Activation and tyrosine phosphorylation of p72^{syk} as well as calcium mobilization after hydrogen peroxide stimulation in peripheral blood lymphocytes

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To determine the regulatory role of p72^{syk} in lymphocyte activation, peripheral blood lymphocytes were treated with 10 mM hydrogen peroxide. Hydrogen peroxide induced a rapid elevation of p72^{syk} activity (4–6-fold) and a dramatic increase in tyrosine phosphorylation of a number of cellular proteins, including phospholipase C γ 1 (PLC γ 1) and p72^{syk}. Monoclonal antibodies to PLC γ 1 co-precipitated p72^{syk} from hydrogen peroxide-stimulated cell lysates, but not from unstimulated cell lysates. Furthermore, we observed a rise in intracellular Ca²⁺,

corresponding to the combination of extracellular Ca²⁺ influx and the release from intracellular Ca²⁺ stores. Extracellular Ca²⁺ influx was necessary for the sustenance of p72^{syk} activity, but not for the initiation of p72^{syk} activation induced by hydrogen peroxide. Taken together, these data suggested that one possible role of p72^{syk} was to activate PLC γ 1, at least in part through tyrosine phosphorylation, and then to trigger calcium mobilization in pig peripheral blood lymphocytes in response to hydrogen peroxide stimulation.

INTRODUCTION

Under normal conditions, mammalian cells exist in a redox environment which is homeostatically stable and which reflects a balance between the activities of oxidant and reductant chemical species. Oxidant stress might be operationally defined as that condition in which one or more oxidant moieties elicited a measurable biological response. Oxidant stress with hydrogen peroxide induced moderate tyrosine phosphorylation that was strongly potentiated by the combination with vanadate [1]. The mechanism was believed to be, at least in part, through inhibiting protein-tyrosine phosphatases [1] or activating protein-tyrosine kinases (PTKs) [2] or both. Up to now, the identified PTKs, activated by hydrogen peroxide itself, were insulin-receptor kinase in rat hepatoma cells [2] and p72^{syk} in a human early B-cell line, Ramos cells [3]. Hydrogen peroxide was also capable of modulating the inositol phospholipid signalling system in a variety of cell types [4,5], a likely pathway was by activating phospholipase C (PLC), which in turn hydrolysed phosphatidylinositol 4,5-bisphosphate to produce two intracellular second-messenger molecules: inositol 1,4,5-trisphosphate (IP₃) and 1,2-diacylglycerol [6].

Tyrosine phosphorylation has been implicated in the activation of phosphatidylinositol-specific PLC γ 1 [7–9]. Upon addition of epidermal growth factor (EGF) or platelet-derived growth factor (PDGF), PLC γ 1 was phosphorylated by, and became physically associated with, EGF and PDGF receptors containing intrinsic PTK activities [10,11]. The tyrosine-phosphorylation-dependent activation of PLC γ 1 was also observed in response to ligation of the T-cell receptor complex on T cells [12,13], the membrane IgM on B cells [14], and the high- or low-affinity Fc receptor for IgG in U937 monocytic cells [15]. These observations indicated that activation of PLC γ 1 by tyrosine phosphorylation was not restricted to receptor PTKs, but might also be achieved through non-receptor PTK(s). In fact, Weber et al. [16] reported that p56^{lck} was associated with PLC γ 1 after stimulation of the T-cell

antigen receptor. In addition, viral and cellular *src* kinases were reported to associate with PLC γ 1 [17]. Therefore, p72^{syk}, which is identified as a non-receptor PTK, and has the characteristics of bearing two SH2 domains, no N-terminal myristoylation site and a C-terminal negative regulatory site [18], was also expected to have some physiological functions in the phosphatidylinositol pathway. Based on these documented data, we hypothesize that hydrogen peroxide-activated p72^{syk} may be one of the candidates responsible for activating PLC γ 1 through tyrosine phosphorylation, perhaps promoting association of p72^{syk} with PLC γ 1, and subsequently triggering calcium mobilization. In the present study, pharmacological doses of hydrogen peroxide is employed to investigate this speculation; the resulting data show that hydrogen peroxide-activated p72^{syk} probably stimulates PLC γ 1 activity through tyrosine phosphorylation of PLC γ 1, and then triggers calcium mobilization in pig peripheral blood lymphocytes (PBLs).

MATERIALS AND METHODS

Materials and chemicals

Pig blood was obtained at a local slaughterhouse. Anti-Syk antibody raised against the synthetic partial polypeptide of p72^{syk} was prepared as previously described [18]. Pansorbin cells were from Calbiochem Corp. Lymphocyte separation solution ($d = 1.077$) was from Nacalai Tesque Inc. Percoll solution was from Pharmacia. Hydrogen peroxide and fura 2-AM were from Wako Pure Chemicals. Anti-phosphotyrosine antibody (4G10) and monoclonal anti-PLC γ 1 antibody were from Upstate Biotechnology Inc.

Cell isolation and stimulation

Protocols were as previously described [19]. Briefly, pig PBLs were harvested from fresh blood by using lymphocyte separation

Abbreviations used: PTK, protein-tyrosine kinase; HBSS, Hanks' balanced salt solution; PBLs, peripheral blood lymphocytes; [Ca²⁺]_i, intracellular calcium concentration; IP₃, inositol 1,4,5-trisphosphate; PLC, phospholipase C.

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solution and Percoll-gradient centrifugation, followed by erythrocyte sedimentation with physiological saline solution containing 3% dextran. Residual erythrocytes were lysed in hypotonic conditions. PBLs were washed twice and finally suspended $[(5-10) \times 10^7 \text{ cells/ml}]$ in Hanks' balanced salt solution (HBSS: 136.7 mM NaCl, 5.4 mM KCl, 0.81 mM MgSO_4 , 1.3 mM CaCl_2 , 0.33 mM Na_2HPO_4 , 0.44 mM KH_2PO_4 , 5.6 mM dextrose, 4.2 mM NaHCO_3 , pH 7.4). For extracellular Ca^{2+} depletion, cells were suspended in Ca^{2+} -free HBSS containing 1 mM EGTA. PBLs were stimulated by hydrogen peroxide at 37 °C with gentle stirring.

Measurement of intracellular calcium concentration ($[\text{Ca}^{2+}]_i$)

The cells were pretreated with fura 2-AM at a final concentration of 5 μM for 30 min at 37 °C. Washed cell suspension (2×10^6 cells/ml) was incubated in a cuvette for 5 min at 37 °C and stimulated by hydrogen peroxide under gentle stirring. Fluorescence was monitored by using a Shimadzu fluorescence spectrometer RF-5000 with standard monitor settings of 340 nm and 380 nm excitation and 490 nm emission.

Immunoblot procedures

After cells ($1 \times 10^7/\text{ml}$) were stimulated at 37 °C, the reaction was terminated by boiling with electrophoresis sample buffer for 3 min and separated by SDS/PAGE (12.5%-acrylamide gel). The separated proteins were transferred electrophoretically to polyvinylidene difluoride membranes. Immunoblot analysis was carried out with anti-phosphotyrosine antibody as described previously [17].

Immunoprecipitation and kinase assays

Stimulated cells (1×10^8 cells/ml) were lysed in 500 μl of RIPA buffer (5 mM EDTA, 150 mM NaCl, 2% Triton X-100, 100 μM Na_3VO_4 , 2 mM phenylmethylsulphonyl fluoride, 10 $\mu\text{g}/\text{ml}$ leupeptin, 50 mM Tris, pH 7.4) after quick centrifugation. The lysates were pre-cleared by incubation for 30 min with Pansorbin cells. Nuclei and cellular debris were removed by centrifugation at 16000 g for 15 min at 4 °C. The supernatant was immunoprecipitated with 0.4 μg of anti-Syk antibody or 2.5 μg of anti-PLC γ 1 antibody and 40 μl of Pansorbin for 1 h at 4 °C. Immunoprecipitates were washed three times with lysis buffer, once with 10 mM Hepes (pH 8.0) buffer containing 500 mM NaCl, and once with the same Hepes buffer without NaCl; then immunoprecipitates were phosphorylated as previously described [18]. The gels were treated with 1 M KOH at 56 °C for 1 h to remove phosphoserine and phosphothreonine before exposure when indicated. Autoradiography was obtained with a PhosphoImager (Fuji BAS 2,000).

Peptide mapping

All procedures were carried out as described by Cleveland et al. [20]. The p72^{syk} band from a 7.5% preparative gel was cut out with a razor blade, trimmed to 5 mm wide and then soaked for 30 min with occasional swirling in 10 ml of a solution containing final concentrations of 125 mM Tris/HCl, pH 6.8, 0.1% SDS and 1 mM EDTA. The final sample wells were filled with this same buffer, and each gel slice was pushed to the bottom of a well with a spatula. Spaces around the slices were filled by overlaying each slice with 10 μl of this buffer containing 20% glycerol. Finally, 10 μl of this buffer containing 10% glycerol and 0.1 μg of V8 protease was overlaid into each slot, and SDS/PAGE (18%-acrylamide gels) was performed by routine procedures,

with the exception that the current was turned off for 30 min when the Bromophenol Blue dye neared the bottom of the stacking gel.

Cell viability

Trypan Blue exclusion was used to monitor cell viability. Under the experimental conditions used, hydrogen peroxide was not cytotoxic to the cells, and the cell viability was always > 95%.

RESULTS

Hydrogen peroxide triggers rapid tyrosine phosphorylation in pig PBLs in a time- and dose-dependent manner

Tyrosine phosphorylation was an early and essential stage during the T-cell and B-cell activation. To address this phenomenon in pig PBLs activated by hydrogen peroxide, we analysed tyrosine-phosphorylated proteins upon addition of hydrogen peroxide using anti-phosphotyrosine-antibody immunoblotting. As shown in Figure 1, when PBLs were stimulated with 10 mM hydrogen peroxide for various periods of time (lanes 2–4), major tyrosine-phosphorylated proteins were at approx. 170, 135, 92, 76, 68–62, 58, 56, 52, 48, 42, 35 and 25–23 kDa. Among them, almost all the proteins except four bands (52, 48 and 25–23 kDa) appeared at an early time (lane 2, 1 min). The maximal effect was reached at 5 min of stimulation (lane 3) and was sustained within 10 min (lane 4). Exposure of PBLs to hydrogen peroxide also resulted in the dose-dependent tyrosine phosphorylation of proteins (Figure 1, lanes 5 and 6). Immunoblot assessment showed that tyrosine phosphorylation in response to hydrogen peroxide was observed at a dose of 1 mM (lane 5), and this tyrosine-phosphorylation response was further enhanced by stimulation with 10 mM hydrogen peroxide (lane 6).

Hydrogen peroxide stimulates kinase activity of p72^{syk}

Hydrogen peroxide was believed to induce tyrosine phosphorylation through the inhibition of protein-tyrosine phosphatase(s) [1], which was the counterpart of PTKs. To check

Figure 1 Hydrogen peroxide stimulation triggers rapid tyrosine phosphorylation in a time- and dose-dependent manner

PBLs (1×10^7 cells/ml) were stimulated with 10 mM hydrogen peroxide for the indicated times (lanes 2–4) or with various concentrations of hydrogen peroxide for 5 min at 37 °C (lanes 5 and 6). Western blotting using a monoclonal anti-phosphotyrosine antibody (4G10) was then performed as described in the Materials and methods section. Lane 1, control; lane 2, 1 min; lane 3, 5 min; lane 4, 10 min; lane 5, 1 mM H_2O_2 ; lane 6, 10 mM H_2O_2 . The positions of molecular-mass markers are shown on the left (kDa), and in Figures 2 and 3.

Figure 2 Time-course and concentration-dependence of p72^{syk} kinase activation by hydrogen peroxide in PBL cells

(a) After 1×10^8 cells/sample were stimulated with 10 mM hydrogen peroxide for the indicated times, they were processed for immunoprecipitation kinase assay as described in the Materials and methods section. The samples were subjected to SDS/PAGE (12.5% gels) and were revealed by autoradiography. Lane 1, control; lane 2, 15 s; lane 3, 1 min; lane 4, 2 min; lane 5, 5 min; lane 6, 10 min. (b) PBL cells were stimulated with various concentrations of hydrogen peroxide for 1 min, and immunoprecipitation kinase assays *in vitro* were performed as described above. Lane 1, control; lane 2, 1 mM; lane 3, 2 mM; lane 4, 5 mM; lane 5, 10 mM. Arrowheads indicate the positions of p72^{syk}.

the postulation that p72^{syk} might be involved in tyrosine phosphorylation by hydrogen peroxide, we prepared immunoprecipitates from untreated and treated cells and performed kinase assays *in vitro*. The data showed that p72^{syk} activity was rapidly and transiently elevated by stimulation with 10 mM hydrogen peroxide (Figure 2a). The p72^{syk} activity was detected within 15 s (lane 2), reached a peak at 1 min (lane 3), and then declined to the basal level after 5 min. The increase in p72^{syk} activity was also confirmed by phosphorylating the exogenous substrate histone H2B (results not shown).

We also observed that hydrogen peroxide-induced elevations of p72^{syk} activity were dose-dependent. As shown in Figure 2(b), maximal augmentation (6-fold) of p72^{syk} activity was obtained at 5 mM hydrogen peroxide (Figure 2b, lane 4). Use of 10 mM hydrogen peroxide did not enhance p72^{syk} activity further (Figure 2b, lane 5). These observations strongly indicated that hydrogen peroxide was able to activate p72^{syk}, thus leading to tyrosine phosphorylation of several proteins in pig PBLs.

p72^{syk} kinase activity exists in anti-PLC γ 1 immunoprecipitates after hydrogen peroxide stimulation

We assayed the anti-PLC γ 1 immunoprecipitates for the presence of p72^{syk} kinase activity, comparing hydrogen peroxide-stimulated and unstimulated cell lysates. In Figure 3(a), anti-PLC γ 1 immunoprecipitates from stimulated cells, but not from

Figure 3 Activity of p72^{syk} kinase *in vitro* co-precipitates with anti-PLC γ 1 antibodies, and peptide mapping

(a) Cells exposed to 10 mM hydrogen peroxide for 0 (lane 2) or 1 min (lanes 1 and 3) were lysed, and immunoprecipitations with anti-Syk antibody (lane 1) or with anti-PLC γ 1 antibody (lanes 2 and 3) and immunoprecipitation kinase assays were performed as described in the Materials and methods section. Reaction products were separated by SDS/PAGE (7.5% acrylamide), and the autoradiograph was obtained with a PhosphorImager. An arrowhead indicates the position of p72^{syk}. (b) p72^{syk} and 72-kDa protein bands in (a), lane 1 and lane 3 respectively, were cut, sliced and applied to a second SDS gel (18% acrylamide) in the presence of the *Staphylococcus aureus* V8 protease as described in the Materials and methods section. Lane 1, pattern of p72^{syk} immunoprecipitated by anti-Syk antibody; lane 2, pattern of a 72-kDa protein immunoprecipitated by anti-PLC γ 1 antibody.

unstimulated cells, possessed kinase activity that phosphorylated a protein co-migrating with p72^{syk}. The radioactive phosphate signal in this 72-kDa protein resisted alkaline hydrolysis (1 M KOH at 56 °C for 60 min), suggesting that phosphate incorporation was almost entirely on tyrosine residues. To obtain more evidence to define whether the 72-kDa protein recovered with anti-PLC γ 1 antibody was p72^{syk}, the gel containing the 72-kDa protein was cut and subjected to limited proteolysis with *Staphylococcus aureus* V8 protease. The resulting autoradiograph was shown in Figure 3(b). The digestion pattern of 72-kDa protein recovered with anti-PLC γ 1 antibody (Figure 3b, lane 2) was the same as that of p72^{syk} recovered by anti-Syk antibody (Figure 3b, lane 1), implying that the 72-kDa protein immunoprecipitated by anti-PLC γ 1 antibody was p72^{syk}. These findings suggested that hydrogen peroxide stimulation probably promoted association of p72^{syk} with PLC γ 1.

Hydrogen peroxide stimulates intracellular calcium mobilization and tyrosine phosphorylation of PLC γ 1 as well as p72^{syk}

In the next experiment, the capacity of hydrogen peroxide to stimulate calcium mobilization was examined. Cells were loaded with fura 2-AM and then stimulated with various doses of hydrogen peroxide: 1 mM hydrogen peroxide induced a gradual

Figure 4 Hydrogen peroxide stimulates intracellular calcium mobilization

Cells were loaded with 5 μ M fura 2-AM. $[Ca^{2+}]_i$ was quantified as described in the Materials and methods section. An asterisk means cells suspended in Ca^{2+} -free HBSS/1 mM EGTA. An arrow represents the starting point of hydrogen peroxide stimulation. The H_2O_2 concentrations used are shown on the right.

increase in $[Ca^{2+}]_i$ (Figure 4). When 10 mM hydrogen peroxide was applied, elevation of $[Ca^{2+}]_i$ reached a plateau and remained in a steady state within the time examined. In the absence of extracellular calcium, hydrogen peroxide was also able to induce calcium mobilization (Figure 4) corresponding solely to Ca^{2+} release from intracellular stores, since the contribution from Ca^{2+} influx was abrogated. Stimulation of the T-cell receptor led to an increase in tyrosine phosphorylation of PLC γ 1, which was likely to induce an augmentation of its catalytic activity [13,21] and subsequent generation of IP_3 as one of the second messengers [6]. Because IP_3 was a major mediator of Ca^{2+} release from intracellular stores, together with the observed coexistence of p72^{syk} activity in anti-PLC γ 1 immunoprecipitates after stimulation, we postulated that PLC γ 1 was tyrosine-phosphorylated by PTKs, of which p72^{syk} might be regarded as the most potential candidate, since hydrogen peroxide itself was incapable of activating src-like PTKs [3]. By Western blotting with anti-phosphotyrosine antibodies, the data in Figure 5 showed that several tyrosine-phosphorylated proteins were detectable in anti-PLC γ 1 immunoprecipitates from stimulated PBLs. However, tyrosine phosphorylation of PLC γ 1 was detectable only in immunoprecipitates from PBLs treated with hydrogen peroxide, although immunoprecipitates from control and stimulated PBLs contained similar levels of PLC γ 1. Hydrogen peroxide stimulation also induced tyrosine phosphorylation of p72^{syk} (Figure 5a, lane 4) without affecting the amount of p72^{syk} immunoprecipitated from unstimulated and stimulated PBLs (Figure 5b, lanes 3 and 4).

Sustained p72^{syk} kinase activity requires the influx of extracellular calcium

Addition of hydrogen peroxide resulted in p72^{syk} activation as well as an increase in $[Ca^{2+}]_i$ that was mainly due to the influx of extracellular Ca^{2+} into pig PBLs. This raised the argument whether influx of extracellular calcium has some effects on p72^{syk} activation. Therefore, a comparative experiment was performed to investigate the possible effect of calcium influx. Cells were suspended in HBSS or calcium-free HBSS buffer, and p72^{syk} kinase activities were assayed after stimulation with 10 mM

Figure 5 Hydrogen peroxide stimulation induces tyrosine phosphorylation of PLC γ 1 and p72^{syk}

Cell lysates from 10^6 unstimulated (lanes 1 and 3) or stimulated (lanes 2 and 4) PBLs were immunoprecipitated with anti-PLC γ 1 antibody (lanes 1 and 2) or anti-Syk antibody (lanes 3 and 4), respectively. Immunoblotting was performed as described in the Materials and methods section. (a) Immunoblotted by 4G10. (b) Immunoblotted by anti-PLC γ 1 antibody (lanes 1 and 2) or anti-Syk antibody (lanes 3 and 4). Arrowheads and arrows indicate the positions of PLC γ 1 and p72^{syk} respectively. Abbreviations: IP, immunoprecipitation; IB, immunoblot.

hydrogen peroxide for the indicated periods of time. As shown in Figure 6, initiation of p72^{syk} activation occurred at the same level both with and without extracellular calcium (Figure 6, lanes 2 and 3); however, disappearance of p72^{syk} kinase activity was rapid with no influx of extracellular calcium (Figure 6, lanes 5 and 7). In the unstimulated condition, chelation of extracellular calcium by EGTA did not affect p72^{syk} activity (results not

Figure 6 Sustenance of p72^{syk} kinase activity requires extracellular calcium influx

Cells were stimulated with 10 mM hydrogen peroxide at the indicated times, and then immunoprecipitation kinase assay was processed as described in the Materials and methods section. Lane 1, unstimulated cells; lanes 2 and 3, 15 s stimulation; lanes 4 and 5, 1 min; lanes 6 and 7, 2 min; lane 8, hydrogen peroxide was added immediately after cell lysis. Arrowhead indicates the position of p72^{syk}.

shown). These observations implied that influx of extracellular calcium was not required for p72^{syk} activation, but had some role in sustaining p72^{syk} kinase activity by an unknown mechanism dependent on extracellular Ca²⁺ influx. To address the mechanism of p72^{syk} activation, we also investigated whether p72^{syk} was activated in the cell-free system. The result showed that hydrogen peroxide could activate p72^{syk} in the cell lysate before immunoprecipitation (Figure 6, lane 8). In contrast, p72^{syk} activity was slightly lower than the control when hydrogen peroxide was directly added to anti-p72^{syk} immunoprecipitates (results not shown). Taken together, these data suggested that hydrogen peroxide was incapable of activating p72^{syk} directly.

DISCUSSION

The combined use of hydrogen peroxide and vanadate triggered strong tyrosine phosphorylation in various types of cells [22–25], however, hydrogen peroxide itself had little effect on tyrosine phosphorylation or required a prolonged exposure to induce tyrosine phosphorylation [26]. In the present paper, we reported evidence to show that hydrogen peroxide alone could induce tyrosine phosphorylation at an early time (Figure 1, lane 2, 1 min) and at low dose (Figure 1, lane 5, 1 mM) in pig PBLs. The tyrosine-phosphorylated proteins might be classified into two groups on the basis of kinetic difference of tyrosine phosphorylation on these proteins. The first group consisted of 170, 135, 92, 76, 68–62, 58, 56, 42 and 35 kDa proteins which appeared at an early time and with low-dose treatment with hydrogen peroxide; the second group included 52, 48 and 25–23 kDa proteins, which were observed after incubation for 5 min or treatment with 10 mM hydrogen peroxide. In contrast, O'Shea et al. [23] observed trace tyrosine phosphorylation at the dose of 10 mM hydrogen peroxide in human PBLs. Possible explanations for this difference were: (a) PTKs and protein-tyrosine phosphatases existed in human PBLs were resistant to oxidant stress; (b) endogenous catalase activity, which could act against the effect of hydrogen peroxide, was higher in human PBLs; (c) the difference in nutritional levels of vanadate, which potentiated tyrosine phosphorylation induced by hydrogen peroxide, between pig and human PBLs.

As an insulinomimetic agent, a series of documented pieces of evidence showed that hydrogen peroxide itself was capable of activating insulin-receptor intrinsic PTK activity [2] and of markedly potentiating insulin-induced tyrosine phosphorylation [26]. In this study, hydrogen peroxide was demonstrated to be able to activate p72^{syk} rapidly and transiently in pig PBLs (Figure 2a). In addition, Schieven et al. [3] also recently reported that hydrogen peroxide alone could activate p72^{syk} in Ramos cells, but not any member of *src*-family PTKs, such as p56^{lck}, p56/59^{lyn} and p59^{yn}. These data strongly indicated the notion that p72^{syk} was more sensitive to oxidant stress than were members of the *src*-family. Tyrosine phosphorylation was a balanced result between the interaction of PTKs and protein-tyrosine phosphatases. The status of tyrosine phosphorylation would be enhanced by breaking this balance through either stimulating PTK activity or inhibiting protein-tyrosine phosphatase activity. Hydrogen peroxide was a potential inhibitor of protein-tyrosine phosphatases, and inhibition of protein-tyrosine phosphatases partly contributed to the hydrogen peroxide-induced tyrosine phosphorylation [1]. Combined with our demonstration in this study, the considerable tyrosine phosphorylation elicited by hydrogen peroxide in pig PBLs was, to some extent, due to the activation of PTK p72^{syk} and the inhibition of protein-tyrosine phosphatases. Further investigation showed that p72^{syk} activation occurred when hy-

drogen peroxide was added to cell lysates before immunoprecipitation (Figure 6, lane 8), but not directly to anti-p72^{syk} antibody immunoprecipitates (results not shown), implying that hydrogen peroxide activated p72^{syk} through an indirect pathway. However, at present the molecular mechanism of the activation of p72^{syk} by oxidant stress is unknown.

In this study, we observed that hydrogen peroxide stimulation promoted the co-precipitation of p72^{syk} with anti-PLCγ1 antibody. The evidence was from the experiment demonstrating that p72^{syk} activity was detected in immunoprecipitates using anti-PLCγ1 antibody in hydrogen peroxide-treated PBLs (Figure 3a, lane 3). The co-precipitation of p72^{syk} with anti-PLCγ1 antibody might be significant for our understanding of how activated p72^{syk} exerts its function. Immunoblot analysis revealed that both PLCγ1 and p72^{syk} were tyrosine-phosphorylated after stimulation. These observations indicated the possibility that PLCγ1 was one of the substrates phosphorylated by activated p72^{syk}. The following evidence supported this interpretation. (1) We did not observe any radioactive phosphate signal around the 60-kDa position, where *src*-family PTKs appeared, if possible, in anti-PLCγ1 immunoprecipitates after electrophoresis and exposure, except a 72-kDa band (Figure 3a). In the case of association of p56^{lck} with PLCγ1 after stimulation of the T-cell antigen receptor, the SH2 domains of PLCγ1 have been implicated to play a key role in directing the interaction of p56^{lck} with PLCγ1 [16]. (2) At 10 mM, hydrogen peroxide alone could not activate *src*-family kinases [3]. Recently several reports also implicated that p72^{syk} was responsible for tyrosine phosphorylation of PLC-γ1 or -γ2, because tyrosine phosphorylation of p72^{syk} and PLCγ1 by FcγRIIA cross-linking was lost in the FcγRIIA mutants [27,28]. In addition, Kolanus et al. [29] demonstrated that a CD16/Syk chimera increased the tyrosine phosphorylation of PLCγ1 through receptor stimulation, whereas cross-linking CD16/Fyn or CD16/Zap70 was not able to produce this increase. In a chicken B-cell line, DT40, p72^{syk} was required to couple the B-cell receptor to the phosphatidylinositol pathway, since tyrosine phosphorylation of PLCγ2, IP₃ generation and calcium mobilization were lost in p72^{syk}-negative mutants [30]. However, whether that interaction of p72^{syk} with PLCγ1 is direct or indirect, through a protein tightly bound to either p72^{syk} or PLCγ1, is still obscure; further investigation is required to elucidate the precise mechanism.

Phosphorylation of tyrosine residues of PLCγ1 has been implicated in activation of PLCγ1 [7–9, 12–15], which in turn hydrolysed phosphatidylinositol 4,5-bisphosphate to generate the intracellular second messenger IP₃ responsible for calcium mobilization. As expected, we observed the elevation of [Ca²⁺]_i, an early phenomenon in activated lymphocytes, after addition of hydrogen peroxide. When cells were suspended in calcium-free 1 mM EGTA buffer, hydrogen peroxide was able to induce a weak calcium signal (one-quarter of that with calcium present). This observation suggested that the increase in [Ca²⁺]_i was from both influx of extracellular calcium and release of stored calcium. Hydrogen peroxide-triggered elevations of [Ca²⁺]_i were abolished by pretreatment with the PTK inhibitor genistein, but not by the protein kinase C inhibitor H7 (results not shown), indicating that calcium elevation by hydrogen peroxide was dependent on PTK activation, but was not due to cell membrane damage. Schieven et al. [4] recently reported that challenge with hydrogen peroxide in human B cells induced rapid but transient IP₃ accumulation, which reached a peak at 1 min. The kinetics of IP₃ accumulation in human B-cells was closely related to that of p72^{syk} activity in pig PBLs after hydrogen peroxide stimulation. Moreover, stimulation by hydrogen peroxide for 1 min triggered heavy tyrosine phosphorylation of PLCγ1 (Figure 5, lane 2). Therefore it was

reasonable to speculate, but we could not prove, that the observed calcium release from internal stores was likely to be regulated by IP₃ derived from hydrolysis of phosphatidylinositol by activated PLC γ 1. However, interleukin-2 stimulation was only capable of activating p72^{syk}, but failed to induce calcium mobilization in PBLs [31], implicating that either different signalling pathways were used or additional signals were necessary for inducing downstream effectors. Next, the effect of the influx of extracellular calcium on p72^{syk} activity was assessed; the data presented demonstrated that influx of extracellular calcium was not required for the initiation of p72^{syk} activation, but was essential to sustain its activity, suggesting that a calcium-dependent mechanism was involved in regulating p72^{syk} activity. Zhao et al. [32] reported that c-Src tyrosine kinase activity was up-regulated by elevation of [Ca²⁺]_i due to the influx of extracellular calcium in cultured keratinocytes; however, c-Yes, another member of the *src* family, was inactivated in the same cells [33]. We also observed that p72^{syk} activity was negatively regulated by influx of extracellular calcium in platelets after thrombin stimulation [34]. This discrepancy reflected the complexity with which different calcium-dependent mechanisms participate in modulating the activities of PTKs.

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