Activation of S6 kinase in human neutrophils by calcium pyrophosphate dihydrate crystals: protein kinase C-dependent and phosphatidylinositol-3-kinase-independent pathways

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Phosphatidylinositol 3-kinase (PI 3-kinase) has been shown previously to be a central enzyme in crystal-induced neutrophil activation. Since activation of the 70 kDa S6 kinase (p70^{S6K}) has been shown to be dependent on PI 3-kinase activation in mammalian cells, and since the former is a key enzyme in the transmission of signals to the cell nucleus, activation of p70^{S6K} was investigated in crystal-stimulated neutrophils. Cytosolic fractions from calcium pyrophosphate dihydrate (CPPD)-crystal-activated neutrophils were separated by Mono Q chromatography and analysed for phosphotransferase activity using a range of substrates and probed by Western analysis using antibodies to p70^{S6K} and mitogen-activated protein kinase (MAP kinase). CPPD crystals induced a robust, transient activation

(peak activity at 2 min) of p70^{S6K} that was fully inhibited by pretreatment with rapamycin. This is the first report of the activation of p70^{S6K} in neutrophil signal transduction pathways induced by an agonist. This crystal-induced activation of p70^{S6K} could also be inhibited by a protein kinase C (PKC) inhibitor (Compound 3), but not by the PI 3-kinase inhibitor wortmannin. CPPD crystals also activated the ERK1 and ERK2 forms of MAP kinase (wortmannin insensitive), PKC (Compound 3 sensitive) and protein kinase B (wortmannin sensitive) in neutrophils. These data suggest that activation of p70^{S6K} may proceed through a PI 3-kinase- and protein kinase B-independent but PKC-dependent pathway in crystal-activated neutrophils.

INTRODUCTION

The inflammatory disease known as acute pseudogout arises from the deposition of calcium pyrophosphate dihydrate (CPPD) crystals in the synovial joints of humans [1]. Both monoclinic and triclinic [CPPD(T)] crystalline forms of CPPD are able to activate neutrophils in the joint, a process that is thought to be a key factor in the pathophysiology of the disease [1–3]. *In vivo*, CPPD crystals adsorb synovial fluid proteins (synovial fluid is an ultrafiltrate of plasma), so that surface-adsorbed proteins may modulate the ability of crystals to activate neutrophils. *In vitro*, both uncoated and plasma protein coated crystals are able to activate neutrophils. The effect of plasma precoating of CPPD(T) crystals is to amplify neutrophil activation levels relative to those with uncoated crystals, probably via the adsorption of opsonizing proteins such as IgG and complement components [2]. These absorbed proteins may bind to specific receptors on the neutrophil plasma membrane, leading to the activation of signalling pathways within the neutrophil that are common to the pathways activated by agents such as opsonized microbial pathogens, the main target of neutrophils [4].

The biochemical mechanisms by which either uncoated or plasma coated CPPD crystals activate neutrophils are poorly understood. Activation of phospholipase C or phospholipase D, elevations in phosphotyrosine levels and increases in intracellular calcium concentrations have been reported in CPPD-activated

neutrophils [5–10]. Recently we showed that the enzyme phosphatidylinositol 3-kinase (PI 3-kinase) is activated in crystal-stimulated neutrophils [11]. Two specific inhibitors of PI 3-kinase, wortmannin and LY294002, were shown to completely suppress neutrophil activation induced by plasma-coated CPPD crystals at concentrations lower than the known IC₅₀ of these inhibitors for PI 3-kinase. The involvement of PI 3-kinase activity in chemoattractant-stimulated neutrophils has been reported previously [12,13]. Wortmannin and LY294002 have also been shown to inhibit activation of the 70 kDa ribosomal S6 kinase (p70^{S6K}) in many cell types in response to a variety of mitogenic stimuli [14–16].

p70^{S6K} is responsible for the multiple phosphorylation of the 40 S ribosomal protein S6 [17,18], which is thought to play a role in the initiation of protein synthesis required for proliferation or mitosis [19–21]. p70^{S6K} is activated by complex phosphorylation events and lies on a distinct pathway from mitogen-activated protein kinases (MAP kinases) and ribosomal S6 kinase. Activation of p70^{S6K} may occur through either protein kinase C (PKC)-dependent or -independent pathways [14,15,18], and can be completely blocked by the immunosuppressant drug rapamycin.

Since activation of PI 3-kinase and p70^{S6K} has been demonstrated in mitogen-induced signalling, we have investigated whether p70^{S6K} might be activated in CPPD(T)-stimulated neutrophils. We report that both uncoated and plasma coated CPPD crystals induced a transient activation of p70^{S6K}; the

Abbreviations used: CPPD, calcium pyrophosphate dihydrate; CPPD(T), triclinic form of CPPD; PI 3-kinase, phosphatidylinositol 3-kinase; p70^{S6K}, 70 kDa ribosomal S6 kinase; MAP kinase, mitogen-activated protein kinase; PKC, protein kinase C; PKB, protein kinase B; TBS, Tris-buffered saline; TTBS, TBS containing 0.05% Tween 20; ERK, extracellular-signal-regulated kinase; MBP, myelin basic protein; TOR, target of rapamycin.

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activation was significantly enhanced when plasma-coated CPPD crystals were used compared with uncoated crystals. $p70^{S6K}$ activation was completely blocked by pretreatment of the cells with rapamycin. The robust activation of $p70^{S6K}$ by CPPD crystals described in this study offers an opportunity to investigate the signal transduction pathway(s) leading to activation of this enzyme by non-mitogenic stimuli in terminally differentiated cells. A number of studies using specific inhibitors of PI 3-kinase have shown that $p70^{S6K}$ lies downstream of PI 3-kinase [14–16]. Recently, however, several reports have dissociated $p70^{S6K}$ from the known components of the PI 3-kinase signalling pathway. Scheid et al. [22] found that PI 3-kinase, but not $p70^{S6K}$, was required for cytokine-regulated haemopoietic cell survival, and Dudek et al. [23] showed that protein kinase B (PKB), but not $p70^{S6K}$, mediated growth-factor-induced neuronal cell survival. MAP kinase lies on a pathway that is distinct from $p70^{S6K}$ [24]; it is unaffected by wortmannin and LY294002 and hence is independent of PI 3-kinase activation. In the present study, wortmannin was found to have no effect on CPPD-induced activation of MAP kinase, in agreement with previous reports. However, $p70^{S6K}$ activation was also unaffected by wortmannin or LY294002 at concentrations as high as $1 \mu\text{M}$ or $50 \mu\text{M}$ respectively, but was strongly inhibited by Compound 3 (a specific inhibitor of PKC). Therefore crystal-induced activation of $p70^{S6K}$ in neutrophils appears to proceed via PI 3-kinase-independent and PKC-dependent mechanisms.

EXPERIMENTAL

All experiments were repeated on three separate occasions, and results describe means $\pm$ S.D.

Antisera and inhibitors

Antibodies to the N-terminus of $p70^{S6K}$ and 4G10 antibodies were obtained from UBI (Lake Placid, NY, U.S.A.). All other antibodies were obtained from Kinetek Pharmaceuticals (Vancouver, BC, Canada). Compound 3 (RO-31-8220), bis-indolyl-maleamide and calphostin C were obtained from Calbiochem (San Diego, CA, U.S.A.). All other inhibitors were obtained from Sigma (St. Louis, MO, U.S.A.).

Preparation and opsonization of crystals

CPPD(T) crystals were prepared as previously described [25]. Opsonized crystals were incubated with 50% (v/v) heparinized plasma (50% Hanks buffered salt solution) at 37°C for 30 min immediately prior to experiments. Samples of 20 mg of CPPD(T) crystals were weighed into individual 1.5 ml Eppendorf tubes, followed by the addition of 0.5 ml of 50% (v/v) fresh human plasma. The tubes were capped and tumbled end-over-end at 30 rev./min for 30 min at 37°C . Tubes were then centrifuged at 1000 *g*, the plasma supernatants were discarded and the crystals were washed with excess Hanks buffered salt solution.

Preparation of neutrophils

Human neutrophils were prepared from fresh whole blood by dextran [1% (w/v) T500; Pharmacia] sedimentation for 20 min followed by Ficoll Paque density centrifugation of the dextran supernatant as previously described [6]. Neutrophils prepared under these conditions yielded cell suspensions that contained over 95% neutrophils, with over 99% cell viability (assessed by Trypan Blue exclusion).

Mono Q chromatography

Cells treated as described were washed in cold lysis buffer (20 mM Hepes, pH 7.5, 0.34 M sucrose, 2 mM 2-mercaptoethanol, $100 \mu\text{M}$ sodium orthovanadate, $0.1 \mu\text{M}$ NaF, 60 mM β -glycerophosphate, 1 mM di-isopropylfluorophosphate, 1% Triton X-100, $10 \mu\text{g/ml}$ each of aprotinin, leupeptin and soybean trypsin inhibitor, $1 \mu\text{M}$ pepstatin). The homogenate was centrifuged at 4°C for 10 min at 8000 *g*, and 2 mg of the supernatant was loaded on to a 1 ml Mono Q (Pharmacia) column equilibrated with buffer A (25 mM β -glycerophosphate, 10 mM Mops, pH 7.2, 5 mM EGTA, 2 mM MgCl_2 , 1 mM dithiothreitol and 2 mM orthovanadate). The column was developed with a 10 ml linear 0–0.8 M NaCl gradient in buffer A at a flow rate of 0.8 ml/min with an FPLC system (Pharmacia LKB Biotechnology), and 250 μl fractions were collected.

Electrophoresis and immunoblotting

SDS/PAGE of the crude and Mono Q-fractionated neutrophil homogenates was performed on 1.5 mm-thick gels with the buffer system described by Laemmli [26]. A 10% (w/v) separating gel and a 4% (w/v) stacking gel were used. Samples were boiled for 5 min in the presence of $5\times$ concentrated SDS sample buffer (125 mM Tris/HCl, pH 6.8, 4% SDS, 0.01% Bromophenol Blue, 10% 2-mercaptoethanol and 20% glycerol) and electrophoresed for 18 h at 10 mA. Subsequently, the separating gel was soaked in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 10 min and then sandwiched with a nitrocellulose membrane. Proteins were transferred for 3 h at 300 mA. The membrane was blocked with TBS (Tris-buffered saline) containing 5% (w/v) skimmed milk (except for the anti-phosphotyrosine immunoblots, which were blocked with 5% BSA) for 3 h at room temperature, and then washed three times in TBS containing 0.05% Tween 20 (TTBS) for 15 min. The membranes were probed overnight at room temperature with affinity-purified rabbit polyclonal antibodies for $p70^{S6K}$ (S6K-NT) and MAP kinase (ERK1-CT, where ERK is extracellular-signal-regulated kinase), and with the anti-phosphotyrosine antibody 4G10 in 2.5% skimmed milk/TTBS. The membranes were washed three times with TTBS before a 1 h incubation at room temperature with secondary antibody (goat anti-mouse IgG coupled to alkaline phosphatase in 2.5% skimmed milk/TTBS). The membranes were then rinsed three times in TTBS and once in TBS before incubation with a 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium colour development solution (mixture of 3% Nitro Blue Tetrazolium in 1 ml of 70% dimethylformamide and 1.5% 5-bromo-4-chloro-3-indolyl phosphate in 1 ml of 100% dimethylformamide before being added to 100 ml of 0.1 M NaHCO_3 /10 mM MgCl_2 , pH 9.8). The colour was developed in 2–10 min, and the reaction was stopped by rinsing the membranes in water.

Kinase assays

$p70^{S6K}$ in the Mono Q fractions (5 μl aliquots) was assayed at 30°C with a final concentration of 0.08 mg/ml S6-10 peptide (AKRRRLSSLRASKSESSQK), 0.08 mg/ml MBP-NT peptide (AAQKRPSQRKTYLA), or 0.2 mg/ml myelin basic protein (MBP) in assay buffer [20 mM Mops (pH 7.2), 25 mM β -glycerophosphate, 5 mM EGTA, 2 mM EDTA, 20 mM MgCl_2 , 2 mM sodium orthovanadate, 1 mM dithiothreitol, 500 nM cAMP-dependent protein kinase inhibitor peptide (Sigma) and, in some cases, the PKC inhibitor Compound 3 (20 μM ; Roche)] and 50 μM [γ - ^{32}P]ATP (~ 2000 c.p.m./pmol) in a final volume of 25 μl . The reactions were allowed to proceed for 10 min, after

which 20 μ l aliquots were spotted on to a 1.5 cm $\times$ 2 cm P-81 phosphocellulose filter paper. The filter paper was washed extensively with 1 % phosphoric acid with 10 changes, after which the adsorbed radioactivity was quantified by liquid scintillation counting in a Packard TriCarb 4530 instrument.

For p70^{S6K}, 200 μ g of total protein from extract supernatants was diluted into immunoprecipitation buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 5 % glycerol, 10 mM NaF, 5 mM EGTA, 1 mM EDTA, 30 mM β -glycerophosphate) and the solutions were incubated with antibodies for p70^{S6K} (S6K-NT) for 4 h at 4 °C. Protein A-agarose beads (Sigma) that had been pre-incubated in cold (4 °C) immunoprecipitation buffer were added and the samples were further incubated for 1 h at 4 °C. The beads were washed twice with immunoprecipitation buffer and twice with KII buffer (12.5 mM Mops, pH 7.5, 12.5 mM β -glycerophosphate, 5 mM EGTA, 7.5 mM MgCl₂, 50 mM NaF and 0.25 mM dithiothreitol). The beads were resuspended in KII buffer supplemented with 10 mM MgCl₂ and 5 % glycerol, and the reactions were initiated with 10 μ l of [γ -³²P]ATP ($\sim$ 2000 c.p.m./pmol) in a final volume of 40 μ l and incubated for 20 min at 30 °C. The reactions were terminated with 5 $\times$ SDS loading buffer, boiled for 5 min and loaded on to an SDS/PAGE gel (11 %). Following transfer of protein as described above, the membrane was immunoblotted for the appropriate protein and exposed to film, and the protein was subjected to Čerenkov counting.

Immunoprecipitation of PKB/PKC activities

Samples of 5 μ l each of antibodies to the α and β catalytic portion of PKC and the pleckstrin homology domain of PKB were adsorbed on to Protein A-Sepharose beads. Then 200 μ g of neutrophil extract was added, followed by 200 μ l of buffer containing 50 mM NaF, 100 mM NaCl, 50 mM Tris/HCl (pH 7.4), 5 mM EDTA and 3 % Nonidet P40. This was incubated for 3 h at 4 °C, then washed twice in the same buffer and twice in buffer without Nonidet P40. The beads were then suspended in 20 μ l of the same buffer and MgCl₂ was added to a final concentration of 10 mM. MBP was used as a substrate for PKB, and histone H1 was used as a substrate for PKC. For PKC determinations, diacylglycerol and phosphatidylserine were added to estimate conventional PKC activity. ATP was added at a concentration of 250 μ M and an activity of 2 μ Ci/reaction. The reaction was allowed to proceed for 30 min at 30 °C and terminated by the addition of 5 $\times$ sample buffer. After boiling (2 min), the samples were loaded on to a 11 % polyacrylamide gel and electrophoresed at 8 mA overnight. The proteins were transferred at 300 mA for 3 h on to nitrocellulose membranes, stained with Ponceau S stain, and the protein substrate bands were excised and radioactivity was counted in a liquid scintillation counter.

RESULTS AND DISCUSSION

To investigate the crystal-induced activation of p70^{S6K}, we fractionated cytosolic extracts from neutrophils incubated with crystals by anion-exchange chromatography on Mono Q. Phosphotransferase activity of the fractions was resolved using three different substrates of p70^{S6K}: MBP-NT peptide, S6K-10 peptide and MBP. Assays with the MBP-NT peptide included the PKC inhibitor Compound 3, since MBP-NT is also a substrate for PKC. Extracts from neutrophils treated for 2 min with plasma coated CPPD crystals gave a major phosphotransferase peak at approx. 0.4 M NaCl for each substrate (Figures 1A–C). To identify this activity, the Mono Q fractions were immunoblotted

Figure 1 Mono Q fractionation of p70^{S6K} activation in neutrophils stimulated with CPPD crystals

Neutrophils (5×10^6 /ml) were incubated with (●) or without (□) plasma-opsonized CPPD(T) crystals (50 mg/ml) for 2 min. Cytosolic extracts were subjected to Mono Q chromatography, and phosphotransferase activity was measured using the substrates (A) MBP-NT, (B) S6K-10 peptide and (C) MBP. The same fractions were subjected to SDS/PAGE for Western blotting with affinity-purified rabbit polyclonal antibodies that recognize the N-terminal region of p70^{S6K} (αS6-NT). Immunodetection was conducted for both control cells (D) and CPPD-treated extracts (E).

with the anti-p70^{S6K}-NT antibody. The major immunoreactive protein co-eluted with the phosphotransferase activity and migrated as a protein of approx. 70 kDa on SDS/polyacrylamide gels (Figure 1D).

Previous work has established that p70^{S6K} is activated through phosphorylation on multiple serine and threonine residues, probably by more than one upstream kinase [17,27–29]. The most highly phosphorylated and active form of p70^{S6K} migrates more slowly than the basally (partially) phosphorylated or dephosphorylated inactive enzyme [17,18]. The corresponding band shift that can be visualized on immunoblotting of the

Figure 2 Time course of p70^{S6K} activation by CPPD crystals

(A) Cytosolic extracts from neutrophils treated for 1, 2, 5, 10 or 15 min with plasma-opsonized crystals (50 mg/ml) and uncoated crystals (control) were subjected to Mono Q fractionation and assayed for MBP-NT phosphotransferase activity for p70^{S6K} activity detection (Compound 3 at 10 nM was included). p70^{S6K} activity within the S6 kinase immunodetected peak was determined. (B) Separate experiments were conducted to determine p70^{S6K} activity in neutrophils incubated for 2 min without crystals (□), with plasma-opsonized crystals (■) or with uncoated crystals (▨).

enzyme correlates with p70^{S6K} activity in many systems. In neutrophils not treated with crystals (control), no phosphotransferase activity was observed and p70^{S6K} was present as a hypophosphorylated species (Figure 1D). Following 2 min of neutrophil incubation with plasma coated CPPD, p70^{S6K} activity was robust, and the hyperphosphorylated form of the kinase could be seen by immunoblotting (Figure 1E). Hei et al. [30] performed similar immunological studies on Mono Q fractions from rat skeletal muscle extracts with antibodies generated against the C-terminus and subdomain III of p70^{S6K}, and the major immunoreactive protein co-eluted with the p70^{S6K} activity in an identical manner to that illustrated in Figure 1. These results indicate that stimulated p70^{S6K} is present in the Mono Q fractions, and this protocol was used in the rest of the present study to investigate the effects of crystal incubation of neutrophils on this kinase.

To determine the kinetics of p70^{S6K} activation following crystal incubation, cytosolic extracts from neutrophils incubated with plasma coated CPPD crystals for 1, 2, 5, 10 and 15 min were separated by anion-exchange chromatography and the MBP-NT phosphotransferase activity was determined. The total phosphotransferase activity recovered in the column fractions that contained p70^{S6K} immunoreactivity was standardized to the amount of protein loaded on to the column and plotted against incubation time. The time-course study showed that there was a marked and transient activation of cytosolic p70^{S6K} activity following 2 min of crystal incubation (Figure 2A). We have shown previously that both uncoated and plasma coated CPPD

crystals activate neutrophils, and that plasma precoating amplifies neutrophil responses to CPPD [2]. Figure 2(B) shows that plasma precoating of CPPD crystals enhanced activity by at least 2-fold over that with uncoated CPPD crystals. Although PI 3-kinase and PKC are known to be activated in neutrophils, this is the first documented observation of neutrophil activation of p70^{S6K}. p70^{S6K} activation and regulation has generally been studied in mammalian cell systems treated with mitogens [17,24], although Tsai et al. [31] suggested a vital role for p70^{S6K} in mast cells relative to stimulation of the Fc receptor, where IgE was shown to stimulate p70^{S6K} at 30 min, presumably through the FcεRI receptor. This p70^{S6K} activation was blocked by rapamycin, but rapamycin did not inhibit 5-hydroxytryptamine release.

The immunosuppressive compound rapamycin has become one of the most important and useful tools for dissecting the pathway leading to p70^{S6K} activation. Rapamycin is a specific and potent inhibitor of p70^{S6K}, blocking phosphorylation and activation of the enzyme by all known mitogens, including epidermal growth factor, v-Src, phorbol esters, calcium ionophore, heat shock, cycloheximide, interleukin-2 and insulin [51,29,30,32,33]. Although rapamycin inhibits p70^{S6K}, it does not block the activation of other MAP kinases such as ERK1 and ERK2, p90rsk or p74raf-1 [15,34]. The drug does not interact directly with p70^{S6K}, but binds to a protein termed FKBP12 (FK506-binding protein 12), and the complex interacts with the target of rapamycin (TOR, also known as FRAP). TOR proteins show significant sequence similarity with the catalytic subunit of PI 3-kinase, and have lipid kinase as well as protein kinase activity, so inositol phosphate derivatives may play a role as second messengers upstream of p70^{S6K}. p70^{S6K} has been shown previously to be activated by both PKC-dependent and -independent routes [15,18], both of which are blocked completely by rapamycin. Rapamycin treatment has no effect on PI 3-kinase activity [15,29], but a recent report suggests that PI 3-kinase activity is required for PMA-induced AP-1 activation and transformation in mouse epidermal cells, linking PI 3-kinase to activation of PKC [35].

Neutrophils were treated with rapamycin for 10 min prior to incubation with or without plasma coated CPPD(T) crystals for 1, 2 and 5 min. Cytosolic extracts from treated neutrophils and non-treated controls were fractionated on Mono Q and phosphotransferase activity was determined (with Compound 3) as described previously. The activation of p70^{S6K} after 2 min of crystal treatment was completely blocked by pretreatment with rapamycin (Figure 3A). The activity in the peak Mono Q fraction (fraction 30) was assayed, and the means of three separate experiments are shown in Figure 3(B). Han et al. [18] suggested that hyperphosphorylation of p70^{S6K} was responsible for the mobility shift observed in Western blots following activation of this enzyme, and that rapamycin may inhibit this phosphorylation. It is therefore likely that the activation of p70^{S6K} by plasma coated CPPD crystals is due to the phosphorylation of p70^{S6K}, since a mobility shift was observed (Figure 1E) and the activation was rapamycin sensitive (Figure 3).

Increased tyrosine phosphorylation of several proteins has been shown to correlate with neutrophil activation, and it has been proposed that tyrosine kinases and phosphatases play a pivotal role in stimulus-response coupling in neutrophils [6]. MAP kinase is a protein serine/threonine kinase that is activated via tyrosine phosphorylation, and it has been reported that ERK1 (p42mapk) is stimulated in neutrophils by chemo-attractant peptides [36,37]. The involvement of MAP kinase as a signal transduction enzyme in crystal-induced neutrophil activation has been reported previously by our group [38]. Immuno-

Figure 3 Effects of rapamycin on CPPD-crystal-induced $p70^{S6K}$ activation in neutrophils

Neutrophils ($5 \times 10^6/\text{ml}$), with or without rapamycin pretreatment (10 min, 100 nM) were incubated with plasma-opsonized CPPD crystals (50 mg/ml) for 2 min and cytosolic extracts were prepared followed by Mono Q fractionation. (A) $p70^{S6K}$ activity was determined by phosphotransferase determinations using the MBP-NT substrate. □, Control cells; ●, CPPD-stimulated (no rapamycin); ■, CPPD-stimulated (rapamycin at 10 mM). (B) $p70^{S6K}$ phosphotransferase activity within the S6 kinase immunodetected peak was determined. □, Control cells; ▨, CPPD-stimulated (no rapamycin); ■, CPPD-stimulated with rapamycin (10 nM) pretreatment.

blotting with the anti-ERK1-CT antibody specific to both ERK1 and ERK2 confirmed our previous findings that both of the MAP kinase isoforms co-eluted with the first MBP phosphotransferase peak in fractions 21–26 (Figure 1C). Both isoforms had a more slowly migrating band that is characteristic of the activated form of these kinases. These fractions were subjected to Western blot analysis with the anti-phosphotyrosine antibody 4G10, and the major immunoreactive tyrosine-phosphorylated band co-migrated with the hyperphosphorylated forms of the ERK proteins, suggesting that the first MBP peak contained active forms of both MAP kinases.

Neither CPPD-induced MAP kinase activation (Figure 4A) nor $p70^{S6K}$ activation (Figure 4B) in neutrophils was significantly inhibited by wortmannin, indicating that PI 3-kinase activity is not required for their activation in this system. The PI 3-kinase-independent activation of MAP kinase has been reported previously [16], but studies of $p70^{S6K}$ suggested that PI 3-kinase is required for its activation [13,15,17,39,40]. Wortmannin at 0.1–1 mM (concentrations that are 10–100-fold greater than that necessary to suppress crystal-induced PI 3-kinase activation in neutrophils [11]) failed to inhibit the crystal-induced activation

Figure 4 Effects of PI 3-kinase inhibitors on CPPD-crystal-induced activation of $p70^{S6K}$ and MAP kinase in neutrophils

Neutrophils ($5 \times 10^6/\text{ml}$) with or without wortmannin or LY294002 pretreatment (5 min) were incubated with plasma-opsonized CPPD crystals (50 mg/ml) for 2 min and cytosolic extracts were prepared by Mono Q fractionation. (A) MAP kinase activity from fraction 24 was assayed using MBP as a substrate. (B) $p70^{S6K}$ activity from fraction 30 was assayed using MBP-NT as a substrate.

of $p70^{S6K}$ (Figure 4B). LY294002 at 50 μM (a concentration determined to inhibit PI 3-kinase and $p70^{S6K}$ in HepG2 cells following treatment with platelet-derived growth factor [15]) was also ineffective at inhibiting CPPD-induced $p70^{S6K}$ activation. CPPD crystals also induced the strong activation of both PKB and PKC after a 2 min crystal/neutrophil incubation (Figures 5A and 5B). Wortmannin was able to fully suppress the activation of PKB, indicating that activation of PKB in neutrophils is downstream of PI 3-kinase, consistent with other reports. In other cells PKB (also known as Akt or Rac) has been proposed as the target of PI 3-kinase and a possible link to $p70^{S6K}$ activation [40,42,43]. In fact, PKB has recently been shown to be activated by interaction of its pleckstrin homology domain with phosphatidylinositol 3,4-bisphosphate generated by the action of PI 3-kinase [44]. The wortmannin-sensitive activation of PKB and wortmannin-insensitive activation of $p70^{S6K}$ described in the present study show that, in neutrophils, crystal-induced $p70^{S6K}$ activation is not dependent on PKB activation. However, activation of both PKC and $p70^{S6K}$ was inhibited by Compound 3 (Figures 5B and 5C), clearly implicating PKC as a putative mediator of $p70^{S6K}$ activation in neutrophils.

Interestingly, CPPD-induced activation of PKB was also inhibited by Compound 3 at 10 nM (Figure 5A). This result was unexpected, since Compound 3 (also known as RO-31-8220) is reported to be a specific inhibitor of PKC activation. We investigated this result further using two other 'specific' inhibitors of PKC, calphostin C and bis-indolylmaleamide, and found that both compounds inhibited PKB activation, by approx. 65% and 60% respectively. Since it is unlikely that all three inhibitors have non-specific inhibitory activity against PKC, it must be

Figure 5 Effects of Compound 3 on CPPD-crystal-induced PKB, PKC or p70^{S6K} activation in neutrophils

Neutrophils (5×10^6 /ml) with or without Compound 3 pretreatment (10 nM, 5 min) or wortmannin pretreatment (100 nM, 5 min) were incubated with plasma-opsonized CPPD (50 mg/ml) for 2 min and cytosolic extracts were prepared. (A) PKB and (B) PKC were immunoprecipitated and assayed with MBP and histone H1 respectively in the presence of [γ -³²P]ATP. (C) p70^{S6K} activity was determined by phosphotransferase determinations using MBP-NT as a substrate.

assumed that in neutrophils there is some dependency or cross-talk between PKB activation and PKC activation. However, the inhibition of PKB activation by wortmannin and the lack of inhibition of p70^{S6K} by wortmannin shows that PKB is not involved in p70^{S6K} activation in neutrophils. However, the results in Figure 5 show that Compound 3 inhibits activation of both PKC and p70^{S6K}, directly implicating PKC in p70^{S6K} activation. If Compound 3 was inhibiting PKB and if this inhibition was responsible for p70^{S6K} inhibition, then wortmannin would have also inhibited p70^{S6K}. A fuller investigation of the cross-talk between PKC and PKB is under investigation in this laboratory.

It has been reported that there are at least two kinase signalling pathways that lead to activation of p70^{S6K}, and that only one pathway is sensitive to wortmannin [18]. Furthermore, it has been reported that p70^{S6K} is activated by PKC-dependent and -independent mechanisms, and that rapamycin blocks both [15]. The present study describes a rapamycin-sensitive but wortmannin (and LY294002)-insensitive activation of p70^{S6K} in non-mitogenic signalling induced by crystal stimulation of neutrophils. We present data that show p70^{S6K} activation without the requirement for either PI 3-kinase or PKB activation, but with

the requirement for PKC activation. Interestingly, phospholipase C has been reported to activate p70^{S6K} in mitogenic signalling via receptor tyrosine kinase activation [43,45]. Crystals of CPPD are known to activate both phospholipase C and PKC [5–10] and PI 3-kinase [11] in non-mitogenic signalling pathways leading to respiratory burst and degranulation effects in neutrophils. We therefore suggest that the crystal-induced activation of p70^{S6K} in neutrophils, described in the present studies, proceeds through a phospholipase C/PKC-dependent pathway. Although a PI 3-kinase/PKB signalling pathway is activated by CPPD crystals in neutrophils, this pathway is not essential for the activation of p70^{S6K}.

The present paper describes the non-mitogenic, rapamycin-sensitive activation of p70^{S6K} in non-proliferating cells. The physiological role of this activation of p70^{S6K} in neutrophils is unknown, but it is unlikely to be related to rapid responses such as the respiratory burst or degranulation activity, since rapamycin had no inhibitory effects on these neutrophil functions (results not shown). The activation of p70^{S6K} in proliferating cells is associated with protein synthesis, and the non-mitogenic activation of this enzyme in neutrophils may be related to more long-term neutrophil responses that are dependent on protein synthesis, such as increases in membrane receptor expression.

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