Specific activation of β -casein kinase by the inflammatory cytokines interleukin 1 and tumour necrosis factor

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Increases (5-fold) in the rate of phosphorylation of β -casein were observed in extracts of human gingival fibroblasts that had been stimulated by interleukin 1 (IL-1) or tumour necrosis factor (TNF). The induced kinase was cytosolic and had little activity on α -casein. Its chromatographic behaviour on anion-exchange and gel-filtration columns was similar to that of β -casein kinase, an enzyme detected originally in MRC-5 cells stimulated by IL-1 and TNF. Phosphopeptide maps of β -casein confirmed that the kinase activated in gingival fibroblasts had the same substrate specificity as β -casein kinase. In gingival fibroblasts, β -casein kinase activity was maximum after 15 min of stimulation by IL-1 or TNF, and remained activated for several hours. Activations of small heat-shock protein (hsp27) kinase and mitogen-activated

protein (MAP) kinase were also maximum 15 min after stimulation, but decreased to background levels within the next 30 min. Study of the effects of 21 agents other than IL-1 or TNF showed that none activated β -casein kinase, whereas several activated MAP kinase or hsp27 kinase. β -Casein kinase was also detected in extracts of bovine articular chondrocytes and human endothelial cells stimulated by IL-1 or TNF. Semi-purified preparations of fibroblast β -casein kinase were not inactivated by phosphatases *in vitro*. Our results suggest that it may be involved in responses specific to IL-1 and TNF in a wide range of cell types and that its activation probably involves mechanisms other than its phosphorylation.

INTRODUCTION

Interleukin 1 (IL-1) and tumour necrosis factor (TNF) are major inflammatory cytokines which mediate many local and systemic features of inflammation. Each can cause endothelial cell activation, leucocyte accumulation, fever and the acute-phase response [1,2]. These responses involve the production by the target cells of prostaglandins and proteins whose nature depends on the cell type. Despite their functional similarities, IL-1 and TNF are structurally unrelated and bind to distinct receptors [1,3].

The signal-transduction pathways triggered by IL-1 and TNF are still unclear. However, there is ample evidence that, in many cell types, the cytokines increase within minutes the phosphorylation of certain proteins [4–12], of which the most extensively studied has been the small heat-shock protein, hsp27 [4–6,9,12]. More recently, IL-1 and TNF have been shown to activate several protein kinases [12–21]. Two which have been definitely identified are the mitogen-activated protein (MAP) kinase [15–18] and its activator MAP kinase kinase [18]. MAP kinase is activated via phosphorylation by its activator in response to a wide range of stimuli other than IL-1 or TNF, such as phorbol myristate acetate (PMA), insulin, nerve growth factor, epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) [22]. It is able to activate directly other protein kinases and may also phosphorylate transcription factors and the arachidonic acid-producing enzyme, phospholipase A_2 [22].

We have previously reported the activation by IL-1 and TNF of two cytosolic protein kinases distinct from MAP kinase and

its activator in MRC-5 fibroblasts. One is a hsp27 kinase [13,16], and the other phosphorylates *in vitro* the bovine milk protein β -casein [16]. Both enzymes may be novel. hsp27 kinase is presumably involved in the phosphorylation of hsp27, which is believed to be a chaperonin [23] and an inhibitor of actin polymerization [24]. The function of β -casein kinase is less clear. It phosphorylates β -casein on serine and threonine residues distinct from those targeted by the constitutively active casein kinases and, unlike them, has little activity on α -casein [16]. Its physiological substrates and its mechanisms of activation are not known.

We have now investigated the possible significance of β -casein kinase in cytokine signalling by studying its activation by potential stimuli in different cell types in comparison with MAP kinase and hsp27 kinase. Our results show that β -casein kinase is activated by IL-1 or TNF not only in fibroblasts, but also in primary cultures of endothelial cells and articular chondrocytes. In contrast with MAP kinase and hsp27 kinase, which are activated by many stimuli, β -casein kinase seems to be specifically activated by IL-1 or TNF. Our results suggest that it belongs to a signalling pathway activated exclusively by the two cytokines in connective-tissue cells.

EXPERIMENTAL

Reagents

Human hsp27 was purified from cultured ME-180 cells as described previously [16] except that chromatofocusing was omitted. Recombinant human IL-1 α , TNF- α and MAP kinase

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; hsp27, small heat-shock protein; IL-1, interleukin 1; MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein; PDGF, platelet-derived growth factor; PMA, phorbol myristate acetate; PMSF, phenylmethanesulphonyl fluoride; PP2A, protein phosphatase 2A; PTP, phosphotyrosine phosphatase; TGF, transforming growth factor; TNF, tumour necrosis factor.

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(erk2) were purified from extracts of *Escherichia coli* expressing the proteins [11,25]. MAP kinase kinase prepared by anion-exchange chromatography and gel filtration from PMA-stimulated KB cells [18,26] was kindly given by Dr. H. L. Thompson, of this laboratory. Recombinant human IL-1-receptor antagonist and phosphotyrosine phosphatase (PTP) 1B were generously given by Dr. Robert C. Thomson, Synergen Inc., Boulder, CO, U.S.A., and Dr. D. Barford, Department of Molecular Biophysics, University of Oxford, Oxford, U.K., respectively. The catalytic subunit of phosphoprotein phosphatase 2A (PP2A) was purified from rabbit muscle as described [27]. Its specific activity was 3300 units/mg, 1 unit being the amount of enzyme releasing 1 nmol of phosphate/min from phosphorylase *a* [27]. Okadaic acid was from Calbiochem-Novabiochem (U.K.). Transforming growth factor (TGF) β 1, basic fibroblast growth factor (bFGF), granulocyte-macrophage colony-stimulating factor and interferons α 1, β (Ser-17) and γ were human recombinant proteins from the National Institute for Biological Standards and Control (Potters Bar, Herts., U.K.). PMA, EGF, bradykinin, insulin, substance P, histamine, forskolin, prostaglandin E_2 , dibutyl cyclic AMP, A23187, ATP, sodium arsenite, myelin basic protein (MBP), dephosphorylated bovine α - and β -caseins, pig heparin, L-1-tosylphenylalanyl-chloromethane-treated trypsin, collagenase, molecular-mass standard proteins, NaF, β -glycerophosphate, EGTA, EDTA and proteinase inhibitors were from Sigma. PDGF (BB) and [γ - 32 P]ATP (3200 Ci/mmol) were from Amersham International (Little Chalfont, Bucks., U.K.). Other chemicals were from BDH (Poole, Dorset, U.K.) or Fisons (Loughborough, Leics., U.K.).

Cell culture

Human lung MRC-5 fibroblasts were from the National Institute for Biological Standards and Control. Human gingival fibroblast cultures were established from explants obtained during surgery [10]. The fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% heat-inactivated foetal-calf serum (GIBCO). Human vascular endothelial cells prepared from umbilical veins [28] and grown in medium 199 (GIBCO) supplemented with 15% heat-inactivated serum in gelatin-coated dishes were generously given by A. Wren, Department of Pharmacology, University of Cambridge (U.K.). Calf articular chondrocytes were prepared by digestion with collagenase of cartilage dissected from ankle joints (16 h, 1 mg/ml in DMEM). The cells were then plated at 0.6×10^6 cells/cm² in DMEM supplemented with 10% heat-inactivated serum. After reaching confluence, they were made quiescent by incubation for 24 h in serum-free DMEM.

Preparation of cell extracts

Confluent cells grown in 10 cm² dishes were used. Resting and stimulated cells were washed twice with ice-cold PBS supplemented with 0.1 mM EGTA, then scraped in 500 μ l of lysis buffer (20 mM Tris/HCl, pH 8.5, 20 mM β -glycerophosphate, 50 mM NaF, 0.1 mM sodium orthovanadate, 2 mM dithiothreitol, 0.5 mM EDTA, 0.5 mM EGTA and 0.1% Nonidet P40) freshly supplemented with 1 mM *o*-phenanthroline, 1 mM phenylmethanesulphonyl fluoride (PMSF), 10 μ M *trans*-epoxy-succinyl-L-leucylamido(4-guanidino)butane and 1 μ M pepstatin. The suspensions were stirred for 30 s by repeated pipetting, kept on ice for 20 min and cleared by centrifugation at 11000 *g* for 2 min (4 °C). The supernatants (extracts) were kept on ice until assayed.

Purification of β -casein kinase

Cytosolic preparations of resting and IL-1-stimulated fibroblasts grown in 175 cm² flasks were prepared as described previously [16] and loaded on an anion-exchange Mono Q HR 5/5 column (Pharmacia) equilibrated in Mono Q buffer (20 mM Tris/HCl, pH 8.5, 20 mM β -glycerophosphate, 10 mM NaF, 0.1 mM sodium orthovanadate, 2 mM dithiothreitol, 0.5 mM EDTA, 0.5 mM EGTA and 0.02% NaN₃). Elution was by a linear gradient of NaCl (0–0.5 M over 28.5 ml, 1 ml/min) in Mono Q buffer, the collection of fractions (1 ml each) beginning 3 min before starting the gradient [16]. For gel filtration, Mono Q fractions of interest were pooled and concentrated 5–6-fold by ultrafiltration. Samples (150 μ l) were then loaded on a Superose 12 HR 10/30 column (Pharmacia) equilibrated in Mono Q buffer supplemented with 320 mM NaCl and 0.05% Brij 35. The column was eluted at 0.3 ml/min, and 0.25 ml fractions were collected. Chromatography fractions were kept at 4 °C.

Protein kinase assays

Assays for phosphorylation of α - and β -casein (each at 0.25 mg/ml), hsp27 (70 μ g/ml) or MBP (0.17 mg/ml) were carried out in 96-well microtitre plates in the presence of 10 mM MgCl₂, 20 μ M ATP and 0.2 μ Ci/ μ l [γ - 32 P]ATP as described previously [16]. Heparin, an inhibitor of casein kinase II, was added to the casein phosphorylation reactions (10 μ g/ml), except for assay of chromatography fractions. Reactions were stopped by addition of 1 vol. of boiling twice-concentrated electrophoresis sample buffer [13]. The mixtures were resolved on SDS/PAGE slab gels (12.5% acrylamide) and the gels were stained, dried and autoradiographed [9]. Densitometric scanning of autoradiographs was carried out as described previously [9].

Phosphopeptide mapping

Phosphorylation of β -casein was done as described above for assays, except that ATP was at 2 μ M, the reaction mixture volume was 240 μ l and the incubation lasted 45 min. After extraction of the labelled protein from electrophoresis gels, carrier β -casein was added and precipitation with trichloroacetic acid was carried out [9]. The precipitated protein was washed in a mixture of ethanol/ether (1:1, v/v) at –20 °C, left to dry and resuspended at 1 mg/ml in 50 mM NH₄HCO₃; 0.2 mg/ml trypsin was added and the mixture was left at 37 °C for 16 h. A further 0.1 mg/ml trypsin was then added and the incubation was extended for another 6 h. It was stopped by addition of 2 mM PMSF and heating at 100 °C for 5 min. The mixture was then freeze-dried. Phosphopeptides were separated on cellulose plates by electrophoresis and chromatography [9].

Preparation of recombinant MAP kinase and β -casein kinase for experiments with phosphatases

Recombinant MAP kinase (200 μ g) was incubated for 16 h at room temperature in the presence of 100 μ l of the MAP kinase preparation, 50 mM Tris/HCl, pH 7.5, 10 mM MgCl₂ and 1 mM ATP in a total volume of 300 μ l. The mixture was then diluted 10-fold in volumes of phosphatase buffer [25 mM Tris/HCl, pH 7.6, 10% (v/v) glycerol, 0.1% (v/v) β -mercaptoethanol and 0.1 mM EGTA], loaded on to the Mono Q column equilibrated in the same buffer and eluted by a linear gradient of NaCl in phosphatase buffer (0–0.5 M over 28.5 ml). The fractions active for MBP phosphorylation were pooled, made 60% (v/v) in glycerol and stored at –20 °C. Samples were diluted 6–10-fold in phosphatase buffer before use. Superose 12 fractions containing β -casein kinase were pooled and incubated for 1 h at 4 °C

in the presence of 5 mM $\text{Na}_4\text{P}_2\text{O}_7$ to inactivate irreversibly possible contaminating phosphatases. The mixture was then diluted 5-fold in phosphatase buffer and loaded on to the Mono Q column equilibrated in the same buffer. The column was then washed with 5 ml of phosphatase buffer supplemented with 150 mM NaCl, and β -casein kinase was eluted in buffer containing 500 mM NaCl. The eluate (2–3 ml) was concentrated 5–6-fold by ultrafiltration and used immediately.

Phosphatase treatments

Samples (15 μl) of the β -casein kinase or MAP kinase preparations were mixed with 1.7 μl of 10-fold-concentrated phosphatases and incubated at 30 °C for 30 min. The reactions were stopped by addition of 1.9 μl of solutions of 500 μM okadaic acid (for PP2A) or 20 mM sodium orthovanadate (for PTP-1B). Control incubations with inhibitors or from which phosphatases were omitted were carried out simultaneously.

RESULTS

Detection of a cytokine-activated β -casein kinase in extracts of human gingival fibroblasts

Extracts of human gingival fibroblasts that had been stimulated by IL-1 or TNF for 15 min before lysis phosphorylated β -casein more strongly than did extracts of resting cells (Figure 1a). In contrast, the phosphorylation of α -casein was not increased in

Figure 1 Activation of a β -casein-phosphorylating enzyme by IL-1 or TNF in gingival fibroblasts

(a) Fibroblasts that had been either pre-treated for 20 min with 20 $\mu\text{g}/\text{ml}$ IL-1-receptor antagonist (lane 4) or not (lanes 1–3) were incubated for 15 min with 20 ng/ml IL-1 α (lanes 2 and 4), 200 ng/ml TNF- α (lane 3) or vehicle (lane 1). Extracts were then prepared and assayed for kinase activities as described in the Experimental section. Phosphorylation of casein was detected after gel electrophoresis of the reaction mixture. An autoradiograph of the dried gel is shown. The migration positions of α - and β -casein are indicated. (b) Fibroblasts grown in 175 cm^2 flasks were either stimulated by 20 ng/ml IL-1 for 15 min (lanes 2, 4 and 6) or left resting (lanes 1, 3 and 5). The cells were then scraped in 3 ml of ice-cold lysis buffer without Nonidet P40. Samples (500 μl) of each suspension were then supplemented with 0.1% Nonidet P40 to prepare lysates in the usual manner (lanes 1 and 2). The rest of the suspensions were passed repeatedly through injection needles to break the cells [16], then centrifuged at 100 000 g for 1 h (4 °C). After collection of the supernatants (lanes 3 and 4), the pellets were resuspended in the original volume of lysis buffer (with 1% Nonidet P40), left on ice for 20 min and clarified by centrifugation at 11 000 g for 2 min (lanes 5 and 6). Assays were carried out as in (a). The positions of the substrates are indicated.

Figure 2 Chromatography of fibroblast casein kinases

(a)–(d) Anion-exchange chromatography. Cytosols from resting (a, c) and IL-1-stimulated (b, d) gingival (a, b) and MRC-5 (c, d) fibroblasts were chromatographed on a Mono Q column eluted with a NaCl gradient as described in the Experimental section. The fractions were assayed for casein phosphorylation. Strips of the autoradiographs showing the phosphorylation of the two substrates are shown. The fraction numbers and the position of the caseins are indicated. (e, f) Gel filtration. Samples of the Mono Q fractions from IL-1-stimulated gingival (e) or MRC-5 (f) fibroblasts containing β -casein kinase were pooled, concentrated and chromatographed on Superose 12 as described in the Experimental section and the fractions were assayed as in (a–c), except that ATP was at 10 μM . The positions of the substrates are indicated. Standards to calibrate the column were Blue Dextran (k_0), apoferritin (443 kDa), yeast alcohol dehydrogenase (141 kDa), phosphorylase b (94 kDa) and transferrin (78 kDa).

extracts of cytokine-stimulated cells (Figure 1a). This indicated the presence of an activated protein kinase specific for β -casein in the extracts of stimulated cells, which could correspond to the β -casein kinase previously described [16]. Its activation by IL-1 was prevented by preincubating the cells with a 1000-fold excess of the IL-1-receptor antagonist (Figure 1a). When gingival fibroblasts were fractionated by mechanical disruption, followed by centrifugation at 100 000 g for 1 h, nearly all the IL-1-induced casein kinase activity was recovered in the supernatant (Figure 1b), indicating that the enzyme was mainly cytosolic.

Chromatographic properties of β -casein kinase

To check whether or not the β -casein kinase activated in gingival fibroblasts was similar to the enzyme previously found in MRC-5 cells, we compared their chromatographic behaviour. After chromatography of the cytosolic preparations of gingival fibroblasts on the anion exchanger Mono Q, the IL-1-activated β -casein kinase was eluted in fractions 20–27 (Figures 2a and 2b). These results were similar to those obtained with MRC-5 fibroblasts (Figures 2c and 2d). In both cell types, the Mono Q chromatograms also showed another IL-1-activated casein

Figure 3 Phosphopeptide maps of β -casein

β -Casein was phosphorylated by the pooled Mono Q fractions 20–26 from stimulated MRC-5 cells (a) and by extracts of resting (b) and IL-1-stimulated (c) gingival fibroblasts. Maps were then obtained as described in the Experimental section. Autoradiographs of the cellulose plates are shown. The origins (O) are indicated on all panels and the directions of electrophoresis (—, +) and of ascending chromatography (arrow) in (a). Phosphopeptides of interest are designated by numbers 1–5.

kinase, which was eluted in fractions 15–19 and phosphorylated both α - and β -casein (Figures 2a–2d). This activity, called casein kinase B in our previous paper [16], was usually activated less than 2-fold, and could not have contributed significantly to the increases in casein kinase activity measured in extracts of gingival fibroblasts.

Further chromatography on a Superose 12 gel-filtration column separated β -casein kinase into two components (Figures 2e and 2f). Most of the activity was recovered in fractions 34–40, which corresponded to molecular masses of about 500 kDa. Some activity was also eluted in fractions 46–48 and could correspond to an enzyme of molecular mass near 90 kDa. Both species were absent from fractions prepared in parallel from resting cells (results not shown). These results suggested that β -casein kinase existed mainly as a multimeric enzyme, or associated with other proteins. The 90 kDa species could be an active subunit of the high-molecular-mass form.

Substrate specificity of the IL-1-induced β -casein kinase

The electrophoretic mobility of β -casein was slightly decreased after phosphorylation by semi-purified β -casein kinase from either MRC-5 or gingival fibroblasts (see Figures 2b and 2d, fractions 19 and 20). This, and their inability to phosphorylate α -casein, strongly suggested that the β -casein kinases of MRC-5 and gingival fibroblasts had the same substrate specificity. To check whether or not this was indeed the case, tryptic peptide maps of β -casein phosphorylated by a preparation of β -casein kinase from MRC-5 cells and by extracts of gingival fibroblasts were compared (Figure 3).

The map obtained with enzyme prepared by chromatography from stimulated MRC-5 cells showed two major tryptic phosphopeptides, designated 1 and 2, and three minor fragments, designated 3–5 (Figure 3a). These peptides were absent from maps obtained with corresponding fractions from resting MRC-5 cells (results not shown). This confirmed that all five spots resulted from phosphorylation by the IL-1-induced enzyme. Maps obtained after phosphorylation of β -casein by extracts of resting gingival fibroblasts showed three major labelled spots, distinct from spots 1–5, which had little mobility during ascending chromatography (Figure 3b). When extracts of gingival fibroblasts stimulated by IL-1 were used, no significant change in the intensity of these spots was seen but the five phosphopeptides 1–5

Figure 4 Dose-dependency of β -casein kinase activation by the cytokines

Extracts of gingival fibroblasts that had been stimulated by various doses of IL-1 or TNF for 15 min were assayed for phosphorylation of (○) α - and (●) β -casein. The results were quantified by densitometric scanning of the autoradiographs and expressed as fold increases relative to values obtained for each substrate with extracts of resting cells.

were now labelled (Figure 3c). Maps obtained with extracts of TNF-stimulated cells also showed spots 1–5 (results not shown).

Although spots 1 and 2 were always strongly labelled, the intensities of spots 3 and 4 tended to vary between experiments and were on average lower than in Figure 3(c). Variations in the activity of trypsin may have caused these differences. The similarities between the phosphopeptide maps generated by the cytokine-stimulated kinase of extracts of gingival fibroblasts and the β -casein kinase from MRC-5 cells confirmed that the two kinases had the same substrate specificity: they were likely to be identical or closely related enzymes.

Dose-dependency of β -casein kinase activation

The dose-dependency of the activation of β -casein kinase by IL-1 and TNF were then investigated. The results were quantified by densitometric scanning of the autoradiographs and expressed as fold increases in the absorbance of the substrate bands, relative to values obtained with extracts of resting cells (Figure 4). Increases (3-fold) in the rate of phosphorylation of β -casein were detected after stimulation with 0.2 ng/ml IL-1 or 2 ng/ml TNF. The responses were maximal at 50 ng/ml IL-1 and 200 ng/ml TNF, with 9-fold increase in substrate phosphorylation being measured. No increase in the phosphorylation rate of α -casein was detected, which showed that β -casein kinase was the only casein kinase being activated by the cytokines over the range of concentrations used (Figure 4).

Time course of β -casein kinase activation

In cells exposed to IL-1 or TNF, the activation of β -casein kinase was undetectable after 1 min of stimulation, but was detected clearly after 5 min (Figure 5a). It was maximum (5-fold increase) 15 min after addition of the cytokines to the medium, decreased slightly by 30 min and stabilized at a level corresponding to a 3.5-fold increase in substrate phosphorylation. In other experiments, this intermediate level of activation remained stable for up to 4 h after stimulation (results not shown). Longer stimulation times were not tested. The protein kinase C activator PMA induced no detectable increase in the phosphorylation rates of α - and β -casein over stimulation times of up to 45 min (Figure 5a).

The time courses of activation of hsp27 kinase and MAP

Figure 5 Time courses of the activations of β -casein kinase, hsp27 kinase and MAP kinase in gingival fibroblasts stimulated by IL-1, TNF or PMA

Extracts of cells that had been stimulated by 20 ng/ml IL-1 (●), 200 ng/ml TNF (▲) or 100 ng/ml PMA (□) for various times were assayed for activities phosphorylating β -casein (a), hsp27 (b) or MBP (c). The results were quantified as in Figure 4.

kinase were also investigated. The enzymes were assayed on purified hsp27 and MBP as substrates, respectively. The onset of their activation followed a course similar to that of β -casein kinase, with no stimulation being detected at 1 min and maximum activation being measured at 15 min (Figures 5b and 5c). However, unlike β -casein kinase, the activities of hsp27 kinase and MAP kinase decreased thereafter and returned to background levels 45 min after stimulation by IL-1 or TNF. PMA induced a slower and weaker activation of hsp27 kinase than did the cytokines, but its effect was more sustained (Figure 5b). MAP kinase was also activated in a more sustained manner by PMA than by IL-1 or TNF (Figure 5c). In the experiment shown here, the cytokines activated MAP kinase as strongly as PMA, but in most cases they caused a much weaker response (Figure 6c).

Specificity of activation of β -casein kinase

To characterize the stimuli leading to the activation of β -casein kinase, extracts of gingival fibroblasts that had been challenged by 21 different agents other than IL-1 or TNF were assayed. These included serum, growth factors, other cytokines and inflammatory agents, drugs mimicking second messengers or inducing their release, and cellular stresses. Experiments involving growth factors, insulin or serum were carried out on cells made quiescent by incubation for 24 h in medium containing only 0.5% serum. This treatment had no influence on the magnitude of the responses to IL-1 or TNF (results not shown).

Figure 6 Effects of various agents on β -casein kinase, hsp27 kinase and MAP kinase in gingival fibroblasts

Cells were challenged for 15 min by 20 ng/ml IL-1 (1), 200 ng/ml TNF (2), 100 ng/ml PMA (3), 10% foetal-calf serum (4), 100 ng/ml PDGF (5), 1 μ M bradykinin (6), 100 ng/ml EGF (7), 5 ng/ml TGF- β 1 (8), 10 ng/ml bFGF (9), 300 international units/ml interferon- β (10), 100 international units/ml interferon- γ (11), 10 μ M histamine (12), 10 μ M prostaglandin E_2 (13), 1 μ M forskolin (14) or 10 μ M A23187 (15), or for 30 min by a 44 $^{\circ}$ C heat shock (16) or for 1 h by 200 μ M sodium arsenite (17). The cells were then lysed and the extracts assayed for phosphorylation of β -casein (a), hsp27 (b) or MBP (c). The results were quantified as in Figure 4. IL-1 and TNF were tested in seven different experiments involving different batches of cells. The other agents were tested three or four times. Means and S.E.M. are shown.

Figure 6 shows the results obtained after 15 min stimulations by IL-1, TNF, PMA, foetal-calf serum, PDGF, bradykinin, EGF, TGF β 1, bFGF, interferon- β , interferon- γ , histamine, prostaglandin E_2 , forskolin or the Ca^{2+} ionophore A23187, a 30 min heat shock (44 $^{\circ}$ C), and a 1 h exposure to sodium arsenite. Apart from IL-1 and TNF, none of these agents induced any detectable increase in the phosphorylation of β -casein by cell extracts (Figure 6a). Recombinant human interferon- α (300 international units/ml), ATP (100 μ M), substance P (20 ng/ml), insulin (50 ng/ml), granulocyte-macrophage colony-stimulating factor (10 ng/ml) and dibutyryl cyclic AMP (1 μ M) were also negative (results not shown). In other experiments, the cells were exposed for up to 45 min to PMA (Figure 5), serum, bradykinin or interferon- γ (results not shown), without any change in the phosphorylation rates of caseins being detected.

This specific pattern of activation was in sharp contrast with those of hsp27 and MAP kinases. The strongest activators of hsp27 kinase were IL-1 and TNF, but PMA, serum, PDGF, bradykinin, EGF, A23187, heat shock and arsenite also increased

Figure 7 Activation of β -casein kinase in articular chondrocytes and vascular endothelial cells

β -Casein was phosphorylated by extracts of bovine articular chondrocytes (a–d) and human umbilical-vein endothelial cells (e, f) that had been left resting (a, e) or stimulated for 15 min by 20 ng/ml IL-1 (b, f), 200 ng/ml TNF (c) or 10% foetal-calf serum (d). Phosphopeptide maps of the substrate were then made. The directions of electrophoresis and chromatography are as in Figure 3. The phosphorylated peptides produced by β -casein kinase are designated 1–5.

hsp27 phosphorylating activity to a lesser extent (Figure 6b). MAP kinase was more strongly activated by PMA, EGF, PDGF and serum than by IL-1 or TNF (Figure 6c).

Activation of β -casein kinase in articular chondrocytes and endothelial cells

We looked for the activation of β -casein kinase in other cell types. IL-1 and TNF activated a β -casein-phosphorylating enzyme in cultures of bovine articular chondrocytes. Phosphopeptide maps of β -casein showed that the activated kinase generated the same pattern of five labelled fragments as did the MRC-5 β -casein kinase (Figures 7a–7c and 3a). This indicated that the chondrocyte enzyme had the same substrate specificity as fibroblast β -casein kinase. Phosphopeptide maps of β -casein showed no detectable activation of the enzyme in chondrocytes

Figure 8 Lack of effect of phosphatases on β -casein kinase

The high- (a) and low- (b) molecular-mass forms of β -casein kinase were prepared from IL-1-stimulated MRC-5 fibroblasts as described in the Experimental section. Samples were then left on ice (lane 1) or incubated at 30 °C without phosphatase (lane 2), with 200 units/ml PP2A (lanes 3, 4, 7, 8) or with 100 μ g/ml PTP-1B (lanes 5–8) in the absence (1–3, 5, 7) or in the presence of phosphatase inhibitors (4, 6, 8) as described in the Experimental section. The incubations were ended after 30 min and the mixtures were assayed for β -casein-phosphorylating activity in the presence of 10 μ M ATP. Strips of the autoradiographs showing the phosphorylated casein are shown. The position of the Coomassie-Blue-stained substrate is indicated (β). (c), (d), Activated recombinant MAP kinase was incubated at 30 °C for 30 min with various amounts of PP2A (c) or PTP-1B (d) in the absence (●) or in the presence (○) of okadaic acid (c) or vanadate (d) as described in the Experimental section. Samples were then assayed for MBP-phosphorylating activity. The results were quantified by counting the Čerenkov radiation from gel fragments containing the substrate.

stimulated by serum (Figure 7d), bFGF or TGF β 1 (results not shown). β -Casein kinase was also activated in human umbilical-vein endothelial cells stimulated for 15 min by IL-1 (Figures 7e and 7f) or TNF (results not shown).

Lack of effect of phosphoprotein phosphatases on β -casein kinase

To investigate whether or not the activity of β -casein kinase depended upon its phosphorylation state, we tested the effects of the phosphoserine/phosphothreonine phosphatase, PP2A, and the phosphotyrosine phosphatase, PTP-1B, on preparations of the high- and low-molecular mass forms of β -casein kinase. No change in activity of either form of the MRC-5 enzyme was observed after incubation with 200 units/ml PP2A or 100 μ g/ml PTP-1B, individually and in combination (Figures 8a and 8b). Similar results (not shown) were obtained with preparations from gingival fibroblasts.

The activity of MAP kinase requires its simultaneous phosphorylation on two nearly adjacent threonine and tyrosine residues [22]. This property enabled us to check whether the phosphatases and their inhibitors were active or not under our experimental conditions. It was found that active recombinant MAP kinase was fully inactivated by only 10 units/ml PP2A or 30 μ g/ml PTP-1B, and that okadaic acid and vanadate were able to inhibit PP2A and PTP-1B completely at concentrations of up to 200 units/ml and 100 μ g/ml respectively (Figures 8c and 8d). Our results thus show that our preparations of β -casein kinase are not inactivated by these phosphatases.

DISCUSSION

β -Casein kinase had been detected initially in anion-exchange-chromatography fractions of cytosols of MRC-5 fibroblasts [16]. Since chromatography was not practicable for large numbers of

samples, it was necessary to measure β -casein kinase directly in cell extracts in order to assess its possible physiological significance. The casein-phosphorylation assays were carried out in the presence of heparin to decrease the background phosphorylation caused by casein kinase II.

Initial experiments showed that an activated kinase phosphorylating β -casein could be detected in extracts of gingival fibroblasts that had been stimulated by IL-1 or TNF. The activated enzyme was cytosolic and its chromatographic properties were the same as those of the MRC-5 β -casein kinase. Gel filtration on Superose 12 separated two active components of apparent molecular masses near 500 and 90 kDa. In previous investigations with a low-pressure gel-filtration column, the high-molecular-mass form had been detected only occasionally [16]. Our new results strengthen our initial interpretation that β -casein kinase may be a multimeric enzyme. The 90 kDa form seems to be the smallest active component. Whether it corresponds to a monomer or to a smaller complex remains to be investigated.

The dose-dependency of the activation of β -casein kinase by the cytokines was commensurate with the dissociation constants of the ligands for their receptors, 20 pM for IL-1 and 320 pM for TNF [10]. The response to IL-1 was prevented by a 1000-fold excess of the IL-1-receptor antagonist. These observations were consistent with the enzyme being activated via the known receptors in a physiological manner.

The time course of activation of β -casein kinase was compared with those of hsp27 kinase and MAP kinase. All were maximally activated 15 min after stimulation by IL-1 or TNF. The activity of β -casein kinase then remained detectable for several hours after stimulation. This contrasted strongly with the transient activations of hsp27 and MAP kinases, which were consistent with the behaviour of these enzymes in other cells [13,15,17–19]. The IL-1-increased phosphorylations of hsp27 [5,9,13] and the EGF receptor [10,11] and the activation of other kinases [15,18] and the nuclear factor NF- κ B [29] are also all transient, with maxima 15–20 min after stimulation.

Cytokine-activated β -casein kinases were also detected in articular chondrocytes and vascular endothelial cells. Their lack of activity on α -casein and the phosphopeptide maps of β -casein that they generated showed that their substrate specificities were the same as those of the β -casein kinases of MRC-5 and gingival fibroblasts. IL-1 and TNF therefore appeared to activate β -casein kinase in several types of connective-tissue cell.

Activation of β -casein kinase was induced in gingival fibroblasts only by IL-1 or TNF. Additionally, phosphopeptide maps of β -casein showed that no activation of the kinase was occurring in chondrocytes challenged with serum, bFGF or TGF β 1. Phosphopeptide mapping was more specific and sensitive for detecting the enzyme than measuring the phosphorylation of the whole protein. This evidence strongly suggested that β -casein kinase was part of a signalling pathway specifically activated by IL-1 or TNF.

The regulation of hsp27-phosphorylating activity was less specific. It was stimulated most strongly by IL-1 or TNF, but also to a lesser extent by some growth factors, PMA and metabolic stress. This was consistent with studies of the phosphorylation of hsp27 in intact cells [6,9,30]. MAP kinase was usually activated by IL-1 or TNF less strongly than by mitogens (serum, PDGF, EGF and PMA) and, of the three kinases under study, was the least specific for IL-1 and TNF.

The IL-1-stimulated hsp27 kinase detected in fibroblasts may be related to a similar enzyme in KB cells [19], and may be the human homologue of the hsp27 kinase from rabbit muscle which can be activated by MAP kinase, MAP kinase-activated protein

kinase II [31]. However, the main activator of hsp27 kinase in IL-1-treated cells is unlikely to be MAP kinase, which is activated by the cytokine only weakly in fibroblasts [16,18] and not at all in KB cells [18,21].

β -Casein kinase was not inactivated by the phosphatases PP2A and PTP-1B *in vitro*, even though they were used at several times the concentrations needed to inactivate MAP kinase fully. This could indicate that the activity of the kinase does not depend on direct phosphorylation. Alternatively, β -casein kinase could be regulated via phosphorylation on a site inaccessible to the phosphatases. In any case, its insensitivity to phosphatases may be a possible reason for its prolonged activation.

The specificity of activation of β -casein kinase distinguishes it from most other inducible serine/threonine kinases, such as MAP kinase, the p54 stress-activated protein kinase [20], protein kinase C or cyclic AMP-dependent protein kinase. It might be part of a signalling pathway used specifically by IL-1 and TNF, either upstream of or parallel to the MAP kinase and hsp27 kinase systems. Because of its cytosolic location, it is unlikely to be directly coupled to the cytokine receptors. Unravelling its mechanism of activation and identifying its physiological substrates may be important to our understanding of the intracellular signalling mechanisms of IL-1 and TNF.

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