SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1

Ana Cuenda[a],[**], John Rouse[a],[**], Yair N. Doza[a], Roger Meier[a], Philip Cohen[a],[*],
Timothy F. Gallagher[b], Peter R. Young[b], John C. Lee[b]

[a]MRC Protein Phosphorylation Unit, Department of Biochemistry, University of Dundee, Dundee DD1 4HN, Scotland, UK
[b]SmithKline Beecham Pharmaceuticals, King of Prussia, PA 19406, USA

Abstract A class of pyridinyl imidazoles inhibit the MAP kinase homologue, termed here reactivating kinase (RK) [Lee et al. (1994) Nature 372, 739–746]. We now show that one of these compounds (SB 203580) inhibits RK in vitro ($IC_{50} = 0.6 \mu M$), suppresses the activation of MAPKAP kinase-2 and prevents the phosphorylation of heat shock protein (HSP) 27 in response to interleukin-1, cellular stresses and bacterial endotoxin in vivo. These results establish that MAPKAP kinase-2 is a physiological RK substrate, and that HSP27 is phosphorylated by MAPKAP kinase-2 in vivo. The specificity of SB 203580 was indicated by its failure to inhibit 12 other protein kinases in vitro, and by its lack of effect on the activation of RK kinase and other MAP kinase cascades in vivo. We suggest that SB 203580 will be useful for identifying other physiological roles and targets of RK and MAPKAP kinase-2.

Key words: MAP kinase; Protein kinase inhibitor; Cytokine; Osmotic stress; LPS; HSP27

1. Introduction

We have previously identified and characterised a protein kinase which is active only after phosphorylation by the p42 or p44 isoforms of mitogen-activated protein (MAP) kinase [1,2] and termed it MAP kinase-activated protein (MAPKAP) kinase-2 to distinguish it from the RSK family of ribosomal protein S6 kinases (MAPKAP kinases-1) which are also activated by MAP kinase. However, we recently made the surprising finding that MAPKAP kinase-2 is not activated in vivo by growth factors which are potent activators of p42/p44 MAP kinases, but instead by chemical or osmotic stress and heat shock [3]. These cellular stresses stimulate a distinct MAP kinase homologue, termed the 'reactivating kinase' (RK), which also activates MAPKAP kinase-2 in vitro [3]. Other laboratories also identified the RK in a B-cell line transfected with the bacterial endotoxin (LPS) receptor and in murine macrophages where it is activated in response to LPS (termed p38 in [4]), and in KB cells where it is activated by interleukin-1 (IL-1) (termed p40 in [5]).

The amino acid sequence of the RK [3,4] is most similar to HOG-1, a MAP kinase homologue which lies in a signalling pathway that restores the osmotic gradient across the cell membrane of S. cerevisiae in response to increased external osmolar-

ity [6]. These enzymes are distinguished by the sequence TGY in the activation domain, which differs from the TEY sequence found in p42 and p44 MAP kinases and the TPY sequence found in the MAP kinase homologue JNK. JNK is activated in vivo by similar stimuli to those which activate the RK, including heat shock [8], osmotic stress [9] and IL-1 [10] and one of its cellular roles is to activate the transcription factor c-Jun [7,8]. A MAP kinase kinase homologue capable of activating both the RK and JNK in vitro has recently been identified [11,12] This enzyme is most similar to PBS2, which lies above HOG-1 in the yeast osmoregulation pathway.

Two homologues of the RK, termed CSAID binding proteins 1 and 2 (CSBP1, CSBP2), have recently been identified in human monocytes as proteins that interact specifically with, and are inhibited by, CSAIDs, a novel class of pyridinyl imidazoles ([13]; Fig. 1). These drugs inhibit the LPS-induced synthesis of inflammatory cytokines (IL-1 and tumour necrosis factor (TNF)) at the translational level [13,14] and may be useful in the treatment of inflammatory diseases, such as rheumatoid arthritis. CSBP2 is the human homologue of murine RK [4,13], while the nucleotide and amino acid sequence of CSBP1 is identical, except for 75 nucleotides corresponding to amino acid residues 230–254. Here we report that the pyridinyl imidazole SB 203580 is a specific inhibitor of the RK in vitro, which prevents the activation of MAPKAP kinase-2 and the phosphorylation of HSP27 in vivo by cellular stresses, IL-1 and LPS. These results suggest that SB 203580 is a powerful new tool to identify physiological roles of the RK signalling pathway.

2. Materials and methods

2.1. Materials

IL-1 was purchased from Boehringer, and HSP27 protein and anti-HSP27 antibodies from Stressgen. Anti-MAPKAP kinase-2 antibodies were raised in sheep against a mixture of three peptides HVKSGLQI-KKNA, KEDKERWEDVKEEMTS and STKVPQTPLHTSR corresponding to sequences found in human MAPKAP kinase-2 [2] and affinity purified on a peptide-Affigel 15 column. CSBP-2 was expressed in E. coli as a His-tagged fusion protein, purified on nickel nitrilotriacetate-agarose [13] and activated with the RK kinase (RKK) from arsenite-stimulated PC12 cells [3]. p42 MAP kinase [1] and the first 194 residues of c-Jun were expressed in E. coli as GST fusion proteins and purified on glutathione-Sepharose. Beads containing 0.35 mg GST-cJun per ml of glutathione-Sepharose were stored at −20°C. Pyridinyl-imidazoles were synthesized at SmithKline Beecham (T. Gallagher et al., in preparation) and dissolved in sterile dimethylsulphoxide (Sigma) to give 20 mM solutions.

2.2. Partial purification and assay of stress-activated protein kinases

PC12 [15], HeLa [3], THP-1[13] and KB cells [5] were cultured, exposed to cellular stresses or cytokines, lysed in the presence of protein

SB203580
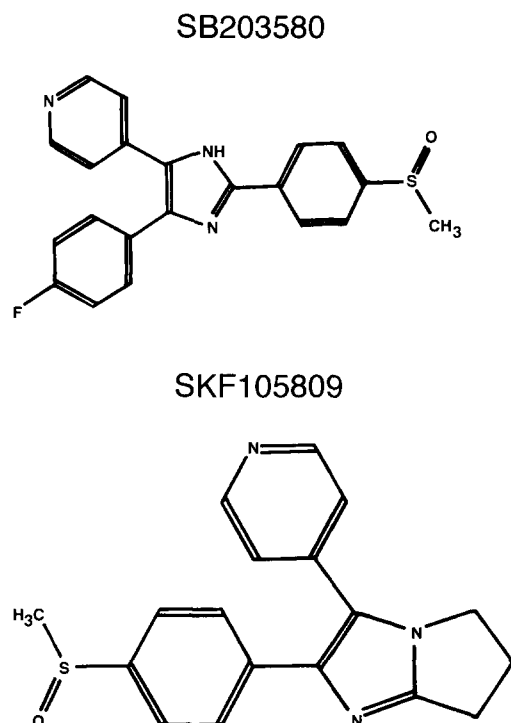
SKF105809

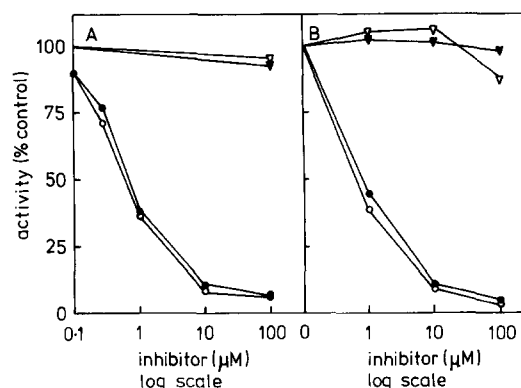Fig. 1. The structures of SB 203580 and SK&F 105809.

Fig. 3. Effect of pyridinyl imidazoles on RK activity in vitro. (A) Effect of SB 203580 (circles) and SK&F 105809 (triangles) on RK activity from chemically stressed (0.5 mM sodium arsenite) PC12 cells (closed symbols) and on bacterially expressed human CSBP-2 (open symbols) which had been activated with RKK from arsenite-stimulated PC12 cells. (B) Effect of SB 203580 on the RK from heat-shocked (10 min, 45°C) HeLa cells (closed circles) and osmotically shocked (5 min with 0.5 M sorbitol) PC12 cells (open circles), and on bacterially expressed p42 MAP kinase activated in vitro by MAP kinase kinase (closed triangles) and JNK from arsenite-stimulated PC12 cells (open triangles).

phosphatase inhibitors and chromatographed on Mono Q as in [3]. The major peak of c-Jun kinase (JNK) eluted at 60–70 mM NaCl in fractions 4/5 (data not shown), the RKK at 170–180 mM NaCl in fractions 8–10 and the RK at 0.35 M NaCl in fractions 19/20 [3]. MAPKAP kinase-2 is not retained by Mono Q and was partially purified by chromatographing the flowthrough fractions on Mono S [3].

The RK and activated CSBP2 were assayed by the activation of MAPKAP kinase-2 [3] and its drug binding activity by photoaffinity labelling with radiolabelled SB 206718 [13]. MAPKAP kinase-2 was assayed using the synthetic peptide substrate KKLNRTLSVA [2] and JNK with GST-cJun [16]. c-Raf, MAPKK and p42^{mapk} were assayed as in [17], p90 and p70 S6 kinases as in [18]. Protein kinase A and casein kinase-2 were assayed using the synthetic peptides LRRASVA and RRREEESEEE as described for MAPKAP kinase-2 and phosphorylase kinase as in [19]. Cyclin-dependent protein kinases were assayed with histone H1 [20] and protein phosphatases 1 and 2A as in [21].

2.3. HSP27 phosphorylation in KB and PC12 cells

Confluent cells grown on 6 cm diameter dishes were washed four times with phosphate-free DMEM containing 10% dialysed foetal calf serum, then incubated for 3 h in the same medium supplemented with 0.5 mCi (KB) or 0.3 mCi (PC12) of ^{32}P-labelled orthophosphate before exposure to cellular stresses, growth factors or cytokines. After stimulation, each dish of cells was washed three times with ice-cold 20 mM sodium phosphate, pH 7.4, 0.15 M NaCl and lysed in 0.4 ml of 20 mM

Tris acetate, pH 7.0 (20°C), 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 1% (by mass) Triton X-100, 10 mM sodium β-glycerophosphate, 25 mM sodium fluoride, 2.5 mM sodium pyrophosphate, 0.1% (by vol.) 2-mercaptoethanol, 1 mM benzamidine and 4 μg/ml leupeptin. After centrifugation for 5 min at 13,000 × g, the supernatant from PC12 cells (10 μl) was electrophoresed on 10% polyacrylamide gels and autoradiographed to locate HSP27. For KB cells, 0.05 ml aliquots were incubated for 2 h at 4°C with 0.01 ml of 50% (by mass) Protein G-Sepharose (Pharmacia) conjugated to 10 μg of anti-HSP27 antibody. The suspension was centrifuged for 1 min at 13,000 × g, the supernatant discarded and the antibody/Protein G-Sepharose pellet washed three times with lysis buffer containing 0.5 M NaCl and twice in lysis buffer. After denaturation in 1% SDS and heating for 5 min at 100°C, the samples were electrophoresed on 15% polyacrylamide gels and autoradiographed to localise HSP27.

3. Results

3.1. SB 203580 is a specific inhibitor of the RK in vitro

The structures of two pyridinyl imidazoles employed in this study are shown in Fig. 1. SB 203580 is a potent inhibitor of LPS-induced cytokine synthesis in the human monocyte cell line THP-1 (IC$_{50}$ = 50–100 nM), while the analogue SK&F 105809 does not inhibit cytokine synthesis.

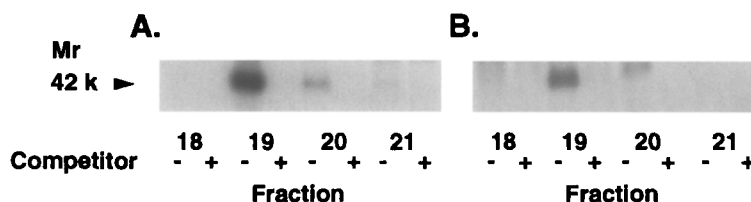The RK was partially purified from arsenite-stimulated PC12

Fig. 2. SB 206718, a radiolabelled photoaffinity analogue of SB 203580, binds covalently to the RK. Lysates from arsenite-stimulated PC12 cells (A) or heat-shocked (10 min, 45°C) HeLa cells (B) were chromatographed on Mono Q and, as described previously, RK activity was detected only in fractions 19 and 20 [3]. Aliquots of the 40 fractions from each column (0.5 ml) were incubated with SB 206718, electrophoresed on SDS/polyacrylamide gels and autoradiographed [13]. A single radiolabelled band co-migrating with the RK (42 kDa) was detected in fractions 19 and 20, and binding was specific because an excess of unlabelled ligand (+) abrogated the radiolabelled band.

Fig. 4. SB 203580 inhibits the in vivo activation of MAPKAP kinase-2 by a variety of stimuli. (A) PC12 cells were incubated for 15 min in the absence (open symbols) or presence (closed symbols) of 25 μM SB 203580 and then for 15 min with 0.5 mM sodium arsenite in the continued presence of SB 203580. The cells were lysed, and the lysates (8 mg protein) chromatographed on 5 × 0.5 cm column of Mono S as in [3]. The flow rate was 1 ml/min and fractions of 1.0 ml were collected and assayed for MAPKAP kinase-2 activity using the peptide KKLNRTLSVA. The NaCl gradient is shown by the broken line. (B) THP-1 cells were incubated for 60 min in the absence (open circles) or presence (closed circles) of 10 μM SB 203580 and then for 15 min with 100 ng/ml LPS in the continued presence of the inhibitor. The cells were lysed and 0.3 mg chromatographed on a 5 × 0.16 cm column of Mono S using a Pharmacia Smart System. The flow rate was 0.1 ml and fractions of 0.1 ml were collected and assayed for MAPKAP kinase-2 as in (A). The open triangles show an experiment in which both SB 203580 and LPS were omitted. (C) KB cells were stimulated for 15 min with 0.5 mM sodium arsenite or 20 ng/ml IL-1 or 0.5 M sorbitol. After lysis, 0.3 mg protein was chromatographed on Mono S as in (B), and the activity associated with major peak of MAPKAP kinase-2 was quantitated (filled bars). The hatched bars show experiments in which cells were incubated for 1 h with 10 μM SB 203580 prior to stimulation and the open bars the results from unstimulated cells not preincubated with SB 203580. The results are shown ±S.E.M. for 3 experiments.

→
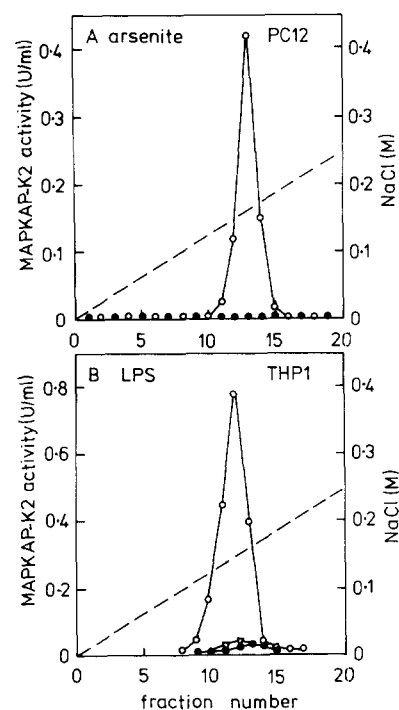
cells and heat-shocked HeLa cells by chromatography on Mono Q, eluting in fractions 19 and 20 at 0.35 M NaCl as described previously [3]. The fractions from each column were incubated with a radiolabelled photoaffinity ligand SB 206718, an analogue of SB 203580 [13]. Upon photoactivation, a single competable radiolabelled band of the expected molecular mass (42 kDa) was detected in fractions 19 and 20 from both columns (Fig. 2A,B), and in none of the other 40 fractions, further establishing the identity of RK and CSBP.

SB 203580 inhibited the RK from heat-shocked HeLa cells (Fig. 3A), osmotically stressed PC12 cells (Fig. 3B) or bacterially expressed CSBP-2 (Fig. 3A) with an IC_{50} of 0.6 μM. SB 203580 (0.1 mM) did not inhibit 12 other protein kinases tested in vitro, including the MAP kinase homologues JNK and p42 MAP kinase (Fig. 3B, Table 1), nor did it inhibit two protein serine/threonine phosphatases (Table 1). SK&F 105809, an analogue of SB 203580 (Fig. 1), which does not inhibit LPS-induced IL-1 and TNF synthesis in monocytes, did not inhibit the RK in vitro (Fig. 3A).

3.2. SB 203580 prevents the activation of MAPKAP kinase-2 in vivo

Preincubation with SB 203580 prevented the activation of MAPKAP kinase-2 by arsenite in PC12 cells (Fig. 4A) and by LPS in THP-1 cells (Fig. 4B). The same concentration of SB 203580 (25 μM) did not inhibit the activation of the RK kinase (the upstream activator of RK) by arsenite in PC12 cells or by IL-1 in KB cells. SB 203580 (25 μM) also did not inhibit the activation of JNK by arsenite in PC12 cells or by IL-1 in KB cells, or the activation of p42 MAP kinase by NGF in PC12 cells or EGF in KB cells (data not shown).

SB203580 strongly suppressed the activation of MAPKAP kinase-2 by IL-1, osmotic stress or arsenite in KB cells (Fig. 4C). The MAPKAP kinase-2 activity induced by all three stimuli eluted from Mono S as one major peak at the same NaCl concentration as MAPKAP kinase-2 from PC12 or THP1 cells

and could be immunoprecipitated quantitatively by anti-MAPKAP kinase-2 antibodies (data not shown).

3.3. SB 203580 prevents the phosphorylation of heat shock protein 27 by cellular stresses or IL-1

MAPKAP kinase-2 phosphorylates the small heat shock proteins (hereafter termed HSP27) in vitro at the same serine residues [22] which are phosphorylated in vivo in response to heat shock, IL-1 and other stimuli which activate MAPKAP kinase-2 [5,23,24]. Moreover, HSP27 kinase activity co-purifies with MAPKAP kinase-2 from skeletal muscle [22] and the major HSP27 kinase activity induced by IL-1 in KB cells co-elutes with MAPKAP kinase-2 on Mono S and is immunopre-

cipitated quantitatively and specifically by HSP27 antibodies (data not shown).

The in vivo phosphorylation state of HSP27 was low in unstimulated KB cells, but increased strikingly after stimulation with IL-1 or exposure to chemical or osmotic stress (Fig. 5A). Prior incubation of cells with SB 203580 suppressed the phosphorylation of HSP27 by every stimulus with an IC_{50} value of <1 μM (Fig. 5A). The phosphorylation of HSP27 induced by arsenite in PC12 cells was also prevented by SB 203580 (Fig. 5B). The effect of SB 203580 on the phosphorylation of HSP27 was highly specific because this drug had no effect on the phosphorylation state of any of the major ^{32}P-labelled proteins in PC12 and KB cells that were resolved by one-dimensional SDS/polyacrylamide gel electrophoresis (data not shown).

3.4. Phosphorylation of HSP27 in PC12 cells is not induced by NGF

The results presented in section 3.2 and [3] indicated that MAPKAP kinase-2 was activated by the RK in vivo, and not by p42/p44 MAP kinase. In this case, an agonist like NGF in PC12 cells, which strongly activates p42/p44 MAP kinases but does not activate MAPKAP kinase-2, should not stimulate the phosphorylation of HSP27. The results shown in Fig. 5C show that this is indeed the case. In contrast, the expected increase in HSP27 phosphorylation occurred in response to arsenite in a parallel experiment (Fig. 5B).

4. Discussion

In this paper we show that SB 203580 is a remarkably specific inhibitor of the RK in vitro (Table 1, Fig. 3) and that it does not prevent the activation of RK kinase or other MAP kinase pathways in vivo. We have therefore exploited the specificity of this compound to establish that MAPKAP kinase-2 is a physiological substrate of the RK (Fig. 4) and that HSP27 is a physiological target for MAPKAP kinase-2 (Fig. 5). Thus the stimuli which activated MAPKAP kinase-2 also promoted the phosphorylation of HSP27, and SB 203580, which prevented the activation of MAPKAP kinase-2 by each agonist, also blocked the phosphorylation of HSP27 in vivo (Fig. 5). NGF, a potent activator of p42 and p44 MAP kinases in PC12 cells [15], does not activate MAPKAP kinase-2 [3] and did not stimulate HSP27 phosphorylation (Fig. 5C). This finding, in conjunction with the suppression of HSP27 phosphorylation by SB 203580, demonstrates that p42 and p44 MAP kinases are not required for the activation of MAPKAP kinase-2 or the phosphorylation of HSP27 in the cells we have studied so far. The role of HSP27 phosphorylation is not yet established, but recent evidence suggests that it may promote actin polymerisation and so counteract the disruptive effects of stress on actin microfilament structure, thereby aiding cell survival [25].

SB 203580 was originally discovered as an inhibitor of LPS-induced cytokine synthesis in THP-1 monocytes [13]. The observations that LPS activates MAPKAP kinase-2 in these cells and that this is prevented by SB 203580 (Fig. 4B) is therefore of particular interest, since it implies that the protein phosphorylation event(s) which triggers cytokine synthesis may be catalysed by either the RK or by MAPKAP kinase-2. The concentration of SB203580 which inhibits LPS-induced cytokine synthesis by 50% in THP-1 cells (50–100 nM) is somewhat

lower than that found to inhibit activation of MAPKAP kinase-2 in vitro in the present study. The most likely explanation for this apparent discrepancy is that prolonged incubation with SB 203580 leads to its accumulation in cells. The assays for inhibition of cytokine synthesis in monocytes involve an 18 h incubation with SB 203580 [13], and indeed we have found that if THP-1 cells are incubated for 18 h with SB 203580 prior to stimulation with LPS (instead of the brief 15–60 min incubations employed throughout this study), the IC_{50} for inhibition of MAPKAP kinase-2 activation is reduced to about 100 nM (A. Cuenda, unpublished observations).

IL-1 was shown previously to activate a protein kinase cascade in KB cells in which an HSP27 kinase (termed p50) is activated by another protein kinase (p40) [5]. The sequences of several peptides from p40 identified it as the RK, but the relationship of IL-1-activated p50 to the stress-activated MAPKAP kinase-2 was uncertain. Here we have established that p50 is either MAPKAP kinase-2 or a very closely related isoform. IL-1 and cellular stresses both induced a MAPKAP kinase-2 activity in KB cells (Fig. 4C) which coeluted during Mono S chromatography with HSP27 kinase and which was immunoprecipitated with anti-MAPKAP kinase-2 antibodies (data not shown). Moreover, the anti-MAPKAP kinase-2 antibodies recognised a single 50 kDa species in human KB cell extracts which coeluted with MAPKAP kinase-2 on Mono S (data not shown). Finally, the activation of MAPKAP kinase-2 in this cell line by either IL-1 or cellular stresses was suppressed by SB 203580 (Fig. 4C).

The vast array of protein kinases in mammalian cells, which are all members of the same superfamily, has led many to doubt whether compounds that are really specific inhibitors of a single protein kinase can be synthesized. However, the recent identification of rapamycin as a specific inhibitor of the activation of p70 S6 kinase [26], the synthesis of a potent and specific inhibitor of the EGF receptor kinase [27] and the present finding that

Table 1
Effect of SB 203580 on the activities of protein kinases and phosphatases

Protein kinase	SB 203580 Concn. (μM)	Activity (% control)
RK/p38	1	35 ± 1
RK/p38	10	5 ± 1
MAPKAP kinase-2	100	92 ± 3
JNK/SAP kinase	100	86 ± 4
p42 MAP kinase	100	97 ± 3
MAP kinase kinase	100	93 ± 4
c-Raf	100	87 ± 2
p90 S6 kinase	100	103 ± 2
p70 S6 kinase	100	97 ± 4
protein kinase A	100	99 ± 2
phosphorylase kinase	100	97 ± 1
cyclin A/cdk2	100	91 ± 4
cyclin E/cdk2	100	112 ± 6
casein kinase-2	100	99 ± 2
protein phosphatase-1	100	97 ± 4
protein phosphatase 2A	100	106 ± 6

Activities are given ± S.E.M. for three separate determinations relative to control incubations in which the inhibitor was omitted. We thank colleagues in the MRC Protein Phosphorylation Unit (Dario Alessi, Craig Jones, Ian Leighton and Robert MacKintosh for carrying out the assays of c-Raf, cyclin-dependent protein kinases, p70 S6 kinase and protein phosphatases, respectively, and Ted Hupp (Biochemistry Department, University of Dundee) for supplying casein kinase-2.
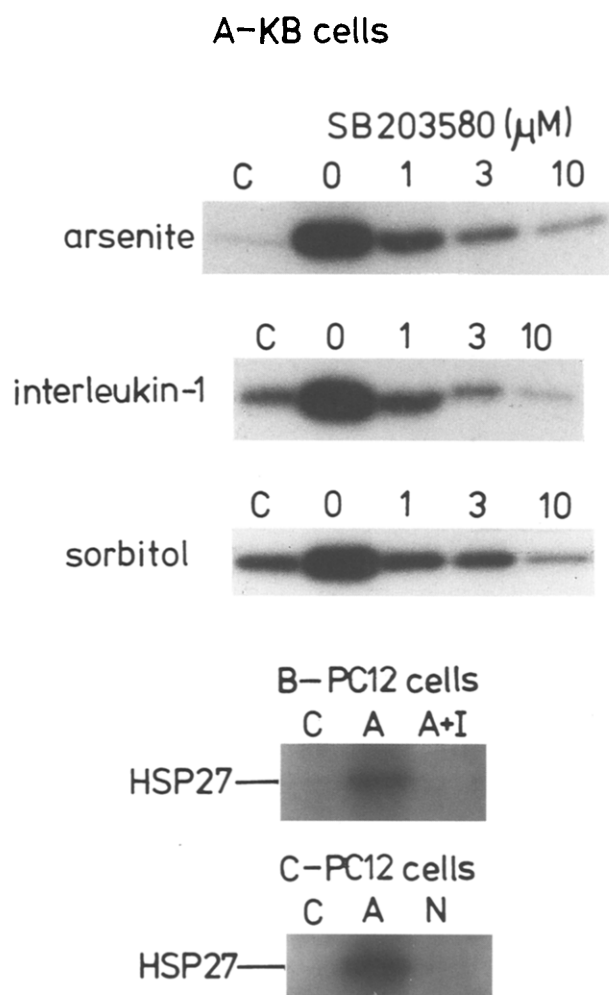
A–KB cells

SB 203580 (μM)

Fig. 5. SB 203580 blocks the phosphorylation of HSP27 induced by several agonists in KB and PC12 cells. Cells were incubated with [³²P]orthophosphate, and for 1 h with the indicated concentrations of SB 203580 and stimulated as described below. (A) KB cells were stimulated for 20 min with 0.5 mM sodium arsenite or 20 ng/ml IL-1, or for 15 min with 0.5 M sorbitol. The cells were washed and lysed, and HSP27 immunoprecipitated, then denatured in SDS and electrophoresed on 15% polyacrylamide gels. The figure shows autoradiographs of the gels. The lanes marked C (control) show the labelling of HSP27 in unstimulated cells where SB 203580 was omitted. The different intensities of the lanes marked C is not due to a variable basal level of HSP27 phosphorylation in different experiments, but to different lengths of autoradiography. (B) PC12 cells were incubated for 30 min in the absence or presence of 10 μM SB 203580 (I, inhibitor) and then stimulated for 15 min with arsenite in the continued presence of the inhibitor (A). SB 203580 and arsenite were omitted in a control (C) experiment. After lysis, aliquots were electrophoresed on 10% polyacrylamide gels and autoradiographed. (C) Same as B except that PC12 cells were stimulated for 15 min with 50 ng/ml NGF (N) or 0.5 mM sodium arsenite (A) or buffer (C, control).

SB 203580 is a specific inhibitor of the RK, has demonstrated that highly specific inhibitors can indeed be developed. We suggest that SB 203580 will be useful for identifying novel physiological roles and substrates of the RK and MAPKAP kinase-2.

References

[1] Stokoe, D., Campbell, D.G., Nakielny, S. Hidaka, H., Leevers, S., Marshall, C. and Cohen, P. (1992) EMBO J. 11, 3985–3994.
[2] Stokoe, D., Caudwell, F.B., Cohen, P.T.W. and Cohen, P. (1993) Biochem. J. 296, 842–849.
[3] Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Llamazares, A., Zamanillo, D., Hunt, T. and Nebreda, A. (1994) Cell 78, 1027–1037.
[4] Han, J., Lee, J.D., Bibbs, L. and Ulevitch, R.J. (1994) Science 265, 808–811.
[5] Freshney, N.W., Rawlinson, L., Guesdon, F., Jones, E., Cowley, S., Hsuan, J. and Saklatvala, J. (1994) Cell 78, 1039–1049.
[6] Brewster, J.L., de Valoir, T., Dwyer, N.D., Winter, E. and Gustin, M.C. (1993) Science 259, 1760–1762.
[7] Derijard B., Hibi, M., Wu, I.-H., Barrett, T., Su, B., Deng, T., Karin, M. and Davis, R.J. (1994) Cell 76, 1025–1037.
[8] Kyriakis, J.M., Banarjee, P., Nikolakaki, E., Dai, T., Rubie, E.A., Ahmad, M.F., Avruch, J. and Woodgett, J.R. (1994) Nature 369, 156–160.
[9] Galcheva-Gargova, Z., Derijard, B., Wu, I.-H. and Davis, R.J. (1994) Science 265, 806–808.
[10] Kracht, M., Truong, O., Totty, N.F., Shiroo, M. and Saklatvala, J. (1994) J. Exp. Med. 180, 2017–2025.
[11] Sanchez, I., Hughes, R.T., Mayer, B.J., Yee, K., Woodgett, J.R., Avruch, J., Kyriakis, J.M. and Zon, L.I. (1994) Nature 372, 794–798.
[12] Derijard, B., Raingeaud, J., Barrett, T., Wu, I.-H, Han, J., Ulevitch, R.J. and Davis, R.J. (1995) Science 267, 682–684.
[13] Lee, J.C., Laydon, J.T., McDonnell, P.C., Gallagher, T.F., Kumar, S., Green, D., McNulty, D., Blumenthal, M.J., Heys, J.R., Landvatter, S.W., Strickler, J.E., McLaughlin, M.M., Siemens, I., Fisher, S., Livi, G.P., White, J.R., Adams, J.L. and Young, P.R. (1994) Nature 372, 739–746.
[14] Lee, J.C., Badger, A.M., Griswold, D.E., Dunnington, D., Truneh, A., Votta, B., White, J.R., Young, P.R. and Bender, P.E. (1993) Ann. NY Acad. Sci. 696, 149–170.
[15] Gomez, N., Tonks, N.K., Morrison, T., Harmar, A.R. and Cohen, P. (1990) FEBS Lett. 271, 119–122.
[16] Hibi, M., Lin, A., Smeal, T., Minden, A. and Karin, M. (1993) Genes and Development 7, 2135–2148.
[17] Alessi, D.R., Cohen, P., Ashworth, A, Cowley, S., Leevers. S.J. and Marshall, C.J. (1995) Methods Enzymol. (in press).
[18] Lavoinne, A., Erikson, E., Maller, J.L., Price, D.J., Avruch, J. and Cohen, P. (1991) Eur. J. Biochem. 199, 723–728.
[19] Smythe, C.W. and Newport, J. (1991) Methods Cell Biol. 35, 449–468.
[20] Cohen, P. (1988) Methods Enzymol. 99, 243–249
[21] Cohen, P., Alemany, A., Hemmings, B.A., Resink, T.J., Stralfors, P. and Tung, H.Y.L. (1988) Methods Enzymol. 159, 390–408.
[22] Stokoe, D., Engel, K., Campbell, D.G., Cohen, P. and Gaestel, M. (1992) FEBS Lett. 313, 307–313.
[23] Hepburn, A., DeMolle, D., Boeynaems, J.M., Fiers, W. and Dumont, J.E. (1988) FEBS Lett. 227, 175–178.
[24] Landry, J., Lambert, H., Zhou, M., Lavoie, J.N., Hickey, E., Weber, L.A. and Anderson, C.W. (1992) J. Biol. Chem. 267, 794–803.
[25] Lavoie, J.N., Lambert, H., Hickey, E., Weber, L.A. and Landry, J. (1995) Mol. Cell. Biol. 15, 505–516.
[26] Chung, J., Kuo, C.J., Crabtree, G.R. and Blenis, J. (1992) Cell 69, 1227–1236.
[27] Fry, D.W., Kraker, A.J., McMichael, A., Ambroso, L.A., Nelson, J.M., Leopold, W.R., Connors, R.W. and Bridges, A.J. (1994) Science 265, 806–808.