Activation of human platelets by peroxovanadate is associated with tyrosine phosphorylation of phospholipase C γ and formation of inositol phosphates

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Vanadate ions in the presence of H₂O₂ (peroxovanadate) induce a marked increase in the degree of tyrosine phosphorylation of proteins in human platelets. This increase preceded the onset of platelet shape change and aggregation, and is associated with activation of phospholipase C and increased [³²P]phosphorylation of proteins of 47 kDa, a substrate for protein kinase C, and 20 kDa, a substrate for both myosin light-chain kinase and protein kinase C. The non-selective inhibitor of protein kinases, staurosporine, inhibits the increase in tyrosine phosphorylation

of nearly all proteins and inhibits completely all other functional responses, suggesting that these events may be linked. In support of this, peroxovanadate stimulates tyrosine phosphorylation of phospholipase C γ 1, suggesting that this may underlie its mechanism of platelet activation. Staurosporine also inhibited activation of phospholipase C by collagen, suggesting that tyrosine phosphorylation has an important role in the early stages of collagen-induced platelet activation.

INTRODUCTION

During platelet activation a number of proteins undergo tyrosine phosphorylation (Ferrell and Martin, 1988; Golden and Brugge, 1989; Nakamura and Yamamura, 1989). Several reports indicate that aggregation contributes to this increase in tyrosine phosphorylation, and implicate the major fibrinogen receptor, glycoprotein IIb/IIIa, in this process (Ferrell and Martin, 1989; Golden et al., 1990). The non-integrin gpIV, a putative thrombospondin/collagen receptor, has also been implicated in tyrosine kinase activation (Huang et al., 1991). In addition, activation of protein kinase C and mobilization of Ca²⁺ is also associated with increased tyrosine phosphorylation (Takayama et al., 1991).

Phospholipase C γ (PLC γ) contains the *src* homology domain SH2, which is involved in protein clustering through binding to tyrosine-phosphorylated regions of other proteins (Matsuda et al., 1990; Mayer et al., 1991). These interactions potentially regulate the activity of PLC γ . In addition, PLC γ 1 has been shown to undergo tyrosine phosphorylation at three distinct sites (Rhee, 1991), and this has been linked to an increase in catalytic activity (Nishibe et al., 1990). PLC γ 1, and in lower levels PLC γ 2, have been identified in human platelets (Banno et al., 1992) although it is not known whether either species undergoes tyrosine phosphorylation on platelet activation.

We sought a method of inducing tyrosine phosphorylation in platelets in order to study its consequences for regulation of PLC and functional responses. Inazu et al. (1990) demonstrated that a peroxovanadate complex {diperoxo-orthovanadate; [VO₂(O₂)₂]³⁻}, formed in solutions of orthovanadate and H₂O₂ (Greenwood and Earnshaw, 1984), induces high levels of protein tyrosine phosphorylation in association with platelet activation. We have therefore used peroxovanadate to investigate whether protein tyrosine phosphorylation stimulates PLC activity in platelets and whether this underlies the mechanism of platelet activation by the vanadate complex.

MATERIALS AND METHODS

Reagents and antibodies

Ro 31-8220 was a gift from Roche Products (Welwyn Garden

City, Herts., U.K.). Anti-pp60^{src} and rabbit anti-PLC γ 1 antibodies were gifts from Dr. J. Brugge (Philadelphia, U.S.A.) and Dr. T. Pawson and Dr. C. Ellis (Toronto, Canada) respectively. Anti-phosphotyrosine mouse monoclonal antibody was purchased from ICN Flow (High Wycombe, Bucks., U.K.). Staurosporine was from Sigma (Poole, Dorset, U.K.). *myo*-[³H]inositol (sp. radioactivity 18.2 Ci/mmol), [³²P]P_i (sp. radioactivity 8500–9120 Ci/mmol) and [³H]5-hydroxytryptamine (5-HT) (sp. radioactivity 27 Ci/mmol) were from Amersham International (Cardiff, U.K.). Collagen was purchased from Hormon-Chemie (Munich, Germany). U44069 was purchased from Sigma. All other reagents were of analytical grade.

Platelet preparation and experimentation

Blood was drawn on the day of the experiment from aspirin-free volunteers, and sterile 20 mM sodium citrate was used as anti-coagulant. Platelet-rich plasma was obtained by centrifugation at 200 *g* for 20 min. Platelets were isolated from platelet-rich plasma by centrifugation at 1000 *g* for 10 min in the presence of prostacyclin (0.1 μ g/ml), used to increase intracellular cyclic AMP. Platelets were resuspended at 37 °C in 1 ml of modified Tyrode–Hepes medium (composition in mM: NaCl 138, NaH₂PO₄ 0.36, KCl 2.9, NaHCO₃ 12, Hepes 20, glucose 5, MgCl₂ 1; pH 7.3) and labelled with [³H]inositol (50 μ Ci/ml for 3 h), [³²P]P_i (2 mCi/ml for 1 h) or [³H]5-HT (0.5 μ Ci/ml for 1 h). Platelets were then centrifuged in the presence of prostacyclin (0.1 μ g/ml) at 1000 *g* for 10 min and resuspended at a concentration of (2–8) $\times$ 10⁸/ml in the above buffer. LiCl (10 mM), which inhibits conversion of inositol monophosphate into free inositol, was added to [³H]inositol-labelled platelets. Platelets were left for at least 30 min before experimentation.

Platelet suspensions (0.29 ml) were prewarmed at 37 °C for 5 min before addition of compounds (10 μ l) as appropriate. Experiments were stopped by transfer to 940 μ l of chloroform/methanol/HCl (50:100:1, by vol.) for analysis of [³H]inositol phosphates, to 0.3 ml of 6% (v/v) glutaraldehyde in phosphate buffer (pH 7.3) for analysis of [³H]5-HT secretion, or to an equal volume of Laemmli buffer (containing 4% SDS and 5% 2-

mercaptoethanol) for analysis of protein phosphorylation. Protein phosphorylation, formation of [^3H]inositol phosphates and [^3H]5-HT secretion were measured as described previously (Nunn and Watson, 1987).

Immunoblotting

Samples were heated in Laemmli buffer at 80 °C for 30 min. Proteins were separated by SDS/PAGE (10% gels) and transferred to polyvinylidene difluoride (PVDF; Immobilon P; Millipore) blotting membranes by semi-dry transfer (90 min, 15 V). Non-specific binding was blocked with 5% BSA in TBS-T (Tris 20 mM, NaCl 137 mM, Tween-20 0.1%, pH 7.6), before probing with the primary antibodies PY20 (anti-phosphotyrosine) or $\alpha\text{PLC}\gamma$ (Decker et al., 1990). Blots were washed five times in TBS-T before detection of bound antibody with horseradish-peroxidase-conjugated sheep anti-mouse IgG (NA 931; Amersham), or donkey anti-rabbit IgG (NA 934). After further washing, the membranes were treated with Amersham ECL reagents, and exposed to ECL-hyperfilm.

Blots were stripped of bound antibody by washing in TBS-T containing 2% SDS at 80 °C for 30 min. The blot was then washed five times with TBS-T, and the stripping of antibody was verified by re-probing with horseradish-peroxidase-conjugated anti-IgG secondary antibody and ECL detection. Blots were then re-blocked and re-probed as detailed above.

Immunoprecipitation

Platelets were lysed on ice for 1 h with an equal volume of ice-cold extraction buffer consisting of 2% Triton X-100, 0.25% sodium deoxycholate, 10 mM EDTA, 100 μM Na_3VO_4 , 100 mM Tris, 1 mM phenylmethanesulphonyl difluoride, 10 mM NaF, 1 mg/ml DNAase 1 and 20 μM cytochalasin B at pH 7.6. All subsequent steps were carried out at 4 °C. Insoluble cell debris was removed by centrifuging in a Microfuge (13000 g) for 30 min, and the platelet lysate was pre-cleared with 200 μl of protein A-Sepharose CL 4B. Each sample was incubated with 30 μl of rabbit anti-PLC γ antibody for 1 h, before addition of 100 μl of Protein A-Sepharose CL 4B. The sample was incubated overnight with continual mixing. Protein A-Sepharose CL 4B was pelleted in a Microfuge (13000 g), and washed six times with a 1:1 mixture of extraction buffer (without DNAase 1 or cytochalasin B) to Tyrode-Hepes buffer. Immunoprecipitated protein was solubilized in Laemmli sample buffer and separated by SDS/PAGE (10% gels) and analysed by immunoblotting.

Analysis of results

In most cases, results are shown as means $\pm$ S.E.M. of at least three separate experiments performed in quadruplicate. Statistical analyses were performed by Student's *t* test, with *P* < 0.05 taken as the level of significance.

RESULTS

Protein phosphorylation

Preliminary studies showed that maximal increases in tyrosine phosphorylation were observed with a combination of orthovanadate (400 μM) and H_2O_2 (4 mM), although neither agent had any effect on their own. Figure 1(a) shows the time course of tyrosine phosphorylation induced by orthovanadate (400 μM) and H_2O_2 (4 mM). Tyrosine phosphorylation increased in a number of proteins, with major increases observed in proteins of molecular masses approx. 36–40, 54, 60, 65, 70, 85, 98, 120 and

140 kDa. This pattern of increased tyrosine phosphorylation is in reasonable agreement with that described by Inazu et al. (1990) in platelets challenged with peroxovanadate. The major band of 54 kDa co-migrated with a protein detected by a monoclonal antibody to pp60^{src} (Golden et al., 1986) and may represent a proteolytic degradation product of pp60^{src} (Rendu et al., 1991). Increased protein phosphotyrosine was detectable within 5 s of addition of H_2O_2 and vanadate (Figure 1), and preceded the increase in phosphorylation of pleckstrin (47 kDa), the major substrate of protein kinase C, and myosin light chains (MLC; 20 kDa), which are phosphorylated at distinct sites by MLC kinase and protein kinase C (Figure 1b). Tyrosine phosphorylation appeared to reach an early plateau within 20 s, which was maintained for up to 120 s, followed by a further increase between 120 and 300 s (Figure 1a).

We used the kinase inhibitors staurosporine and Ro 31-8220 to investigate whether peroxovanadate directly induces tyrosine phosphorylation or whether this is secondary to activation of protein kinase C. Staurosporine is a relatively non-specific

Figure 1 Tyrosine and [^{32}P]phosphorylation in platelets stimulated by peroxovanadate

^{32}P -labelled platelets were stirred in an aggregometer at 37 °C and challenged with peroxovanadate (400 μM sodium vanadate/4 mM H_2O_2) for the times indicated. Proteins were solubilized and separated on a 10% polyacrylamide gel, and either transferred to PVDF membranes or dried for autoradiography. The Western blot was probed with anti-phosphotyrosine antibody as described in the Materials and methods section. Protein tyrosine phosphorylation is shown in (a), and [^{32}P]P_i incorporation into pleckstrin, the major PKC substrate (47 kDa), and MLC (20 kDa) are shown in (b). Results are representative of three separate experiments.

Figure 2 Effects of staurosporine and Ro 31-8220 on protein-tyrosine, pleckstrin and MLC phosphorylation

^{32}P -labelled platelets were stirred in an aggregometer at 37°C and challenged with peroxovanadate [$400\ \mu\text{M}$ sodium vanadate (V)/ $4\ \text{mM}$ H_2O_2 (H)] for 60 s. Staurosporine (Stauro.; $10\ \mu\text{M}$) or Ro 31-8220 ($10\ \mu\text{M}$) was added 1 min before peroxovanadate. Proteins were solubilized and separated on a 10% polyacrylamide gel, and either transferred to PVDF membranes or dried for autoradiography. The Western blot was probed with anti-phosphotyrosine antibody as described in the Materials and methods section. The same samples were used for the anti-phosphotyrosine Western blot (a) and for autoradiography, showing pleckstrin (47 kDa) and MLC (20 kDa) phosphorylation (b). Results are representative of two experiments.

inhibitor of both serine/threonine and tyrosine kinases (Watson et al., 1988; Geissler et al., 1990), whereas Ro 31-8220 is an analogue of staurosporine with increased selectivity for protein kinase C (Davis et al., 1989; Walker and Watson, 1993). Consistent with this, a maximally effective concentration of staurosporine ($10\ \mu\text{M}$) completely blocked peroxovanadate-induced pleckstrin and MLC phosphorylation, and caused a full inhibition of tyrosine phosphorylation of nearly all proteins,

Figure 3 Aggregation and secretion responses to peroxovanadate

$[\text{^3H}]\text{5-HT}$ -labelled platelets were stirred in an aggregometer at 37°C and challenged with peroxovanadate [$400\ \mu\text{M}$ sodium vanadate (V)/ $4\ \text{mM}$ H_2O_2 (H)] for 60 s. Staurosporine (St.; $10\ \mu\text{M}$) or Ro 31-8220 (Ro 31.; $10\ \mu\text{M}$) was added 1 min before addition of peroxovanadate. Shown are aggregation traces and $[\text{^3H}]\text{5-HT}$ secretion values representative of four separate experiments performed in triplicate.

although the 54 kDa protein was a notable exception in that its phosphorylation was only inhibited partially (Figure 2). In contrast, a maximally effective concentration of Ro 31-8220 ($10\ \mu\text{M}$) completely inhibited phosphorylation of the 47 kDa protein induced by peroxovanadate, but only decreased partially the phosphorylation of MLC and had no apparent effect on tyrosine phosphorylation (Figure 2). The partial inhibition of phosphorylation of MLC by Ro 31-8220 is thought to reflect inhibition of the protein kinase C-mediated component of phosphorylation (Walker and Watson, 1993). These results provide further evidence for the selectivity of Ro 31-8220 as an inhibitor of protein kinase C and confirm the non-selectivity of staurosporine.

Functional responses

The increase in tyrosine phosphorylation induced by orthovanadate ($400\ \mu\text{M}$) and H_2O_2 ($4\ \text{mM}$) was associated with shape change, aggregation and secretion responses (Figure 3). On its own, vanadate ($400\ \mu\text{M}$) had no effect on functional responses, whereas occasionally H_2O_2 ($4\ \text{mM}$) appeared to produce a transient decrease in light transmission, which may reflect effervescence. The onset of shape change and aggregation lagged behind the early increase in tyrosine phosphorylation and appeared to occur in parallel with phosphorylation of 20 kDa and 47 kDa proteins (Figures 1 and 3). Similar results (not shown) were seen in platelets treated with indomethacin.

Aggregation, shape change and $[\text{^3H}]\text{5-HT}$ secretion induced by peroxovanadate were inhibited completely by staurosporine ($10\ \mu\text{M}$), suggesting that these responses are initiated by tyrosine phosphorylation (Figure 3). In contrast, Ro 31-8220 ($10\ \mu\text{M}$) had no apparent effect on peroxovanadate-induced shape change, but slowed the rate of aggregation and markedly decreased the

Table 1 Measurements of PLC activity

Results are expressed as percentage (mean $\pm$ S.E.M.) of basal values, induced by peroxovanadate (400 μ M vanadate, 4 mM H_2O_2), U44069 (20 μ M) or collagen (30 μ g/ml) during 5 min stimulation ($n = 6-12$). The mean basal value of [3H]inositol phosphates was 411 ± 15 d.p.m. Indomethacin (10 μ M) was present in all collagen and U44069 experiments. Platelets were treated with 10 μ M staurosporine or 10 μ M Ro 31-8220 for 5 min before stimulation with peroxovanadate. Values significantly different from basal are indicated by * ($P < 0.05$). Relevant values significantly different from collagen-stimulated platelets are indicated by † ($P < 0.05$).

	PLC activity (% of basal)
Basal	100 $\pm$ 7
Basal and Ro 31-8220	107 $\pm$ 3
Basal and staurosporine	119 $\pm$ 4
Vanadate	102 $\pm$ 9
H_2O_2	110 $\pm$ 10
Peroxovanadate	177 $\pm$ 11*
Peroxovanadate and Ro 31-8220	193 $\pm$ 11*
Peroxovanadate and staurosporine	89 $\pm$ 8
Collagen	147 $\pm$ 9*
Collagen and Ro 31-8220	203 $\pm$ 16*†
Collagen and staurosporine	108 $\pm$ 6†
U44069	130 $\pm$ 7*
U44069 and staurosporine	138 $\pm$ 10*

Figure 4 Tyrosine phosphorylation of PLC γ induced by peroxovanadate

Platelets (2×10^9) were challenged with peroxovanadate (400 μ M sodium vanadate/4 mM H_2O_2) for 20 s (+) and compared with control samples (-). Platelets were lysed and proteins immunoprecipitated with anti-PLC γ antibody. The protein in the immunoprecipitate was separated by PAGE (10% gel), transferred to PVDF membranes and treated with an antibody to either PLC γ or phosphotyrosine (PY) (see the Materials and methods section). Antibodies were detected by using Amersham ECL reagents and exposure to ECL-hyperfilm. An antibody-stripping protocol enabled the same blot to be probed for both tyrosine-phosphorylated protein and PLC γ sequentially.

degree of secretion (Figure 3). The inhibitory action of Ro 31-8220 is similar to that described previously against thrombin-induced activation of platelets (Walker and Watson, 1993).

Formation of [3H]inositol phosphates

The ability of peroxovanadate to stimulate functional responses in platelets in association with increased phosphorylation of 20 kDa and 47 kDa proteins resembles the characteristics of platelet activation by PLC-coupled receptors such as those for thrombin or platelet-activating factor. We therefore investigated whether peroxovanadate induces activation of PLC. Peroxovanadate stimulated marked formation of total [3H]inositol phosphates, which was not altered in the presence of Ro 31-8220 but was inhibited completely by staurosporine (Table 1). In contrast,

we have reported previously that staurosporine has no effect on thrombin-induced formation of [3H]inositol phosphates (Watson et al., 1988) and observed a similar result with the thromboxane A_2 mimetic U44069 (Table 1). In contrast, formation of [3H]inositol phosphates by collagen in indomethacin-treated platelets was inhibited completely by staurosporine, although this was potentiated in the presence of Ro 31-8220 (Table 1).

Tyrosine phosphorylation of PLC γ

In view of the ability of peroxovanadate to stimulate formation of total [3H]inositol phosphates, we sought to determine whether PLC γ was among the proteins which undergo tyrosine phosphorylation, using a polyclonal antibody raised against the C-terminus of bovine brain PLC γ 1 (Decker et al., 1990). Several tyrosine-phosphorylated proteins were detectable in anti-PLC γ immunoprecipitates from control and stimulated platelets. However, tyrosine phosphorylation of PLC γ 1 was detectable only in immunoprecipitates from platelets treated with peroxovanadate (20 s), despite the fact that immunoprecipitates from control and stimulated platelets contain similar levels of PLC γ 1 (Figure 4).

DISCUSSION

Vanadate behaves as a phosphate mimetic and therefore is relatively non-specific in its action. Nevertheless, the present study demonstrates that vanadate induces a marked increase in tyrosine phosphorylation in platelets, which is associated with stimulation of functional responses. The aim of this study has therefore been to investigate whether this increase in phosphorylation initiates platelet activation.

Stimulation of tyrosine phosphorylation and functional responses induced by peroxovanadate are inhibited completely by the non-specific protein kinase inhibitor staurosporine, apart from a protein of 54 kDa identified as *src*, whose state of phosphorylation is decreased partially. In contrast, staurosporine slows the rate of aggregation induced by thrombin and inhibits markedly secretion of 5-HT, but does not cause full inhibition, despite a complete absence of an increase in ^{32}P incorporation into 47 and 20 kDa proteins (Watson et al., 1988; Walker and Watson, 1993). These data provide evidence therefore that tyrosine phosphorylation, protein [^{32}P]phosphorylation and functional responses induced by peroxovanadate are associated rather than separate events, and that tyrosine phosphorylation of *src* may not play a role in platelet activation.

It is possible that the increase in tyrosine phosphorylation leads to the activation of PLC, and that this underlies the later increase in ^{32}P incorporation into 20 kDa and 47 kDa proteins and platelet activation. Consistent with this, formation of [3H]inositol phosphates induced by peroxovanadate was inhibited completely by staurosporine, and ^{32}P incorporation and platelet activation appeared to lag behind the early increase in tyrosine phosphorylation. Further, the staurosporine analogue Ro 31-8220, which is claimed to be a selective inhibitor of protein kinase C (Davis et al., 1989), had no apparent effect on tyrosine phosphorylation and formation of inositol phosphates induced by peroxovanadate, despite complete inhibition of phosphorylation of 47 kDa protein (the major substrate for protein kinase) and a marked decrease in aggregation and secretion. The inhibition of aggregation and secretion by Ro 31-8220 is consistent with a role of protein kinase C in these functional responses (Walker and Watson, 1993). The results therefore demonstrate that the early increase in tyrosine phosphorylation induced by peroxovanadate is not mediated through activation of protein kinase C, nor through the onset of aggregation or secretion.

Activation of platelets by peroxovanadate is associated with increased tyrosine phosphorylation of PLC γ 1, suggesting that this may underlie the increase in formation of inositol phosphates and onset of functional responses. The ability of various receptor tyrosine kinases, e.g. receptors for epidermal growth factor and platelet-derived growth factor, to induce formation of inositol phosphates is also associated with increased tyrosine phosphorylation of PLC γ (Meisenhelder et al., 1989; Decker et al., 1990; Goldschmidt-Clermont et al., 1991), and tyrosine phosphorylation of PLC γ has been reported to increase its catalytic activity (Nishibe et al., 1990). Alternatively, the general increase in tyrosine phosphorylation in platelets induced by peroxovanadate may provide a suitable membrane anchor for the SH2 domain of PLC γ 1, thereby leading to its activation. Tyrosine phosphorylation of certain platelet proteins during physiological stimulation requires the integrity of platelet integrin receptors and other receptors which bind the RGD peptide sequence (Ferrell and Martin, 1989; Golden et al., 1990). It is an attractive hypothesis that binding of fibrinogen, thrombospondin or other proteins to these receptors during primary aggregation stimulates secondary PLC activity through tyrosine phosphorylation of PLC γ 1. Similarly, the inhibition of collagen-stimulated PLC activation by staurosporine observed in the present study may suggest that tyrosine kinase activity has an important role in the early stages of collagen-induced platelet activation.

In conclusion, we have demonstrated that peroxovanadate causes the tyrosine phosphorylation of platelet PLC γ 1, stimulation of PLC activity, and platelet activation. The physiological significance of this remains to be determined.

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REFERENCES

- Banno, Y., Nakashima, T., Kumada, T., Ebisawa, K., Nonomura, Y. and Nozawa, Y. (1992) *J. Biol. Chem.* **267**, 6488–6494
- Davis, P. D., Hill, C. H., Keech, E., Lawton, G., Nixon, J. S., Sedgwick, A. D., Wadsworth, J., Westmacott, D. and Wilkinson, S. E. (1989) *FEBS Lett.* **259**, 61–63
- Decker, S. J., Ellis, C., Pawson, T. and Thierry, V. (1990) *J. Biol. Chem.* **265**, 7009–7015
- Ferrell, J. E. and Martin, S. G. (1988) *Mol. Cell. Biol.* **8**, 3603–3610
- Ferrell, J. E. and Martin, S. G. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2234–2238
- Geissler, J. F., Traxler, P., Regenass, U., Murray, B. J., Roesel, J. L., Meyer, T., McGlynn, E., Storni, A. and Lydon, N. (1990) *J. Biol. Chem.* **265**, 22255–22261
- Golden, A. and Brugge, J. S. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 901–905
- Golden, A., Nemeth, S. P. & Brugge, J. S. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 852–856
- Golden, A., Brugge, J. S. and Shattil, S. J. (1990) *J. Cell Biol.* **111**, 3117–3217
- Goldschmidt-Clermont, P. J., Kim, J. W., Machesky, L. M., Rhee, S. G. and Pollard, T. D. (1991) *Science* **251**, 1231–1233
- Greenwood, N. N. and Earnshaw, A. (1984) *Chemistry of the Elements*, p. 1157, Pergamon Press, Oxford
- Huang, M. M., Bolen, J. B., Barnwell, J. W., Shattil, S. J. and Brugge, J. S. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 7844–7848
- Inazu, T., Taniguchi, T., Yanagi, S. and Hirohei, Y. (1990) *Biochem. Biophys. Res. Commun.* **170**, 259–263
- Matsuda, M., Mayer, B. J., Fukui, Y. and Hanafusa, H. (1990) *Science* **248**, 1537–1539
- Mayer, B. J., Jackson, P. K. and Baltimore, D. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 627–631
- Meisenhelder, J., Suh, P. G., Rhee, S. G. and Hunter, T. (1989) *Cell* **57**, 1109–1122
- Nakamura, S. and Yamamura, K. (1989) *J. Biol. Chem.* **264**, 7089–7091
- Nishibe, S., Wahl, M. I., Hernandez-Sotomayor, S. M. T., Tonks, N. K., Rhee, S. G. and Carpenter, G. (1990) *Science* **250**, 1253–1256
- Nunn, D. L. and Watson, S. P. (1987) *Biochem. J.* **243**, 809–813
- Rendu, F., Eldor, A., Grelac, F., Levy-Toledano, S. and Levitzki, A. (1991) *Blood Coagulation Fibrinolysis* **1**, 713–726
- Rhee, S. G. (1991) *Trends Biochem. Sci.* **16**, 297–301
- Takayama, H., Nakamura, T., Yanagi, S., Taniguchi, T., Nakamura, S. and Yamamura, H. (1991) *Biochem. Biophys. Res. Commun.* **174**, 922–927
- Walker, T. R. and Watson, S. P. (1993) *Biochem. J.* **289**, 277–282
- Watson, S. P., McNally, J., Shipman, L. and Godfrey, P. P. (1988) *Biochem. J.* **249**, 345–350