RESEARCH COMMUNICATION

The activation of distinct mitogen-activated protein kinase cascades is required for the stimulation of 2-deoxyglucose uptake by interleukin-1 and insulin-like growth factor-1 in KB cells

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The uptake of 2-deoxyglucose into KB cells was stimulated about 2-fold by interleukin-1 (IL1), anisomycin or insulin-like growth factor-1 (IGF1). Stimulation by IL1 and anisomycin was prevented by SB 203580, a specific inhibitor of the mitogen-activated protein (MAP) kinase homologue termed 're-activating kinase' [RK; also known as p38, p40 and CSBP (cytokine synthesis anti-inflammatory-drug-binding protein)], but was unaffected by PD 98059, a specific inhibitor of the activation of the classical MAP kinase pathway. In contrast, the stimulation of 2-deoxyglucose uptake by IGF1 was blocked by PD 98059 and unaffected by SB 203580. Consistent with these

observations, IL1 and anisomycin were potent activators of MAP kinase-activated protein (MAPKAP) kinase-2, a physiological substrate of RK, whereas IGF1 was only a very weak activator of MAPKAP kinase-2. Conversely, IGF1 was a stronger activator of p42 MAP kinase than IL1 or anisomycin. These results imply that the activation of distinct MAP kinase pathways is required for the stimulation of glucose transport by IL1/anisomycin and IGF1 in KB cells, and suggest that the combined use of SB 203580 and PD 98059 is a powerful new approach to explore the roles of different MAP kinase cascades in cell regulation.

INTRODUCTION

Growth factors whose receptors are protein tyrosine kinases trigger the activation of several signal-transduction pathways including the 'classical' MAP kinase cascade (for brevity the abbreviations are not redefined in the text). In this pathway, the formation of GTP-Ras at the plasma membrane is followed first by the activation of c-Raf and then by the activation of MAP kinase kinase-1 and MAP kinase kinase-2 (MAPKK-1 and MAPKK-2; also known as MEK1 and MEK2). MAPKK-1 and MAPKK-2 in turn activate the p44 and p42 MAP kinases (also known as ERK1 and ERK2 respectively), which then phosphorylate (activate) variety of proteins, including isoforms of MAPKAP kinase-1 (also known as Rsk).

More recently, cellular stresses, such as chemical, osmotic and heat shock, UV radiation and protein-synthesis inhibitors, bacterial endotoxin (LPS) and the cytokines IL1 and TNF, have been shown to trigger the activation of two different homologues of MAP kinase, termed SAP kinase and RK (also known as p38, p40 and CSBP) [1–8]. One physiological substrate of SAP kinase (also termed JNK) is the transcription factor c-Jun [1,9], whereas one physiological target of RK has been identified as MAPKAP kinase-2 [5]. MAPKAP kinase-2 phosphorylates HSP 27 [7,10], the phosphorylation of which appears to stimulate the polymerization of actin. This may help to repair the actin microfilament network after its disruption by cellular stresses, thereby aiding cell survival [11].

Several stimuli which activate SAP kinase and RK, such as LPS, IL1 and heat shock, are also reported to activate the p42 and p44 MAP kinases [12–14], raising the question of which

signalling network triggers each of the responses evoked by these agonists. One way to resolve this question would be to develop specific inhibitors of these pathways, and two such compounds have recently been identified and characterized. The pyridinylimidazole SB 203580 (Figure 1) inhibits RK *in vitro* (IC_{50} =

Figure 1 The structures of SB 203580 and PD 98059

Abbreviations used: MAP, mitogen-activated protein; MAPKK, MAP kinase kinase; MKK4, MAP kinase kinase-4; ERK, extracellular-signal-regulated kinase; MEK, MAP kinase or ERK kinase; MAPKAP, MAP-kinase-activated protein; SAP, stress-activated protein; SEK, SAP kinase or ERK kinase; RK, re-activating kinase; JNK, Jun kinase; LPS, lipopolysaccharide; HSP, heat-shock protein; CSBP, cytokine synthesis anti-inflammatory-drug-binding protein; IL1, interleukin-1; TNF, tumour necrosis factor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; IGF1, insulin-like growth factor-1; PMA, phorbol 12-myristate 13-acetate.

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0.6 μ M) and prevents the activation of MAPKAP kinase-2 and the phosphorylation of HSP27 by osmotic shock or IL1 in KB cells and by chemical stress in PC12 and KB cells, but has no effect on the activities of SAP kinase, p42 MAP kinase and many other protein kinases tested, even at concentrations 100-fold higher than those which inhibit RK [7]. Moreover, SB 203580 does not inhibit the activation of SAP kinase by IL1 in KB cells or by chemical stress in PC12 cells, the activation of p42 and p44 MAP kinases by EGF in KB cells and by nerve growth factor in PC12 cells, or the activation of the 'upstream' activator of RK (RK kinase) in IL1-stimulated KB cells [7]. These findings indicate that SB 203580 shows a very high degree of specificity for RK.

Another compound, PD 98059 (Figure 1), inhibits the activation of MAPKK-1 by c-Raf *in vitro* ($IC_{50} = 4 \mu$ M) and exerts its effect by binding to MAPKK-1, which may explain its exquisite specificity. PD 98059 does not inhibit the phosphorylation of myelin basic protein or casein by c-Raf, and does not inhibit the active (phosphorylated) forms of MAPKK-1 and MAPKK-2, nor does it inhibit 20 other protein serine/threonine kinases and protein tyrosine kinases tested [15]. These include other MAPKK homologues, such as SEK1 (also known as MKK4), which is thought to be an activator of SAP kinase *in vivo* [16,17], or the upstream activator of RK in KB cells (RK kinase), which is distinct from SEK1 (A. Cuenda, unpublished work). PD 98059 also failed to prevent the activation of SEK1 *in vitro* [15] by its activator MEK kinase [17–20] and did not affect the activation of SEK1 induced by osmotic shock in PC12 cells, or the activation of SAP kinase induced by IL1 in KB cells [15]. Similarly PD 98059 did not prevent the activation of RK kinase or MAPKAP kinase-2 by IL1 in KB cells. Moreover, PD 98059 also had no effect on the activation of p70 S6 kinase by EGF or insulin in Swiss 3T3 cells [15]; this enzyme lies in a distinct growth-factor-stimulated signal-transduction pathway. However, consistent with its effect *in vitro*, PD 98059 inhibited (by 80–90%) the activation of MAPKK-1 by insulin, IGF1, EGF, PDGF and PMA in Swiss 3T3 [15] and the activation of MAPKK-1 by insulin and IGF1 in L6 cells (D. A. Cross and P. Cohen, unpublished work). PD 98059 completely blocked the activation of p42 MAP kinase and MAPKAP kinase-1 by insulin (a weak activator of MAPKK-1) in Swiss 3T3 or L6 cells ([15]; D. A. Cross and P. Cohen, unpublished work), but only caused partial inhibition of the activation of p42 MAP kinase and MAPKAP kinase-1 by EGF, PDGF and PMA (which are strong activators of c-Raf and MAPKK-1) in Swiss 3T3 cells [15].

The finding that SB 203580 and PD 98059 are specific inhibitors of RK and the activation of MAPKK-1, respectively, suggested that they might be useful for probing the physiological roles of the classical MAP kinase pathway and the stress- and cytokine-activated RK pathway. Indeed, the class of pyridinylimidazoles, which include SB 203580, were originally found to be inhibitors of the synthesis of IL1 and TNF by LPS in monocytes before RK was identified as their intracellular target [22]. Here we use these inhibitors to demonstrate that the stimulation of glucose uptake, an important early response to growth factors and cytokines, requires activation of the classical MAP kinase pathway or the RK pathway, depending on the nature of the extracellular agonist.

MATERIALS AND METHODS

Materials

Sheep antibodies that immunoprecipitate MAPKAP kinase-2 were raised in the Scottish Antibody Production Unit (Carlisle,

Ayrshire, Scotland) against the peptide MTSALATMRVDYE-QIK, which corresponds to a sequence towards the C-terminus of human MAPKAP kinase-2 [23] and affinity-purified on a peptide-CH-Sepharose column by Dr. N. Morrice at the University of Dundee. Anti-(p42 MAP kinase) antibody (antisera 122) was kindly provided by Dr. C. J. Marshall (Institute for Cancer Research, London, U.K.). SB 203580 was a gift from Dr. J. Lee (SmithKline Beecham, King of Prussia, PA, U.S.A.) and PD 98059 was a gift from Dr. A. Saltiel, Parke-Davis, Detroit, MI, U.S.A.). Human IL1 α and human IGF1 were purchased from Boehringer (Lewes, East Sussex, U.K.) and anisomycin from Sigma Chemical Co. (Poole, Dorset, U.K.).

Cell culture and protein kinase assays

KB cells were cultured, exposed to different agonists and lysed in the presence of protein phosphatase inhibitors as described in [7]. p42 MAP kinase was immunoprecipitated from the lysates and the immunoprecipitates washed and assayed as described in [24]. One unit of MAP kinase activity was that amount which incorporated 1 nmol of phosphate into myelin basic protein in 1 min. MAPKAP kinase-2 was immunoprecipitated in an identical manner and assayed as described in [23]. One unit of MAPKAP kinase-2 was that amount which incorporated 1 nmol of phosphate into the peptide KKLNRTLSVA in 1 min. The immunoprecipitation of MAPKAP kinase-2 was judged to be specific, because it did not occur if the antibody was first preincubated with the peptide immunogen.

Glucose-transport assays

Transport was determined by measuring the uptake of 2-deoxy-D-[2,6- 3 H]glucose as described in [25]. Confluent plates of KB cells were incubated for 3 h in serum-free Dulbecco's modified Eagle's medium prior to assay, then washed three times with 3 ml of Krebs-Ringer phosphate (KRP; 136 mM NaCl/4.7 mM KCl/1.25 mM $MgSO_4$ /1.25 mM $CaCl_2$ /5 mM NaH_2PO_4 , pH 7.4) at 37 °C, covered with 1 ml of KRP containing 1% BSA and placed on a 37 °C hotplate. Agonists/inhibitors were present in 1% BSA/KRP for the times indicated in the Tables. Uptake was initiated by the addition of 2-deoxyglucose (final concn. 100 μ M; 0.25 μ Ci/assay), and transport was determined by exposure to the labelled compound for 3 min. After this time, the cells were washed twice with a large volume of ice-cold PBS, solubilized by incubation with 1 ml of 1% Triton X-100, and the radioactivity associated with each well was determined by liquid-scintillation counting. Uptake values were corrected for non-specific association of 2-deoxyglucose with the cells by subtracting uptake measured in the presence of 10 μ M cytochalasin B, an inhibitor of glucose transport [26]. This value typically amounted to < 10% of the radioactivity associated with the cells in the absence of cytochalasin B. In control experiments, we have established that the uptake of 2-deoxyglucose is linear over a 30 min exposure to labelled 2-deoxyglucose (both in the presence or absence of agonists and inhibitors), indicating that transport of the sugar, and not its subsequent phosphorylation, is rate-limiting under these conditions.

RESULTS AND DISCUSSION

The cytokines IL1 and TNF stimulate several signal-transduction pathways in mammalian cells, but which pathway(s) is involved in mediating the different effects of these cytokines is unknown. In KB cells, IL1 triggers the activation of at least three protein kinase cascades, namely those leading to the activation of the

Table 1 Effect of SB 203580 and PD 98059 on the stimulation of deoxyglucose transport by IL1 and IGF1

KB cells were incubated for 1 h in the presence of 25 μ M SB 203580 or 50 μ M PD 98059 or in the absence of either inhibitor, then stimulated for a further 1 h in the absence (basal) or presence of 250 nM IGF1 and/or 20 ng/ml IL1 in the continued presence (or absence) of the inhibitors. Values are presented as mean $\pm$ S.D. of triplicate determinations and are from a representative experiment. Similar results were obtained in several different experiments. Values are expressed as a percentage increase over the basal rate, which was 388 pmol/min per 35 mm well in this experiment. *Significant difference from basal rate ($P < 0.05$). **Significant difference from rate in the absence of SB 203580. ***Not significantly different from rate in the absence of PD 98059.

	No inhibitor	SB 203580	PD 98059
Basal	1.0	0.80 $\pm$ 0.14	0.77 $\pm$ 0.21
IL1	2.1 $\pm$ 0.23*	0.79 $\pm$ 0.13**	1.76 $\pm$ 0.33***
IGF1	2.4 $\pm$ 0.13*	2.05 $\pm$ 0.20	1.01 $\pm$ 0.10**
IGF1 plus IL1	2.1 $\pm$ 0.21*		

MAP kinase homologues, termed p42 MAP kinase [27], SAP kinase [28] and RK [6,7], raising the question of which pathway is required for any given IL1 response. Recently, specific cell-permeant inhibitors of two of these kinase cascades have been described (see the Introduction), and prompted us to examine their effects on cellular processes which are known to be stimulated by IL1, as well as by growth factors which stimulate the classical MAP kinase pathway. In the present study we examined the effect of SB 203580 and PD 98059 on 2-deoxyglucose transport stimulated by IL1 and IGF1. IGF1 was selected in preference to other growth factors because its effects on glucose transport are well documented and because it is a relatively weak activator of the classical MAP kinase cascade. Consequently, the activation of p42 MAP kinase by IGF1 can be completely suppressed by PD 98059 in Swiss 3T3 cells [15] and L6 cells (D. A. Cross and P. Cohen, unpublished work). In contrast, the activation of p42 MAP kinase by EGF, PDGF or PMA is only partially inhibited [15]. KB cells were used because their responsiveness to IL1 and because many of the experiments used to establish the *in vivo* specificity of PD 98059 and SB 203580 had been carried out using this epithelial-cell line [7,15]. Moreover, the stimulation of glucose uptake is comparable with other cell lines in which growth-factor- and cytokine-stimulated sugar transport has been measured.

IL1 and IGF1 both increased the rate of 2-deoxyglucose uptake into KB cells, the maximal stimulation by either agonist being about 2-fold (Table 1). The effect of IL1 was blocked by SB 203580, whereas PD 98059 had little or no effect. In contrast, 2-deoxyglucose uptake stimulated by IGF1 was unaffected by SB 203580, but prevented by PD 98059. SB 203580 or PD 98059 only decreased the basal rate of glucose transport slightly (Table 1).

The ability of SB 203580 and PD 98059 to prevent the stimulation of glucose transport by IL1 and IGF1 respectively correlated with the extent to which IL1 and IGF1 were able to stimulate the RK pathway and the classical MAP kinase pathway respectively. IL1 was a potent activator of MAPKAP kinase-2, a physiological substrate of RK, but only activated p42 MAP kinase slightly (Table 2). Conversely, IGF1 stimulated a much larger increase in p42 MAP kinase activity, but was only a very weak activator of MAPKAP kinase-2 (Table 2). As expected, SB 203580 suppressed the activation of MAPKAP kinase-2, but did not prevent the activation of p42 MAP kinase by either agonist, while PD 98059 blocked the activation of p42 MAP kinase, but not the activation of MAPKAP kinase-2 (Table 2).

The finding that SB 203580 prevented the stimulation of glucose transport by IL1 prompted us to investigate whether another activator of the RK pathway would also stimulate 2-deoxyglucose uptake and, if so, whether stimulation would also be prevented by SB 203580. The protein-synthesis inhibitor anisomycin has been shown to be a potent activator of SAP kinase [1], even at concentrations below those which inhibit protein synthesis [29]. In the present study we found that MAPKAP kinase-2 was activated when KB cells were stimulated with anisomycin. Stimulation was prevented by SB 203580, but not by PD 98059 (Table 2). Anisomycin induced only a slight (Table 2) and very transient (results not shown) activation of p42 MAP kinase. Anisomycin stimulated the uptake of 2-deoxyglucose into KB cells similarly to IL1, and no further stimulation was observed if IL1 and anisomycin were combined. The effect of anisomycin on sugar uptake, like that of IL1, was prevented by SB 203580 (Table 3).

The concentration of PD 98059 which inhibits the IGF1-stimulated increase in 2-deoxyglucose uptake ($IC_{50} = 5 \mu$ M; results not shown) was similar to that which inhibits the activation of MAPKK-1 by c-Raf *in vitro* (results not shown). In contrast, the concentration of SB 203580 which inhibits the IL1-stimulated increase in 2-deoxyglucose uptake ($IC_{50} = 5 \mu$ M; results not shown) was 5–10-fold higher than that required to prevent the activation of MAPKAP kinase-2 by RK [7]. This suggests that RK activity must be inhibited by about 80% before the stimu-

Table 2 Effect of SB 203580 and PD 98059 on the stimulation of MAPKAP kinase-2 and p42 MAP kinase by IGF1, IL1 and anisomycin in human KB cells

KB cells were incubated for 1 h in the presence of 25 μ M SB 203580 or 50 μ M PD 98059 or in the absence of either inhibitor, then stimulated in the absence (control) or presence of 250 nM IGF1, 20 ng/ml IL1 or 50 ng/ml anisomycin in the continued presence (or absence) of the inhibitors. The cells were lysed, and p42 MAP kinase and MAPKAP kinase-2 immunoprecipitated from the cell lysates and assayed as described in the Materials and methods section. The cells were stimulated for the times found to produce maximal activation of each enzyme. These were 5 min with IGF1 and 30 min with IL1 (for both MAPKAP kinase-2 and p42 MAP kinase), 30 min with anisomycin for MAPKAP kinase-2 and 15 min with anisomycin for p42 MAP kinase.

	p42 MAPK (m-units/mg)		MAPKAP kinase 2 (m-units/mg)			
	No inhibitor	SB 203580	PD 98059	No inhibitor	SB 203580	PD 98059
Basal	22 $\pm$ 14	22 $\pm$ 12	20 $\pm$ 11	11 $\pm$ 2.5	5.2 $\pm$ 2.5	18 $\pm$ 3.8
IL1	54 $\pm$ 20	63 $\pm$ 10	29 $\pm$ 5	388 $\pm$ 39	12.5 $\pm$ 8	293 $\pm$ 45
IGF1	122 $\pm$ 15	160 $\pm$ 30	46 $\pm$ 18	41 $\pm$ 7	5.6 $\pm$ 3	20 $\pm$ 6
Anisomycin	42 $\pm$ 6	44 $\pm$ 5	25 $\pm$ 3	600 $\pm$ 50	20 $\pm$ 7	574 $\pm$ 16

Table 3 Effect of anisomycin and IL1 on the stimulation of 2-deoxyglucose transport in KB cells

The experiments were carried out as described in Table 1. After incubation for 1 h in the presence or absence of 25 μ M SB 203580, the cells were stimulated with 50 ng/ml anisomycin or 20 ng/ml IL1 for the times indicated, in the presence or absence of SB 203580. The results are the mean $\pm$ S.D. of triplicate determinations at each point. Similar results were obtained in a second separate experiment. * $P < 0.05$; ** $P > 0.05$.

	Incubation time (h)	SB 203580 (25 μ M)	Transport rate (pmol/min/35 mm well)
Basal	1	—	320 $\pm$ 29
	1	+	279 $\pm$ 31**
Anisomycin	1	—	510 $\pm$ 36*
	2	—	515 $\pm$ 20*
	1	+	242 $\pm$ 46**
	2	+	260 $\pm$ 21**
IL1	1	—	534 $\pm$ 44*
IL1 $\pm$ anisomycin	1	—	552 $\pm$ 28*

lation of deoxyglucose uptake is inhibited by 50 %. However, an alternative explanation that cannot be excluded is that the effect of IL1 on glucose transport is mediated by an RK homologue which is less sensitive to SB 203580.

Immunoblotting experiments have shown the presence of GLUT-1 in KB cells and revealed that its level is unaffected by IL1 in the presence or absence of SB 203580 or PD 98059 over the duration of the experiment in Table 1 (results not shown). Since no other glucose-transporter isoform could be detected in KB cell extracts by immunoblotting, and stimulating KB cells with IL1 plus IGF1 did not increase 2-deoxyglucose uptake any more than either agonist added alone (Table 1), it is likely that IL1 and IGF1 both exert their effects by activating pre-existing GLUT-1 molecules.

The effects of SB 203580 and PD 98059 demonstrate that the stimulation of 2-deoxyglucose uptake/GLUT-1 by IL1 and IGF1 requires the activation of distinct signal-transduction pathways. These are presumably the kinase cascades in which RK and p42 MAP kinase participate, although the precise molecular mechanisms by which these MAP kinase homologues exert their effects on sugar uptake remain to be elucidated. We have reported previously that glucose transport in *Xenopus* oocytes, also thought to be mediated by GLUT-1, is stimulated by micro-injection of Mos (an activator of MAPKK-1), by MAPKK-1 itself or by 42 MAP kinase itself [30], consistent with the effects of IGF1 reported here. However, the stimulation of glucose transport by insulin in adipocytes or in the skeletal-muscle cell line L6, which results from the translocation of GLUT-4 to the plasma membrane rather than from the activation of GLUT-1, is unaffected by PD 98059 [31]. Thus the classical MAP kinase pathway does not mediate the translocation of GLUT-4 induced by insulin.

Finally, it is important to emphasize that the requirement for activation of the classical MAP kinase pathway by IGF1 or the

requirement for activation of the RK pathway by IL1 and anisomycin to stimulate glucose transport does not exclude the involvement of other signal-transduction pathways in this process. It remains possible that more than one kinase cascade needs to be activated in order to trigger the stimulation of glucose transport by these agonists.

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