAPKAP-K1 is present in the cytosol after 5 min but, as reported previously [29], translocates to the nucleus after 10 min, where it becomes physically separated from the largely cytosolic GSK3. Such compartmentalisation may account for the inability of MAPKAP-K1 to phosphorylate GSK3 at these later time points.

Interestingly, inhibition of the MAPK cascade actually stimulated the PI 3-kinase-dependent phosphorylation of GSK3 induced by EGF after 15 min (Fig. 4). This unexpected observation was explained when the EGF-dependent activation of PKB was found to become much less transient in the presence of inhibitors of the MAPK cascade. This implies that the MAPK cascade controls the duration of PKB activation after stimulation with EGF. To our knowledge, this is the first report of 'crosstalk' between these two signal transduction pathways. The point at which this 'crosstalk' takes place is currently under investigation.

In summary, our results demonstrate that PKB plays a major role in the regulation of GSK3 activity by EGF and that the classical MAPK cascade plays two roles in this process. Firstly, it contributes to the EGF-induced inhibition of GSK3 after 5 min. Secondly, it controls the inactivation of PKB and time of re-activation of GSK3 thereafter.

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